LABORATORY ASSESSMENT OF THE THERAPEUTIC POTENTIAL OF NEW PENICILLINS AND OTHER ANTIBIOTICS

by

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This thesis, submitted for the degree of Doctor of Science of the University of Edinburgh, is my own work and has been composed by me alone.

The papers incorporated describe studies which have been conducted in my laboratory, either by me alone, or in collaboration with colleagues. In these latter studies, my contribution has been significant and substantial, varying from the inception and design of study, the conduct and supervision of the practical work to the analysis and reporting of data.

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INTRODUCTION

The introduction of a novel antibiotic or antibacterial agent into clinical practice is the culmination of several years work by relatively large numbers of specialists in widely different fields of science. The details of the testing procedures employed in the selection of the compound of choice from a large number of candidates will vary, depending upon the nature of the substance being evaluated, but the general principles remain the same.

Thus, although the final proof of the value of an antibacterial agent is the clinical result obtained when the drug is used to treat infection it is clearly impracticable to evaluate new drugs by clinical trial alone. Consequently, the therapeutic potential of a novel antibiotic must be assessed by study of selected parameters in the laboratory. These include measurement of antibacterial activity *in vitro* and *in vivo*, and studies of the absorption, distribution and metabolism of the drug in animals and healthy volunteers. The toxicology of the compound is also of great importance and is a factor which calls for the generation of vast amounts of data from animal studies.

In addition to the evaluation data generated during the selection or assessment procedures, other parameters are of importance. For instance, knowledge of the interaction of the test compound with other available drugs, of the mode of action, and of the rate of development of resistance, provides information to be considered in the assessment of activity. Moreover, the potential value of a compound which is in clinical use may be greatly affected by the emergence of resistant bacteria and understanding of this is important as part of ongoing evaluation.

The sections below illustrate the factors involved in the laboratory evaluation of new antibiotics and in the assessment of the therapeutic potential of these drugs, by reference to experimental data obtained in my laboratory in investigations with novel penicillins or other antibacterial substances.

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I. PRINCIPLES OF LABORATORY EVALUATION PROCEDURES

1. Antibacterial activity in vitro

The primary test of antibacterial activity is usually determination of the minimum concentration of drug required to prevent growth of the test organism in liquid or in solid medium after overnight incubation. The minimum inhibitory concentration (MIC) determined in this way is the basic measure of antibacterial activity *in vitro*, and the relative activities of antibacterial compounds are measured by comparison of MIC values. However, the MIC may be greatly influenced by test conditions, and for meaningful evaluation it is essential that these factors should be taken into account. For example, the antibacterial activity of certain antibiotics is affected by pH or by composition of the medium, but this is not usually the case with penicillins. In addition, the activity of some antibiotics may be greatly influenced by the presence of blood or serum as a result of protein binding (1, 6), and the significance of this is discussed separately (1, 2). Similarly, the size of inoculum used in the test can have a marked effect on the MIC, and this may be an important factor in the measurement of the activity of β-lactam antibiotics against β-lactamase-producing bacteria. For instance, a penicillinase-labile penicillin, such as benzylpenicillin, shows a high level of activity against a small inoculum of a penicillinase-producing staphylococcus but is inactive when the inoculum is large, whereas the activity of penicillinase-stable penicillins is barely influenced by changes in inoculum size (6). Likewise, the activity of broad-spectrum penicillins against Gram-negative bacilli is often dependent upon the numbers of cells in the

test inoculum as a result of β-lactamase activity (24, 44). However, an increase in MIC with increase in inoculum size does not necessarily indicate drug destruction even with β-lactam antibiotics. A small number of resistant cells may be present in the bacterial population, and these may grow to visible proportions when a large inoculum is used but may not be seen when the inoculum is small (14, 42, 44). The rate at which resistant mutants can be selected in vitro is an important parameter in the assessment of the therapeutic potential of a new drug as the rapid development of resistance in vitro is often paralleled in clinical practice.

The relationship between the MIC as determined in vitro and the activity of the antibiotic at the site of infection is not entirely clear. It is generally assumed that the concentration required for inhibition of bacterial growth at the site of infection is the same as that required in vitro but this may not be the case as the activity of the compound in vivo may be influenced by a number of factors. For example, the growth of the organism at the site of infection may be very different from that in vitro or its response to the antibiotic may have been altered by exposure to the immune mechanism of the host. In addition, the antibiotic concentrations present in vivo may vary extensively as the compound is absorbed and excreted resulting in a situation very different to that in vitro where the drug is present at a given concentration for the whole of the test, usually 18-24 hours.

Nevertheless, despite these limitations, measurement of the antibacterial activity of an antibacterial agent by determination of minimum inhibitory concentrations is an important step in the evaluation procedure. Experience has shown that the efficacy of a

drug in clinical practice is clearly related to its intrinsic antibacterial activity, when other parameters such as protein binding and bioavailability are taken into account.

2. Protein binding

Most antibiotics are bound to some extent to proteins present in plasma and tissues but a good deal of confusion still exists over the significance of protein binding. The binding of antibiotics in serum is usually readily reversible, and the extent of this binding depends on the structure of the drug in question and the species of animal from which the serum has been obtained (1). Marked differences occur in the extent of binding in the sera of different animals and man, and for this reason it is important to use human serum rather than serum from some other species for laboratory work on antibiotic evaluation.

Protein binding has two important consequences from the chemotherapeutic point of view. First, the protein-bound drug is essentially without antibacterial activity and, as a result, the activity displayed *in vitro* in the presence of serum is that of the unbound fraction of the drug (1). Second, protein-bound drug is relatively nondiffusible. The effect of protein binding *in vivo*, therefore, is that a proportion of the drug is sequestered in a temporarily inactive and nondiffusible form within the vascular system. As a result, the diffusion gradient from the plasma to the tissue fluid is determined by the level of free drug in the plasma and not by the total level of drug and, hence, the concentration of free antibiotic in the tissues will not be expected to exceed the peak level of free drug reached in the plasma (2). Binding occurs not

only to serum proteins but also to the proteins present in lymph and other fluids and, thus, depots of bound drug may exist in various extravascular compartments. However, because of the reversible nature of the binding even highly bound compounds can be cleared rapidly from the body (2, 6).

Since the levels of free active antibiotic in extravascular sites are determined by the levels of free drug reached in the plasma, it would be expected that in order to achieve a therapeutic effect the level of unbound drug in the plasma should at least equal the MIC of the pathogen in question. Examination of data available for a number of antibiotics which are known to be clinically effective in the treatment of staphylococcal infections indicates that this is indeed the case. Thus, it can be shown that despite marked differences in the extent of binding, level of antibacterial activity, and total serum levels reached with normal dosage, the peak levels of free antibiotic in the plasma do exceed the MIC (4).

3. Antibacterial activity in vivo

Activity against experimental infections in animals is considerably more complex than activity in vitro, and interpretation of in vivo activity in a quantitative sense may be difficult unless the factors involved are determined separately (3, 38, 39, 40). For example, activity against an experimental infection in a laboratory animal depends not only on the intrinsic antibacterial activity of the compound but also on the absorption, distribution, extent of protein binding, and metabolism of the drug in that particular animal species. In man these factors might be very different and, as a result, the clinical value of the drug might not correspond with the findings

obtained in the laboratory animal. Differences between laboratory animals and man are particularly marked with respect to absorption and rates of excretion of antibiotics. With many antibiotics, for example, the rate of elimination in mice is particularly rapid, and an inhibitory level is maintained in the animal for a very short time compared with man. Marked differences also occur in the extent to which antibiotics are bound to plasma proteins in man and in laboratory animals (1) and may complicate the correlation between the activity in the experimental animal and the probable activity if administered to man.

Nevertheless, studies in experimental animals are of significance in providing a demonstration of activity against an infection in a living host. Moreover, if the individual factors concerned in activity in the animal are measured, i.e. the levels of drug reached in the blood and tissues, the extent of binding of the drug in plasma, and the rate at which the drug is eliminated, the determination of the protective dose may be of undoubted value in comparing one compound with another. In particular, with knowledge of these parameters it may be possible to demonstrate real differences in the activities of related compounds and to draw meaningful conclusions regarding their potential clinical usefulness which may not be apparent from studies <u>in vitro</u> alone (38, 39).

4. <u>Bioavailability studies in human volunteers</u>

A crucial step in the assessment of the therapeutic potential of a novel antibacterial agent is the initial study in which the drug is administered to human volunteer subjects as there is no sure way of predicting beforehand to what extent the pharmacological data

obtained in animals will resemble those in man. Attempts to assess from animal data the probable relative oral bioavailabilities of related compounds in man are particularly hazardous. Favourable results in animals may or may not be reflected in man and vice versa. For instance, the penicillinase-stable penicillin, flucloxacillin, is absorbed to a significantly greater extent than its analogue, cloxacillin, in man but not in animals. Likewise, esters of carbenicillin, which itself is not absorbed by the oral route in man (14), may be well absorbed in man and animals but not necessarily to the same extent in the various test species (21).

In addition to the measurement of antibiotic levels in serum and urine of volunteer subjects, in the case of penicillins it is possible to measure the excretion in urine of inactive metabolites, namely, penicilloic acids, as a guide to metabolism (4, 5). Similarly, conversion of the administered compound in the body to another form or metabolite may be detected by use of suitable chromatographic or electrophoretic techniques (11, 12, 18, 21).

Overall, knowledge of peak serum concentrations, rate of elimination of the compound and extent of metabolism in human subjects, coupled with the results of studies of antibacterial activity *in vitro* and *in vivo*, should enable a reliable assessment to be made of the likely value of the new compound in clinical practice.

II. LABORATORY EVALUATION OF NEW PENICILLINS AND OTHER ANTIBIOTICS

1. Classification of penicillins

For convenience penicillins may be classified into three groups according to their antibacterial spectra (4):

(a) Narrow-spectrum penicillins. This group comprises penicillins that have a spectrum of activity essentially similar to penicillin G (benzylpenicillin) and include compounds such as penicillin V, phenethicillin, propicillin and azidocillin. These penicillins are active mainly against Gram-positive bacteria and show little or only moderate activity against Gram-negative bacilli.

(b) Penicillinase-stable penicillins. These are penicillins that are active against penicillin-resistant strains of Staphylococcus aureus as a consequence of their stability to staphylococcal β-lactamase (penicillinase). This group includes methicillin, oxacillin, cloxacillin, dicloxacillin, flucloxacillin and nafcillin.

(c) Broad-spectrum penicillins. The antibacterial spectra of these penicillins encompasses Gram-positive and many Gram-negative bacteria. Examples in clinical use are the aminopenicillins, ampicillin and amoxycillin, the α-carboxy-penicillins, carbenicillin and ticarcillin, and the ureido-penicillins, azlocillin and mezlocillin. In addition, there are a number of derivatives or analogues of ampicillin which hydrolyse to release the latter in vivo, for example, hetacillin, metampicillin, pivampicillin, and talampicillin.

2. Penicillinase-stable penicillins

The mechanism of penicillin resistance in Staphylococcus aureus is inactivation of the penicillin molecule by production of the enzyme β-lactamase (penicillinase) which opens the β-lactam ring to form the antibacterially-inactive penicilloic acid. Although cultures of these strains are resistant to penicillin the individual cells are intrinsically sensitive. Consequently, when the penicillin nucleus, 6-aminopenicillanic acid, was isolated it was considered likely that the choice of suitable side chain structures might result in penicillins which would be stable to staphylococcal penicillinase and active against resistant strains.

In 1960, methicillin, the first of the penicillinase-stable penicillins, was introduced into clinical practice. However, although this changed significantly the problem of the penicillin-resistant staphylococcus, methicillin itself is intrinsically much less active than benzylpenicillin against Staphylococcus aureus and must be given by injection in relatively large doses (4). Accordingly, penicillin derivatives were sought that would be more active and could be taken by mouth. These properties were discovered in the isoxazolyl series of penicillins which ultimately led to the clinical use of oxacillin, cloxacillin, dicloxacillin and flucloxacillin.

Early studies with the first of the series, oxacillin (BRL 1400) showed that the penicillin was active against penicillin-resistant staphylococci but was rather less active than a later member, cloxacillin (BRL 1621), which was more stable to staphylococcal β-lactamase than oxacillin (5). The results of later studies to compare the activities of four isoxazole penicillins, oxacillin, cloxacillin, dicloxacillin (BRL 1702) and flucloxacillin (BRL 2039)

confirmed that oxacillin was the least active of the compounds against β-lactamase-producing staphylococci (6). However, as there were no marked differences in the levels of antibacterial activity of the four penicillins, assessment of their therapeutic potential was based upon protein-binding characteristics and bioavailability data measured after administration to human volunteers. Thus, flucloxacillin and dicloxacillin were shown to produce significantly higher antibiotic concentrations in serum and urine after oral administration to man than did cloxacillin which was itself distinctly superior to oxacillin in this respect (6). However, because dicloxacillin was bound to serum protein to a greater extent than was flucloxacillin it was predicted that the latter would be expected to compare favourably in clinical use with the other three isoxazole penicillins (6).

3. Broad-spectrum penicillins

(i) Aminopenicillins

Ampicillin (α-aminobenzylpenicillin), the first of the broad-spectrum penicillins, became available for clinical use in 1961, since when the compound has achieved very considerable success in the treatment of a variety of infections. As a result, several related aminopenicillins have been introduced into clinical practice including amoxycillin, cyclacillin and epicillin. In addition, a number of drugs that hydrolyse in the body to form ampicillin have been reported, namely, hetacillin, metampicillin and thiazolidine esters of ampicillin.

(a) Amoxycillin

Amoxycillin (α-amino-p-hydroxybenzylpenicillin), is closely related to ampicillin and initial studies showed that the antibacterial spectra and levels of activity of both penicillins were essentially

similar (7, 9). Thus, both compounds were almost as active as benzylpenicillin against penicillin-sensitive Gram-positive cocci but the aminopenicillins were distinctly more active against certain Gram-negative bacilli. In combination with aminoglycosides or with the penicillinase-stable penicillin, flucloxacillin, synergistic effects were observed against certain bacteria (29, 30, 37).

The major difference between amoxycillin and ampicillin is that the former was found to be absorbed in man to a significantly greater degree when administered by mouth, and as a result, the antibiotic concentrations achieved in the serum of human volunteers were about twice as high as those of ampicillin (8, 9). Similarly, the urinary excretion of amoxycillin was about double that of ampicillin. It was predicted that amoxycillin could be expected to compare favourably with ampicillin in clinical use as a consequence of its good activity and favourable absorption.

Amoxycillin was also found to be significantly more effective than ampicillin in the treatment of experimental infections. The comparative therapeutic effects produced by these penicillins in animals were studied in detail and are discussed separately (39, 40).

(b) Hetacillin

Hetacillin is the generic name for the compound obtained by reaction of ampicillin with acetone. The antibacterial activity of hetacillin was found to be indistinguishable from that of ampicillin but it was also shown that hetacillin hydrolysed rapidly in aqueous solution to liberate ampicillin and acetone (11). Consequently, it was concluded that the antibacterial activity of hetacillin was due to the ampicillin formed by hydrolysis, a supposition which appeared to be supported by the fact that the activities of analogues of

hetacillin (condensation products of ampicillin and formaldehyde) corresponded closely with the rates at which the compounds hydrolysed to ampicillin. After oral administration to human volunteers electrophoretic studies showed that hetacillin hydrolysed rapidly in the body and that only small quantities of unchanged hetacillin were found in the blood of the subjects (11). Moreover, the serum concentrations of ampicillin obtained after administration of hetacillin were found to be notably lower than those measured with ampicillin. Consequently, it was concluded that as the therapeutic activity of hetacillin was due to the formation of ampicillin in the body and as the blood levels obtained with hetacillin were lower than those of ampicillin, there would appear to be no advantage to be claimed for hetacillin.

(c) Metampicillin

Metampicillin is the product produced by the reaction of ampicillin with formaldehyde. As was the case with hetacillin, metampicillin was shown to hydrolyse readily in aqueous solution to liberate ampicillin and so it was not unexpected that the drug showed antibacterial activity *in vitro* similar to that of ampicillin (12). After oral administration, metampicillin was not found in the blood of volunteer subjects and the levels of ampicillin obtained with metampicillin were lower than those produced by ampicillin (12). On the other hand, unchanged metampicillin was detected in significant quantities in blood after intramuscular injection. On the basis of these properties it was concluded that it was unlikely that metampicillin would offer any therapeutic advantage compared with ampicillin.

(d) Esters of ampicillin

Attempts to improve the absorption characteristics of ampicillin have included the preparation of esters of ampicillin, in

which the ester is in the C3-position of the thiazolidine ring, Such esters are devoid of antibacterial activity and depend upon hydrolysis in vivo to the parent penicillin after absorption from the gastro-intestinal tract. Studies with a series of lactonyl esters of ampicillin and other penicillins revealed that certain phthalidyl esters of ampicillin were hydrolysed readily by animal tissues and produced satisfactory blood concentrations of ampicillin in rodents and squirrel monkeys (13). In contrast, the phthalidyl esters of penicillin V and cloxacillin produced lower antibiotic concentrations in animals than the parent compounds. The phthalidyl ester of ampicillin (talampicillin) was shown to produce significantly higher concentrations of ampicillin after administration to man than did ampicillin and the compound was recommended for clinical trial studies (13).

(ii) Antipseudomonas penicillins

Carbenicillin, and related compounds such as ticarcillin and sulbenicillin with acidic groups in the side chain, have a broad-spectrum of antibacterial activity, but the most important property of these compounds is the activity displayed against Pseudomonas aeruginosa, an opportunistic pathogen which is resistant to penicillins and most antibiotics. Carbenicillin and its analogues have proved to be of great value in the treatment of serious infections due to Pseudomonas aeruginosa and other Gram-negative bacilli. In addition, a number of acylated derivatives of ampicillin have been shown to possess anti-pseudomonas activity of significance and two compounds, azlocillin and mezlocillin, have been selected for clinical use.

(a) Carbenicillin

The antibacterial spectrum of carbenicillin includes a wide range of Gram-negative bacilli and Gram-positive cocci. For instance,

carbenicillin was as active as ampicillin against Escherichia coli, Proteus mirabilis, Salmonella species, Shigella species and Haemophilus influenzae, but was less active than the latter against Gram-positive cocci (14). The activity displayed by carbenicillin against Pseudomonas aeruginosa was of a moderate order only (15, 16) but as the compound was shown to possess a low order of toxicity to experimental animals (15) it was concluded that it would be possible to treat systemic clinical infections with high dosage (14, 15). Synergy was shown against Pseudomonas aeruginosa when carbenicillin was combined with the aminoglycoside antibiotic, gentamicin (16), a finding of considerable clinical importance. Apart from its anti-pseudomonas activity, carbenicillin was active against certain ampicillin-resistant strains of Escherichia coli, indole-positive Proteus species, Enterobacter species, Citrobacter species and Serratia marcescens (16, 17), most of which were resistant also to cephalosporins. Unlike the aminopenicillins, carbenicillin was not absorbed by the oral route and had to be administered by injection (14). An important finding was that special assay procedures were required for the assay of carbenicillin in body fluids because of the presence in the material of a small amount of benzylpenicillin formed during the manufacturing process (14, 18). Relatively high levels of carbenicillin were found in the blood and urine of volunteers after intramuscular and intravenous injection (14) and it was predicted that the compound would be likely to be effective in the treatment of infections due to Gram-negative bacilli.

(b) Ticarcillin

A close chemical analogue of carbenicillin, ticarcillin, was shown to resemble the former penicillin closely in its biological

properties, the most important difference between the compounds being that ticarcillin showed a higher level of activity against Pseudomonas aeruginosa (19, 20). As was the case with carbenicillin, combinations of ticarcillin with aminoglycosides produced synergistic effects in vitro and against experimental infections (32, 33).

(c) Carfecillin

Efforts to overcome the lack of absorption of carbenicillin after oral administration to man resulted in the preparation of esters of the compound, but unlike the ampicillin esters described earlier, the ester group was incorporated in the α-carboxy side chain and not in the thiazolidine ring. Extensive studies with a series of simple alkyl and aromatic derivatives showed that a number of α-esters were absorbed by the oral route (21). In general, the aromatic esters produced higher carbenicillin concentration in the blood of monkeys and volunteer subjects than did alkyl esters. In addition, the alkyl compounds were hydrolysed relatively slowly by animal and human tissues and unhydrolysed ester was found in significant quantities in the blood of volunteer subjects (21). On the basis of these observations, the α-phenyl ester of carbenicillin (carfecillin) was selected for clinical trial.

The antibacterial activity of carfecillin in antibacterial tests in vitro was shown to be a composite of the intrinsic activity of carfecillin and of the carbenicillin formed by hydrolysis during the course of the test. Carfecillin was shown to be more active in vitro in tests against Gram-positive cocci than the parent compound, but to be rather less active than the latter against Gram-negative bacilli (22). However, these findings were largely of academic interest, as unhydrolysed carfecillin was not found in vivo because of the rapid hydrolysis of the ester moiety by body tissues (22, 23).

In the treatment of experimental infections, the compound was almost as active by the oral route as was parenteral carbenicillin (23), and it was concluded that carfecillin would be an effective oral broad-spectrum penicillin.

(d) Ureidopenicillins

Two ureidopenicillins, azlocillin and mezlocillin have been introduced into clinical practice, the former with claims to be especially active against Pseudomonas aeruginosa and the latter to be a broad-spectrum penicillin with significant activity against Klebsiella aerogenes. However, the antibacterial activities of the ureidopenicillins were affected greatly by changes in inoculum size employed in the antibacterial tests and the compounds were shown to be relatively unstable to the β-lactamases produced by Pseudomonas aeruginosa and Klebsiella aerogenes (24). The significance of these findings is not clear but pose some questions regarding the significance of the activities of the compounds.

III. SYNERGY OF PENICILLIN COMBINATIONS

Combined antibiotic therapy is often used in the treatment of serious infections. In many cases the intention is to extend the antibacterial spectrum but in some instances antibiotics are combined to produce enhancement of activity. As part of the laboratory evaluation procedure, an important stage is determination of the results of interaction of new agents with established drugs. Penicillins may produce synergistic activity in combination with other antibiotics (28), but the reverse may also be the case and antagonistic effects may be demonstrated when a penicillin is combined with a bacteriostatic antibiotic such as tetracycline or chloramphenicol (10).

1. Synergy between amoxycillin and aminoglycosides

Enhancement of the bactericidal activity of benzylpenicillin against streptococci is one of the best known examples of antibiotic synergy, and the use of penicillin/aminoglycosides for the treatment of enterococcal endocarditis is a widely accepted instance of clinical synergy. Amoxycillin was shown to be more active than ampicillin and benzylpenicillin against Streptococcus faecalis and to produce synergistic bactericidal effects against this organism when combined with aminoglycoside antibiotics (29, 30). During these tests it was observed that a significant number of strains of Streptococcus faecalis possessed a high level of resistance to the older aminoglycosides, streptomycin and kanamycin, but were relatively sensitive to the newer aminoglycosides, gentamicin and tobramycin. Enhanced activity was shown by the amoxycillin + aminoglycoside combination only when the

organism was sensitive to the test aminoglycoside. In a later study, strains of Streptococcus faecalis resistant to gentamicin and tobramycin were found in recent clinical isolates (31) emphasizing the need for appropriate laboratory tests when combined therapy is being considered for enterococcal infections. Combinations of amoxycillin and aminoglycosides also produced increased bactericidal effects against streptococci other than enterococci, namely, viridans streptococci and Streptococcus bovis (30). On the basis of these studies it was considered that amoxycillin could have advantages over ampicillin and benzylpenicillin in the treatment of enterococcal infections and that combined therapy with amoxycillin and aminoglycoside antibiotics might be expected to be efficacious against severe streptococcal infections.

2. Synergy between α-carboxypenicillins and aminoglycosides

In a similar fashion, combinations of α-carboxypenicillins and aminoglycosides have been shown to produce synergistic effects in the laboratory against Pseudomonas aeruginosa and other Gram-negative bacilli. Thus, initial studies with carbenicillin showed that the growth of Pseudomonas aeruginosa was inhibited by concentrations of carbenicillin + gentamicin which were themselves not inhibitory for the test organism (16). Later studies comparing the effects produced by combinations of carbenicillin or ticarcillin with the aminoglycoside antibiotic tobramycin, showed that similar synergistic effects were produced by the penicillins in bacteriostatic and bactericidal tests in vitro (32). It was found, for instance, that enhanced antibacterial activity was demonstrated by the combinations of antibiotics against the majority of strains tested. Similar results were obtained when

ticarcillin was combined with newer aminoglycoside antibiotics including amikacin, dibekacin and sisomicin (33). As ticarcillin was more active than carbenicillin against Pseudomonas aeruginosa, combinations of ticarcillin + tobramycin were more effective than corresponding combinations containing carbenicillin but this was not the case against other Gram-negative bacilli. The synergy demonstrated in vitro was observed also in tests in vivo and combined therapy of experimental mouse infections was significantly more effective than treatment with the individual antibiotics alone (32, 33). It was concluded from these studies that combined therapy with ticarcillin and aminoglycosides would be justified in the treatment of serious infections caused by Gram-negative bacilli.

3. Synergy between penicillins

An entirely different example of synergy involving penicillins, for which the mechanism appears to be clearly established, is that where enhanced activity may be demonstrated between pairs of β-lactam antibiotics as a result of competitive inhibition of β-lactamase activity. Thus, the resistance of many Gram-negative bacilli to the broad-spectrum penicillins is often associated with β-lactamase activity and the ability of these bacteria to destroy the compounds (44). The penicillinase-stable penicillins such as methicillin or cloxacillin are not destroyed by the β-lactamases of Gram-negative bacilli but are ineffective because these organisms are intrinsically insensitive (44). However, it was observed that the inactivation of ampicillin by β-lactamases of certain Gram-negative bacilli was significantly reduced in the presence of methicillin or cloxacillin and that synergistic effects were observed when ampicillin was combined with methicillin

or cloxacillin (34). It was concluded that the synergy observed was due to the protection of the broad-spectrum penicillin from destruction as a result of inhibition of the β-lactamase activity of the organism by the penicillinase-stable penicillin. In a later study it was confirmed that the enhanced activity of a combination of ampicillin and cloxacillin was shown to be due to protection of ampicillin from destruction and it was demonstrated that the combination was effective against experimental infections (35). Significant synergistic effects were demonstrated against a limited range of Gram-negative bacilli, presumably because of the relatively large variety of β-lactamases produced by these organisms (36). However, because the synergy demonstrated <u>in vitro</u> was reflected also in the treatment of experimental infections it was concluded that treatment of selected infections caused by certain β-lactamase-producing bacteria with combinations of penicillins might well be justified (35, 37).

IV. MODE OF ACTION: IN VIVO

Although the therapeutic efficacy of a new antibacterial agent may be predicted with some confidence from knowledge of factors such as antibacterial activity *in vitro*, extent of binding to serum protein, and metabolism and bioavailability in man, there are examples where this is not the case and where a better understanding of other parameters is required.

1. Comparative activities of spiramycin and erythromycin

In comparing the antibacterial activities of the macrolide antibiotics, spiramycin and erythromycin, it was apparent that the latter was notably more active in bacteriostatic and bactericidal tests *in vitro* (38). However, spiramycin was found to be at least as effective as erythromycin against staphylococcal infections in mice when the drugs were administered after the onset of infection. Moreover, spiramycin was the more effective of the compounds in tests in which the drugs were administered prior to infection of the animals. Investigations into the distribution of the antibiotics in the mouse showed that spiramycin was present in lower concentrations than erythromycin in the blood, but that spiramycin produced higher concentrations than erythromycin in the organs. This effect was particularly marked at 24 hours and 48 hours after injection when erythromycin was hardly detected in any of the specimens in contrast to findings with spiramycin. These results suggested that the efficacy of spiramycin against experimental infections was related to its persistence in the animal body, and that assessment of the therapeutic activity of spiramycin based solely on *in vitro* and blood level data might be misleading.

2. Comparative activities of amoxycillin and ampicillin

Comparative studies with amoxycillin and ampicillin showed that both penicillins produced similar levels of antibacterial activity *in vitro* (9) but that amoxycillin was usually significantly more effective than ampicillin in the treatment of experimental infections (39). Amoxycillin produced higher antibiotic blood concentrations in mice than did ampicillin after oral administration and this was a possible explanation for the superior activity of amoxycillin by the oral route, but this would not explain the results obtained by injection where the antibiotic blood concentrations were the same for both compounds. However, it was found that amoxycillin was more effective than ampicillin in reducing the bacterial counts in the peritoneal cavity and in the blood of infected animals (39). Subsequently, microscopic observation of specimens from mice infected with *Escherichia coli* showed marked differences in the effects produced by the two penicillins on the morphology of bacteria present in blood and peritoneal fluid. Thus, when the antibiotics were present in the animal body at concentrations close to their minimum inhibitory concentrations against the infecting organism, amoxycillin caused the formation of spheroplast forms which lysed readily, whereas ampicillin produced long cells or filaments which were relatively stable (40). On the basis of these observations it was concluded that the superior therapeutic activity of amoxycillin was due to the greater bacteriolytic and bactericidal effects produced *in vivo*.

V. RESISTANCE OF BACTERIA TO PENICILLINS AND OTHER ANTIBIOTICS

Knowledge of the incidence and likely rate of emergence of resistant bacteria to a novel antibacterial agent is of considerable importance in assessing its therapeutic potential. Also, information regarding the mechanism or nature of bacterial resistance to antibiotics may be of value in understanding the mode of action of a compound or its interrelationships with other drugs. A traditional method of investigating development of antibiotic-resistant bacteria is by repeated sub-culture of sensitive bacteria in the presence of sub-inhibitory concentrations of the drug, the process being continued until resistant organisms are selected. However, caution must be observed in interpreting the results of such tests, as the factors responsible for selection *in vitro* may not apply *in vivo*, and the antibiotic-resistant bacteria obtained in this fashion may differ radically from naturally-occurring antibiotic-resistant strains (41). Nevertheless, data of this kind are usually sought and interpreted as part of the evaluation procedure.

1. Methicillin-resistant staphylococci

The mechanism of resistance of wild type strains of staphylococci to penicillin is that of drug inactivation brought about by the enzyme, β-lactamase, and consequently these strains were found to be sensitive to penicillinase-stable penicillins such as methicillin, cloxacillin or flucloxacillin. At the time methicillin was first introduced, naturally-occurring strains of *Staphylococcus aureus* resistant to the drug had not been encountered but soon after, a number of strains with increased resistance were isolated from

clinical sources. These organisms produced β-lactamase but did not inactivate methicillin or cloxacillin, and the resistance of these strains was shown to be due to the presence in each culture of a minority population of cells with a high level of resistance to the penicillins (42). The heterogeneous nature of the resistance of naturally-occurring staphylococci, in which the resistant cells grew slowly in the presence of the penicillinase-stable penicillin, led to difficulties in the routine detection of these organisms. It was found that under normal conditions of testing, methicillin-resistant staphylococci were not detected if a dilute inoculum was tested, and even with a large inoculum the presence of resistant cells might not be observed until after 48 hours of incubation (42, 43). However, as the minority resistant cells were known to grow more rapidly in media containing added sodium chloride, it was suggested that routine sensitivity testing for methicillin-resistant staphylococci could be performed satisfactorily utilising agar containing 5% sodium chloride (43).

2. Mechanism of resistance of Gram-negative bacilli to penicillins

The penicillinase activity of bacteria was first described in 1940 in a strain of Escherichia coli but this was overshadowed by the clinical significance of the subsequent demonstration of penicillinase-producing strains of Staphylococcus aureus. The development of the broad-spectrum penicillins with significant activity against many Gram-negative bacilli resulted in renewed interest in their resistance to penicillins.

In general, the narrow-spectrum penicillins, phenethicillin and propicillin, and the penicillinase-stable penicillins were not

active against Gram-negative bacteria but the resistance of the organisms was not associated with inactivation (44). In contrast, the resistance of Gram-negative bacilli to ampicillin and benzylpenicillin was often due to destruction of the penicillins, although a number of these organisms also possessed an intrinsic insensitivity to the compounds (44). Investigation of the mechanism of inactivation suggested that this was due to β-lactamase activity rather than to the activity of penicillin acylase (44). More detailed studies confirmed that penicillin-acylase activity was not a significant factor in the resistance of Gram-negative bacilli to penicillins, not solely because only a limited number of bacteria produced the enzyme but also because the cultural conditions of routine antibacterial tests favoured the production of β-lactamase rather than penicillin acylase (45).

The resistance of Gram-negative bacilli to carbenicillin was also often associated with β-lactamase activity, but this was not the case with typical strains of Pseudomonas aeruginosa; β-lactamase activity could be demonstrated but carbenicillin was not inactivated (14, 16). Similarly, strains of Klebsiella which showed a relatively high level of resistance to penicillins, and which produced significant quantities of β-lactamase, inactivated carbenicillin to only a limited extent (46).

3. Development of antibiotic-resistant Gram-negative bacilli

The discovery in the early 1960's that transferable antibiotic resistance was common and world-wide, led to extensive speculation regarding the possibility of the rapid development of widespread antibiotic resistance among Gram-negative bacilli. However, in an extensive study of enteropathogenic strains of Escherichia coli

isolated in the U.K. between 1948 and 1968 it was found that transferable resistance was almost as common among strains isolated between 1957 and 1960 as in strains isolated in 1967 and 1968 (47). Moreover, strains of E. coli capable of transferring sulphonamide resistance were observed among cultures isolated as early as 1951. It was also observed that ampicillin-resistant strains were common between 1948 and 1951, but that they differed from resistant strains isolated from 1957 onwards in that the β-lactamases produced by the early strains were chromosomally-mediated, and were not transferable until the extrachromosomally-mediated β-lactamases of the later strains (48). These data showed that antibiotic-resistant strains of E. coli capable of transferring antibiotic resistance became established in the U.K. at some time between 1951 and 1957 and suggested that it was unlikely therefore that there would be a rapid and widespread development of antibiotic resistance among Gram-negative bacilli.

This supposition appears to be supported by the results of three surveys to investigate the incidence of resistance to ampicillin and other antibiotics among Gram-negative bacilli isolated in 1961, 1967 and 1975. Overall, there appeared to have been no great changes in the resistance of bacteria to ampicillin between 1961 and 1967 (49, 50) and that there had been only a relatively small decrease in the sensitivity of Escherichia coli isolated in 1975 (51).

VI. ANTIBACTERIAL ACTIVITY OF ANTIBIOTICS IN BONE CEMENT

Incorporation of antibiotics in acrylic bone cement has been said to reduce the incidence of infection in patients undergoing total joint replacement. The original studies reported data with gentamicin and tetracycline but no results had been described for β-lactam antibiotics. Accordingly, tests were initiated to compare the antibacterial activities of penicillins and other antibiotics incorporated in acrylic bone cement. Surprisingly (in view of the heat evolved during the polymerisation process) the amounts of penicillins released from the hardened bone cement (2.5-10% of the dose incorporated) were similar to those of heat stable antibiotics such as gentamicin, fusidic acid and clindamycin and, in general, the penicillins and non-penicillin antibiotics showed similar diffusion characteristics (52). The initial rapid release of antibiotic activity *in vitro* and *in vivo*, followed by a relatively prolonged production of low antibiotic concentrations, was consistent with the leaching of the antibiotics from, or near, the surface of the bone cement. It was concluded that penicillins incorporated in acrylic bone cement would demonstrate antibiotic activity *in vivo* and could be considered for use under clinical conditions.

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THE BINDING OF ANTIBIOTICS TO SERUM PROTEINS

BY

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The published work on the binding of antibiotics to serum proteins is extensive. Nevertheless, data are still incomplete in certain respects. For example, there is little information on the rate at which antibiotics are bound to the proteins and the rate at which this complex breaks down. Similarly, although the binding of antibiotics to serum proteins is known to be essentially reversible it is not certain whether there is any proportion of the drug which is bound irreversibly. On certain aspects, published data are available but are conflicting and some lack of agreement exists on the precise extent of binding of certain antibiotics, particularly of tetracyclines. There is also a general lack of comparative results on the extent of binding for related antibiotics obtained from experiments carried out by the same method.

In the present paper, results are given for the effect of certain factors on the binding of penicillins and other antibiotics in serum and comparative data are also given for the extent of binding in human serum for all the penicillins at present in clinical use.

METHODS

Measurement of binding of antibiotics to serum proteins

The extent of binding of penicillins and other antibiotics to the proteins of serum was measured by ultrafiltration through Visking viscose-cellulose dialysis tubing. Preliminary experiments indicated that there was no significant difference between the extent of binding in plasma obtained from heparinized blood and that in serum. The antibiotic present in the protein-free ultrafiltrate was measured by microbiological assay and this quantity represented the free, unbound, fraction of antibiotic in serum. The amount of antibiotic bound to protein was derived by subtracting the level of free antibiotic from the known total concentration in serum. Before comparative experiments were made to measure the extent to which different antibiotics were bound to the proteins of human serum, preliminary investigations were carried out into the effects of various experimental factors thought likely to influence the extent of binding. These included the effects of antibiotic concentration, nature of protein, temperature, individual variation, rate of binding and nature of binding.

Ultrafiltraton techniques

Suitable lengths of Visking tubing (0.25 in. internal diameter) were knotted at one end and attached at the other to a manifold connected to compressed air. Serum samples were introduced into the tubing before connexion to the manifold and ultrafiltration was then carried out at room temperature at a positive pressure of 15 lb/in^2. The ultrafiltrate was collected in a glass tube which closely surrounded the Visking sac. In most experiments the volume of serum used was 5 ml., and the volume of ultrafiltrate collected for assay was about 0.5 ml. In some experiments a volume of 10 to 12 ml. of serum was used which permitted an adequate sample of ultrafiltrate to be collected in about 7 min.

Horse, sheep, rabbit, and calf sera were obtained as commercial samples from Burroughs Wellcome. Human serum was obtained from healthy volunteers and pooled before use. The *p*H of the fresh human serum was approximately 7.4, but on standing in the refrigerator for a few days the *p*H rose to as high as 8.0 or above. Because of this change in *p*H, in all the experiments the *p*H of the serum was adjusted to 7.4 with carbon dioxide before use, although in a limited number of experiments with benzylpenicillin and cloxacillin the extent of binding did not appear to be greatly influenced by changes in *p*H over the range 7.4 to 8.2.

With each antibiotic it was confirmed that there was free passage of the compound through the membrane without selective filtration when aqueous solutions were placed inside the Visking tube and air pressure was applied. It was also shown that the ultrafiltrate from normal serum was devoid of protein. There was no evidence that blocking of the membrane took place during filtration, and in repeat experiments with different penicillins consecutive samples of ultrafiltrate showed no significant difference in assay results. In contrast, with the tetracyclines there was some evidence that the concentration in the first sample of ultrafiltrate was erroneously low and a subsequent sample was routinely used for assay. This phenomenon with tetracycline has already been reported by Remington & Finland (1962).

Measurement of free and total antibiotic

The antibiotic content of samples of serum or serum ultrafiltrate was determined by microbiological assay. Rectangular glass plates (12 × 15 in.) were poured with a layer of nutrient agar (Oxoid No. 2) inoculated with a suspension of a suitable assay organism. For assay of penicillins and cephalothin, *Bacillus subtilis* (N.C.T.C. 8236) or *Sarcina lutea* (N.C.T.C. 8340) was employed. The tetracyclines were assayed with *Bacillus cereus* (N.C.TC 8035), and novobiocin with the Oxford strain of *Staphylococcus aureus* (N.C.T.C. 6571). Holes (7 mm) were punched in the seeded agar with a No. 4 cork-borer, and these were filled with the test samples or appropriate standard solutions.

For estimation of free concentrations of penicillins in ultrafiltrate samples, standard solutions were prepared in 0.05 M-phosphate buffer (*p*H 7.4), preliminary experiments having shown that standard penicillin solutions in phosphate buffer yielded results identical to equivalent standard solutions prepared in ultrafiltrate from normal human serum. With the tetracycline antibiotics, however, preliminary experiments showed that standard solutions in buffer produced inhibition zones which were significantly larger than those caused by the equivalent solutions prepared in serum ultrafiltrate. Accordingly, with this group of antibiotics, and with novobiocin and fusidic acid, standard solutions were prepared in normal serum ultrafiltrate. Total antibiotic concentrations in serum were measured against standard solutions prepared in normal human serum, *p*H 7.4. Inhibition zone diameters were measured after incubation overnight at 30° C and the concentrations of antibiotic were estimated by reading from a graph derived from similar assays of standard solutions.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations required to prevent growth of bacteria for 24 hr at 37° C were determined by serial dilution of the antibiotic in 1 ml. volumes of nutrient broth (Oxoid No. 2). In addition, minimum inhibitory concentrations were also determined in serum in the same fashion by serial dilution in 1 ml. volumes of normal human serum, *p*H 7.4. For the determinations of minimum inhibitory concentrations in serum, aqueous solutions of the antibiotic were added to give the desired range of antibiotic concentrations with a final serum concentration of 95%. One drop of a 1/1,000 dilution of an overnight culture was added to each tube to give an inoculum of about 10^4 organisms. Visual determination of minimum inhibitory concentrations in serum was not always definite, and with the penicillins the end points were checked by subculturing from the serial dilution onto antibiotic-free agar, and observing growth after a further 24 hr incubation.

RESULTS

Factors affecting the binding of antibiotics to serum proteins

Concentration of antibiotic. Fig. 1 illustrates the effects of variation of antibiotic concentration on the extent of binding of certain penicillins in human serum. With

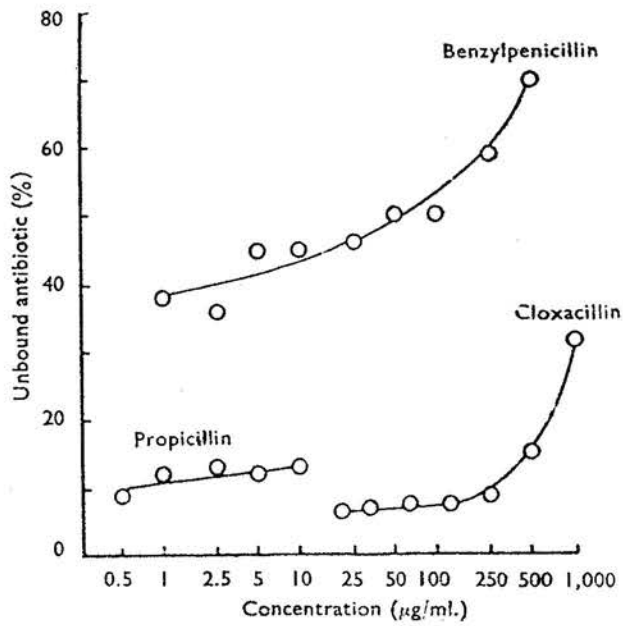

Fig. 1. Effect of variation in the concentration of penicillin on the extent of binding in serum. Human serum containing various concentrations of benzylpenicillin, propicillin or cloxacillin was filtered through Visking membranes. The protein-free ultrafiltrates, containing the unbound compound, were assayed for antibiotic content.

benzylpenicillin and cloxacillin the percentage of unbound drug in serum increased greatly with increase in concentration above approximately 200 μg/ml. of penicillin. Below this value changes in concentration appear to have a relatively slight effect on the extent of binding.

TABLE 1

EXTENT OF BINDING OF BENZYLPENICILLIN AND CLOXACILLIN IN SERA OF DIFFERENT ANIMAL SPECIES

Sera containing benzylpenicillin (1 μg/ml.) or cloxacillin (50 μg/ml.) were filtered through Visking membranes. The ultrafiltrates, containing the free, unbound penicillin of each serum specimen, were assayed for antibiotic content. Unbound penicillin was calculated as the ratio of the concentration in ultrafiltrate to the total concentration in serum, × 100

	Unbound penicillin (%) for	
Species	Benzylpenicillin	Cloxacillin
Man	49·0	6·5
Horse	59·0	30·0
Sheep	70·0	19·5
Rabbit	65·0	22·0
Calf	63·0	25·0

Nature of protein. Table 1 shows the extent of binding of benzylpenicillin and cloxacillin to sera of different animal species. The extent of binding of a particular penicillin to serum may differ greatly from one species to another, the results with cloxacillin in human and horse serum being particularly marked. Thus, in horse serum the unbound proportion of cloxacillin was about five-times greater than in human serum.

With both benzylpenicillin and cloxacillin the degree of binding was greatest with human serum, but the relative binding capacities of the other sera were not the same for both penicillins.

Concentration of protein. The relationship between concentration of human serum and extent of binding is shown in Fig. 2 for benzylpenicillin and cloxacillin. Human serum was diluted to the appropriate concentration with distilled water and the determinations

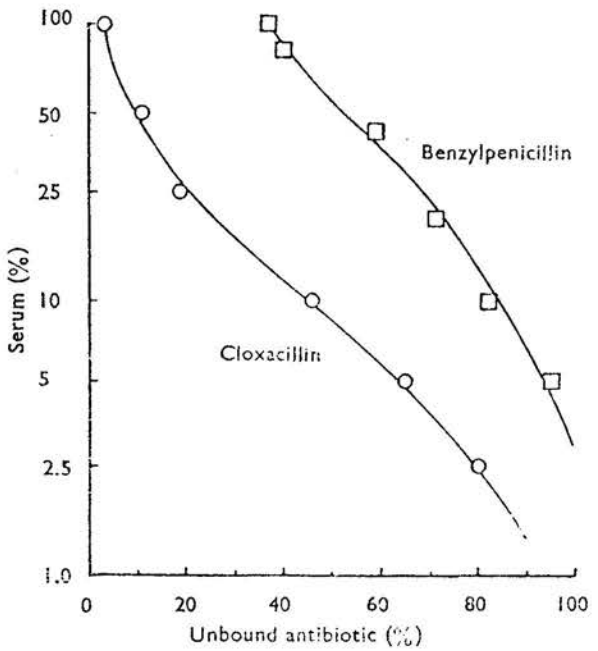

Fig. 2. Effect of variation in the concentration of serum protein on the extent of binding of benzylpenicillin and cloxacillin. Human serum was diluted with 0.05 M-phosphate buffer (*p*H 7.4) to give solutions of various protein concentrations. These solutions containing benzylpenicillin (1 μg/ml.) or cloxacillin (50 μg/ml.) were filtered through Visking membranes and the protein-free ultrafiltrates, containing the unbound penicillin of each solution, were assayed for antibiotic content.

of extent of binding were made by ultrafiltration of samples of aqueous sera containing benzylpenicillin at 2 μg/ml. and cloxacillin at 50 μg/ml. of serum. As would be expected, the extent of binding of both penicillins decreased as the concentration of protein was reduced, and the relative increase in unbound antibiotic with decreasing serum concentration was very similar for benzylpenicillin and cloxacillin. Nevertheless, with cloxacillin only about 50% of the penicillin was available as unbound antibiotic even after a ten-fold dilution of the serum.

Temperature. The percentage binding of benzylpenicillin and cloxacillin in human serum was determined by ultrafiltration at 37, 22 and 4° C. Solutions of benzylpenicillin (1 μg/ml. of serum) and cloxacillin (50 μg/ml. of serum) were adjusted to the required temperature before filtration and the experiments were carried out in temperature controlled rooms. The temperatures of the samples of ultrafiltrate were checked as they

were collected and did not differ significantly from the initial temperatures. In several experiments with benzylpenicillin and cloxacillin, variation in temperature had little effect on the extent of binding of the penicillins, and with both compounds results obtained at 37° C differed little from those obtained at 4° C.

Binding in sera of individual human subjects. Serum was obtained from each of six healthy subjects. Benzylpenicillin was added to give a concentration of 2 μg/ml. and the extent of binding was determined by ultrafiltration. Ultrafiltration was repeated the following day using a further aliquot of each of the samples of sera. One week later serum was again obtained from the same six subjects and the determination of binding with benzylpenicillin was carried out on two successive days as before. The results are shown in Table 2. Differences in binding capacity between one subject and another were slight and might well be less than the experimental errors inherent in the methods. Further

TABLE 2

VARIATION IN THE EXTENT OF BINDING OF BENZYLPENICILLIN IN SERA OF INDIVIDUAL HUMAN SUBJECTS

Serum was obtained from each of six normal subjects and benzylpenicillin added to give a concentration of 2 μg/ml. The extent of binding was determined by ultrafiltration and the experiment was repeated the following day using a further aliquot of each serum sample (Experiment 1). One week later serum was again obtained from the same six subjects and the determination of binding carried out on two successive days as before (Experiment 2)

	Unbound antibiotic (%) in			
	Experiment 1		Experiment 2	
Subject	First determination	Repeat determination	First determination	Repeat determination
1	47·5	41·0	39·0	36·5
2	47·5	46·0	41·0	39·5
3	49·0	49·0	40·0	40·0
4	57·0	49·5	42·0	40·0
5	49·0	44·5	33·5	35·0
6	50·0	41·5	39·0	40·5

experiments with another group of six subjects using both benzylpenicillin and cloxacillin confirmed these results and failed to indicate marked differences in binding capacity between one person and another.

Rate of binding and dissociation of penicillin in human serum. Experiments were carried out with benzylpenicillin, phenoxymethylpenicillin, phenethicillin, methicillin, cloxacillin and ampicillin to determine the rate at which binding takes place in human serum. Ultrafiltration was carried out immediately after the addition of the penicillin to the serum and further samples were also taken at intervals of time up to 1 hr. The time taken to obtain the first sample of ultrafiltrate was about 8 min after adding the penicillin to the serum. With all the penicillins tested the assay of the initial sample did not differ significantly from any of the subsequent samples indicating that equilibrium had been reached by the time the first sample had been collected.

The following experiments were also carried out to determine the rate of breakdown of the penicillin-protein complex. Cloxacillin was dissolved in serum to give a penicillin concentration of 400 μg/ml. and a sample of ultrafiltrate was obtained. At this concentration 13% of the penicillin was present in the serum as free, unbound, cloxacillin. The

serum was then diluted fortyfold with 0.05 M-phosphate buffer (*p*H 7.4) to give a serum concentration of 2.5%, and samples of ultrafiltrate were taken immediately after dilution and also at intervals of time up to 1 hr. Assay of the sample taken immediately after dilution showed 49% unbound antibiotic, and assays of the subsequent samples showed that the new equilibrium had been established by the time the first sample was taken.

Extent of irreversible binding in serum. Cloxacillin was dissolved in human serum at a concentration of 400 μg/ml. and a sample of ultrafiltrate was obtained in the usual way. Under these conditions 87% of the antibiotic was bound to protein. An aliquot of the original serum solution was then diluted fortyfold with 0.05 M-phosphate buffer, *p*H 7.4, which resulted in dissociation of the antibiotic-protein complex and yielded a solution consisting of 2.5% serum in buffer and cloxacillin at a concentration of 10 μg/ml. A sample of ultrafiltrate was then obtained and compared with ultrafiltrate from a solution of 10 μg/ml. cloxacillin in 2.5% serum prepared directly by adding cloxacillin to 2.5% serum. Results, typical of those obtained when the experiment was repeated, are shown in Table 3. After dilution of the serum with buffer the concentration of unbound antibiotic was slightly lower than in the solution prepared directly at the same

TABLE 3

THE EXTENT OF IRREVERSIBLE BINDING OF CLOXACILLIN IN HUMAN SERUM

Human serum containing cloxacillin (400 μg/ml.) was diluted fortyfold in 0·05 M-phosphate buffer (*p*H 7·4), and the unbound penicillin present was measured by ultrafiltration. The extent of binding of a solution of cloxacillin (10 μg/ml.) prepared directly in 2·5% human serum was also determined. The amount of irreversible binding of cloxacillin to serum is indicated from a comparison of the amounts of free cloxacillin in the two solutions

Solution	Cloxacillin concentration (μg/ml.)	Serum concentration (%)	Antibiotic	
			Bound (%)	Unbound (%)
Original	400	100	87	13
Diluted fortyfold	10	2·5	49	51
Prepared directly	10	2·5	40	60

serum concentration. Further experiments were also carried out with oxacillin at two concentrations, 400 and 20 μg/ml., before dissociation by dilution with buffer. As in the experiments with cloxacillin the concentration of unbound drug was slightly higher in the solution prepared directly in 2.5% serum than in the corresponding solution obtained by dilution of normal serum with buffer. These results suggest that some degree of irreversible binding may take place, although it appears that this is only a small proportion of the total antibiotic in normal serum.

Competitive binding between cloxacillin and other substances. The results of experiments to determine the extent of binding of cloxacillin in human serum in the presence of substances which are themselves highly bound to serum proteins are shown in Table 4. In these experiments various concentrations of compounds were added to serum containing 50 μg/ml. cloxacillin. Ultrafiltrates of serum containing these compounds alone showed no antibacterial activity which would have obscured the activity of unbound cloxacillin, except in the experiments with novobiocin and sulphonamides. To eliminate activity due to novobiocin the assay of cloxacillin was performed with a novobiocin-resistant strain of *Staph. aureus*; sulphonamide activity was neutralized by the addition of *p*-aminobenzoic acid to the assay agar.

Table 4 shows that the level of unbound cloxacillin in serum was four- to fivefold greater than normal in the presence of 500 μg/ml. of sodium salicylate, γ-resorcylic acid, sulphamethoxypyridazine or phenylbutazone, but that at a concentration of 100 μg/ml. these substances resulted only in a slight increase in the amount of unbound cloxacillin. These results were not influenced by the order in which the cloxacillin was added to the serum,

TABLE 4

COMPETITIVE BINDING BETWEEN CLOXACILLIN AND OTHER SUBSTANCES

Cloxacillin was added, to give a concentration of 50 μg/ml., to aliquots of human serum containing various compounds at concentrations of 100 and 500 μg/ml. The sera were filtered through Visking membranes and the amount of unbound cloxacillin was measured by assay of the ultrafiltrates. Ultrafiltrates of serum containing these compounds alone showed no antibacterial activity which would have obscured the activity of unbound cloxacillin, except in the experiments with novobiocin and sulphonamides. To eliminate activity due to novobiocin the assay of cloxacillin was performed with a novobiocin-resistant strain of *Steph. aureus*; sulphonamide activity was neutralized by the addition of *p*-aminobenzoic acid to the assay agar

Competitive compound	Unbound cloxacillin (%) for concentration of competitive compound	
	100 μg/ml.	500 μg/ml.
None	6·6	6·6
Sodium salicylate	8·8	33·0
γ-Resorcylic acid	10·8	32·0
Sulphamethoxypyridazine	8·0	23·5
Phenylbutazone	10·4	23·0
Sulphadimidine	8·9	20·6
Novobiocin	—	7·0

either before or after the addition of the competitive compound to the serum. The addition of novobiocin at a concentration of 500 μg/ml. to serum containing cloxacillin had no significant effect on the extent of binding of cloxacillin although novobiocin is known to be highly bound to serum proteins.

Measurement of the extent of binding of various antibiotics to the proteins of human serum

Results for the extent of binding in human serum are given in Table 5 for all the penicillins in clinical use at present. Results are also given for cephalothin, tetracycline, chlortetracycline, demethylchlortetracycline, novobiocin and fusidic acid. The determinations of protein binding were carried out by ultrafiltration of human serum, as described in Methods, at room temperature and *p*H 7.4. The concentrations of the antibiotics were governed by the extent of binding of each compound and the sensitivity of the assay but, in general, these concentrations were of the same order as those reached in serum in humans following the recommended dosage schedules. In many instances, determinations were also made at more than one drug concentration, and for any particular antibiotic no significant differences in protein binding could be detected over the ranges of concentration used. Data for groups of closely related antibiotics such as phenoxymethylpenicillin, phenethicillin and propicillin and tetracycline, chlortetracycline and demethylchlortetracycline were obtained in comparative experiments using the same batches of pooled serum, although there was no evidence of variation in binding capacity between one batch of serum and another.

TABLE 5

THE EXTENT OF BINDING OF VARIOUS PENICILLINS AND OTHER ANTIBIOTICS IN HUMAN SERUM

Aliquots of human serum containing the respective antibiotics were subjected to ultrafiltration and the ultrafiltrate was assayed for antibiotic content. The percentage of bound antibiotic in serum was calculated as the difference between the total concentration in serum and the concentration in ultrafiltrate divided by the total concentration in serum, × 100. The concentrations of the antibiotics used were governed by the extent of binding of each compound and the sensitivity of the assay, but in general these concentrations were of the same order as those reached in serum in humans following the recommended dosage schedules

Antibiotic	Concentration in serum (μg/ml.)	Unbound (%)	Bound (%)
Benzylpenicillin	1	41	59
Phenoxymethylpenicillin	5	20·3	79·7
Phenethicillin	10	17·3	82·7
Propicillin	10	11·5	88·5
"Rixapen"	10	8·4	91·6
Phenbenicillin	10	2·8	97·2
Nafcillin	50	13·2	86·8
Oxacillin	50	6·9	93·1
Cloxacillin	50	6·0	94
Methicillin	10	50·7	49·3
Ampicillin	5	82	18
Tetracycline	20	30	70
Chlortetracycline	20	13	87
Demethylchlortetracycline	20	11	89
Novobiocin	100	0·8	99·2
Fusidic acid	50	2·8	97·2
Cephalothin	5	23	77

Among the penicillins, ampicillin was the least affected by the presence of human serum, 82% of the compound being present as free antibiotic. The acid-stable penicillins, phenoxymethylpenicillin and phenethicillin were both bound to about the same extent, the extent of binding being such that the free level of each drug was about half that of benzylpenicillin. Propicillin was bound somewhat more than phenoxymethylpenicillin and phenethicillin while phenbenicillin was bound to a significantly greater extent. Among the group of penicillinase-stable penicillins, nafcillin, oxacillin and cloxacillin were all highly bound to the proteins of human serum, whereas methicillin was bound to a relatively small extent, about 50% of the drug being present in the free form.

Like the penicillins, the tetracycline group of antibiotics showed differences in the relative extent of binding to serum proteins. Both chlortetracycline and demethylchlortetracycline showed a high degree of binding and were bound to a significantly greater extent than was tetracycline. With the cephalosporin derivative, cephalothin, about one-quarter of the compound was present in serum as free antibiotic.

Both novobiocin and fusidic acid were highly bound to the proteins of human serum. In the case of novobiocin, only 0.8% of the drug was available as unbound antibiotic, this being the highest degree of binding of antibiotic to serum measured in this series of experiments.

Effect of serum on antibacterial activity

Results are given in Table 6 for the minimum inhibitory concentrations of benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin and cloxacillin, against *Staph. aureus* (Smith) in nutrient broth and in 95% human serum. These compounds

TABLE 6

CORRELATION BETWEEN THE EXTENT OF BINDING OF VARIOUS PENICILLINS IN HUMAN SERUM WITH THE DIMINUTION OF THE ANTIBACTERIAL ACTIVITY OF THESE COMPOUNDS IN THE PRESENCE OF HUMAN SERUM

Minimum inhibitory concentrations of the penicillins against *Staph. aureus* (Smith) were determined in nutrient broth and in 95% human serum. The percentage of penicillin bound to serum was calculated as the difference between the minimum inhibitory concentration in serum and the minimum inhibitory concentration in nutrient broth, divided by the minimum inhibitory concentration in serum, ×100; this was compared with values obtained by ultrafiltration

Penicillin	No. of expts.	Average minimum inhibitory concentration (μg/ml.)		Extent of binding (% bound) determined	
		Nutrient broth	95% Human serum	From the minimum inhibitory concentration	By ultra-filtration
Benzylpenicillin	2	0·02	0·04	50	59
Phenoxymethylpenicillin	4	0·017	0·067	75	79
Phenethicillin	4	0·045	0·18	76	82
Propicillin	4	0·055	0·36	85	88
Cloxacillin	2	0·21	3·1	93	94

differ markedly with regard to the effect of serum on antibacterial activity. In each instance activity was diminished, but whereas with benzylpenicillin activity was depressed only twofold, with cloxacillin the decrease was approximately fifteenfold. In the presence of serum the proportion of the drug bound to protein is generally believed to be without antibacterial activity (Goldstein, 1949). The percentage of the drug bound can therefore be estimated from the ratio of the activities in the absence and in the presence of serum. These values have been calculated and are shown in Table 6, together with values for the extent of protein binding as determined in the subsequent experiments using ultrafiltration. There is a fairly close correlation between the diminution of activity in serum and the proportion of the drug bound as determined by ultrafiltration.

DISCUSSION

Although some evidence is presented here which suggests that a small proportion of drug may be irreversibly bound in human serum, the phenomenon of binding appears to be essentially reversible. Of the factors which affect the equilibrium between protein and free drug on the one hand and protein-bound antibiotic on the other, the nature of the drug itself and the type of serum appear to be the most important. For example, with different antibiotics the amount of unbound drug in human serum may range from 82% with ampicillin to as low as 0.8% with novobiocin. Similarly, for a given antibiotic the extent of binding in the serum of one animal species may differ greatly from that in another. With cloxacillin, for example, the proportion of unbound drug in human serum was 6.5% compared with 30% in horse serum. Variation in binding capacity for sulphonamides between sera of different animal species has been reported also by Anton (1960). As with the penicillins reported here, binding of the sulphonamides was generally highest in human serum compared with that of other species. It is of some interest that sera from different species appear to have different relative binding capacities for different antibiotics. It is of particular importance, when a number of substances are evaluated experimentally in one animal species, that the assumption is not made that the relative extent of binding of the compounds will be the same in some other species.

Temperature has been reported as a factor influencing the extent of protein binding of antibiotics. Klotz, Urquhart & Weber (1950) reported that the extent of binding of penicillin decreased with increase in temperature and Scholtan & Schmid (1962, 1963) reported the binding of penicillin and tetracyclines to be lower at 37 than at 4° C. On the other hand, Wozniak (1960) reported binding of tetracycline to be slightly greater at 37 than at 2 to 5° C. In the case of sulphonamides, Davis (1943) reported binding to be little influenced by temperature. In the experiments reported here with two penicillins, binding was not significantly influenced by temperature in the range 4 to 37° C.

With regard to the effect of antibiotic concentration on the extent of binding, it is known that the proportion of unbound drug increases with increase in drug concentration (Davis, 1943 ; Goldstein, 1949). In the case of certain penicillins a significant difference in the extent of binding was reported by Scholtan & Schmid (1962) in a range of concentrations as low as 0.5 to 8.0 μg/ml. On the other hand, from the data given by Kunin (1961) for certain penicillins there appeared to be little effect of concentration in the range 5 to 20 μg/ml., and Verwey & Williams (1962), using dog plasma, reported that variation in penicillin concentration from 5 to 100 μg/ml. did not alter the percentage of the drug bound. In experiments reported here with benzylpenicillin and cloxacillin, the percentage unbound drug increased sharply at concentrations over approximately 200 μg/ml. but at the levels normally achieved in the body during therapy it appears that changes in concentration do not greatly influence the extent of binding. It appears that in serum containing penicillin at concentrations of 200 μg/ml. or greater a significant proportion of the binding sites on the serum proteins are occupied, and consequently with further increase in penicillin concentration the proportion of the drug which can be bound must fall. As is shown in Table 4 the same binding sites can also be occupied by other substances such as sodium salicylate or a sulphonamide and when this occurs the extent of binding of a penicillin, such as cloxacillin, is diminished. These substances, however, must be present at a concentration greater than 100 μg/ml. in order to occupy a significant proportion of the binding sites on the serum proteins and thereby diminish the extent of binding of a penicillin.

Competitive binding in serum between different substances has been described by Anton (1960) who reported displacement of protein-bound sulphonamide with a number of substances including phenylbutazone, and the displacement of plasma-bound bilirubin by sulphonamides is well established (Odell, 1959). More recently, Kunin (1964) has reported competitive binding between penicillins and other substances with results similar to those described here. Competition does not always occur between compounds which are extensively bound to protein ; in our experiments, novobiocin had no significant influence on the binding of cloxacillin, presumably because different binding sites were involved.

Bond, Lightbown, Barber & Waterworth (1963) have reported that the extent of binding of penicillin in human serum varied from subject to subject and that this difference was sufficiently marked to make the use of pooled serum unsuitable for the preparation of standards for the measurement of penicillin in the sera of individual subjects. The experiments reported here have failed to confirm this, and suggest that differences in binding capacity between one subject and another are slight and not sufficient to influence signifi-

cantly the assays of serum levels. For all practical purposes the use of normal pooled serum should give reliable results in the measurement of antibiotic activity.

The results in Table 5 showing the extent of binding of different antibiotics in human serum emphasize the great variation which exists between one compound and another. Among the analogues of phenoxymethylpenicillin the differences in binding between phenoxymethylpenicillin and phenethicillin appear to be slight, but with phenbenicillin the binding in serum is significantly greater. With the penicillinase-stable penicillins, binding with cloxacillin is much higher than with methicillin. However, cloxacillin is considerably more active than methicillin and, from a consideration of the total serum levels obtained with the recommended therapeutic doses of these compounds, the activity of the unbound fraction of cloxacillin in serum is at least equal to that obtained with methicillin. With oxacillin the extent of binding is similar to that of cloxacillin but the total serum levels obtained are significantly lower for equivalent dosage (Knudsen, Brown & Rolinson, 1962). Consequently, the levels of unbound oxacillin are also lower than those obtained with cloxacillin.

With the broad-spectrum antibiotics, differences in binding are particularly marked. For example, with ampicillin, 82% of the drug in human serum is free penicillin, whereas with tetracycline only 30% of antibiotic is unbound and with demethylchlortetracycline the corresponding value is as low as 11%. Total serum concentrations obtained with ampicillin are of the same order as those obtained with tetracyclines, and antibacterial activities *in vitro* are also comparable against many pathogens. In such instances the differences in extent of binding of these compounds to the proteins of human serum may be a significant factor in determining therapeutic effect *in vivo*.

Our results relating the activity of certain penicillins in nutrient broth with the activity of these substances in human serum show that there is a fairly close correlation between the diminution of antibacterial activity in serum and the extent to which the drug is bound as determined by ultrafiltration. These observations agree with results already reported by others (Tompsett, Schultz & McDermott, 1947; Colville & Quinn, 1961; Quinn, Colville, Ballard, Jones & Debnam, 1962; Kirby, Rosenfeld & Brodie, 1962) and support the view that the protein-bound drug is inactive (Davis, 1942, 1943; Goldstein, 1949; Goodman & Gilman, 1955; Lambert, 1964), and that the activity in serum is that of the unbound fraction. Direct determination of activity of the protein-bound antibiotic is not possible because dissociation of the antibiotic-protein complex to give an equilibrium between bound and free drug is unavoidable. From the indirect evidence, however, it seems that the activity of the bound drug is negligible. Since the protein-bound drug is essentially inactive and also relatively non-diffusible (Davis, 1943; Goldstein, 1949; Scholtan & Schmid, 1962) the levels of unbound drug obtained in the serum are likely to be more significant than are the levels of total antibiotic (Tompsett *et al.*, 1947; Kunin, 1961; Colville & Quinn, 1961; Bond *et al.*, 1963). Therefore, in the evaluation of an antibiotic, due consideration should be given to the extent of binding to serum proteins, as well as to data relating to *in vitro* and *in vivo* activity and to studies on absorption and excretion.

SUMMARY

1. The binding of penicillins and other antibiotics in serum was studied by ultrafiltration techniques.

2. The nature of the binding was shown to be essentially reversible. The extent of binding was largely independent of the concentration of antibiotic below 100 μg/ml., but above this concentration binding decreased with increase in drug concentration. Binding of penicillins was little influenced by temperature in the range 4 to 37° C.

3. Results were obtained on the relationship between the extent of binding and the concentration of serum. The extent of binding of benzylpenicillin and cloxacillin was also determined in the serum of different animal species. With both compounds, binding was greatest in human serum. No significant variation was found in the extent of serum binding between different human subjects.

4. Competitive binding was demonstrated between cloxacillin and other compounds bound to serum proteins including phenylbutazone, sodium salicylate and sulphonamides.

5. Diminution of antibacterial activity of penicillins in the presence of serum was directly proportional to the extent of binding as determined by ultrafiltration.

6. Results are presented for the extent of binding in normal human serum of various antibiotics including all the penicillins at present in clinical use.

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EFFECT OF PROTEIN BINDING ON LEVELS OF AMPICILLIN AND CLOXACILLIN IN SYNOVIAL FLUID

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Effect of protein binding on levels of ampicillin and cloxacillin in synovial fluid

The penetration of ampicillin and cloxacillin into synovial fluid has been measured after oral administration of these penicillins to patients with osteoarthritis or rheumatoid arthritis. The results show that both penicillins diffuse readily into synovial joint fluid and that concentrations inhibitory for bacteria commonly associated with septic arthritis can be readily attained in infected synovial joints following systemic administration of ampicillin or cloxacillin. The penicillins differed with respect to the levels appearing in the synovial fluid relative to the levels in serum; in the case of ampicillin (which is not highly bound to serum) the total levels in synovial fluid were similar to the total serum levels, whereas with cloxacillin (which is highly bound to serum) the total levels in synovial fluid were notably lower than in serum. However, the unbound levels of cloxacillin in synovial fluid and serum were similar, as with ampicillin, indicating that the degree of binding is important in distribution between serum and tissue.

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Pyogenic arthritis is still seen frequently enough to present a therapeutic problem. This occurs most frequently in rheumatoid arthritis[11] but may complicate other arthritic conditions. Patients with debilitating diseases such as diabetes[1] and chronic leukemia[5] are also susceptible to pyogenic arthritis.

The most common infecting organism is *Staphylococcus aureus*, and a high proportion of strains are penicillin resistant. Argen, Wilson, and Wood[1] found this organism to be responsible for 25 of 42 cases of suppurative arthritis, and 12 out of 17 staphylococcal infections treated medically were found to be penicillin resistant. Rimoin and Wennberg[18] reported that, of 32 cases of rheumatoid arthritis complicated by septic arthritis, 23 were due to *S. aureus*, and of the 18 cases in which antibiotic sensitivities were carried out 11 were found to be penicillin resistant. More recently, Myers, Miller, and Pinals[14] reported that, of 16 patients with rheumatoid arthritis complicated by pyarthrosis, 12 were infected with *S. aureus*. Numerous other organisms may also be associated with joint disease, including the gonococcus, meningococcus, α- and β-hemolytic streptococci, *Escherichia coli*, Proteus sp., and *Pseudomonas aeruginosa*. Klebsiella

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sp., Salmononella sp., and *Hemophilus influenzae* are also encountered, but less commonly, and in a certain number of cases an organism cannot be isolated from the synovial fluid although infection may be suspected. Here broad-spectrum antibiotics have proved useful.

Adequate antibiotic therapy must be given promptly in this disease or severe joint damage may ensue. There is a fairly high mortality rate if the condition is not treated effectively.[10] Systemic administration of antibiotics is usually preferred since intra-articular injection can cause complications including synovitis.[3, 8] It is essential therefore that the antibiotic used should pass freely into the joint cavity.

Studies on the penetration of penicillins into synovial fluid have been reported by a number of authors. Balboni, Shapiro, and Kydd[2] found that levels reached in the synovial fluid were only slightly lower than those present in the serum following intramuscular administration of benzylpenicillin. The levels in the synovial fluid tended to lag slightly behind those in the plasma and this would be consistent with the diffusion first from the plasma into the fluid, and then as the drug is cleared from the plasma, with back diffusion from the synovial fluid into the vascular system. Ready diffusion of benzylpenicillin into synovial fluid is also evident from the data of Hirsh, Feffer, and O'Neil,[8] Jocson,[9] Rapp, Griffiths, and Hebble,[17] and Drutz and associates.[6] Viek and Santangelo[24] have reported the penetration of penicillin V and phenethicillin into synovial fluid following oral dosage. The levels in the synovial fluid one hour after dosing were approximately 20 per cent those in the plasma. Penetration of nafcillin into synovial fluid has also been reported by Viek[23] who found that one hour after intramuscular administration the mean serum to mean synovial fluid level ratio was 5.7:1. Levels of methicillin in synovial fluid comparable with those reached in plasma have been reported by Rapp, Griffiths, and Hebble[17] and penetration of oxacillin into synovial fluid has been reported by Myers, Miller, and Pinals.[14] Recently, Nelson[15] and Parker and Schmid[16] have reported that various penicillins, including ampicillin, methicillin, and cloxacillin, entered synovial fluid in therapeutic concentrations following systemic administration.

It seems clear that benzylpenicillin and other penicillins do penetrate readily into synovial fluid. However, the levels in the synovial fluid relative to those in plasma vary in the different studies reported and they also vary for the different penicillins examined. Chemical structure, extent of protein binding, and degree of permeability of the synovial membrane may be expected to influence the relative serum–synovial fluid levels of antibiotics. Of these it seemed likely that the differences in the extent of binding to protein would have the greatest effect on the distribution of different penicillins between serum and synovial fluid.

In view of the variation in results in the literature for the penetration of penicillins into synovial fluid, it was considered of interest to examine in detail the distribution of ampicillin and cloxacillin between the plasma and synovial fluid in patients and especially to examine the effect that protein binding might have on the drug levels reached in the synovial fluid. The reason for comparing these 2 penicillins in this respect is that ampicillin is not highly bound in normal human serum (18 per cent bound) whereas cloxacillin binding to the serum proteins is as high as 94 per cent.[20]

Methods

Thirteen patients (6 women and 7 men, with an age range of 27 to 62 years) took part in these studies. All were suffering from either osteoarthritis (5) or rheumatoid arthritis (8), and all had chronic effusions of one or both knees. As far as possible all drugs were stopped 48 hours before the studies were carried out, but in some patients corticosteroid treatment was continued. The penicillins were given

as capsules of ampicillin trihydrate (Penbritin) and cloxacillin (Orbenin) by the oral route, and samples of blood and synovial fluid were taken over the subsequent 6 hour period. Antibiotic levels were measured by a standard agar-diffusion microbiologic assay.[12] In this method it is essential that standard solutions be prepared in the same fluid as that presented in the test samples, or, alternatively, a diluent must be used for which it has been shown experimentally that the zone diameter–concentration relationship is the same as that given by the fluid of the test samples. In the work described here, standards of ampicillin and cloxacillin were prepared in pooled human serum for the assay of serum samples. Samples of synovial fluid containing ampicillin were assayed against ampicillin standards prepared in 0.05M phosphate buffer pH 7.0, while synovial fluid samples containing cloxacillin were assayed against cloxacillin standards prepared in 50 per cent pooled human serum diluted with 0.05M phosphate buffer pH 7.0. In both cases it was established by prior experiment that these diluents were appropriate. In addition, a limited number of standard concentrations of ampicillin and cloxacillin were prepared in synovial fluid drawn from each patient prior to penicillin dosage and agreement between the zone diameters of these standards and those used to prepare the standard line was verified.

The extent of binding of ampicillin and cloxacillin in the serum and synovial fluid taken from patients before penicillin administration was measured by ultrafiltration of the specimens and microbiologic assay of the protein-free ultrafiltrate.[20] After penicillin was administered, the levels of free antibiotic in serum and synovial fluid were calculated from the known total concentrations and the extent of binding.

Results

Ampicillin. Results are shown in Fig. 1 for the levels of ampicillin in serum and in synovial fluid in 6 patients following a single oral dose of 500 mg. ampicillin taken in the fasting state. It will be seen that the levels in the synovial fluid followed those in the serum quite closely. The mean peak level in the synovial fluid for the 6 patients was 70 per cent that reached in the serum, while the levels in the synovial fluid only lagged slightly behind those in the serum suggesting ready diffusion of the drug. The levels shown in Fig. 1 are those of total ampicillin present, both bound and free. However, the extent of binding of ampicillin in human serum is known to be low,[20] and in the study reported here the extent of binding of ampicillin was measured in the serum from 4 patients and 86 to 87 per cent was found to be free. The extent of binding was also measured in the synovial fluid from 9 patients, and 94 to 100 per cent of the drug was found to be unbound. It can be assumed therefore that the levels in synovial fluid shown in Fig. 1 were essentially all in the form of free drug and that at least 80 per cent of the drug in the serum was also present as free antibiotic.

Cloxacillin. Results are shown in Fig. 2 for the levels of cloxacillin in serum and in synovial fluid in 5 fasting patients following a single oral dose of 500 mg. It will be seen that, in contrast with the results for ampicillin, the levels of cloxacillin in synovial fluid were substantially lower than those reached in the serum. The mean peak level in the synovial fluid was 22 per cent that reached in the serum compared with 70 per cent in the case of ampicillin. It will be seen however that the levels of cloxacillin in synovial fluid were not markedly more prolonged than those in the serum and the peak values in the synovial fluid occurred only slightly later than those in the serum. This suggested that the lower levels of cloxacillin in the synovial fluid were not the result of a slower rate of diffusion of this penicillin between the serum and the synovial fluid but were due to some other cause.

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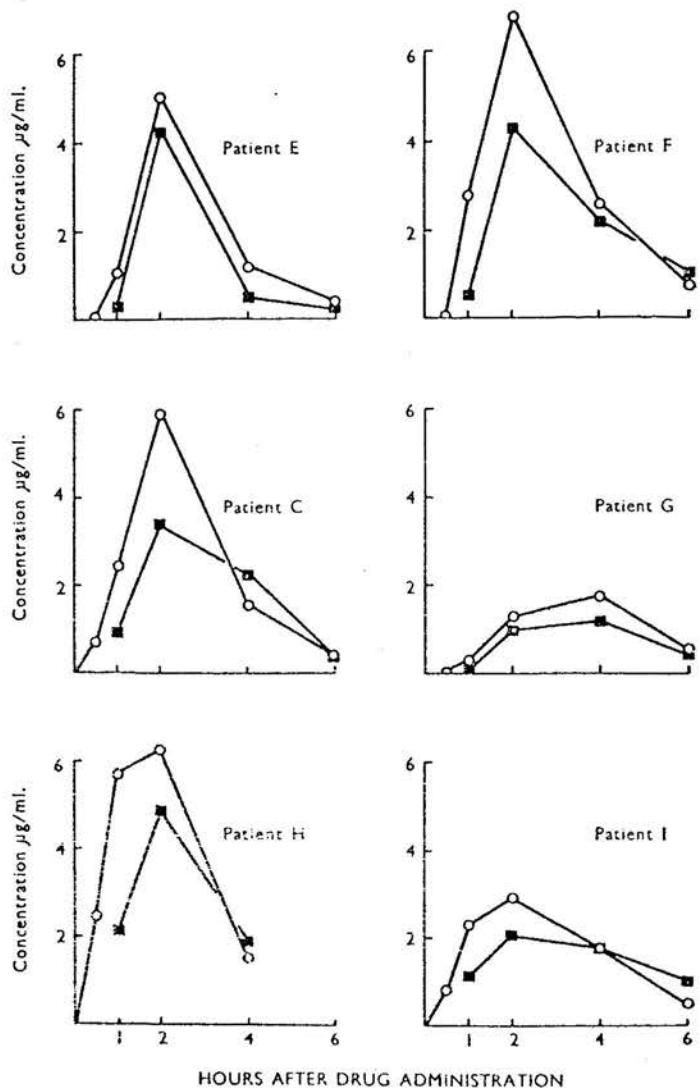

Fig. 1. Serum (○——○) and synovial fluid (■——■) levels following a single oral dose of 500 mg. ampicillin.

Cloxacillin is known to be highly bound to serum protein, and a second study was carried out to examine the relationship between the extent of protein binding and the relative levels of cloxacillin in serum and synovial fluid. The extent of binding of cloxacillin was measured in serum and synovial fluid obtained from each of 6 fasting patients prior to dosage with 500 mg. of cloxacillin, and the serum and synovial fluid cloxacillin levels were assayed over the 4 hour period after dosage. The mean values for the 6 patients are shown in Fig. 3. It will be seen that the total level of cloxacillin in the synovial fluid was again substantially lower than that reached in the serum, the mean peak level in the synovial fluid being 21 per cent of the peak serum level. On the other hand, the levels of free, unbound cloxacillin in serum and synovial fluid were much closer, and from 2 hours onward the free levels in the synovial fluid even exceeded those in the serum.

Ampicillin and cloxacillin. In order to take into account variation from patient

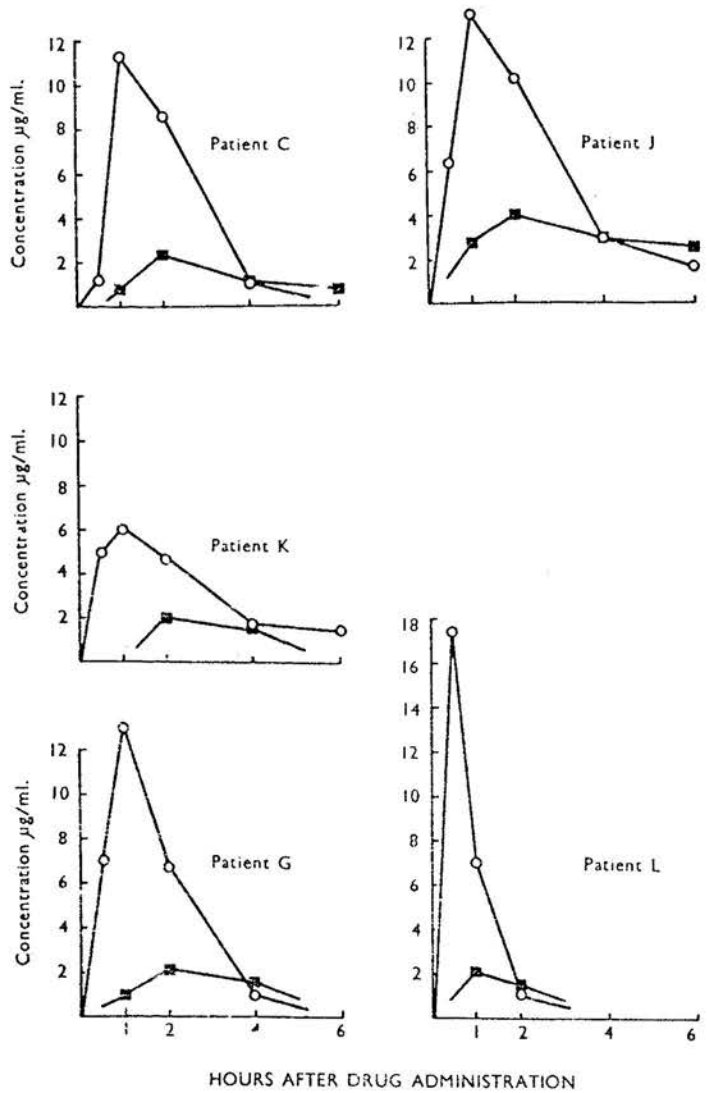

Fig. 2. Serum (○——○) and synovial fluid (■——■) levels following a single oral dose of 500 mg. cloxacillin.

to patient and to make a more direct comparison between ampicillin and cloxacillin with regard to penetration into the synovial fluid, both drugs were administered at the same time to 4 patients and the resulting levels of ampicillin and cloxacillin were measured.[21] A single oral dose of 1 Gm. of cloxacillin together with 500 mg. of ampicillin was used in this study. Because cloxacillin is absorbed more rapidly than ampicillin, breakfast was given before dosage in order to delay the absorption of cloxacillin and thus bring the time of peak serum levels for the 2 penicillins closer together. The extent of binding of cloxacillin in serum and synovial fluid was again measured in specimens taken from each patient before penicillin dosage. The results are shown in Table I and Fig. 4. It will be seen that the total levels of cloxacillin in the synovial fluid were again considerably lower than those in the serum but that the levels of free cloxacillin in serum and synovial fluid were very similar indeed. In 3 of the patients the free cloxacillin level in the synovial

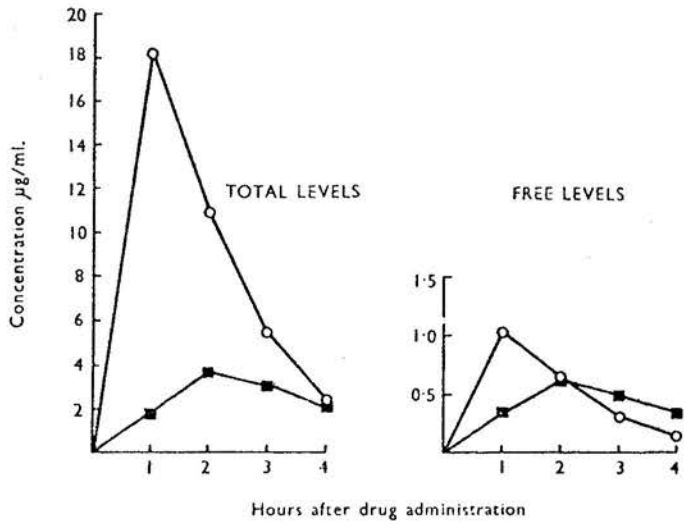

Fig. 3. Mean levels of total and free cloxacillin in serum (○——○) and synovial fluid (■——■) in 6 patients following a single oral dose of 500 mg.

Table I. *Ampicillin and cloxacillin serum and synovial fluid levels in patients at the Middlesex Hospital, London*

Patient	Time (hr.)	Cloxacillin (µg/ml.)						Ampicillin (µg/ml.)	
		Serum			Synovial fluid				
		Total	% (Free)	Free (calculated)	Total	% (Free)	Free (calculated)	Serum (total)	Synovial fluid (total)
A	1	7.4		0.44	1.0		0.23	0.71	0.16
	2	17.0	(6.0)	1.02	2.8	(23)	0.64	1.56	0.68
	3	17.0		1.02	4.0		0.92	2.47	1.45
	4	17.0		1.02	4.5		1.04	2.95	1.58
B	1	24.5		1.62	1.9		0.53	2.05	<0.05
	2	17.6	(6.6)	1.17	2.9	(28)	0.81	2.8	0.8
	3	12.5		0.83	4.1		1.15	3.25	1.95
	4	6.4		0.42	4.8		1.34	3.35	2.38
C	1	6.5		0.39	<1.0		—	1.16	0.2
	2	11.5	(6.0)	0.69	1.0	(21)	0.21	1.38	0.48
	3	9.0		0.54	2.7		0.57	1.02	0.73
	4	7.45		0.45	2.8		0.59	1.02	0.80
D	1	17.0		1.02	1.0		0.21	2.2	0.66
	2	18.3	(6.0)	1.10	6.6	(21)	1.39	3.45	2.0
	3	8.65		0.52	4.0		0.84	2.8	1.7
	4	4.55		0.27	3.0		0.63	1.83	1.47

Oral dosage was 500 mg. of ampicillin and 1 Gm. of cloxacillin for nonfasting patients.

fluid followed that in the serum very closely, and the peak levels were also very similar. In the fourth patient absorption of cloxacillin was considerably more rapid and the serum and synovial fluid levels were correspondingly more out of phase. Nevertheless, in all 4 patients the levels of free cloxacillin in the synovial fluid would clearly seem to be related to the levels of free cloxacillin in the serum, whereas the levels of total drug in synovial fluid and serum were markedly dif-

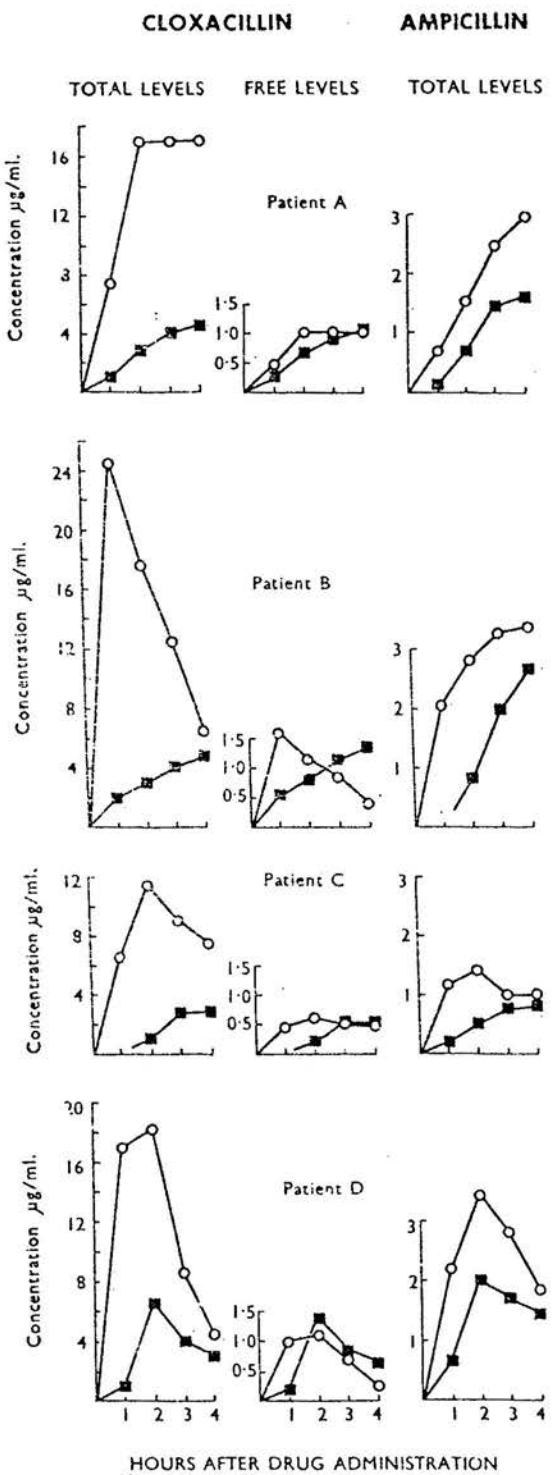

Fig. 4. Levels of total ampicillin, total cloxacillin, and free cloxacillin in serum (O——O) and synovial fluid (■——■) of patients following a single oral dose of one gram of cloxacillin together with 500 mg. ampicillin.

ferent. In contrast, with ampicillin, which is not highly bound to serum protein, the levels of total ampicillin in the synovial fluid followed those in the serum relatively closely and were generally similar to the results obtained in the studies in which ampicillin alone was administered (Fig. 1).

Discussion

The results reported here show that ampicillin and cloxacillin diffuse readily into synovial joint fluid following oral administration to patients with joint disease. There is, however, a marked difference between the 2 penicillins with respect to the levels of antibiotic appearing in the synovial fluid relative to the levels in serum, and this may be explained by the differences in the extent of binding to serum protein of the 2 penicillins. Thus, with ampicillin, which is not highly bound to serum protein, the total levels of bound and unbound drug in synovial fluid are similar to those appearing in the serum. In contrast, with cloxacillin which shows a high degree of binding to serum protein, the total levels in synovial fluid are substantially lower than those in serum. However, the levels of unbound cloxacillin in synovial fluid are very similar to the levels of free drug in serum and appear to be in equilibrium with each other.

Cloxacillin, like other penicillins, is bound reversibly to protein in serum and other tissue fluids with the result that an equilibrium exists between protein and free drug on the one hand and protein-bound drug on the other. While antibiotic molecules are bound to proteins they are relatively nondiffusible. The primary effect of protein binding therefore is to confine drug molecules within the vascular system so that the concentration gradient for diffusion of drugs from the vascular system into extravascular fluids is determined by the level of unbound drug present in the serum and not by the level of total drug. When drug molecules diffuse into an extravascular fluid, for example into synovial

fluid, binding may also occur with protein present in that fluid and an equilibrium will again occur between bound and free drug. The effect of protein binding on antibiotic distribution between serum and an extravascular fluid such as synovial fluid is shown diagrammatically in Fig. 5.

In the process of diffusion of cloxacillin from serum into synovial fluid and in the reverse direction, it would be expected that the levels of unbound drug would tend to reach equilibrium while bound drug in both compartments would represent depots of nondiffusible drug. This is borne out by the results of this study which show that despite large differences in the levels of total cloxacillin in serum and in synovial fluid, the levels of unbound drug in these fluids were very similar. In contrast, in the case of ampicillin, the levels of *total* drug in the synovial fluid and serum were quite similar and this is consistent with the low level of protein binding shown by ampicillin. In the synovial fluid and also in the serum, ampicillin was present almost entirely as free drug, and depots of nondiffusible protein-bound drug in this case were negligible.

In addition to being relatively nondiffusible, protein-bound drug is devoid of antibacterial activity. Levels of free drug are therefore more meaningful than total levels when considering antibiotic concentrations present in tissue fluid in relation to the sensitivity of particular pathogens. Following a single oral dose of 500 mg. of cloxacillin the mean peak level of free drug in the synovial fluid was 0.63 μg per milliliter and a level of 0.36 μg per milliliter was still present 4 hours after dosage. These levels, which are similar to the levels of unbound cloxacillin in serum, may well be adequate for the treatment of staphylococcal infections, including penicillin-resistant strains which are usually sensitive to 0.25 to 0.5 μg per milliliter of cloxacillin. With ampicillin the levels present in synovial fluid are virtually all as free drug. Following a single 500 mg. oral dose, the peak levels were generally between 3 and 5 μg per milliliter of ampicillin. Such levels are greatly in excess of the sensitivity values for penicillin-sensitive staphylococci, α- and β-hemolytic streptococci, gonococci, and meningococci and may be expected to be adequate also in the case of many strains of *H. influenzae*, *E. coli* and Proteus. Moreover, it would be expected that higher levels of ampicillin or cloxacillin could readily be achieved in the synovial fluid following higher dosage or parenteral administration.

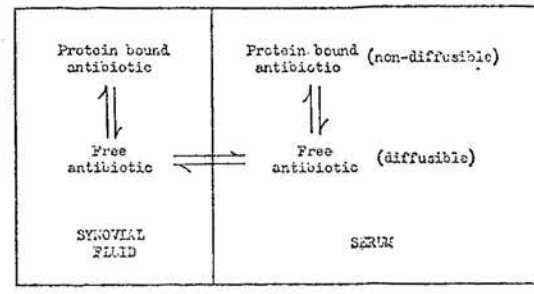

Fig. 5. Effect of protein binding on antibiotic distribution between serum and synovial fluid.

The studies reported here confirm data in the literature showing that penicillins penetrate readily into synovial fluid following systemic administration[2, 14, 17, 24] and agree with the conclusions of several authors that there is no need for local or intra-articular administration.[3, 15, 16] Moreover, the results obtained with the highly protein-bound penicillin, cloxacillin, offer an explanation for some of the variation in the data reported in the literature for different penicillins and are consistent with the view that it is the level of unbound antibiotic in serum which determines the tissue concentrations of antibiotics.[4, 7, 13, 19, 22]

We wish to thank Mrs. Eleanor Fairclough for skilled technical assistance and Drs. A. C. Boyle, S. Mattingly, and Mary Corbett for permission to study patients under their care.

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INTRAPERITONEAL CHALLENGE

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Experimental intraperitoneal mouse infections are used universally in the assessment of the therapeutic potential of novel antibacterial agents and in the majority of cases the virulence of the infecting organism is enhanced by the concomitant administration of hog gastric mucin to the animal. Much effort has been devoted to studies of the preparation, biological mode of action, and active constituents of hog gastric mucin and there is a considerable body of knowledge concerning these aspects, as can be seen from the excellent reviews of Olitski (1948, 1957). Surprisingly, there appears to have been much less study of the effects of therapy on the development of the intraperitoneal infection in the animal host, despite the extensive use of these routine tests in the evaluation of compounds.

The influence of hog gastric mucin on the development of infection in mice after intraperitoneal injection of a strain of Escherichia coli is illustrated in Fig. 1, where it can be seen that when an inoculum of 10^5 cells of an overnight culture of the organism was administered in saline, the bacterial count in the peritoneal cavity fell rapidly to a count of about 10^3 cells/ml and remained at about this level for the next 7 hours. There was a rapid appearance of bacteria in the blood following intraperitoneal injection, reaching a count of about 50 bacteria/ml., but thereafter the count barely increased over the next 7 hours. In contrast, when the inoculum was injected as a suspension in 3% hog gastric mucin, the bacterial count in the peritoneal cavity increased rapidly to in excess of 10^9 cells/ml of washings within 8 hours. Similarly, the blood bacterial count rose rapidly to a count of 7×10^8 cells/ml at 8 hours. In the presence of mucin, therefore, the culture was able to proliferate

rapidly in the peritoneal cavity after intraperitoneal injection, resulting in the rapid appearance of bacteria in blood and the production of progressive bacteraemia.

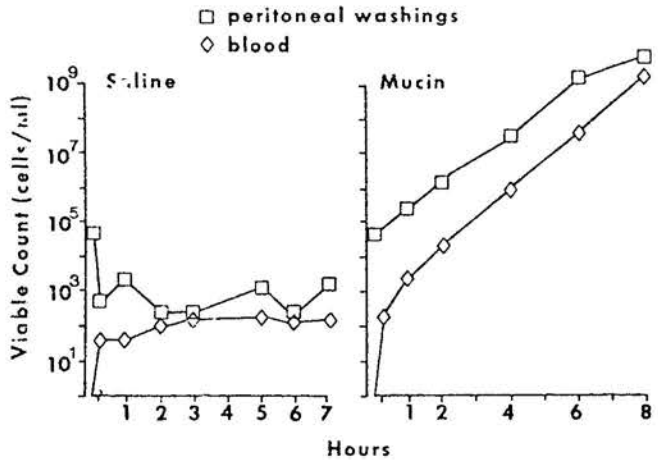

FIG. 1. Effect of mucin on the proliferation of E. coli (8) in mice after intraperitoneal injection

The development of bacterial growth in the animal after intraperitoneal injection of mucin suspensions of different bacteria varies according to the organism. For example, the multiplication of the Smith strain of Staphylococcus aureus in the peritoneal cavity and the blood of mice was very similar indeed to that of Escherichia coli (strain 8), but with Pseudomonas aeruginosa (strain 11), a rather different pattern was observed (Fig.2). Thus, bacterial growth was very much slower, so that 4 hours after injection the peritoneal bacterial count had barely increased by one order of magnitude and by 8 hours the count was less than 10^8 cells/ml, in contrast to the counts of $>10^9$ cells/ml observed with E. coli and Staph. aureus. Likewise, in blood the bacterial numbers increased at a slow rate, and after 8 hours the pseudomonas count had reached a level of only 10^4 cells/ml. With Salmonella typhimurium (strain 10) growth of the organism was relatively slow in the peritoneal cavity, (Fig. 2) reaching a count of only 10^7 cells/ml by 8 hours, but the blood bacterial count rose rapidly to the levels observed in the peritoneum. It is apparent, therefore, that there are distinct differences in the growth of bacteria in the animal after intraperitoneal injection of bacterial suspensions in mucin and it would be expected that these differences would result in varying responses to therapy.

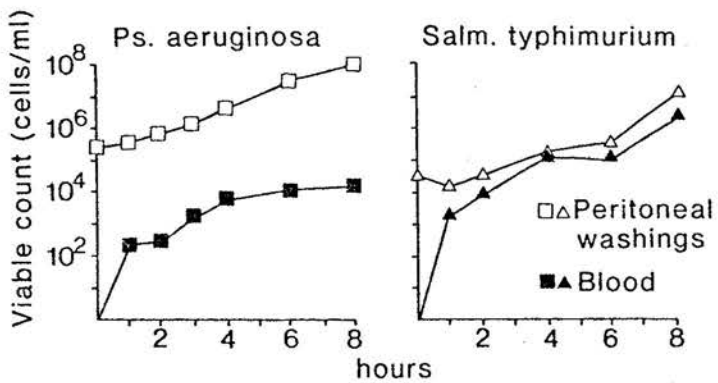

FIG. 2. Bacterial counts in mice after intraperitoneal injection of mucin suspensions of Ps. aeruginosa (11) and Salm. typhimurium (10).

As might be expected, the virulence of an organism may be influenced significantly by the concentration of mucin in which the inoculum is administered. Thus, with Escherichia coli (strain 8) an inoculum of 10^5 cells in 1% mucin was considerably less virulent than the same inoculum in 3% mucin - producing only 40% mortality compared with 100% deaths caused by the latter. With the same inoculum in 5% mucin, no increase in mortality could be expected beyond the 100% deaths obtained with 3% mucin, but response to penicillin therapy was much less pronounced showing that the virulence was increasing in proportion to the quantity of mucin injected into the peritoneal cavity. This is probably due to the prolongation of the length of time the mucin remains in the cavity and consequent persistence of its virulence-enhancing properties in the host. For example, with an inoculum of E. coli (8) in 3% mucin the infection-promoting effects of the mucin disappear within 4 hours after injection. This was demonstrated by administering sterile mucin intraperitoneally to non-infected mice, and subsequently injecting, at intervals, broth suspensions of E. coli 8 by the same route (Table 1). When administration of the bacteria was delayed for 4 hours after injection of the mucin there was a significant reduction in mortality compared with results obtained between 0 and 2 hours, and by 7 hours, the virulence-promoting effects of the mucin were minimal. The rate of disappearance of mucin from the peritoneal cavity has therefore, a marked influence on the virulence of the infection and its response to therapy.

TABLE 1. Effect of intraperitoneal injection of E. coli 8 in mice pre-dosed intraperitoneally with sterile mucin

Time of infection after dosing with sterile mucin (hours)	0	1	2	4	6	7
% survivors	0	10	10	70	70	90

The effects of a single subcutaneous dose of amoxycillin on the multiplication of bacteria in the peritoneal cavity after intraperitoneal injection of E. coli (8) in 3% mucin are shown in Fig. 3. In untreated mice the peritoneal counts rose rapidly to greater than 10^9 cells/ml by 6 hours. Effective doses of amoxycillin (200, 50 and 12.5mg/kg) produced rapid bactericidal effects and caused 99% reduction in viable counts within 1 to 2 hours after infection. Between 2 and 6 hours, following elimination of the antibiotic from the animal, there was a gradual increase in the bacterial count and 6 to 12 hours after infection the count rose sharply to levels of between 5×10^6 and 10^8 cells/ml. Between 12 and 16 hours the count remained stationary and persisted at a level of about 10^6 cells/ml up to 40 hours after injection after which the count decreased to 10^2 cells/ml at 72 hours.

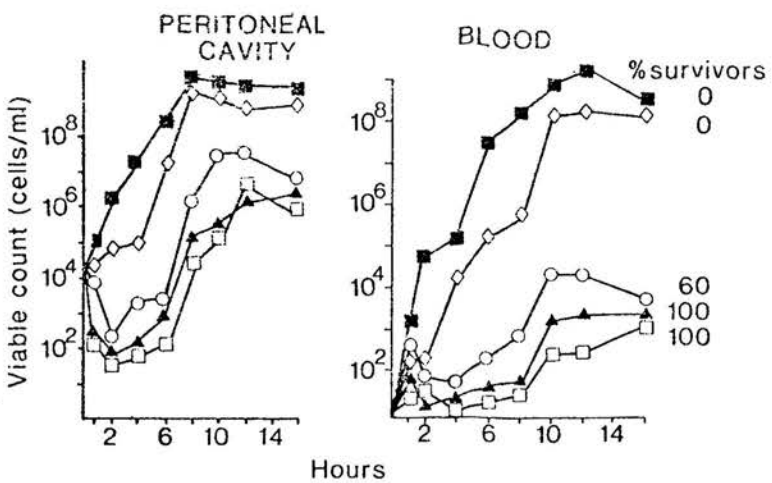

FIG. 3. Effects of a single subcutaneous dose of amoxycillin on the bacterial counts in the peritoneal cavity and blood of mice infected intraperitoneally with E. coli (8)
■, control; □, 200mg/kg; ▲, 50mg/kg; ○, 12.5mg/kg; ◇, 3.1mg/kg

The cessation of growth at 12 hours following the phase of rapid increase in count between 6 and 12 hours was unexpected in view of the high numbers of bacteria reached in the peritoneal cavity during this period and one possibility considered for the apparent lack of virulence of these organisms was that bacteria surviving exposure to the penicillins were in some way deficient. In order to investigate this possibility, healthy mice were infected with E. coli (strain 8) (Table 2) and dosed immediately after infection with a single subcutaneous 200mg/kg or 100mg/kg dose of amoxycillin, doses normally resulting in 90-100% protection of the infected animals. A dose (0.5ml) of sterile mucin was injected intraperitoneally into these mice 6, 8 or 10 hours after infection, and it can be seen that administration of the mucin between 6 and 10 hours to the amoxycillin-treated animals resulted in a significant reduction in survivors, suggesting that the bacteria exposed to amoxycillin in the peritoneal cavity had not been damaged and were capable of showing normal virulence. It would appear then that the decline in the peritoneal growth of treated animals from 12 hours onwards was a direct result of cellular response within the animal.

TABLE 2. Effect of administering a second dose of sterile mucin to mice infected with E. coli 8 and treated with subcutaneous amoxycillin

Time of addition of 2nd dose of mucin (hr. after infection)	% survivors	
	100 mg/kg	200 mg/kg
0*	90-95	100
6	15	35
8	30	10
10	10	10

* No 2nd dose

Examination of bacterial growth in the blood of the infected mice (Fig. 3) showed a similar pattern of bactericidal activity and a similar dose response, reflecting events in the peritoneal cavity. Thus, after injection of amoxycillin, the blood counts in animals treated at the higher dose levels remained between 10^2 cells/ml from 1 to 4 hours after infection, followed by gradual growth between 4 and 8 hours and a phase of more rapid growth from 8 hours to 10 hours. From 10 hours onwards the rate of growth declined and counts remained stationary at about

10^3 cells/ml for up to 24 hours, after which there was a gradual fall until no bacteria were detected in the blood 72 hours after infection.

These findings show that after intraperitoneal injection of bacteria the blood bacterial counts are related to the rate of proliferation in the peritoneal cavity and demonstrate that the inhibitory effects of penicillins on blood bacterial counts depend not only upon the antibiotic concentrations present in blood, but also upon the bactericidal effects of the compounds in reducing the numbers of bacteria in the peritoneal cavity in the early stages of infection. In turn, reduction of the blood bacterial count resulted in greater ability of the immune mechanism of the infected animal to eliminate the high bacterial counts appearing in the peritoneal cavity after excretion of the antibiotic from the animal.

In conclusion, the results reported illustrate some of the factors that influence the development of experimental intraperitoneal mouse infections and demonstrate the value of chemotherapeutic tests in the evaluation of antibacterial agents by measurement of therapeutic effects that could not be predicted from knowledge of *in vitro* antibiotic sensitivity of the organism or of antibiotic blood concentrations alone. The intraperitoneal mouse infection is often criticised as being a highly artificial model with little relevance to the clinical situation but proper understanding of the model and of the effects of treatment of the infection may contribute significantly to the assessment of the therapeutic potential of a novel agent.

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Semisynthetic Penicillins

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I. Introduction

Among the naturally occurring antibiotics it is not unusual to encounter families of substances in which the members possess a common basic structure but differ with respect to some substituent group or groups. The penicillins are an example of such a family, and in this case the common structure is a fused thiazolidine-β-lactam ring system (Fig. 1). Attached to this nucleus at the 6-amino position is an acyl radical, which has become known as the penicillin side chain, and it is the structure of this side-chain group which distinguishes one member of the penicillin family from another.

The penicillins shown in Table I are those that were encountered in the early penicillin fermentations and which, prior to the determination of their structures, were identified by numerals in the United Kingdom and by the letters X, G, F, and K in the United States. Examination of these individual penicillins showed that they possessed considerable similarity in biological properties, notably in the spectrum of antibacterial activity, but significant differences were also apparent, for example, in the potency against particular bacteria and in the extent of binding to serum proteins. Consideration of the differences in antibiotic and pharmacokinetic properties led to the selection of penicillin G (benzylpenicillin) as the preferred compound for clinical use, and commercial production was greatly facilitated by the discovery that this penicillin could be produced almost exclusively by incorporating phenylacetic acid in the fermentation medium thus providing a source of this particular side-chain structure.

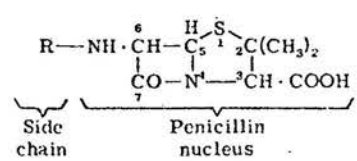
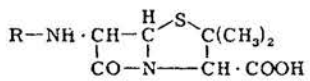
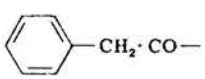
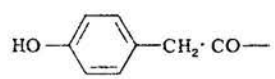


Fig. 1. Structure of penicillin.

TABLE I

NATURALLY OCCURRING PENICILLINS



Penicillin	Structure of side chain R
Penicillin G (benzylpenicillin)	
Penicillin X (*p*-hydroxybenzylpenicillin)	
Penicillin F (pentenylpenicillin)	$CH_3 \cdot CH_2 \cdot CH{:}CH \cdot CH_2 \cdot CO-$
Penicillin dihydro F (pentylpenicillin)	$CH_3(CH_2)_4 \cdot CO-$
Penicillin K (heptylpenicillin)	$CH_3(CH_2)_6 \cdot CO-$

The use of phenylacetic acid in this way as a precursor for the production of benzylpenicillin was followed by an extensive investigation of a large number of monosubstituted acetic acid derivatives as possible precursors. Many of these were found to be acceptable to the mold and resulted in the biosynthesis of penicillins not otherwise available as natural products. The object of this work was to exploit the knowledge that the nature of the side chain could influence the antibacterial and pharmacological properties of the penicillin, and it was hoped that in this way novel penicillins might be obtained with advantageous therapeutic properties. One of the penicillins prepared during a program of work using precursors (Behrens *et al.*, 1948) was α-phenoxymethylpenicillin (penicillin V) (Fig. 2) which shows the important property of acid stability (Brandl and Margreiter, 1954). Apart from phenoxymethylpenicillin, however, none of the other penicillins obtained by this precursor approach appeared to show any significant advantage over benzylpenicillin, and, indeed, the diversity of side-chain structure which can be obtained in this way is severely limited by the fact that many carboxylic acids and their derivatives are not acceptable to the mold as precursors.

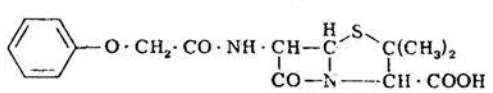


FIG. 2. Structure of phenoxymethylpenicillin (penicillin V).

Another approach to the preparation of penicillins with novel side-chain structures has been the chemical modification of a penicillin that can itself be obtained by fermentation. Semisynthetic penicillins of this type were first prepared from *p*-hydroxybenzylpenicillin (penicillin X) (Table I), the *p*-hydroxy group in the benzene ring facilitating substitution by halogens and various azo radicals (Coghill *et al.*, 1949). Some of the azo penicillins prepared in this way were more active *in vitro* than benzylpenicillin, but the antibacterial spectrum was not improved and none was considered likely to be superior to benzylpenicillin in clinical practice.

Semisynthetic penicillins have also been prepared by chemical modification of the *p*-amino group of *p*-aminobenzylpenicillin (Doyle and Nayler, 1957; Tosoni *et al.*, 1958), although none of these compounds appeared to be promising from the chemotherapeutic point of view. However, during the course of work described by Ballio *et al.* (1959) on the preparation of *p*-aminobenzylpenicillin, certain control fermentations were carried out in which no precursor had been added and in these fermentations a marked discrepancy was observed between the results of the biological and the chemical methods used to assay the penicillin formed. These observations led to the recognition that in the fermentations carried out in the absence of side-chain precursors, the penicillin nucleus, 6-aminopenicillanic acid (Fig. 3) was itself produced as a natural product (Batchelor *et al.*, 1959).

The isolation of 6-aminopenicillanic acid provided the starting point for the preparation of new penicillins by the addition of novel side-chain structures by chemical means at the 6-amino group. Several objectives presented themselves at this time, one of which was the possibility that new penicillins might show a broader spectrum of antibacterial activity than that shown by penicillins G and V, particularly with respect to the gram-negative bacilli. Another possibility was that by the choice of suitable side-chain structures, penicillins that are stable to staphylococcal penicillinase and active against the penicillin-resistant staphylococci might be obtained. These and other objectives have been realized, and it is the penicillins prepared by chemical reaction with 6-aminopenicillanic acid which are now referred to as the new penicillins or semisynthetic penicillins.

Semisynthetic penicillins now comprise an important group of antibacterial agents and no fewer than nineteen of these compounds have found a place in clinical practice. In addition there is a very much larger number

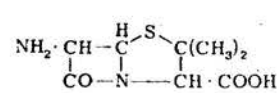


FIG. 3. Structure of 6-aminopenicillanic acid.

which have been evaluated in some detail, and, in view of the considerable mass of published data now relating to semisynthetic penicillins, it may be opportune to review this group of compounds. Reviews dealing primarily with structure–activity relationships of the semisynthetic penicillins are already available (Doyle and Nayler, 1964; Price, 1969; Nayler, 1973). The present review, however, will consider only those penicillins for which substantial evaluation data have been published. There are at least thirty different penicillins that fall within this category, and the purpose of this article is to marshal these compounds into related groups and to present a comparative assessment of the biological properties which have been determined from laboratory and human pharmacology studies. In making this assessment the views presented are very much those of the authors, and no attempt is made to present a complete review of the published work.

II. Laboratory Evaluation

Before reviewing the biological properties of the semisynthetic penicillins, it may be appropriate to consider what criteria are important in the evaluation of these compounds.

The ultimate test of the value of an antibiotic is the clinical result obtained when the drug is used to treat infection. However, evaluation of new drugs by clinical trial alone is clearly impracticable, and laboratory work *in vitro*, in experimental animals, and in human volunteers forms the basis from which compounds are selected for clinical trial. It follows, therefore, that the parameters studied in the laboratory evaluation phase should be as complete and as relevant as possible and that the results should be interpreted with maximum accuracy in relation to the conditions that will apply when the drug is used clinically.

In addition to the role of laboratory studies in the selection of compounds for clinical trial, these data are also of value at a later stage when the drug is in clinical use because differences between closely related compounds may be difficult or time-consuming to prove by clinical trial and the choice of one compound over another in clinical use may depend largely on the data which have been obtained in the laboratory.

With regard to semisynthetic penicillins, and to antibiotics generally, the parameters important in laboratory evaluation include, first, the antibacterial activity of the compound *in vitro* and *in vivo*, and a knowledge of the factors that affect activity, and, second, the absorption, distribution, and metabolism of the drug in animals and in human volunteers. The toxicology of the compound is also of great importance but is outside the scope of this review.

A. Antibacterial Activity *in Vitro*

Antibacterial activity is usually assessed in the first instance by determination of the minimum concentration of drug required to prevent growth of the test organism in liquid or on solid medium after overnight incubation. The minimum inhibitory concentration (MIC) determined in this way is the basic measure of antibacterial activity *in vitro*, and the results of such tests are widely used to compare one antibiotic with another. However, the MIC may be greatly influenced by test conditions, and for meaningful evaluation it is essential that these factors should be taken into account; for example, the antibacterial activity of certain antibiotics is affected by pH or by composition of the medium, but these factors do not appear to be of great importance as far as most penicillins are concerned. The activity of some penicillins may, however, be greatly influenced by the presence of blood or serum as a result of protein binding, and the significance of this is discussed separately. Similarly, the size of inoculum used in the test may have a marked effect on the MIC, and an important aspect of this is the production of β-lactamase (penicillinase) by the test organism which may result in drug inactivation. Thus, a penicillinase-labile penicillin, such as benzylpenicillin, shows a high level of activity against a small inoculum of a penicillinase-producing staphylococcus but is inactive when the inoculum is large. A similar effect can be seen with certain gram-negative bacilli and penicillins such as ampicillin and carbenicillin. In addition to organisms that show this inoculum effect, there are also many bacteria in which β-lactamase production is accompanied by intrinsic resistance and in such cases an inoculum effect may not be very pronounced.

Where inoculum size influences activity as a result of β-lactamase formation the question arises as to how this should be interpreted in relation to the clinical situation. Bacterial numbers and β-lactamase production at the site of infection may vary considerably, and it might be asked whether a partial degree of stability would be adequate. Clinical experience, however, indicates that a high degree of stability to β-lactamase is, in fact, required for reliable therapeutic effect. This is evidenced by the findings that benzylpenicillin, even in high dosage, is ineffective in the treatment of serious infections of penicillinase-producing staphylococci and that ampicillin is not effective in the treatment of *Klebsiella* infections even though many strains are quite sensitive when the inoculum tested is small. In the evaluation of penicillins it would seem wise, therefore, to employ a large inoculum in sensitivity tests and to give preference to those compounds for which the degree of stability to β-lactamase is high.

With penicillins an increase in the MIC with increase in inoculum size is

commonly regarded as indicating drug destruction, but this is not necessarily the case. A small number of resistant cells may be present in the bacterial population, and these may grow to visible proportions when a large inoculum is used but may not be seen when the inoculum is small. Methicillin-resistant staphylococci provide a good example of these effects.

In most cases, drug destruction or the presence of resistant mutants accounts for the effect of inoculum size on activity, but there are also instances where the reason for the effect of inoculum size is not entirely clear. For example, in the case of carbenicillin and *Pseudomonas aeruginosa* in tests using a heavy inoculum, growth of the test organism may be very greatly diminished at concentrations as low as 25–50 μg/ml, but some meager growth may persist even with concentrations of carbenicillin as high as 250 μg/ml; in contrast, using a smaller inoculum, a sharp end point is obtained with complete inhibition of growth at a level of 25 to 50 μg/ml (Knudsen *et al.*, 1967). In this instance drug destruction is not the reason for the effect, and, although it has been shown that resistant members of the population can be isolated (Bell and Smith, 1969), this does not seem to provide the full explanation for this phenomenon of "tailing."

The relationship between the MIC as determined *in vitro* and activity of the antibiotic at the site of infection is also not entirely clear. It is generally assumed that the concentration required for inhibition of bacterial growth at the site of infection is the same as that required *in vitro*, but this may not be the case. It is possible that bacteria that have grown *in vivo* and which have been subjected to body defense mechanisms may show a sensitivity different from that of bacteria grown *in vitro*. The relevance of the *in vitro* MIC may also be complicated by the fact that *in vivo* the level of drug in the body may show marked fluctuations following each dose, and this is particularly true for penicillins that are usually excreted rapidly. As a result, an inhibitory concentration may only be present *in vivo* for a relatively brief period of time, whereas *in vitro* the drug is present at a given concentration for the entire period of the test, usually 18–24 hours. In most cases the MIC's determined after 18 to 24 hours do reflect closely the situation which obtains during the first few hours of the test. Thus, concentrations of drug that fail to inhibit growth overnight also fail to cause inhibition during the early stages of the test and, similarly, concentrations that prove to be inhibitory after 24 hours also show an inhibitory effect during the first few hours. This situation is generally true for penicillins and gram-positive bacteria. With gram-negative bacilli, on the other hand, the results obtained after overnight incubation do not always reflect the degree of activity shown by the drug during the first few hours of the test. There are instances where a period of marked bactericidal action may occur initially

which is followed by a resumption of bacterial growth. After overnight incubation this growth may reach visible proportions and, as a result, penicillin concentrations which appear to be subinhibitory after 24 hours may, nevertheless, have exerted a marked bactericidal effect during the first few hours of the test. This effect is very evident with carbenicillin and *P. aeruginosa* and also with ampicillin and *Escherichia coli*. In such cases the effects observed after a few hours might be more relevant to the clinical situation than the results obtained after incubation overnight.

Despite the limitations discussed above concerning the determination of the MIC *in vitro* and the factors involved in interpretation of the results, this measurement of antibacterial activity is a valid means of comparing one compound with another. There is no doubt that antibacterial activity as indicated by the MIC is one of the factors that determine the therapeutic effect of the drug when used clinically.

B. Protein Binding

Most antibiotics are bound to some extent to proteins present in plasma and tissues but a good deal of confusion still exists over the significance of protein binding despite clarification by a number of authors (Davis, 1943; Goldstein, 1949; Scholtan and Schmid, 1962; Kunin, 1966; Verwey *et al.*, 1966; Rolinson, 1967). The binding of penicillins in serum is readily reversible, and the extent of this binding depends on the structure of the penicillin in question and the species of animal from which the serum has been obtained. Marked differences occur in the extent of binding in the sera of different animals and man, and for this reason it is important to use human serum rather than serum from some other species for laboratory work on penicillin evaluation.

Protein binding has two important consequences from the chemotherapeutic point of view. First, the protein-bound drug is essentially without antibacterial activity and, as a result, the activity displayed *in vitro* in the presence of serum is that of the unbound fraction of the drug. Second, protein-bound drug is relatively nondiffusible. The effect of protein binding *in vivo*, therefore, is that a proportion of the drug is sequestered in a temporarily inactive and nondiffusible form within the vascular system. As a result, the diffusion gradient from the plasma to the tissue fluid is determined by the level of free drug in the plasma and not by the total level of drug and, hence, the concentration of free antibiotic in the tissues will not be expected to exceed the peak level of free drug reached in the plasma. Binding occurs not only to serum proteins but also to the proteins present in lymph and other fluids and, thus, depots of bound drug may exist in various extravascular compartments. However, because of the reversible

nature of the binding even highly bound penicillins can be cleared rapidly from the body.

Since the levels of free active antibiotic in extravascular sites are determined by the levels of free drug reached in the plasma, it would be expected that in order to achieve a therapeutic effect the level of unbound drug in the plasma should at least equal the MIC of the pathogen in question. Examination of data available for a number of antibiotics which are known to be clinically effective in the treatment of staphylococcal infections indicates that this is indeed the case. Thus, it can be seen that despite marked differences in the extent of binding, level of antibacterial activity, and total serum levels reached with normal dosage, the peak levels of free antibiotic in the plasma do exceed the MIC, and in most cases by a factor of 2 to 4 (Rolinson, 1967).

An adequate level of free antibiotic in the plasma does not in itself ensure that the drug will diffuse readily into the tissues. Differences do occur in the rate at which unbound molecules of different antibiotics diffuse across membranes in the body. However, unless the concentration of unbound drug in the plasma is an inhibitory one, it is unlikely that an inhibitory concentration will be reached in the tissues.

C. Antibacterial Activity *in Vivo*

As part of the laboratory evaluation of an antibiotic it is important to show that it is active in the animal body. Thus, if a compound were found to be active *in vitro* but showed little or no activity *in vivo*, it would be necessary to seek an explanation before one could have confidence in the potential clinical usefulness of the drug. Activity against experimental infections in animals, however, is considerably more complex than activity *in vitro*, and interpretation of *in vivo* activity in a quantitative sense may be difficult unless the factors involved are determined separately. For example, activity against an experimental infection in a laboratory animal depends not only on the intrinsic antibacterial activity of the compound but also on the absorption, distribution, extent of protein binding, and metabolism of the drug in that particular animal species. In man these factors might be very different and, as a result, the clinical value of the drug might not correspond with the findings obtained in the laboratory animal. Differences between laboratory animals and man are particularly marked with respect to absorption and rates of excretion of antibiotics. With most penicillins, for example, the rate of elimination in mice is particularly rapid, and, as a result, in single-dose protection tests the resulting activity may be primarily a reflection of the length of time an inhibitory level is maintained in the body rather than a measure of the antibacterial potency of the drug.

Single-dose protection tests can, therefore, give results which may be misleading with respect to the clinical activity in man where the rate of excretion may be slower and where repeated administration of the antibiotic is more usual.

Marked differences also occur in the extent to which antibiotics are bound to plasma proteins in man and in laboratory animals. For example, the extent of binding of cloxacillin in mouse serum is very much lower than that in human serum. Again, in mouse serum, nafcillin is more highly bound than cloxacillin, whereas in man the reverse is the case. Such differences in protein binding may influence the level of activity *in vivo* and may complicate the correlation between the activity in the experimental animal and the probable activity if administered to man.

The real value of the protection test in experimental animals is that it does provide a demonstration of activity against an infection in a living host. The quantitative aspects of such tests, however, are more difficult to interpret. Unless the individual factors concerned in activity in the animal are measured, i.e., the levels of drug reached in the blood and tissues, the extent of binding of the drug in plasma, and the rate at which the drug is eliminated, the determination of the protective dose may be of limited value in comparing one compound with another. Even when these pharmacokinetic data are known for the experimental animal, the corresponding data in man are required in order to assess the likely clinical value of the compound. Fortunately, certain pharmacological data can be obtained from studies in human volunteers including drug levels reached in plasma and urine, the extent of binding in human serum, the extent of metabolism of the compound, and the rate of elimination. These data, together with the results of the *in vitro* and *in vivo* studies, enable an assessment to be made of the probable therapeutic value of the compound in man.

III. Classification of Semisynthetic Penicillins

Semisynthetic penicillins may be conveniently grouped into three classes on the basis of their antibacterial spectra. Thus, penicillins that have a spectrum of activity essentially similar to that of penicillin V and that are active primarily against gram-positive cocci, are often referred to as *narrow-spectrum penicillins*. These compounds have been described as "acid-stable penicillins" because of their similarity to penicillin V, but this term is no longer favored since it has become obvious that acid stability is a property shared by many other penicillins with very different biological activities. The semisynthetic penicillins that are relatively stable to staphylococcal penicillinase are referred to as the *penicillinase-stable penicillins*,

and this group includes all the compounds in clinical use active against penicillin-resistant strains of *Staphylococcus aureus*. The use of the term "penicillinase-stable" in this way is not particularly satisfactory as other penicillins, such as phenethicillin, ampicillin, or carbenicillin, are stable to the β-lactamases produced by certain gram-negative bacilli, although they are destroyed by staphylococcal penicillinase. The third group of penicillins are the *broad-spectrum penicillins*, so called because their antibacterial spectra encompass gram-positive and gram-negative bacteria. The broad-spectrum penicillins at present available may be separated into those compounds resembling ampicillin in activity and those compounds with anti-pseudomonas activity, but it seems probable that there will be developments in the future which will lead to the introduction of semi-synthetic penicillins with widely overlapping properties. For the purposes of this article the semisynthetic penicillins are considered as narrow-spectrum penicillins similar to penicillin V, penicillinase-stable penicillins, and broad-spectrum penicillins, and the antibacterial spectra of representative members of these groups are shown in Table II.

IV. Analogs of Penicillin V and Penicillin G

This group of penicillins includes phenethicillin, propicillin, and phenbenicillin, which are members of the phenoxy series, and clometocillin and azidocillin both of which are analogs of benzylpenicillin. These penicillins are all acid-stable and relatively well-absorbed after oral dosage, giving blood levels that are higher than those obtained with penicillin V. Differences in serum levels, however, tend to be offset by differences in antibacterial activity or degree of protein binding, and a consideration of all these properties suggests that the therapeutic advantages of one member over another may be rather small.

A. Phenoxypenicillins

The phenoxypenicillins are all analogs of penicillin V (Table III). The biological properties of these penicillins also resemble those of penicillin V, but they show some differences in the level of antibacterial activity, the degree of protein binding, and the levels of drug present in the plasma after oral dosage.

1. *Phenethicillin*

As a result of the asymmetric carbon atom in the side chain of phenoxyethylpenicillin, the antibiotic exists as two diastereoisomers, the commercial product, phenethicillin, being a mixture of these two isomers. The anti-

TABLE II

ANTIBACTERIAL SPECTRA OF SEMISYNTHETIC PENICILLINS[a]

Organism	Minimum inhibitory concentration[b] (μg/ml)						
	Narrow-spectrum penicillins			Penicillinase-stable penicillins		Broad-spectrum penicillins	
	Penicillin G	Penicillin V	Phenethicillin	Methicillin	Cloxacillin	Ampicillin	Carbenicillin
Staphylococcus aureus	0.02	0.05	0.05	1.25	0.1	0.05	1.25
Staphylococcus aureus (penicillinase-producing)	R	R	250	2.5	0.25	R	25
Streptococcus pyogenes	0.01	0.02	0.05	0.25	0.1	0.02	0.5
Streptococcus pneumoniae	0.02	0.02	0.05	0.25	0.25	0.05	1.25
Streptococcus faecalis	2.5	5.0	5.0	25	25	1.25	25
Clostridium welchii	0.05	0.1	0.1	1.0	0.5	0.05	0.5
Neisseria gonorrhoeae	0.01	0.02	0.1	0.1	0.1	0.02	0.05
Neisseria meningitidis	0.02	0.05	0.1	0.25	0.25	0.02	0.05
Haemophilus influenzae	0.5	5.0	5.0	2.5	12.5	0.25	0.25
Escherichia coli[c]	50	125	R	R	R	5.0	5.0
Salmonella typhi	5.0	125	R	R	R	0.5	5.0
Shigella sonnei[c]	50	250	R	R	R	2.5	12.5
Proteus mirabilis[c]	5.0	50	250	250	R	1.25	2.5
Indole-positive *Proteus*[c]	R	R	R	R	R	250	5.0
Klebsiella aerogenes	250	R	R	R	R	250	250
Enterobacter species[c]	R	R	R	R	R	250	12.5
Pseudomonas aeruginosa[c]	R	R	R	R	R	R	50

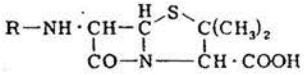
[a] Beecham Research Laboratories, unpublished data.

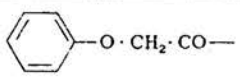
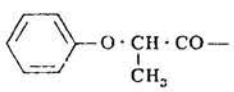
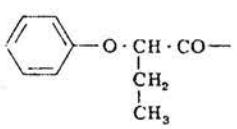
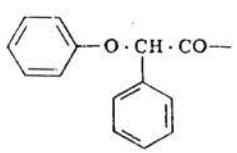
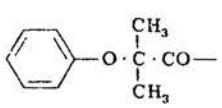
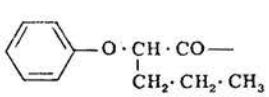
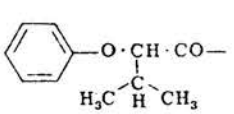
[b] Sensitivity of majority of strains. R = MIC > 250 μg/ml.

[c] Resistant strains are encountered.

TABLE III

ANALOGS OF PENICILLIN V



Penicillin	Structure of side chain R
Penicillin V (phenoxymethylpenicilliin)	
Phenethicillin (DL-α-phenoxyethylpenicillin)	
Propicillin (DL-α-phenoxypropylpenicillin)	
Phenbenicillin (DL-α-phenoxybenzylpenicillin)	
α-Phenoxyisopropylpenicillin	
α-Phenoxybutylpenicillin	
α-Phenoxyisobutylpenicillin	

bacterial activity of the L-isomer is somewhat greater than that of the D-isomer (Gourevitch *et al.*, 1960; McCarthy and Finland, 1960; Wallmark and Finland, 1961; English and McBride, 1962) but does not appear to be clearly superior to that of the DL mixture which in different studies con-

sisted of 30 to 34% of the D-isomer and of 66 to 70% of the L-isomer. Blood level studies reported by Cronk *et al.* (1960) following oral dosage in man showed the levels of the D-isomer to be slightly higher than those of the L-isomer 1 hour after dosage, but at 2 hours the position was reversed and the levels of the L-isomer were higher than those obtained with the D-isomer.

Phenethicillin (DL-α-phenoxyethylpenicillin) was first introduced into clinical practice in 1959. The antibacterial spectrum and level of activity is very similar to that of penicillin V, and the main property of interest in this penicillin is the antibiotic level reached in the blood after oral dosage. Serum levels obtained in adult volunteers after a single dose of 250 mg of phenethicillin are shown in Fig. 4 together with the levels obtained with penicillin V. These results are broadly in agreement with those reported by other workers (Cronk *et al.*, 1960; Morigi *et al.*, 1960; Williamson *et al.*, 1961; Griffiths and Black, 1962; Kirby *et al.*, 1962; Bond *et al.*, 1963) and they show that the serum levels of phenethicillin are considerably higher than those obtained with the same dose of penicillin V. Not only are the serum levels of phenethicillin higher than those of penicillin V but they are also more prolonged, and, as a result, the difference in the levels of these two compounds becomes more marked at the later time intervals. For example, from the results shown in Fig. 4, it will be seen that at 0.5 hour after dosage the level of phenethicillin was only 23% higher than that of penicillin V, but at 1 hour the level was approximately twice as high, and at 2 hours the difference was nearly threefold.

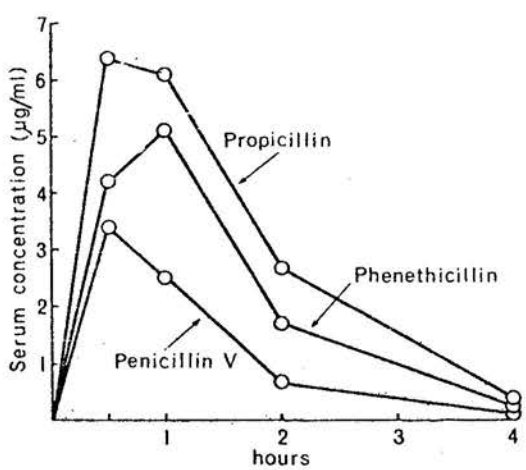


FIG. 4. Blood levels obtained in adult volunteers in the fasting state following a single oral dose of 250 mg of penicillin V (67 subjects), phenethicillin (89 subjects), and propicillin (26 subjects) (Beecham Research Laboratories, unpublished data).

TABLE IV

URINARY EXCRETION OF PENICILLIN AND THE CORRESPONDING PENICILLOIC ACID FOLLOWING AN ORAL DOSE OF VARIOUS PENICILLINS TO ADULT VOLUNTEERS[a]

Penicillin	No. of subjects	Dose (mg)	Dose Recovered in urine from 0 to 12 hr (%)[b]		
			Penicillin	Penicilloic acid	Penicillin plus penicilloic acid
Penicillin V	6	500	26 ± 12	34 ± 20	60 ± 23
Phenethicillin	6	500	50 ± 12	23 ± 14	73 ± 17
Propicillin	6	500	45 ± 21	21 ± 14	66 ± 24
Oxacillin	6	500	17 ± 13	16 ± 13	33 ± 24
Cloxacillin	6	500	38 ± 18	11 ± 13	49 ± 26
Dicloxacillin	6	500	33 ± 20	4 ± 8	37 ± 23
Flucloxacillin	6	500	41 ± 30	4 ± 5	45 ± 28
Ampicillin[c]	10	250	43 ± 18	11 ± 6	54 ± 20
Amoxycillin[c]	10	250	63 ± 19	20 ± 11	83 ± 22

[a] From Cole *et al.* (1973), by permission of *Antimicrobial Agents & Chemotherapy.*

[b] The values given are the means for the number of subjects as indicated with the limits of error at the 95% probability level.

[c] Dose recovered from 0 to 6 hours.

Recent work in these laboratories by Cole *et al.* (1973) indicates that the difference in serum levels with phenethicillin and penicillin V may be the result of a difference in the extent of metabolism rather than a difference in absorption. Data are shown in Table IV for the amount of penicillin and corresponding penicilloic acid recovered in the urine of healthy adult volunteers following a single dose of antibiotic. It will be seen that in the case of penicillin V as much as 34% of the dose administered was recovered in the form of the corresponding penicilloic acid and only 26% was found as unchanged penicillin; in contrast, with phenethicillin, only 22% of the dose was recovered as penicilloic acid and approximately 50% was excreted as unchanged penicillin. From these results it would appear that the degree of absorption of penicillin V and phenethicillin may be very similar but the extent of metabolism of phenethicillin is less. The observation that the serum levels of phenethicillin are more prolonged than those of penicillin V would also be consistent with this difference in the metabolism of the two compounds. Phenethicillin is also metabolized to a small extent in the body without loss of biological activity and the resulting penicillin is excreted in the urine (Rolinson and Batchelor, 1963). This metabolite has not been identified but the corresponding metabolite of penicillin V has been shown to be the *p*-hydroxy derivative (Vanderhaeghe *et al.*, 1963).

Serum levels obtained with phenethicillin are undoubtedly higher than those of penicillin V, and the question arises as to what the clinical significance of this might be. Since most infections are present in extravascular compartments, plasma levels are of importance in so far as they influence the level of antibiotic reached at the site of infection. The effect of protein binding on tissue distribution of penicillin has already been discussed, but this factor can largely be ignored in comparing phenethicillin with penicillin V because the two compounds appear to be bound to almost the same extent. Bond *et al.* (1963) reported the binding of phenethicillin to be slightly lower than that of penicillin V (75.2% compared with 80.1%), whereas Rolinson and Sutherland (1965) reported the binding of phenethicillin to be marginally higher than that of penicillin V (82.7% compared with 79.7%). Diffusion of antibiotic from the plasma into the tissues will be dependent not only on the peak level reached in the plasma but also on the period of time a level in the plasma is maintained, and both factors need to be taken into account when comparing the plasma levels obtained with phenethicillin and penicillin V. Unfortunately, the relative importance of the peak level compared with the duration of plasma concentration is not known and, therefore, it is not easy to arrive at a single numerical value that expresses the significance of the serum levels of phenethicillin compared with those of penicillin V. On the basis of the relative peak plasma levels and the total areas under the curve, the superiority of phenethicillin over penicillin V would appear to be approximately twofold, whereas on the basis of the plasma levels that are present from 2 to 4 hours after dosage, the superiority would appear to be nearer threefold.

The antibacterial spectrum of phenethicillin is similar to that of penicillin V. However, examination of the level of activity of phenethicillin indicates that this is somewhat lower than that of penicillin V, at least against certain pathogens, and the question has arisen as to how far this difference in activity offsets the advantage in blood levels. Against penicillin-sensitive strains of *Staphylococcus aureus*, phenethicillin has been reported to be twofold less active than penicillin V (McCarthy and Finland, 1960; Griffiths and Black, 1962; Kirby *et al.*, 1962; Morrison and Williamson, 1962). On the other hand, Garrod (1960a,b), Shoemaker *et al.* (1961), Colville and Quinn (1962), Rollo *et al.* (1962), Wallmark (1962), Bond *et al.* (1963), and Frisk and Tunevall (1963) have reported the activity of phenethicillin and penicillin V against staphylococci to be very close, indeed, and to differ by less than a factor of 2. With regard to staphylococcal infections, therefore, the superiority of the serum levels of phenethicillin may be partially, but not completely, offset by a marginally lower level of antibacterial activity.

Against the Group A streptococci the published work is quite consistent in reporting phenethicillin to be twofold less active than penicillin V. In this case the difference in activity may largely negate the advantage in blood levels, and against such infections phenethicillin might be expected to be no more than equal to penicillin V. Most workers have also reported phenethicillin to be twofold less active than penicillin V against pneumococci, although Williamson *et al.* (1961) and Rollo *et al.* (1962) have reported results showing phenethicillin and penicillin V to have essentially the same activity. Frisk and Tunevall (1963) also reported phenethicillin to have almost the same activity as penicillin V against α-hemolytic streptococci and pneumococci. Against pneumococcal infections, therefore, a slightly lower level of antibacterial activity may only partly offset the advantage in blood levels which phenethicillin shows compared with penicillin V. Against bacteria other than staphylococci and streptococci, phenethicillin shows activity similar, or slightly lower, than that of penicillin V, and the compound is not indicated in the treatment of infections other than those caused by staphylococci and streptococci.

Phenethicillin does show somewhat greater stability to staphylococcal penicillinase than does penicillin V and, as a result, the MIC's of phenethicillin against penicillinase-producing staphylococci are slightly lower than those of penicillin V. Results with a number of strains of staphylococci, which are also typical of results reported by other workers, are shown in Table V. The degree of stability to staphylococcal penicillinase, however, is not great, and it is doubtful whether the activity shown against penicillin-resistant staphylococci is sufficient to be of clinical value.

2. *Propicillin*

Propicillin (DL-α-phenoxypropylpenicillin) is the next member to phenethicillin in an homologous series in the same way as phenethicillin is the next higher member to penicillin V. In many respects the biological properties of propicillin are a continuation of the trends that phenethicillin shows as compared with penicillin V, that is, higher blood levels after oral administration, lower antibacterial activity against certain organisms, greater stability to staphylococcal penicillinase, and a higher degree of protein binding. As in the case of phenoxyethylpenicillin, phenoxypropylpenicillin exists as two diastereoisomers, the commercial product, propicillin, being a mixture of these isomers. Studies carried out with the two separate isomers (Griffiths and Black, 1962) indicate the L-isomer to be slightly more active than the D-isomer and to give rise to higher blood levels after oral dosage in man. The results of Wick *et al.* (1962), however, indicate that the L-isomer is not clearly more active than the DL-mixture.

TABLE V

MINIMUM INHIBITORY CONCENTRATIONS OF PENICILLIN V, PHENETHICILLIN, AND PROPICILLIN AGAINST PENICILLIN-RESISTANT STAPHYLOCOCCI[a]

Strain	Minimum Inhibitory Concentration[b] (μg/ml)		
	Penicillin V	Phenethicillin	Propicillin
1	0.5	0.25	0.1
2	12.5	0.5	0.5
3	25	1.25	0.5
4	50	2.5	1.25
5	50	50	5.0
6	250	125	25
7	500	125	25
8	500	500	50
9	>500	500	250

[a] Beecham Research Laboratories (unpublished data).

[b] Serial dilution in agar using one drop of undiluted, overnight, broth culture as inoculum.

Serum levels obtained with propicillin in healthy adult volunteers compared with phenethicillin and penicillin V are given in Fig. 4. These results show that the levels reached with propicillin are higher than those of phenethicillin, and this has been confirmed by a number of other workers (Williamson *et al.*, 1961; Bond *et al.*, 1963; Frisk and Tunevall, 1963). Compared with penicillin V, the levels of propicillin at 0.5 and 1 hour after dosing appear to be approximately twice as high, and at 2 and 4 hours the difference is approximately fourfold (Naumann, 1962a). As with phenethicillin, the extent of metabolism of propicillin to the corresponding penicilloic acid is lower than is the case with penicillin V (Table IV), and this may largely account for the higher and more prolonged plasma levels obtained with propicillin (Cole *et al.*, 1973). On the other hand, the extent to which propicillin is metabolized does not appear to be very different from that of phenethicillin and, therefore, the difference in blood levels between propicillin and phenethicillin may well indicate a difference in extent of absorption. Like phenethicillin, propicillin undergoes some metabolism in the body to give rise to an active metabolite (Rolinson and Batchelor, 1963). The amount of this metabolite excreted in the urine appears to be rather greater than occurs with phenethicillin and may amount to as much as 25–30% of the total penicillin excreted. The biological activity of the metabolite, however, appears to be similar to that of the parent compound.

Although the serum levels of propicillin are considerably higher than those of penicillin V, the extent of binding to serum proteins is also greater. Bond *et al.* (1963) reported values of 80.1 and 89.3%, and Rolinson and Sutherland (1965) 79.7 and 88.5%, respectively, for the binding of penicillin V and propicillin. These results are in close agreement and indicate the free levels to be approximately 10% in the case of propicillin compared with approximately 20% with penicillin V, that is, the proportion of unbound propicillin is approximately half that of penicillin V. As a result of this difference in binding, although the levels of total propicillin in serum are 2–4 times higher than those of penicillin V, the levels of free propicillin at 0.5 and 1 hour after dosing are only comparable and at 2 and 4 hours the difference is only twofold. This difference in levels of free antibiotic in the plasma is barely adequate to compensate for the lower level of antibacterial activity which propicillin shows, at least against certain organisms. Thus, against penicillin-sensitive staphylococci, propicillin is two- to fourfold less active than penicillin V (Williamson *et al.*, 1961; Colville and Quinn, 1962; Griffiths and Black, 1962; Kirby *et al.*, 1962; Naumann, 1962a; Wallmark, 1962; Bond *et al.*, 1963; Frisk and Tunevall, 1963), and this difference in activity is sufficient to negate the advantage which propicillin shows in terms of free levels in the plasma. On the other hand, against the Group A streptococci, and particularly against the pneumococcus, the activity of propicillin is only slightly lower than that of penicillin V. Different workers have reported marginally different results but, in general, the difference in activity between propicillin and penicillin V appears to be approximately twofold (Williamson *et al.*, 1961; Griffiths and Black, 1962; Kirby *et al.*, 1962; Naumann, 1962a; Wallmark, 1962; Frisk and Tunevall, 1963). Against these pathogens, therefore, the difference in activity may not entirely offset the advantage shown by propicillin in terms of free levels in the plasma.

Compared with penicillin V, propicillin has a significantly greater degree of stability to staphylococcal penicillinase which is reflected in the MIC's against penicillinase-producing strains of *Staphylococcus aureus*. Typical results are shown in Table V, and it will be seen that the MIC values for propicillin are significantly lower than those of both penicillin V and phenethicillin. In the case of strains of staphylococci that are only slightly resistant to benzylpenicillin, the activity of propicillin might well be of clinical value. However, against highly resistant strains of staphylococci the degree of penicillinase stability of propicillin is inadequate to prevent extensive inactivation of the drug in this type of test, and with these strains the MIC values suggest that propicillin would not be clinically effective. Whether, in fact, the clinical value of propicillin in the treatment of penicil-

lin-resistant staphylococcal infections has been adequately investigated is perhaps debatable because in 1961 when propicillin was first introduced, the activity of methicillin against penicillinase-producing staphylococci was already well established.

3. *Phenbenicillin*

Phenbenicillin (α-phenoxybenzylpenicillin) is well absorbed following oral administration giving plasma levels which are substantially higher than those of penicillin V (Carter and Brumfitt, 1962; Rollo *et al.*, 1962) and which may even be higher than those obtained with propicillin (Bond *et al.*, 1963). The antibacterial activity of phenbenicillin against *Streptococcus pyogenes* and *Streptococcus pneumoniae* appears to be comparable with propicillin, but the level of activity against penicillin-sensitive staphylococci is slightly lower (Bond *et al.*, 1963). The activity of phenbenicillin against penicillin-resistant staphylococci is also slightly lower than that of propicillin and in this respect comparable with that of phenethicillin.

Although phenbenicillin does give rise to high blood levels the compound is very highly bound to plasma proteins. Bond *et al.* (1963) and Rolinson and Sutherland (1965) have both reported that in human serum only 2.8% of the drug is free and this level of binding may well offset any advantage phenbenicillin might have in terms of absorption. Phenbenicillin also undergoes considerable metabolism in the body, and two active metabolites can be detected in serum and in urine samples (Rolinson and Batchelor, 1963). In terms of activity against *Bacillus subtilis*, *Sarcina lutea*, and *Staphylococcus aureus*, the two metabolites appear to contribute more activity than the amount of unchanged phenbenicillin and this may complicate the biological assay of the blood levels obtained with this penicillin.

4. *Other Phenoxyalkylpenicillins*

In addition to phenethicillin and propicillin, data have been reported for certain other phenoxyalkylpenicillins, including phenoxyisopropyl, phenoxybutyl, and phenoxyisobutylpenicillin (see Table III), none of which appear to be clearly superior to phenethicillin or propicillin.

In the case of phenoxyisopropylpenicillin, serum levels after oral dosage are similar (Kirby *et al.*, 1962) or only slightly higher than those obtained with propicillin (Frisk and Tunevall, 1963), but the level of antibacterial activity is three- to fourfold lower than that of propicillin (Kirby *et al.*, 1962; Frisk and Tunevall, 1963). With phenoxybutylpenicillin, serum levels have been reported to be higher than those of propicillin (Frisk and Tunevall, 1963), but antibacterial activity, particularly against staphylococci, was

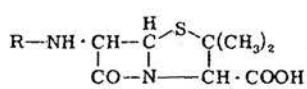
found to be slightly lower. In the case of phenoxyisobutylpenicillin, Kirby *et al.* (1962) reported blood levels to be only equal to those obtained with penicillin V, although Frisk and Tunevall (1963) reported levels higher than penicillin V and even slightly higher than those obtained with propicillin. Both groups of authors, however, reported activity to be lower than that of propicillin. Phenoxyisopropyl- and phenoxyisobutylpenicillin both show somewhat greater activity than propicillin against penicillin-resistant staphylococci but neither compound has found a place in clinical practice.

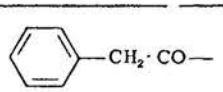
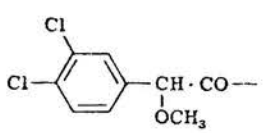
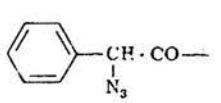
B. Clometocillin

Penicillins that are stable in acid solution are not necessarily well absorbed when given by mouth. However, unless the penicillin is relatively acid-stable, extensive destruction may occur in the stomach before absorption can take place. Benzylpenicillin itself is not stable in acid solution and for this reason it is less suitable for oral administration than is penicillin V which is acid-stable and well absorbed. Acid stability can be achieved by introducing an electron-attracting substituent in the α-position of the side chain of benzylpenicillin, and α-methoxybenzylpenicillin is an example of such a substitution. Claesen *et al.* (1962) reported the chemotherapeutic properties of a number of α-substituted benzylpenicillins from which clometocillin (3,4-dichloro-α-methoxybenzylpenicillin) (Table VI) was

TABLE VI

Analogs of Penicillin G



Penicillin	Structure of side chain R
Penicillin G (benzylpenicillin)	
Clometocillin (DL-3,4-dichloro-α-methoxybenzylpenicillin)	
Azidocillin (D-α-azidobenzylpenicillin)	

selected for detailed study (Van Dijck *et al.*, 1962; Vanderhaeghe *et al.*, 1962).

The biological properties of clometocillin resemble those of penicillin V, phenethicillin, and propicillin, and it is with these penicillins that a comparison should be made. Plasma levels of clometocillin compared with those of penicillin V and phenethicillin following oral dosage in man were reported by Vanderhaeghe *et al.* (1962) but were assayed in terms of activity against a strain of *Streptococcus pyogenes* using a twofold dilution technique in broth, and it is not clear what the relative plasma concentrations were in terms of microgram of drug per milliliter. Studies carried out in the authors' laboratory, however, have indicated that the peak concentrations reached with this penicillin are similar to those with phenethicillin, but the levels at 2 to 6 hours after dosing are somewhat higher than those of phenethicillin and generally comparable with those of propicillin. The extent of binding of clometocillin in human serum, however, is higher than that of phenethicillin (Rolinson and Sutherland, 1965), and the free levels in the plasma may be comparable only with those of phenethicillin. As a result, comparison of clometocillin with phenethicillin rests on antibacterial activity. Vanderhaeghe *et al.* (1962) have reported clometocillin to be slightly more active than phenethicillin against *S. pyogenes*. The same authors have also shown that the serum antistreptococcal titers in volunteers were higher with clometocillin than with phenethicillin. Against *Streptococcus pneumoniae* and penicillin-sensitive staphylococci, clometocillin appears to be equal to phenethicillin in activity but does not show any advantage.

Clometocillin is not stable to staphylococcal penicillinase and, therefore, does not show useful activity against penicillin-resistant strains of *Staphylococcus aureus*. As in the case of the α-phenoxypenicillins, clometocillin undergoes metabolism in the body (Vanderhaeghe *et al.*, 1962). A single metabolite can be detected in the urine which in terms of activity against *Bacillus subtilis* may represent as much as 50% of the unchanged penicillin present in the urine (Rolinson and Batchelor, 1963). The identity of this metabolite has not been established.

C. Azidocillin

Azidocillin (D-α-azidobenzylpenicillin) (Table VI) shows a spectrum and level of antibacterial activity very similar to that of benzylpenicillin (Sjöberg *et al.*, 1968; Tunevall and Frisk, 1968) but differs in that it is acid-stable and well absorbed in man when given by mouth (Hansson *et al.*, 1968). Against *Streptococcus pyogenes*, *Streptococcus viridans*, *Streptococcus*

pneumoniae, and *Streptococcus faecalis*, azidocillin appears to be twofold more active than penicillin V and is marginally more active than benzylpenicillin. Against penicillin-sensitive staphylococci, on the other hand, azidocillin is marginally less active than both penicillin V and benzylpenicillin but the difference is less than twofold. Azidocillin is also as active as benzylpenicillin against *Haemophilus influenzae* and in this respect is only slightly less active than ampicillin Against *Haemophilus parainfluenzae*, however, azidocillin has been reported to be considerably less active than ampicillin (Forsgren, 1969). Like benzylpenicillin, azidocillin is not stable to staphylococcal penicillinase and does not show useful activity against penicillin-resistant strains of *Staphylococcus aureus*.

When given by mouth, azidocillin gives rise to serum levels higher than those of penicillin V (Hansson *et al.*, 1968) and comparable with those obtained with phenethicillin. The level of binding of azidocillin in human serum is also similar to that of phenethicillin (Sjöberg *et al.*, 1968). On the basis of antibacterial activity against staphylococci and streptococci and the levels of free drug obtained in the plasma, azidocillin would appear to show some advantage over both penicillin V and phenethicillin. With regard to *H. influenzae*, azidocillin is clearly superior to both penicillin V and phenethicillin, but, compared with ampicillin, a slightly lower level of activity against this pathogen and a higher degree of binding in serum suggest that azidocillin might be inferior.

V. Penicillins Stable to Staphylococcal Penicillinase

Within a few years of the widespread clinical use of penicillin G, strains of penicillin-resistant *Staphylococcus aureus* were encountered with increasing frequency. By 1948, as many as 50% of strains in hospitals were found to be resistant and, by 1960, the incidence in some hospitals had risen to as high as 80% (Barber and Rozwadowska-Dowzenko, 1948; Thompson *et al.*, 1960; Ridley *et al.*, 1970). These strains of *S. aureus* owe their penicillin resistance to the production of the enzyme, penicillinase (β-lactamase) which inactivates penicillin G by opening the β-lactam ring to give the corresponding penicilloic acid (Fig. 5) which is entirely devoid of antibacterial activity. The correlation between penicillinase production and penicillin resistance in *S. aureus* was established by Kirby (1944) and since that time cross-infection and selection due to penicillin usage has brought about the increase in the number of resistant strains encountered in hospitals. In recent years it has been shown that the genetic determinant for penicillinase production in *S. aureus* is characteristically located extrachromosomally

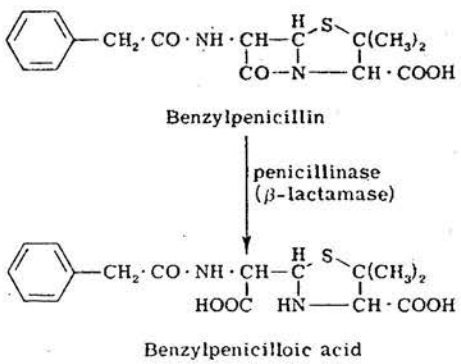


FIG. 5. Inactivation of penicillin by penicillinase.

on a plasmid (Novick, 1963). Transfer of this plasmid from one cell of *S. aureus* to another by transduction can take place *in vivo* (Novick and Morse, 1967), and it is probable that this transfer has been responsible for the spread of penicillin resistance which has occurred among different staphylococcal phage types.

By 1960 the problem of resistant staphylococci in hospitals was made even more difficult by the high incidence of strains resistant not only to penicillin G but also to other antibiotics including tetracycline, streptomycin, erythromycin, chloramphenicol, and novobiocin. In addition, these multiple resistant strains were generally characterised by high virulence and the frequency of these staphylococci in hospitals represented a serious clinical problem.

Although penicillin-resistant staphylococci produce a penicillinase that inactivates penicillin G, the individual bacterial cells are intrinsically sensitive, and resistance is only apparent when the number of bacteria and, hence, the amount of penicillinase, is large. With the ability to prepare novel penicillins from 6-aminopenicillanic acid, it was considered likely that stability to staphylococcal penicillinase could be achieved by the choice of suitable side-chain structures. In 1960 the first of the penicillinase-stable penicillins, methicillin, was introduced into clinical practice and this was followed by nafcillin, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin. All these penicillins show a high degree of stability to staphylococcal penicillinase together with good activity against staphylococci and streptococci, although they do not have any other activity of clinical importance. Two other penicillins, ancillin and quinacillin are also stable to staphylococcal penicillinase and active against penicillin-resistant staphylococci. Both compounds have been taken to the stage of clinical trial but neither has

found a place in clinical practice. Among these penicillinase-stable penicillins, important differences are shown in the level of antibacterial activity, the extent of protein binding, and the levels of drug reached in the blood after both oral and intramuscular administration, and these properties are summarized in Table VII.

In addition to the penicillins already mentioned, certain penicillin amides have been shown to possess significant stability to penicillinase. Amides of phenethicillin and propicillin have been reported to show marked activity against resistant staphylococci *in vitro* (Huang *et al.*, 1964), but these compounds were not active *in vivo*. However, the amide of α-methoxypropylpenicillin has been reported to be active against experimental infections with penicillinase-producing staphylococci (Huang *et al.*, 1964). Another penicillin that has been reported to be stable to penicillinase and active against penicillin-resistant staphylococci *in vivo* is D-α-guanidinobenzylpenicillin (Leanza *et al.*, 1965). However, no reports of clinical trials have appeared with this compound nor with the amide of α-methoxypropylpenicillin.

A. Methicillin, Nafcillin, and Ancillin

1. *Methicillin*

Methicillin (2,6-dimethoxyphenylpenicillin) (Table VIII) is highly stable to staphylococcal penicillinase, and, although the intrinsic activity

TABLE VII

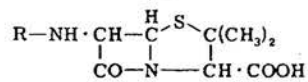
Antistaphylococcal Activity, Serum Binding, and Oral Absorption of Penicillinase-Stable Penicillins[a]

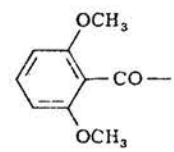
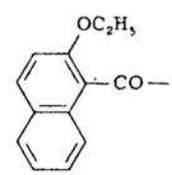
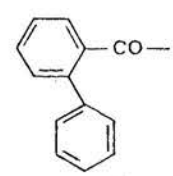
Compound	Date of first report	Minimum Inhibitory Concentration, *S. aureus* (μg/ml)		Extent of binding in human serum (% bound)	Oral absorption in man
		Penicillin-sensitive	Penicillin-resistant		
Methicillin	1960	1.25	2.5	49	None
Ancillin	1961	0.20	0.80	91	Moderate
Nafcillin	1961	0.20	0.40	87	Poor
Oxacillin	1961	0.15	0.40	93	Moderate
Cloxacillin	1962	0.2	0.35	94	Good
Quinacillin	1964	0.5	1.25	40	None
Dicloxacillin	1964	0.15	0.30	97	Very good
Flucloxacillin	1970	0.15	0.30	95	Very good

[a] Beecham Research Laboratories (unpublished data).

TABLE VIII

METHICILLIN, NAFCILLIN, AND ANCILLIN[a]



Penicillin	Structure of side chain R
Methicillin (2,6-dimethoxyphenylpenicillin)	
Nafcillin (2-ethoxy-1-naphthylpenicillin)	
Ancillin (2-biphenylylpenicillin)	

[a] Penicillins stable to staphylococcal penicillinase.

of the compound against staphylococci is relatively low, penicillinase-producing strains are almost as sensitive to methicillin as are non-penicillinase-producing strains (Rolinson *et al.*, 1960). This stability to staphylococcal penicillinase results from an extremely high K_m value (Novick, 1962), that is to say greatly diminished affinity between the penicillin and the penicillinase enzyme molecules.

Most strains of penicillin-resistant *Staphylococcus aureus* are inhibited by methicillin at a concentration of 2 to 3 μg/ml, and strains of *Streptococcus pyogenes* and *Streptococcus pneumoniae* are generally inhibited at a level of 0.2 μg/ml or less. Methicillin is also highly active against the gonococcus and the meningococcus. Strains of *Haemophilus influenzae*, on the other hand, are generally inhibited only at a level of 2 μg/ml or more, and *Streptococcus faecalis* and the gram-negative bacilli are relatively resistant. In contrast with most of the other penicillinase-stable penicillins, methicillin

is not highly bound to serum proteins. Rolinson and Sutherland (1965) have reported the level of binding in human serum to be 49% which is similar to that of benzylpenicillin, and the antibacterial activity of methicillin is not significantly affected by the presence of serum.

Methicillin is unstable in acid solution and is not absorbed in man when given by mouth and must be administered parenterally. Like benzylpenicillin, absorption is rapid and the compound is also excreted rapidly. Thus, following administration of a single 1.0-gm intramuscular dose, Knudsen and Rolinson (1960) reported mean peak serum concentrations of 17 μg/ml 0.5 hour after injection, falling to about 5 μg/ml at 2 hours, and to subinhibitory levels by 4 hours. Approximately 60% of the dose is excreted in the urine within 3 hours, and about 70% can be recovered in the 0–6 hour period after injection (Knudsen and Rolinson, 1960; Foltz *et al.*, 1962). Administration of probenecid results in significantly increased and prolonged serum concentrations following intramuscular injection (Stevenson and Harrison, 1960). After intravenous administration of a 1.0-gm dose, Cronk and Morigi (1961) reported that the serum concentrations of methicillin were 60 μg/ml after 5 minutes but had fallen to 7 μg/ml after 1 hour, indicating rapid excretion of the drug.

Although the intrinsic antistaphylococcal activity of methicillin is very much lower than that of many other penicillinase-stable penicillins, this difference in activity is very much less in the presence of serum and, therefore, methicillin is still regarded by some clinicians as one of the drugs of choice in the treatment of severe staphylococcal infections where parenteral therapy is required.

2. *Nafcillin*

Nafcillin (2-ethoxy-1-naphthylpenicillin) (Table VIII) shows some structural similarity with methicillin. However, the level of activity of nafcillin against penicillin-resistant staphylococci is considerably greater than that of methicillin and is comparable with that of the isoxazolyl penicillins. Most strains of penicillin-resistant *Staphylococcus aureus* are inhibited by nafcillin at a level of 0.2 to 0.8 μg/ml (Klein and Finland, 1963b; Rosenman and Warren, 1963; Hopper *et al.*, 1964). Group A streptococci and pneumococci are also highly sensitive to nafcillin and in this respect the compound is rather more active than the isoxazolyl penicillins (Hopper *et al.*, 1964). The extent of binding of nafcillin to serum proteins is relatively high, and Rolinson and Sutherland (1965) have reported the compound to be 87% bound to human serum. As a result of this degree of protein binding, activity against staphylococci is markedly diminished in the presence of serum. For example, Rosenman and Warren (1962) found that the activity of naf-

cillin in 50% human serum was reduced eightfold, and under these conditions the compound was only twofold more active than methicillin. Similarly, in experiments carried out in 95% human serum, Barber and Waterworth (1964) reported the activity of nafcillin against a penicillin-resistant strain of *S. aureus* to be only equal to that of methicillin.

Unlike methicillin, nafcillin is absorbed when given by mouth but the blood levels obtained are not high, and Whitehouse *et al.* (1963) concluded that serum concentrations following a 1-gm dose of nafcillin would be inadequate for the treatment of staphylococcal infections. Klein and Finland (1963b) reported rather higher levels using a buffered preparation of the sodium salt of nafcillin, but the mean peak level following a 1-gm dose was only 6.6 μg/ml, and only 11% of the dose was recovered in the urine over 0 to 12 hours. Serum levels of nafcillin were also found to be substantially lower when the drug was given after food. Sidell *et al.* (1963) and Kunin (1966) have reported that blood levels following oral dosage of nafcillin are lower than those obtained with oxacillin, and Whitehouse *et al.* (1963) have suggested that the low blood levels obtained with nafcillin may result because the compound is not highly stable at low pH. More recently, Kind *et al.* (1970) have suggested that the low levels may be due to inactivation of nafcillin in the liver and this might also account for the relatively low serum levels and urinary excretion of nafcillin following intramuscular administration (Klein and Finland, 1963b; Kunin, 1966).

In summary, the high activity of nafcillin against gram-positive cocci, including penicillin-resistant staphylococci, appears to be offset to a considerable extent by the degree of binding to serum proteins and the low levels of drug obtained in the blood, particularly after oral dosage.

3. *Ancillin*

Ancillin (2-biphenylylpenicillin) (Table VIII) is also an analog of methicillin, but unlike the latter it is acid-stable and is absorbed when given by mouth (Dolan *et al.*, 1962). Ancillin is more active than methicillin against penicillinase-producing staphylococci and its antibacterial spectrum is generally similar to that of nafcillin and oxacillin. However, ancillin appears to be rather less stable to staphylococcal penicillinase than is nafcillin and oxacillin (Gourevitch *et al.*, 1962; Hogan *et al.*, 1962), and its level of activity against penicillin-resistant staphylococci is also slightly lower (Gourevitch *et al.*, 1962; Hogan *et al.*, 1962; Kirby *et al.*, 1962; Abu-Nassar *et al.*, 1963). The degree of binding of ancillin to serum proteins is high and similar to that of oxacillin (Kirby *et al.*, 1962), and activity is diminished in the presence of serum to a similar extent to that seen with oxacillin and nafcillin (Gourevitch *et al.*, 1962; Sabath *et al.*, 1963).

Ancillin is relatively well absorbed in man when given by mouth (Farquhar *et al.*, 1962), but the peak levels reached in the blood appear to be somewhat lower than those obtained with oxacillin (Gourevitch *et al.*, 1962; Kirby *et al.*, 1962; Sabath *et al.*, 1963). Over the 6 hour period after drug administration, Sabath *et al.* (1963) found that 27% of the dose had been excreted in the urine. The same authors also reported that after intramuscular administration of ancillin the resulting serum levels were only slightly higher than those obtained by oral dosage, and only 35% of a 500-mg dose was recovered in the urine over the 6 hour period following injection.

Ancillin has not found a place in clinical practice, probably because the antibacterial activity and absorption of this compound are inferior to those of other penicillinase-stable penicillins.

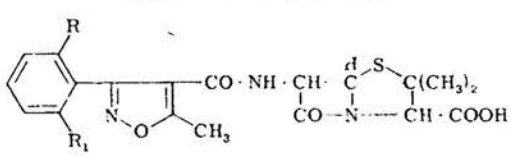
B. Isoxazolyl Penicillins

Four members of this series of penicillins are now in clinical use, namely, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin (Table IX). In addition, some evaluation data concerning another member of this series, BRL 1577, have also been published.

These particular isoxazolyl penicillins are very stable to staphylococcal penicillinase and show high activity against penicillin-sensitive and penicil-

TABLE IX

Isoxazolyl Penicillins[a]



Penicillin	Side chains R	Side chains R_1
Oxacillin (3-phenyl-5-methyl-4-isoxazolylpenicillin)	H	H
Cloxacillin [3-(2-chlorophenyl)-5-methyl-4-isoxazolylpenicillin]	Cl	H
Dicloxacillin [3-(2,6-dichlorophenyl)-5-methyl-4-isoxazolylpenicillin]	Cl	Cl
Flucloxacillin [3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolylpenicillin]	Cl	F

[a] Penicillins stable to staphylococcal penicillinase.

lin-resistant staphylococci. They are also highly active against pyogenic streptococci and pneumococci. When given by mouth, these compounds are well absorbed, although the resulting blood levels are adversely affected by food. All these members of the isoxazole series are highly bound to plasma proteins. Oxacillin, cloxacillin, dicloxacillin, and flucloxacillin differ slightly in their degree of antibacterial activity but primarily in the levels of unbound drug reached in the blood after oral dosage.

Oxacillin, Cloxacillin, Dicloxacillin, and Flucloxacillin

Comparative results are shown in Tables X and XI for the antibacterial activity of oxacillin, cloxacillin, dicloxacillin, and flucloxacillin and the blood levels achieved after oral dosage. The data shown are taken from results reported by Sutherland *et al.* (1970) in a comparative assessment of the properties of these four isoxazolyl penicillins. Flucloxacillin is a relatively new penicillin and so far there are few experimental reports in which this compound has been compared with other isoxazolyl penicillins, but the results shown in Tables X and XI for oxacillin, cloxacillin, and dicloxacillin are in good general agreement with the results of many other investigators including Auhagen *et al.* (1962a), Knox *et al.* (1962), Knudsen *et al.* (1962), Sidell *et al.* (1964), Gravenkemper *et al.* (1965), and Knott *et al.* (1965).

Against penicillin-sensitive staphylococci, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin all show essentially the same activity. Similarly, against penicillin-resistant staphylococci, cloxacillin, dicloxacillin, and flucloxacillin show comparable activity but oxacillin is slightly less active as a result of a lower degree of stability to staphylococcal penicillinase. All four isoxazolyl penicillins are highly active against the β-hemolytic streptococci, although cloxacillin is marginally less active than the other three. Against *Streptococcus pneumoniae*, cloxacillin, dicloxacillin, and flucloxacillin are equal in activity, but oxacillin is significantly more active, the difference being one twofold dilution. There are no important differences in the activity of these isoxazolyl penicillins against organisms other than the pyogenic cocci. Activity against the gonococcus and meningococcus is relatively high but is probably not of clinical importance. Strains of *Haemophilus influenzae* are generally only inhibited by levels of 5 to 10 μg/ml, and against the gram-negative bacilli these isoxazolyl penicillins are virtually inactive.

With regard to comparison with other penicillinase-stable penicillins, Sidell *et al.* (1963) and Hopper *et al.* (1964) reported cloxacillin and nafcillin to be essentially equal in activity against penicillin-resistant staphylococci. However, Barber and Waterworth (1964) reported cloxacillin to be more active than nafcillin, but in tests carried out in 95% human serum the two

TABLE X

ANTIBACTERIAL ACTIVITY OF OXACILLIN, CLOXACILLIN, DICLOXACILLIN, AND FLUCLOXACILLIN[a]

Organism	Minimum inhibitory concentration (μg/ml)[b]			
	Oxacillin	Cloxacillin	Dicloxacillin	Flucloxacillin
Staphylococcus aureus				
Penicillin-sensitive	0.15	0.21	0.17	0.16
Penicillin-resistant	0.41	0.34	0.28	0.31
β-hemolytic streptococci	0.07	0.1	0.08	0.07
Streptococcus pneumoniae	0.09	0.22	0.20	0.21

[a] From Sutherland *et al.* (1970), by permission of the *British Medical Journal*, London.
[b] Mean values.

compounds showed equal activity. Against the β-hemolytic streptococci, cloxacillin and nafcillin show a similar level of activity but, against *S. pneumoniae*, nafcillin appears to be significantly more active than cloxacillin (Sidell *et al.*, 1963; Barber and Waterworth, 1964).

The isoxazolyl penicillins are all highly bound to serum proteins (Table VII), but, whereas oxacillin, cloxacillin, and flucloxacillin are bound to a similar degree, the extent of binding of dicloxacillin is significantly higher. Consequently, the high serum concentrations obtained with dicloxacillin,

TABLE XI

SERUM CONCENTRATIONS OF OXACILLIN, CLOXACILLIN, DICLOXACILLIN, AND FLUCLOXACILLIN IN VOLUNTEERS FOLLOWING A SINGLE ORAL DOSE[a]

Penicillin	Dose (mg)	Mean total level (μg/ml)					Mean free level[b] (μg/ml)	
		½ hr	1 hr	2 hr	3 hr	4 hr	1 hr	2 hr
Oxacillin	500	5.6	4.9	1.5	0.6	0.2	0.34	0.1
Cloxacillin	500	8.4	9.2	4.5	<2.0	<2.0	0.55	0.27
Flucloxacillin	500	13.9	14.5	6.7	4.4	2.2	0.76	0.35
	250	6.9	8.8	5.0	2.2	<1.0	0.46	0.26
Dicloxacillin	250	6.4	9.3	6.7	3.1	1.4	0.29	0.20

[a] From Sutherland *et al.* (1970), by permission of the *British Medical Journal*, London.
[b] Calculated from the extent of binding in human serum.

as a result of its excellent absorption characteristics, are to a large extent offset by its extensive serum binding which limits the availability of active drug in the body (Kunin, 1966; *Medical Letter*, 1968).

The isoxazolyl penicillins are all absorbed after oral administration, but there are significant differences in the serum levels obtained. Data in Table XI show that the total levels obtained with cloxacillin are about twice as high as those obtained with oxacillin, whereas those of dicloxacillin and flucloxacillin are approximately double those of cloxacillin. These differences are also reflected in the proportion of the dose that can be recovered in the urine. In fasting volunteers, approximately 25% of an oral dose of oxacillin is excreted in the urine as active penicillin, compared with 30–40% in the case of cloxacillin and 50–60% with dicloxacillin and flucloxacillin (Knudsen *et al.*, 1962; Sutherland *et al.*, 1970). However, although the total serum concentrations for a given oral dose of oxacillin, cloxacillin, dicloxacillin, and flucloxacillin might be expressed as 1:2:4:4, respectively, because the extent of binding of dicloxacillin is greater than that of the other penicillins, the levels of active unbound drug would be 1:2:2:4.

As with other penicillins, the serum levels of the isoxazolyl penicillins are higher and more prolonged as a result of concomitant administration of probenecid. Absorption of the isoxazolyl penicillins is adversely affected by the presence of food, and the peak serum levels reached are substantially lower than those obtained in the fasting state.

As in the case of the phenoxyalkyl series, differences in blood levels after oral dosage with different members of the isoxazolyl series may be the result of differences in metabolism rather than differences in absorption. For example, Rosenblatt *et al.* (1968) reported that the plasma half-life of oxacillin in patients with almost no renal function was approximately half that of dicloxacillin whereas cloxacillin occupied an intermediate position. These authors also found that in healthy volunteers the amount of oxacillin recovered in the urine after intravenous administration was lower than that of dicloxacillin. These results are consistent with a more rapid rate of degradation of oxacillin compared with dicloxacillin and are further supported by the data of Cole *et al.* (1973) shown in Table IV. In the case of oxacillin after oral dosage in man, approximately 16% of the dose could be recovered in the urine as unchanged penicillin and a further 16% in the form of the corresponding penicilloic acid, but with dicloxacillin and flucloxacillin, some 30–40% was recovered as penicillin and less than 5% as the degradation product. When the amount of penicilloic acid is taken into account the absorption of these isoxazolyl penicillins appears to be rather similar. This difference in extent of metabolism may also account in part for the differences in the blood levels obtained with oxacillin, cloxacillin, and

flucloxacillin after parenteral administration (Rosenblatt *et al.*, 1968; Sutherland *et al.*, 1970).

Data on another member of the isoxazole series, BRL 1577 (5-methyl-3-*p*-chlorophenyl-4-isoxazolylpenicillin) have been reported by Barber and Waterworth (1962) and by Knox *et al.* (1962). This isoxazolyl penicillin is slightly less active than cloxacillin against penicillin-resistant staphylococci, and the blood levels obtained after oral dosage are approximately half those obtained with cloxacillin; as a result the compound has not found a place in clinical practice.

The isoxazolyl penicillins undergo some metabolism in the body to give rise to active metabolites. In the case of cloxacillin the penicillin metabolite has been isolated from urine and shown to have antibacterial activity almost indistinguishable from the parent compound (Nayler *et al.*, 1962). By using this preparation of the metabolite, it was shown that approximately 10% of the penicillin excreted in the urine was in the form of the metabolite (Rolinson and Batchelor, 1963). In the case of oxacillin, paper chromatography indicated that the extent of metabolism was somewhat greater than with cloxacillin. The metabolite that occurs with dicloxacillin has been shown to differ from the parent compound in having a hydroxyl function in the 5-methyl group on the isoxazole ring (Van Harken *et al.*, 1970).

C. Pyrazocillin

Pyrazocillin [1-(2,6-dichlorophenyl)-4-methyl-5-pyrazolylpenicillin] (Fig. 6), is closely related in structure to dicloxacillin and contains a pyrazole ring in the side chain instead of an isoxazole ring. Koczka *et al.* (1970) have reported the activity of pyrazocillin against penicillin-resistant staphylococci to be equal to that shown against penicillin-sensitive strains indicating a high degree of stability to staphylococcal penicillinase. The level of activity of pyrazocillin against penicillin-resistant staphylococci was also reported to be comparable with that of dicloxacillin. Regarding the extent of binding, Koczka *et al.* (1970) reported studies with bovine serum but not

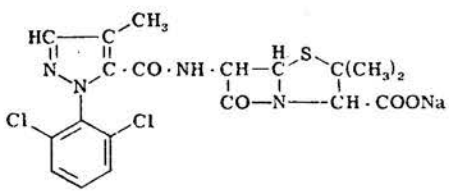


Fig. 6. Structure of pyrazocillin.

with human serum, and it is known that the extent of binding of penicillins may differ in the sera of different species. Koczka *et al.* (1970) also reported data on serum levels of pyrazocillin after oral administration in mice and dogs but not in man.

Data obtained in the authors' laboratory show pyrazocillin to be as active as dicloxacillin against penicillin-resistant staphylococci and to be rather less bound than dicloxacillin in human serum. The serum levels obtained in human volunteers after oral dosage, however, were found to be less than half those of dicloxacillin, and the compound would not appear to offer a clear advantage over existing isoxazolyl penicillins.

D. Quinacillin

Quinacillin (3-carboxy-2-quinoxalinylpenicillin) (Fig. 7) shows an unusual spectrum of antibacterial activity in that the compound is highly active against staphylococci but shows quite low activity against other bacteria which are usually sensitive to penicillins, for example, streptococci, micrococci, and gram-positive bacilli. Against the gram-negative bacilli, quinacillin is almost inactive (Richards *et al.*, 1963).

In subsequent studies on this compound, Hale *et al.* (1964) found quinacillin to be approximately fourfold more active than methicillin against penicillin-resistant staphylococci and almost equal in activity to cloxacillin. However, Barber and Waterworth (1964) reported quinacillin to be only twofold more active than methicillin and in comparison with cloxacillin, quinacillin appeared to be approximately fourfold less active. The activity of quinacillin against staphylococci would, therefore, seem to lie somewhere between that of methicillin and cloxacillin. Quinacillin is not highly bound to serum proteins. Bough *et al.* (1971) reported the extent of binding in human serum to be 39.5% which is similar to that of methicillin and considerably lower than that of the isoxazolyl penicillins. Barber and Waterworth (1964), however, reported that, whereas the minimum inhibitory concentration of methicillin in tests carried out in 95% human serum was the same as that in nutrient broth, the MIC of quinacillin showed a two- to fourfold increase, and the concentration required to inhibit a large inoculum in 95% serum was the same for methicillin, quinacillin, and cloxacillin.

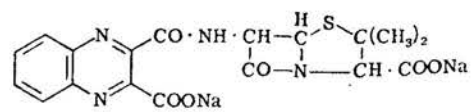


Fig. 7. Structure of quinacillin.

From a biochemical point of view, quinacillin is of particular interest in that it is a poor inducer of staphylococcal penicillinase. However, quinacillin, like methicillin and the isoxazolyl penicillins, is so highly stable to staphylococcal penicillinase that the therapeutic significance of diminished induction of penicillinase formation seems doubtful.

Although quinacillin is acid-stable, it is not absorbed in man when given by mouth (Bough *et al.*, 1971), but when administered by intramuscular injection the serum levels of quinacillin (Christie *et al.*, 1966) appear to be very similar to those obtained with the same dose of cloxacillin (Knudsen *et al.*, 1962). Limited clinical experience with quinacillin was reported to be good (Christie *et al.*, 1966), but the compound has not found a place in medicine. This may be because quinacillin does not show a clear advantage over the existing penicillins with respect to resistant staphylococci and also because the compound can only be used parenterally.

E. Staphylococci Resistant to Penicillinase-Stable Penicillins ("Methicillin-Resistant Staphylococci")

The incidence and rate of emergence of drug-resistant strains of bacteria are important aspects of the clinical usefulness of an antibiotic, and an example of this is the resistance of *Staphylococcus aureus* to the penicillinase-stable penicillins. These staphylococci are commonly referred to as methicillin-resistant staphylococci, but it should be made clear that such strains are also resistant to all the other penicillins and cephalosporins which are in clinical use.

At the time methicillin was first introduced into clinical practice the incidence in the United Kingdom of strains of *S. aureus* resistant to the drug was extremely low and possibly less than 0.1% (Jevons, 1961; Parker and Hewitt, 1970). Jevons *et al.* (1963) reported the results of testing over 27,000 strains of *S. aureus* isolated over the period 1960–1962 and found an incidence of resistant strains of 0.37%. Since that time the numbers of methicillin-resistant staphylococci appear to have increased but this may be partly as a result of the use of more sensitive test methods such as incubation at 30°C (Annear, 1968) or the use of 5% salt agar (Barber, 1964). Some indication of the incidence of methicillin-resistant staphylococci in the United Kingdom may be gained from the data of Parker and Hewitt (1970) showing the frequency of such strains isolated from lesions in patients in eight London hospitals. These results show a frequency of about 1% in the period 1964–1967, 2.5% in 1967–1968, and 5% in 1968–1969. Since 1969, however, the frequency of methicillin-resistant staphylococci in these particular hospitals has not continued to increase and for the years 1969–

1970 and 1970–1971 the frequency was found to be 2.3 and 3.0%, respectively (Parker, private communication).

Although the overall frequency of methicillin resistance is relatively low the distribution of such strains is extremely patchy. In a few hospitals a single strain has become endemic, and in such instances the methicillin-resistant strains account for a substantial proportion of the total staphylococcal isolates. One hospital in the United Kingdom, for example, has reported recently that 58% of all staphylococcal infections were caused by methicillin-resistant strains (Hale and Selkon, 1970). In other hospitals, on the other hand, methicillin-resistant staphylococci have been encountered only rarely (Ridley *et al.*, 1970). In addition to the uneven distribution of strains within a particular country, there is also a marked difference in the frequency of methicillin-resistant staphylococci in the different countries of the world. In the United States, for example, although methicillin-resistant strains were encountered as early as 1960 (Barrett *et al.*, 1968), they have remained relatively uncommon and, up to 1967, there had been only 1 well-documented case of serious infection caused by a methicillin-resistant staphylococcus (Bulger, 1967). More recently, an incidence of methicillin-resistant strains of 1.4% has been reported for the Boston City Hospital (Barrett *et al.*, 1968), and O'Toole *et al.* (1970) have reported an outbreak in Seattle involving 6 cases of infection due to type-84 methicillin-resistant *S. aureus*. In contrast, in the Zurich canton of Switzerland as early as 1966, 17% of staphylococcal infections were caused by methicillin-resistant strains (Benner and Kayser, 1968) and a high incidence of such strains has been reported in other parts of Europe, notably in France (Bastin *et al.*, 1967) and Denmark (Jessen *et al.*, 1969).

The frequency of methicillin-resistant staphylococci in different countries, and in different hospitals within a given country, does not appear to be related to the usage of methicillin and similar penicillins. For example, significant numbers of resistant strains were encountered in Poland (Parnas and Jablonski, 1961) before any of the penicillinase-stable penicillins had been used clinically in that country. Parker and Hewitt (1970) have also drawn attention to the poor correlation between the spread of methicillin-resistant staphylococci and the usage of methicillin and have pointed out that widespread dissemination of such strains has been observed in hospitals that use very little of the drug.

Although naturally occurring strains of *S. aureus* that are resistant to methicillin usually produce large quantities of penicillinase, this is not the mechanism of resistance since the penicillinase-stable penicillins are not inactivated by these staphylococci. Also, it can be shown that penicillinase-negative mutants of methicillin-resistant staphylococci have a reduced

sensitivity to penicillins and cephalosporins compared with penicillinase-negative mutants derived from strains that are sensitive to methicillin (Dyke, 1969). Methicillin-resistant staphylococci typically show a heterogeneous resistance to the drug due to the presence of a small number of cells possessing a high level of intrinsic insensitivity and which are able to grow in the presence of high concentrations of the penicillinase-stable penicillins (Sutherland and Rolinson, 1964). The proportion of resistant cells in each culture is very small and when this feature was recognized it raised the question as to whether infections caused by such strains might successfully be treated with methicillin or cloxacillin since inhibition of the majority of the bacterial population by the penicillin might allow body defense mechanisms to overcome the resistant minority. Ayliffe and Barber (1963), for example, expressed the view that it was possible that infections with these strains would respond to treatment with large doses of methicillin, and it would appear that some infections have, indeed, been treated successfully with methicillin or cloxacillin (Benner and Kayser, 1968; Turner and Cox, 1967). Nevertheless, it is also clear that serious infections do occur with methicillin-resistant staphylococci that do not respond to treatment with the penicillinase-stable penicillins (Harding, 1963; Chabbert *et al.*, 1965; Benner and Kayser, 1968; O'Toole *et al.*, 1970) or cephalosporins (Acar *et al.*, 1971).

Certain of the penicillinase-stable penicillins can appear to be slightly more active than other members of this group particularly if small inocula are tested. However, the presence of resistant cells which can grow in high concentrations of any of these penicillins can readily be demonstrated under appropriate test conditions. From the point of view of comparative evaluation of the compounds and their therapeutic usefulness the penicillinase-stable penicillins and cephalosporins should all be regarded as essentially inactive against the methicillin-resistant strains of *S. aureus*.

VI. Broad-Spectrum Penicillins

A. Aminopenicillins

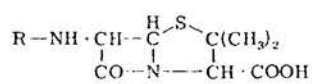
The introduction of an amino group in the α-position of the side chain of benzylpenicillin confers a high degree of acid stability together with enhanced activity against gram-negative bacteria. The resulting penicillin, ampicillin, was introduced in 1961 and has achieved considerable clinical success as an oral broad-spectrum antibiotic. More recently, two compounds closely related to ampicillin, namely, amoxycillin and epicillin, have appeared in clinical practice and a further analog of ampicillin, BL-P 875,

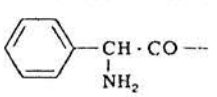
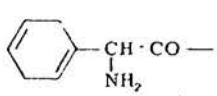
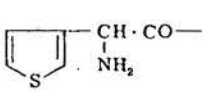
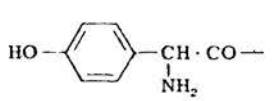
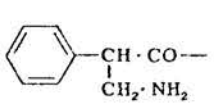
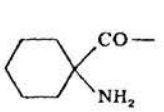
has also been evaluated in some detail. In addition to these penicillins with an α-amino group, data on two other aminopenicillins, betacin and cyclacillin, have been published and the latter compound has recently become available for clinical use. Certain "pro-penicillins" that hydrolyze in the body to give rise to ampicillin have also been introduced clinically; these compounds are hetacillin, metampicillin, and pivampicillin.

The main characteristics of this group of aminopenicillins include a wide spectrum of activity against gram-positive and gram-negative bacteria and a low degree of binding to serum proteins. Most of these penicillins are also well absorbed when given by mouth (Table XII).

TABLE XII

AMINOPENICILLINS



Penicillin	Structure of side chain R
Ampicillin (D(−)-α-aminobenzylpenicillin)	
Epicillin (D-α-amino(1,4-cyclohexadien-1-yl)-methylpenicillin)	
BL-P 875 (D-α-amino-3-thienylmethylpenicillin)	
Amoxycillin (D(−)α-amino-*p*-hydroxybenzylpenicillin)	
Betacin (D(−)α-phenyl-β-aminoethylpenicillin)	
Cyclacillin (1-aminocyclohexylpenicillin)	

1. *Ampicillin*

One of the original objectives of the work on derivatives of 6-aminopenicillanic acid was to obtain penicillins with a broader spectrum of activity than that shown by benzylpenicillin, and this was realized with the preparation of α-aminobenzylpenicillin. This compound was originally prepared as the DL-racemic mixture with the laboratory number BRL 1060, but it was subsequently shown that the D(−) isomer was significantly more active than the L(+) epimer, and the compound in clinical use with the generic name of ampicillin is D(−)-α-aminobenzylpenicillin (Table XII).

Ampicillin differs from benzylpenicillin not only in its antibacterial spectrum but also in the fact that it is highly stable under acid conditions. At pH 2 and 37°C the loss of activity after 2 hours is no more than 5%, and even after storage overnight under these conditions nearly 50% of the activity is retained. For oral administration, ampicillin is generally used in the form of the free acid, either as the trihydrate or as the anhydrous compound, but more recently the potassium salt has also been used as an oral preparation (Cahn and Levy, 1969). The free acid of ampicillin shows low solubility in water and for parenteral administration the sodium salt is used.

The antibacterial activity of ampicillin was first described by Rolinson and Stevens (1961) and these data have subsequently been confirmed and extended by many other workers including Auhagen *et al.* (1962b), Ross *et al.* (1962), Klein and Finland (1963a), Anderson *et al.* (1964). A comparison of the activity of ampicillin with that of benzylpenicillin is shown in Table II.

Against the pyogenic streptococci, pneumococci, and penicillin-sensitive staphylococci, ampicillin is only slightly less active than benzylpenicillin and against *Streptococcus viridans* the level of activity is equal or slightly greater. Ampicillin is also somewhat more active than benzylpenicillin against *Streptococcus faecalis*. As with benzylpenicillin, however, the level of activity of ampicillin against enterococci is considerably lower than against other gram-positive cocci. Against *S. faecalis*, ampicillin shows a synergistic effect in combination with aminoglycoside antibiotics, including streptomycin, kanamycin, and gentamicin as is the case with benzylpenicillin (Sonne and Jawetz, 1968; Wilkowske *et al.*, 1971). Ampicillin is comparable with benzylpenicillin in activity against *Corynebacterium diphtheriae*, *Clostridia*, and *Actinomyces israelii* (Blake, 1963) and is more active than benzylpenicillin against *Listeria monocytogenes* (Seeliger *et al.*, 1967). Ampicillin is not stable to staphylococcal penicillinase and, consequently, is not active against penicillin-resistant staphylococci.

In general, the gram-negative bacteria are less sensitive to ampicillin

than are the gram-positive organisms, but the gram-negative cocci are highly sensitive. The meningococci are sensitive to a concentration of 0.05 μg/ml and in this respect the activity of ampicillin is comparable with that of benzylpenicillin. The gonococci show an interesting pattern of sensitivity in that, although ampicillin is slightly less active than benzylpenicillin against the strains that are fully sensitive to penicillin, against the penicillin-resistant strains showing MIC values of 0.5 μg/ml or greater, ampicillin is 2–4 times more active than benzylpenicillin (Odegaard, 1962; Reyn and Bentzon, 1968; Keys *et al.*, 1969; Johnson *et al.*, 1970). Other gram-negative bacteria against which ampicillin is particularly active include *Haemophilus influenzae*, *Bordetella pertussis*, *Brucella* species, and *Pasteurella* species and these are generally inhibited at concentrations of 0.1 to 0.5 μg/ml.

Against the gram-negative bacilli, including *Escherichia coli*, *Shigella* species, *Salmonella* species, and *Proteus mirabilis*, ampicillin is 5–10 times more active than benzylpenicillin, and most strains of these bacteria are inhibited by concentrations of 1 to 5 μg/ml. To some extent this enhanced activity against gram-negative bacilli, compared with benzylpenicillin, is due to the greater stability of ampicillin to the β-lactamases produced by these organisms (Ayliffe, 1963; Smith, 1963; Sutherland, 1964). However, sensitive strains of *P. mirabilis* and *Salmonella typhi* do not appear to produce β-lactamase, and against these organisms the enhanced activity of ampicillin may be due to greater reactivity with the target site or to better penetration of the bacterial cell wall. In this connection, Izaki *et al.* (1968) have shown that ampicillin and benzylpenicillin were equally effective in inhibiting an isolated transpeptidase system from *E. coli*, whereas the intact bacterial cells were considerably more sensitive to ampicillin than to benzylpenicillin.

Although ampicillin shows a broad spectrum of activity most strains of indole-positive *Proteus*, *Klebsiella/Enterobacter* species, *Serratia*, *Mimeae* and *Herrellea*, *Bacteroides fragilis*, and *Pseudomonas aeruginosa* are resistant. Bacteria resistant to ampicillin are often found to produce β-lactamase which is able to inactivate the drug and in some cases this forms the basis of the resistance. Certain β-lactamases produced by ampicillin-resistant organisms can be competitively inhibited by other penicillins, including methicillin and cloxacillin, which are themselves stable to these β-lactamases (Hamilton-Miller *et al.*, 1964; Sutherland and Batchelor, 1964; Cole *et al.*, 1972). As a result, a combination of one of these penicillins with ampicillin can show a synergistic effect against certain ampicillin-resistant organisms. However, the concentration of the competitive inhibitor required to bring about a therapeutically useful degree of synergism is relatively high and the number of pathogens for which such synergism

can be demonstrated is rather low (Sutherland and Batchelor, 1964; Bach *et al.*, 1967).

In addition to the production of a β-lactamase, many ampicillin-resistant bacteria are intrinsically insensitive and this may be due to decreased permeability of the cell (Smith *et al.*, 1969). The enhanced activity of ampicillin against resistant bacteria in the presence of ethylenediaminetetraacetate (EDTA) is consistent with such a permeability barrier (Weisser *et al.*, 1968).

Despite the fact that ampicillin has been in widespread clinical use since 1961, there are few surveys published concerning changes in the incidence or resistance to the drug. As already mentioned, ampicillin resistance in many bacteria is associated with β-lactamase production and among the Enterobacteriaceae the genetic determinant for β-lactamase synthesis is frequently extrachromosomal and can readily be transferred from one bacterial cell to another by conjugation. Moreover, such ampicillin R factors have clearly existed for some time. For example, transferable resistance to ampicillin has been demonstrated for strains of *E. coli* and *Shigella sonnei* isolated in Mexico in 1956 (Evans *et al.*, 1968) and in *E. coli* isolated in London in 1961 (Slocombe and Sutherland, 1969). The transfer of extrachromosomal R factors by conjugation can be very rapid, and, in view of the prevalence of transferable resistance in the Enterobacteriaceae, it might have been expected that the frequency of ampicillin resistance would have increased very considerably since 1961.

In the case of certain pathogens an increase in ampicillin resistance has, indeed, occurred, although the increase is by no means uniform in different countries nor in different areas of the same country. For example, in the United Kingdom in recent years, a marked increase in ampicillin resistance has occurred in *Salmonella typhimurium* Type 29 isolated from man and animals (Anderson and Lewis, 1965) and this resistance is transferable. Similarly, Winshell *et al.* (1970) reported that in the eastern United States the incidence of ampicillin-resistant *S. typhimurium* had increased over the period 1965–1969 reaching a figure of 23.4%; but, more recently, Cherubin *et al.* (1972) reported that the incidence in the same area in 1970 had dropped to 12.9%. In contrast to these changes in resistance in the United Kingdom and the United States, Guinée (1971) has reported that ampicillin resistance has remained relatively constant at about 10% of strains of *S. typhimurium* isolated from animals in Holland between 1966 and 1969. In *S. sonnei*, a high incidence of ampicillin resistance now exists in strains isolated in the London area (Davies *et al.*, 1970), although it is of interest that the resistance in this case does not appear to be transferable (Davies *et al.*, 1968). In the United States, a rapid increase during the past 4 years in ampicillin-resistant shigellae in the Washington D.C. area has been reported

by Ross *et al.* (1972); whereas Farrar and Eidson (1971) have reported that the incidence of ampicillin-resistant *S. sonnei* in Atlanta between 1967 and 1970 was only 3.7%.

In other pathogens there is little evidence for an increase in the incidence of ampicillin resistance. For example, in *E. coli*, although a high proportion of ampicillin-resistant strains are able to transfer their resistance (Smith and Armour, 1966; Datta, 1969; Slocombe and Sutherland, 1969), surveys indicate that the frequency of resistant strains has not increased markedly since 1961 despite extensive usage of the drug (Slocombe and Sutherland, 1969; Weil and Pagano, 1970; Gillespie *et al.*, 1971; McCracken, 1971). The incidence of ampicillin resistance in *P. mirabilis* and *H. influenzae* also appears to have remained essentially unchanged.

As with other penicillins, ampicillin is bactericidal in action and concentrations close to the minimum inhibitory values result in marked bactericidal effects against gram-positive and gram-negative bacteria. Ampicillin is also not highly bound to serum proteins (20% bound), and the antibacterial activity of the compound is not significantly influenced by the presence of serum. These properties together with high activity *in vitro* and a broad antibacterial spectrum combine to give rise to good activity against experimental infections in animals (Acred *et al.*, 1962).

Ampicillin is relatively well absorbed after oral administration in man, and from 30 to 50% of the drug is excreted as unchanged penicillin in the urine (Knudsen *et al.*, 1961; Auhagen *et al.*, 1962b; Naumann, 1962b; Klein and Finland, 1963a). Peak serum concentrations are reached at about 2 hours, after which there is a gradual fall to low but detectable levels at 8 hours. Increasing the dose results in a corresponding increase in serum concentrations, and administration of probenecid results in markedly higher serum levels. Administration of ampicillin by intramuscular injection results in serum levels 2–3 times higher than those obtained with an equivalent oral dose, and 60–70% of the dose can be recovered in the urine (Eickhoff *et al.*, 1965; Naumann, 1965). Ampicillin is excreted in the urine largely as unchanged ampicillin, and only a small proportion appears in the form of the biologically inactive penicilloic acid (Table IV). Elimination takes place via the kidneys, but a significant amount does appear in the bile and biliary concentrations are several times higher than those in the blood (Ayliffe and Davies, 1965; Mortimer *et al.*, 1969). As with other penicillins, ampicillin does not diffuse readily across the blood–brain barrier in the healthy state (Bunn *et al.*, 1963), but ampicillin may be present in the cerebrospinal fluid in significantly higher concentrations when the meninges are inflamed (Lithander, 1964). Ampicillin appears to diffuse well into other compartments of the body, and good levels have been reported to occur in lymph,

amniotic fluid, pleural fluid, and synovial fluid (Acred *et al.*, 1962, 1970a; Blecher *et al.*, 1966; Nelson, 1971; Howell *et al.*, 1972).

2. Compounds That Hydrolyze to Give Ampicillin

a. *Hetacillin.* This drug [6-(2,2-Dimethyl-5-oxo-4-phenyl-1-imidazolidinyl) penicillanic acid] is the condensation product resulting from the reaction between ampicillin and acetone (Hardcastle *et al.*, 1966) (Fig. 8). In dilute aqueous solution and at neutral pH, hetacillin hydrolyzes rapidly to liberate ampicillin and acetone, the half-life at 37°C being 20–30 minutes (Magni *et al.*, 1967; Sutherland and Robinson, 1967; Brown *et al.*, 1969; Smith and Hamilton-Miller, 1970). It is not surprising, therefore, that the antibacterial activity of hetacillin *in vitro* should appear indistinguishable from that of ampicillin, and it may be that hetacillin is itself devoid of antibacterial activity prior to its hydrolysis to ampicillin (Sutherland and Robinson, 1967; Smith and Hamilton-Miller, 1970; *Medical Letter*, 1971). Hydrolysis of hetacillin to ampicillin also takes place rapidly in the body. Sutherland and Robinson (1967) have reported that following oral administration of hetacillin to man, only trace amounts of unchanged hetacillin could be detected and these were only present during the first 90 minutes after dosing. From 90 minutes onward, only ampicillin could be detected, and at no time was the amount of unchanged hetacillin more than a very small proportion of the total antibiotic present in the blood. Simi-

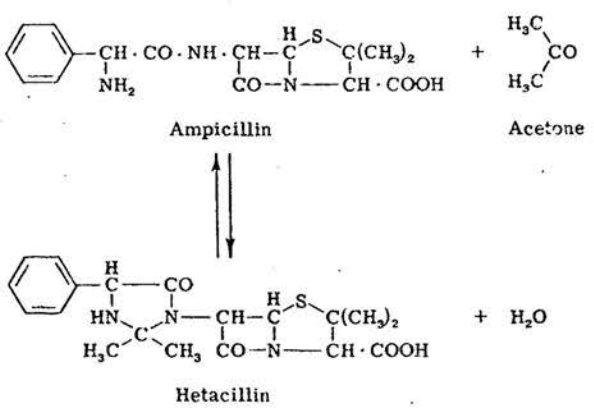


Fig. 8. Reaction between ampicillin and acetone to form hetacillin.

larly, Brown *et al.* (1969) reported that, following intramuscular administration of hetacillin, hydrolysis to ampicillin was rapid.

In the initial studies on absorption of hetacillin given by mouth the peak levels of antibiotic in the blood were reported to be similar to those obtained when ampicillin itself was administered (Bunn *et al.*, 1966; Kirby and Kind, 1967). The peak levels with hetacillin were reached somewhat later than with ampicillin and as a result the blood levels obtained with hetacillin were reported to be slightly more prolonged. These findings have not been entirely confirmed by later work, and the studies reported by Magni *et al.* (1967), Ridley (1967), Sutherland and Robinson (1967), Modr *et al.* (1970), and Kahrimanis and Pierpaoli (1971) show that the peak antibiotic levels following administration of hetacillin are substantially lower than those obtained with ampicillin itself. These studies do confirm, however, that absorption is rather slower with hetacillin and that the blood levels at 6 to 8 hours after dosing are marginally higher than those obtained with ampicillin, but it is questionable whether this is of therapeutic significance.

In concentrated aqueous solutions, such as are used for parenteral administration, hetacillin has been reported to be more stable than ampicillin (Kahrimanis and Pierpaoli, 1971). However, when administered intramuscularly, peak blood levels obtained with hetacillin are considerably lower than those obtained with ampicillin (Bunn *et al.*, 1966; Sutherland and Robinson, 1967), and although they are slightly more prolonged it is doubtful whether this is of clinical importance. The conclusion reached in the *Medical Letter* (1972) is that hetacillin does not offer any advantage over ampicillin.

b. *Metampicillin.* The approved name for the compound obtained by reaction of ampicillin with formaldehyde is metampicillin (Fig. 9). This drug has been described as α-methyleniminobenzylpenicillin, but it is possible that the hydrated form, namely, α-hydroxymethylaminobenzylpenicillin may also be present. Metampicillin was first described as a new broad-spectrum penicillin by Ferrero (1967) who also reported the compound to be more stable than ampicillin to the β-lactamase produced by the bacterial flora of the rat intestine. In subsequent studies, metampicillin was reported to give rise to higher blood levels than ampicillin after oral and intramuscular administration to man (Franchi and Perraro, 1967) and to result in significantly higher levels in bile than obtained with ampicillin when the drugs were given parenterally (de Vecchi Pellati *et al.*, 1970; Lande, 1970).

Metampicillin does, indeed, show a broad spectrum of antibacterial activity, but the report by Ferrero (1967) failed to mention that metampicillin hydrolyzes rapidly in dilute aqueous solution to give ampicillin, the half-

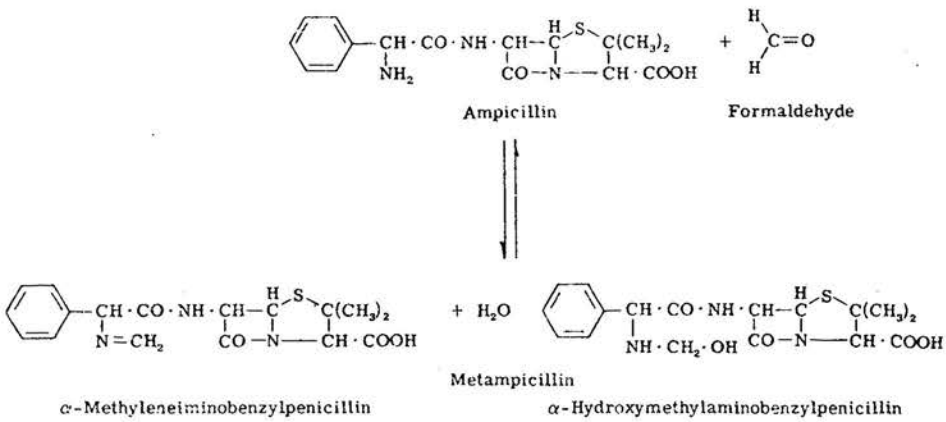


FIG. 9. Reaction between ampicillin and formaldehyde to form metampicillin.

life at neutral pH and 37°C being approximately 60 minutes (Sutherland *et al.*, 1972b). It is probable, therefore, that the activity of metampicillin in such tests was largely due to the liberation of ampicillin. In this connection, Sutherland *et al.* (1972b) have reported that the antibacterial spectrum and level of activity shown by metampicillin is very similar to that of ampicillin and that neither compound showed significant activity against penicillinase-producing bacteria.

Following oral administration to man, Sutherland *et al.* (1972b) reported that unchanged metampicillin could not be detected either in blood or in urine and ampicillin alone was present, although the levels of ampicillin in the blood were somewhat lower than those obtained following an equivalent dose of ampicillin itself. These results are in contrast with those of Franchi and Perraro (1967) who reported that antibiotic levels after administration of metampicillin were higher than those obtained with ampicillin. Franchi and Perraro, however, used metampicillin standards to assay the levels in the blood and, since the resulting antibiotic is ampicillin, which in blood gives rise to larger zones of inhibition than does metampicillin, the assay for metampicillin would be erroneously high.

Following intramuscular injection of metampicillin, Sutherland *et al.* (1972b) have reported that the antibiotic present in the blood was predominantly ampicillin, but small amounts of unchanged metampicillin were detected up to 1 hour after dosing. Urine specimens showed the presence of ampicillin only. The same authors also reported that the levels of ampicillin in the blood following intramuscular administration of metampi-

cillin were lower than those normally obtained following injection of ampicillin itself.

Metampicillin can be detected in the blood more readily after intramuscular injection than after oral dosage, probably because hydrolysis of metampicillin is very rapid under acid conditions and the compound may largely undergo hydrolysis in the stomach before being absorbed. At neutral pH, metampicillin is more stable and after parenteral administration complete hydrolysis does not occur so rapidly. Also metampicillin hydrolyzes slowly in human serum probably as a result of binding to serum proteins. These factors may account for the presence of small quantities of unhydrolyzed metampicillin in blood shortly after intramuscular administration. The exact extent of protein binding of metampicillin is not known, but the antibacterial activity is greatly diminished in the presence of serum.

The relatively slow rate of hydrolysis in serum may account for the fact that antibiotic levels in the bile appear to be higher following parenteral administration of metampicillin than they are with ampicillin dosage (de Vecchi Pellati *et al.*, 1970; Lande, 1970). In these studies, however, it is not clear whether the antibiotic in the bile was assayed in terms of metampicillin or ampicillin and the results reported are, therefore, somewhat difficult to interpret, but it may well be that metampicillin is rapidly eliminated in the bile and then undergoes hydrolysis to ampicillin.

c. *Pivampicillin.* Efforts to improve the absorption characteristics of penicillins have included esterification of the 3-carboxyl group. An example of such a penicillin ester is penamecillin, the acetoxymethyl ester of benzylpenicillin (Agersborg *et al.*, 1966), which has been in clinical use for a number of years, although in this case the levels of benzylpenicillin obtained in the serum after administration of penamecillin are only slightly higher than those obtained with the sodium salt of benzylpenicillin itself (Agersborg *et al.*, 1966; Hulbert, 1972).

More recently a series of hydrolyzable acyloxymethyl esters of ampicillin have been described with the claim for substantial improvement in absorption resulting in significantly higher levels of ampicillin after oral administration than are obtained with ampicillin itself (von Daehne *et al.*, 1970). The preferred compound, the pivaloyloxymethyl ester of ampicillin, pivampicillin (Fig. 10), has been reported to give ampicillin serum concentrations in man 2–3 times greater than those obtained with an equivalent dose of ampicillin (von Daehne *et al.*, 1971; Foltz *et al.*, 1971; Jordan *et al.*, 1971).

Pivampicillin is relatively stable in acid solution and hydrolyzes slowly in neutral solution with the liberation of ampicillin, but hydrolysis is very much more rapid in the presence of serum, blood, or tissue homogenates of various animal species (von Daehne *et al.*, 1971). It is believed that after

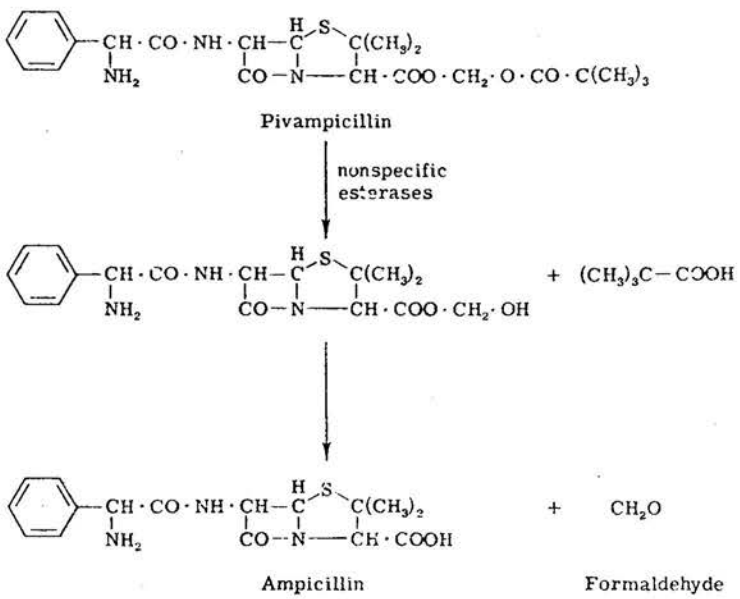


FIG. 10. Structure of pivampicillin and its hydrolysis to ampicillin.

oral administration, pivampicillin is absorbed intact and is then hydrolyzed by nonspecific esterases to give pivalic acid and the unstable hydroxymethyl ester of ampicillin which, in turn, decomposes spontaneously into ampicillin and formaldehyde (Fig. 10) (von Daehne *et al.*, 1971). It is clear that hydrolysis of the ester in the body is very rapid after oral administration, and in the study of von Daehne *et al.* (1971) in man, the concentration of the ester in the blood at no time exceeded 2% of the ampicillin concentration and within 15 minutes more than 99% of the drug was found to be present in the form of ampicillin.

Studies in human volunteers have shown that pivampicillin is absorbed more rapidly and more completely than ampicillin. After oral administration of pivampicillin in man, the resulting peak levels of ampicillin in the serum were reached approximately 1 hour after dosage and were 2–3 times higher than the peak levels obtained with ampicillin itself which were reached 1.5–2 hours after dosage (von Daehne *et al.*, 1971; Foltz *et al.*, 1971; Jordan *et al.*, 1971). It should be noted, however, that these studies were carried out using an equivalent dosage of ampicillin, and 358 mg of pivampicillin are required to contain the equivalent of 250 mg of ampicillin. The superior absorption of pivampicillin, compared with ampicillin, is also

reflected in the proportion of the dose recovered in the urine, the values given by von Daehne *et al.* (1971), Foltz *et al.* (1971), and Jordan *et al.* (1971) range from 29 to 46% with ampicillin and from 56 to 73% with pivampicillin. Foltz *et al.* (1971) and Jordan *et al.* (1971) have reported that food did not adversely affect the absorption of pivampicillin, whereas with ampicillin absorption was significantly depressed. Data obtained in the authors' laboratory, however, indicate that food interferes slightly with the absorption of pivampicillin and ampicillin and to a similar extent with both compounds.

Although pivampicillin hydrolyzes rapidly to ampicillin in the body, Foltz *et al.* (1971) reported evidence of hepatotoxicity in the dog during the course of toxicological studies, but it was noted that an impurity was present in the pivampicillin used. In view of these effects, clinical studies in the United States were being held in abeyance pending further investigations, although the drug has now been approved for clinical use in certain other countries.

3. *Analogs of Ampicillin*

a. *Epicillin.* The generic name epicillin has been assigned to the structural analog of ampicillin [D-amino(1,4-cyclohexadien-1-yl) methylpenicillin] (Table XII) in which the benzene ring has been replaced by the partially reduced 1,4-cyclohexadienyl system (Dolfini *et al.*, 1971). The chemical and biological properties of epicillin, including acid stability, penicillinase stability, serum binding, and oral absorption are essentially the same as those of ampicillin; also the antibacterial spectrum and the level of activity of epicillin are virtually identical with those of ampicillin both *in vitro* (Basch *et al.*, 1971) and against experimental infections in mice (Gadebusch *et al.*, 1971).

Although the two compounds are very similar in antibacterial activity, Basch *et al.* (1971) have reported that epicillin was found to be more active than ampicillin against *Pseudomonas aeruginosa* and that 50% of 56 clinical isolates were sensitive to epicillin at 100 μg/ml, whereas only 12.5% were inhibited by the same concentration of ampicillin. Data obtained in the authors, laboratory confirm that epicillin is very slightly more active than ampicillin against *P. aeruginosa*, but both compounds show a very low level of activity against this organism. Compared with carbenicillin, both epicillin and ampicillin are at least 10 times less active, and it is doubtful whether the difference between epicillin and ampicillin is of any clinical significance.

A number of clinical studies have demonstrated the efficacy of epicillin against infections due to sensitive gram-positive and gram-negative bacteria, but it remains to be seen whether epicillin will show any therapeutic advantage compared with ampicillin.

b. *BL-P 875.* This drug (α-amino-3-thienylmethylpenicillin) is a close analog of ampicillin in which the benzene ring has been replaced by a thiophene ring (Table XII). The antibacterial spectrum and level of activity *in vitro* are indistinguishable from those of ampicillin, but against experimental infections in mice the compound has been reported to be up to twice as active as ampicillin (Price *et al.*, 1969a). Blood levels following oral administration to mice were also higher than those obtained with ampicillin, and Price and his colleagues concluded that if this proved to be also the case in man the compound might justify clinical trial. No further reports have appeared in the literature.

c. *Amoxycillin.* This drug [D(−)-α-amino-*p*-hydroxybenzylpenicillin trihydrate] is a close analog of ampicillin (Table XII) in which a hydroxy group has been introduced in the para position of the benzene ring of the side chain. Like ampicillin, amoxycillin is acid-stable and shows an antibacterial spectrum and a level of activity very similar to those of ampicillin (Neu and Winshell, 1971b; Sutherland and Rolinson, 1971). The extent of binding to serum proteins is also the same as that of ampicillin.

Amoxycillin differs from ampicillin in that it is better absorbed when given by mouth, both in animals (Acred *et al.*, 1971b) and in man (Neu and Winshell, 1971c; Bodey and Nance, 1972; Gordon *et al.*, 1972; Kosmidis *et al.*, 1972; Sutherland *et al.*, 1972a), and, as a result, the levels reached in the blood and in the urine, are considerably higher than those obtained with ampicillin. In studies in human volunteers, following oral administration, the peak serum levels are reached at the same time as those of ampicillin but are about twice as high, and the total area under the serum curve is also approximately double that for ampicillin. Over the first 6 hours after dosing, some 60–80% of the dose can be recovered in the urine as unchanged penicillin (Neu and Winshell, 1971c; Sutherland *et al.*, 1972a).

The presence of food does not significantly decrease the amount of amoxycillin absorbed in volunteers (Neu and Winshell, 1971c; Sutherland *et al.*, 1972a), but, if the drug is taken immediately before or after a meal, absorption is delayed slightly, as is the case also with ampicillin. Administration of probenecid results in higher and more prolonged blood levels.

Against experimental infections in mice the activity of amoxycillin is of particular interest (Acred *et al.*, 1971b). When given by mouth, amoxycillin is more active than ampicillin. This may not be unexpected because the

compound is better absorbed in mice as it is in man and the blood levels are considerably higher than they are with ampicillin. When given subcutaneously to mice, however, the blood levels of amoxycillin and ampicillin are essentially the same, yet amoxycillin is again more active. Recent results obtained in the authors' laboratory indicate that, *in vivo*, amoxycillin has a more rapid and more complete bactericidal effect than ampicillin against certain gram-negative bacilli and this may account for the superior activity of amoxycillin against experimental infections even when the blood levels for the two compounds are similar.

4. *Betacin*

This drug [D(−)-α-phenyl-β-aminoethylpenicillin] is a β-amino analog of ampicillin (Table XII). The antibacterial spectrum of this compound is very similar to that of ampicillin (Testa *et al.*, 1964; Acocella *et al.*, 1965), activity against gram-negative bacteria being comparable with that of ampicillin but, against the gram-positive organisms, betacin is somewhat less active.

Betacin differs markedly from ampicillin in that it is poorly absorbed in man when given by mouth (Acocella *et al.*, 1965). The poor oral absorption of betacin is apparently not due to lack of acid stability since the compound is similar to ampicillin in this respect. Nevertheless, the peak serum concentrations obtained in human subjects with a 500-mg dose of betacin were reported to be 10–20 times lower than those normally obtained with an equivalent dose of ampicillin. Poor absorption was also reflected in the fact that only 2.5% of the dose was recovered in the urine over the 9-hour period after dosing. On the other hand, after intramuscular administration, peak serum levels ranging from 2.6 to 5.8 μg/ml were measured 1 hour after a dose of 250 mg of betacin and 55% of the dose was excreted in the urine. Although betacin is relatively well absorbed when given by intramuscular injection the compound would not appear to offer any advantage over ampicillin. No clinical data have been reported for this compound.

5. *Cyclacillin*

Alburn *et al.* (1968) reported the preparation of a number of aminoalicyclic penicillins, one of which was 1-aminocyclohexylpenicillin which has subsequently been given the approved name of cyclacillin (Table XII). These authors considered it would be of interest to prepare such penicillins since it was known that 1-aminocyclopentane carboxylic acid is rapidly

absorbed and transported like natural L-amino acids and is also slowly excreted.

Cyclacillin has been described by Yurchenco *et al.* (1970) as having a broad spectrum of activity against gram-negative and gram-positive bacteria, including penicillin-resistant staphylococci. The level of activity of cyclacillin *in vitro*, however, is relatively low (Roserman *et al.*, 1968; Yurchenco *et al.*, 1970). Compared with ampicillin, for example, cyclacillin is 5–20 times less active against penicillin-sensitive staphylococci and Group A streptococci, and 20–50 times less active against *Streptococcus pneumoniae*. Against the gram-negative bacilli, cyclacillin is only active at levels of 25 μg/ml or greater, and in this respect the compound is some 10–20 times less active than ampicillin. Like ampicillin, cyclacillin is inactive against *Klebsiella* and *Pseudomonas*. Against penicillinase-producing staphylococci, Rosenman *et al.* (1968) reported cyclacillin to be about twice as active as ampicillin and to show MIC values of 3.9 to 125 μg/ml with a mean of 25.3 μg/ml, whereas the data of Yurchenco *et al.* (1970) show cyclacillin to be only equal to ampicillin in activity *in vitro*. Data obtained in the authors' laboratory indicate that cyclacillin is marginally more active than ampicillin against penicillinase-producing staphylococci but activity is greatly influenced by inoculum size.

The binding of cyclacillin to serum proteins is low and similar to that of ampicillin (approximately 20% bound in human serum) and activity is not significantly reduced in the presence of serum.

Against experimental infections in mice, cyclacillin has been reported to be considerably more active than might have been expected on the basis of *in vitro* activity (Hopper *et al.*, 1968; Yurchenco *et al.*, 1968, 1970). Against gram-positive penicillin-sensitive organisms, cyclacillin was found to be comparable with ampicillin in activity *in vivo*, although against gram-negative organisms cyclacillin was two- to fivefold less active. Yurchenco and co-workers offered no explanation for the relatively good activity of cyclacillin *in vivo*, but data obtained in the author's laboratory show that cyclacillin is extremely well absorbed in mice when given by mouth, and peak serum concentrations are obtained which are 5–10 times higher than those of ampicillin (Acred, unpublished data). Blood levels of cyclacillin in the mouse are also slightly more prolonged than those of ampicillin. It seems likely, therefore, that the relatively good activity of cyclacillin in the mouse is due to good absorption which offsets its low *in vitro* activity. Whether this will apply in man is not clear.

Data on absorption and excretion of cyclacillin after oral dosage in man have been published by Mashimo *et al.* (1970), Miki *et al.* (1970), Okubo *et al.* (1970), and Ueda *et al.* (1970). Absorption of cyclacillin was found to

be rapid, and peak levels of 10 to 18 μg/ml were obtained 30–60 minutes after a dose of 500 mg. These levels are 2–3 times higher than those normally obtained with ampicillin. After reaching the peak value, however, the blood levels of cyclacillin were found to fall rapidly and only trace amounts could be detected at 4 and 6 hours. Over the first 6 hours after dosing, approximately 50% of the drug could be recovered in the urine. It seems clear that cyclacillin is well absorbed in man when given by mouth but it remains to be seen whether this is sufficient to compensate for the low level of antibacterial activity.

In studies in man, Martin and White (1971) have reported that, although cyclacillin was effective in suppressing penicillin-sensitive staphylococci in nasal carriers, it failed to inhibit penicillin-resistant strains. These authors concluded that it was unlikely that cyclacillin would be effective against infections caused by penicillin-resistant staphylococci. Similarly, in the monkey, cyclacillin failed to protect against a lethal staphylococcal infection which was treated successfully with nafcillin, although high concentrations of cyclacillin were measured in the experimental animals after oral dosage (Carlisle and Saslaw, 1971).

Further data will be required to assess the clinical value of this penicillin.

B. Penicillins Active against *Pseudomonas*

Activity against *Pseudomonas aeruginosa* is not a property usually associated with penicillin. Certain semisynthetic penicillins containing acidic side chains, however, do show important activity against this pathogen. The penicillins comprising this group (Table XIII), namely, carbenicillin, ticarcillin, sulfocillin, and suncillin, have a number of biological properties in common, of which the most important is the activity against *P. aeruginosa*. In addition, however, these penicillins show significant activity against other gram-negative bacilli, including certain bacteria that are generally resistant to ampicillin, for example, the indole-positive *Proteus* and *Enterobacter*, although strains of *Klebsiella* are generally resistant. A further property in common is a marked degree of stability to staphylococcal penicillinase. This results in some degree of activity against penicillin-resistant staphylococci, but it is doubtful whether this is adequate to be of clinical importance. The penicillins of this group also share the properties of not being highly bound to serum proteins and not being absorbed when given by mouth. In addition to these penicillins with acidic side chains, certain *N*-acylated derivatives of ampicillin also show marked activity against *Pseudomonas*, and data have been published on three compounds of this type in which the α-substituent in the side chain incorporates a ureido group.

1. *α-Carboxypenicillins*

a. *Carbenicillin.* This drug (disodium α-carboxybenzylpenicillin) was the first penicillin to be described showing significant activity against *Pseudomonas aeruginosa*, and the biological properties of this compound have been reported by numerous authors (Brumfitt *et al.*, 1967; Knudsen *et al.*, 1967; Bodey and Terrell, 1968; Smith and Finland, 1968; Silverblatt and Turck, 1969; Standiford *et al.*, 1969). Carbenicillin used clinically is a mixture of two diastereoisomers. The two epimers have been isolated in pure form and found to be indistinguishable in their antibacterial activity (Butler *et al.*, 1970). This may well be due to rapid epimerization under the conditions of the test. In many respects the antibacterial spectrum of carbenicillin resembles that of ampicillin (Table II). The compound is active against *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and penicillin-sensitive staphylococci, although the level of activity is very much lower than that of ampicillin. Against *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*, carbenicillin is highly active and comparable in this respect with ampicillin. Carbenicillin also shows activity close to that of ampicillin against *Escherichia coli*, *Proteus mirabilis*, *Salmonella* species, and *Shigella* species and, in keeping with ampicillin, carbenicillin is not active against most strains of *Klebsiella*. Carbenicillin available commercially contains a small amount of benzylpenicillin present as an impurity. This does not affect significantly the activity of carbenicillin in terms of the MIC's, but for the assay of carbenicillin in serum or other body fluids it is advisable to use a test organism that is sensitive to carbenicillin but resistant to benzylpenicillin (Burnett and Sutherland, 1970).

Carbenicillin is not highly bound to serum proteins. Knudsen *et al.* (1967) reported the extent of binding in human serum to be 47% which is similar to that of benzylpenicillin. Antibacterial activity is not significantly diminished in the presence of serum.

Although the activity of carbenicillin resembles that of ampicillin in many respects, there are also important differences. The greatly diminished level of activity against pyogenic cocci has been referred to and the activity of carbenicillin against *Streptococcus faecalis* is also very low compared with that of ampicillin. Unlike ampicillin, carbenicillin shows a distinct degree of stability to staphylococcal penicillinase. Most strains of penicillin-resistant staphylococci are inhibited by carbenicillin at a level of approximately 25 μg/ml even when a heavy inoculum is used, but this is probably not of clinical value. Carbenicillin does show important activity, however, against most strains of indole-positive *Proteus* species, i.e., *P. morganii*, *P. rettgeri*, and *P. vulgaris*, and the compound is also active against the

majority of strains of *Enterobacter* and *Citrobacter* (Slocombe and Sutherland, 1970). In general, strains of *E. coli* and paracolon resistant to ampicillin are also resistant to carbenicillin, but some strains that are resistant to ampicillin but relatively sensitive to carbenicillin have been encountered (Brumfitt *et al.*, 1967; Rolinson and Sutherland, 1968). Neu and Swarz (1969) have shown that in the case of strains of β-lactamase-producing *E. coli* in which the enzyme is located superficially and is episomally mediated, there is a high level of resistance to both ampicillin and carbenicillin, but strains in which the β-lactamase is controlled chromosomally are somewhat less resistant to ampicillin and are sensitive to carbenicillin.

The activity of carbenicillin also differs notably from that of ampicillin with respect to *P. aeruginosa*. Using a moderately large inoculum, most strains of *P. aeruginosa* are inhibited by carbenicillin at a level of 50–100 μg/ml (Knudsen *et al.*, 1967), whereas ampicillin is virtually inactive against this organism. Ampicillin itself is relatively stable to the β-lactamsae produced by most strains of *P. aeruginosa* but lacks intrinsic activity. Carbenicillin is also highly stable to this β-lactamase and, in addition, is able to reach the target site in the bacterial cell.

The activity of carbenicillin against *P. aeruginosa* is somewhat complicated by the fact that in serial dilution tests a sharp end point is not obtained when the inoculum is very heavy (Knudsen *et al.*, 1967). In tests carried out on agar, growth is usually diminished very markedly at a concentration of approximately 25 μg/ml, but some sparse growth may be seen even at a concentration as high as 125 μg/ml and this meager growth is more readily apparent in broth dilution tests than it is on agar. With a somewhat smaller inoculum, however, a relatively sharp end point is obtained. This phenomenon of "tailing" is not the result of drug inactivation, nor does it appear to be due to the selection of resistant mutants. Sensitivity tests carried out on the growth persisting at high concentrations usually indicated unchanged sensitivity, although Bell and Smith (1969) have reported that by careful colony isolation, resistant variants can be picked off from the film of growth occurring at high concentrations of carbenicillin. However, this would not seem to explain the results obtained in liquid culture in which a period of marked bactericidal activity can occur which is then followed by extensive bacterial growth (Knudsen *et al.*, 1967) which may reach visible proportions. Yet, in tests using this growth as inoculum, the sensitivity appears unchanged and a period of bactericidal action is again followed by regrowth.

Against strains of *P. aeruginosa*, carbenicillin frequently shows a synergistic action in combination with gentamicin and this can be seen *in vitro* (Brumfitt *et al.*, 1967; Rolinson and Sutherland, 1968; Phair *et al.*, 1969;

Sonne and Jawetz, 1969) and *in vivo* (Acred *et al.*, 1971a; Koničková and Prát, 1971). However, some degree of inactivation of gentamicin has been reported to occur in combination with carbenicillin (McLaughlin and Reeves, 1971), but the rate of inactivation is extremely slow and it is doubtful whether it is of any clinical significance when the two drugs are used in combination (Eykyn *et al.*, 1971; Klastersky, 1971; Levison and Kaye, 1971; Lynn, 1971; Riff and Jackson, 1971).

When carbenicillin was first introduced to medicine, it was appreciated that strains of *P. aeruginosa* show a range of MIC values and therefore do not show a uniform level of sensitivity to the drug. Some strains of *P. aeruginosa*, notably those isolated from sputum, are highly sensitive and inhibited by levels of 1–2 μg/ml. The majority of strains, however, show MIC values of 50–100 μg/ml, and some are only inhibited by concentrations as high as 500 μg/ml. In these strains, sensitivity to carbenicillin is not related to drug inactivation. Strains of *P. aeruginosa* typically produce a β-lactamase but this does not inactivate carbenicillin, and the relative insensitivity of some strains is the result of intrinsic resistance. More recently, however, Lowbury *et al.* (1969) and Newsom (1969) have reported the isolation of a small number of carbenicillin-resistant strains of *P. aeruginosa* which produce a β-lactamase that does rapidly inactivate carbenicillin. In the strain reported by Newson (1969) the genetic control appears to be chromosomal, but in the strains isolated by Lowbury *et al.* (1969) the β-lactamase is transferable and is similar to the R-factor-mediated β-lactamase of *E. coli* (Fullbrook *et al.*, 1970; Sykes and Richmond, 1970). It seems probable that in these strains resistance to carbenicillin has been acquired as a result of R-factor transfer from another species (Ayliffe *et al.*, 1972).

The isolation of strains of *P. aeruginosa* showing increased resistance following treatment with carbenicillin has been reported, although in some of these patients the dose of carbenicillin used was relatively low (Darrell and Waterworth, 1969; Holmes *et al.*, 1970) and the use of suboptimum doses may well contribute to the *in vivo* emergence of resistance (Marks and Eickhoff, 1970). By using high dosage of carbenicillin, Bodey *et al.* (1971) have reported that emergence of carbenicillin-resistant *Pseudomonas* has not been a problem despite widespread use of the drug.

Carbenicillin is not absorbed when given by mouth but can be given by intramuscular or intravenous administration. Because of the relatively low level of activity of carbenicillin against *P. aeruginosa*, high intravenous dosage is required in order to achieve inhibitory levels in the blood and tissues. Knudsen *et al.* (1967) reported that levels of 140 μg/ml were reached in the blood after the intravenous administration of 1 gm carbenicillin, but these levels fell rapidly to approximately 50 μg/ml after only 1 hour.

Bodey *et al.* (1969) have reported, however, that serum levels of over 300 μg/ml are achieved following an intravenous dose of 5-gm carbenicillin and that levels in excess of 100 μg/ml are maintained when 5 gm is administered over a 2-hour period every 4 hours, making a daily dose of 30 gm.

Following an intramuscular dose of 1 gm of carbenicillin, peak serum levels of about 20 μg/ml are obtained (Brumfitt *et al.*, 1967; Knudsen *et al.*, 1967; Smith and Finland, 1968), indicating good absorption although these levels would not be expected to be inhibitory against most strains of *Pseudomonas*. Levels of 2000–4000 μg/ml are achieved in the urine, however, following an intramuscular dose of 1 gm, and over 80% of the dose can be recovered in the urine. The use of probenecid results in higher and more prolonged blood levels when carbenicillin is given intramuscularly (Knudsen *et al.*, 1967) but has little effect when given intravenously (Bodey *et al.*, 1969). As with other penicillins, carbenicillin does not diffuse readily into the cerebrospinal fluid in patients with uninflamed meninges (Bodey *et al.*, 1969), but measurement of the levels of carbenicillin present in lymph in animals (Chisholm *et al.*, 1968) and man (Bodey *et al.*, 1969) indicate that the drug diffuses well into the tissues.

b. *Carbenicillin Indanyl Sodium* (*Carindacillin**). Although carbenicillin itself is not absorbed when given by mouth, certain α-carboxy esters of carbenicillin are well absorbed by this route and undergo rapid hydrolysis in the body to yield carbenicillin. One such compound is the 5-indanyl ester (Table XIII), carbenicillin indanyl sodium (English *et al.*, 1972).

In bacteriological media of the type used to determine antibacterial activity, the indanyl ester of carbenicillin hydrolyzes slowly to give carbenicillin. The resulting activity *in vitro*, therefore, is a combination of the activity of the ester per se and the activity of carbenicillin. Against the gram-positive cocci the ester shows greater activity than carbenicillin, but against the gram-negative organisms the activity seen is that resulting from the liberation of carbenicillin (English *et al.*, 1972).

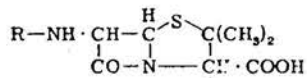
In the animal body the indanyl ester of carbenicillin hydrolyzes very much more rapidly than *in vitro*. As a result the activity of the ester *in vivo* can be regarded as that of carbenicillin, and Butler *et al.* (1971) have reported that only trace amounts of the ester could be detected in serum. Data obtained in the authors' laboratory also show that, in human volunteers, hydrolysis to carbenicillin is rapid but some unhydrolyzed ester could be detected in serum collected 30 minutes after oral dosage and in urine from 0 to 2 hours.

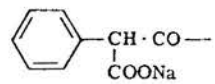
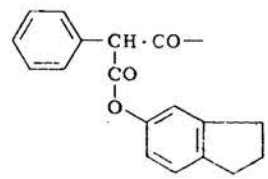
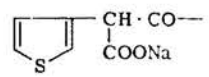
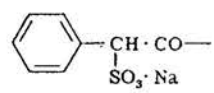
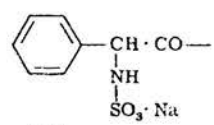
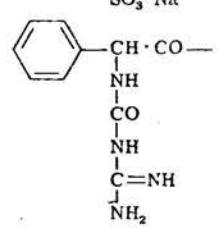
Carbenicillin indanyl sodium is highly acid-stable and is well absorbed from the gastrointestinal tract (English *et al.*, 1972). Bran *et al.* (1971) and

* Approved name, British Pharmacopeia Commission.

TABLE XIII

PENICILLINS ACTIVE AGAINST *Pseudomonas aeruginosa*



Penicillin	Structure of side chain R
Carbenicillin (disodium-α-carboxybenzylpenicillin)	
Carbenicillin indanyl sodium (5-indanyl ester of) carbenicillin)	
Ticarcillin (α-carboxy-3-thienylmethylpenicillin)	
Sulfocillin (α-sulfobenzylpenicillin	
Suncillin (α-sulfoaminobenzylpenicillin)	
BL-P 1654 [D-α-(3-guanylureido)benzylpenicillin]	

Wallace *et al.* (1971) have reported that peak levels of carbenicillin of about 10 μg/ml are obtained 1–2 hours after a single 1-gm dose of the ester in man, although Burgy (1972) has reported levels of only 11.2 μg/ml after a 2-gm dose. About 30–35% of the dose can be recovered in the urine from

0 to 6 hours, and concentrations of carbenicillin of approximately 1000 μg/ml are obtained in the urine with a dose of 1 gm. With regard to *Pseudomonas aeruginosa*, Wallace *et al.* (1971) conclude that, although the serum levels reached with the indanyl ester appear suboptimal the drug might be of clinical value in the treatment of urinary tract infections.

c. *Ticarcillin.* This drug (α-carboxy-3-thienylmethylpenicillin) (Table XIII) is an analog of carbenicillin and resembles the latter very closely in its biological properties (Sutherland *et al.*, 1971). Against the gram-positive cocci the activity of the compound is very similar to that of carbenicillin, including the level of activity against penicillin-resistant staphylococci. Against *Escherichia coli*, ticarcillin appears to be equal or slightly more active than carbenicillin and, similarly, against *Proteus mirabilis*, and also the indole-positive *Proteus* species, activity appears to be equal to that of carbenicillin (Adler *et al.*, 1971; Bodey and Deerhake, 1971; Neu and Winshell, 1971a; Sutherland *et al.*, 1971). The activity of ticarcillin against *E. coli* is also comparable with that of ampicillin. Most strains of *E. coli* resistant to ampicillin are also resistant to ticarcillin but, as with carbenicillin, a certain number of ampicillin-resistant strains are relatively sensitive to this penicillin (Neu and Winshell, 1971a; Sutherland *et al.*, 1971). Ticarcillin is highly active against meningococci and *Haemophilus influenzae* and in this respect the level of activity has been reported to be slightly higher than that of ampicillin (Sabath *et al.*, 1971). Ticarcillin is bound to human serum to the same extent as carbenicillin (Sutherland *et al.*, 1971) and antibacterial activity is not significantly diminished in the presence of serum.

Ticarcillin is of particular interest in that it is 2–4 times more active than carbenicillin against *P. aeruginosa in vitro* (Adler *et al.*, 1971; Bodey and Deerhake, 1971; Neu and Winshell, 1971a; Sutherland *et al.*, 1971) and *in vivo* (Acred *et al.*, 1971a). The majority of strains of *P. aeruginosa* are thus inhibited at a concentration of approximately 25 μg ml, whereas a concentration of 50–100 μg/ml is required for inhibition with carbenicillin. In view of the fact that a very high dosage of carbenicillin is required in order to achieve inhibitory levels in the body, the increased activity of ticarcillin may well be of clinical importance. As with carbenicillin, combinations of ticarcillin with gentamicin show a synergistic effect against *Pseudomonas in vitro* and *in vivo* (Acred *et al.*, 1971a; Neu and Winshell, 1971a; Sutherland *et al.*, 1971).

Ticarcillin is not absorbed when given by mouth. The drug is well absorbed, however, after intramuscular injection, and the resulting serum levels are somewhat higher than those obtained with carbenicillin (Sutherland and Wise, 1971). These levels are further increased by the use of probenecid. Following a 5-gm intravenous dose of ticarcillin, peak serum con-

centrations of approximately 400 μg/ml are obtained and the levels remain in excess of 100 μg/ml for at least 2 hours (L. Lees, unpublished data).

Ready diffusion into extravascular compartments is indicated by results obtained in the dog showing that levels of unbound ticarcillin in lymph follow closely those in plasma (Acred *et al.*, 1971a).

2. *Sulfocillin*

Sulfocillin (α-sulfobenzylpenicillin) (Table XIII) shows some chemical similarity to carbenicillin in that both penicillins have acidic side chains and they also resemble one another very closely in their antibacterial activity (Nakazawa, 1971; Tsuchiya *et al.*, 1971; Yamazaki and Tsuchiya, 1971). Against a number of pathogens including penicillin-sensitive staphylococci, streptococci, pneumococci, *Escherichia coli*, *Proteus* species, *Salmonella* species, *Shigella* species, and *Pseudomonas aeruginosa*, sulfocillin and carbenicillin appear to be equally active. Against penicillin-resistant staphylococci, however, sulfocillin is slightly more active than carbenicillin and this is associated with greater stability to staphylococcal penicillinase. The extent of binding of sulfocillin to serum proteins is slightly higher than it is with carbenicillin, but the difference is probably not important. Sulfocillin does not appear to be absorbed from the gastrointestinal tract, and the distribution and excretion of the drug is reported to be similar to that of carbenicillin (Okubo, 1971).

3. *Suncillin*

Suncillin is the generic name for α-sulfoaminobenzylpenicillin (BL-P 1462) (Table XIII). Evaluation data have been published by Bodey and Stewart (1969), Price *et al.* (1969b), Barza *et al.* (1971), Bodey and Deerhake (1971), and Price *et al.* (1971). The antibacterial spectrum of suncillin resembles that of carbenicillin, but the level of activity against *Escherichia coli* and indole-positive *Proteus* species appears to be substantially lower. On the other hand, against *Proteus mirabilis* the data of Bodey and Deerhake (1971) indicate suncillin and carbenicillin to be equal in activity, and Bodey and Stewart (1969) have also reported suncillin to be more active than carbenicillin against *Enterobacter*. Against *Pseudomonas aeruginosa*, Bodey and Stewart (1969) reported suncillin to be slightly more active than carbenicillin but the authors pointed out that the tests with the two compounds were not carried out at the same time, and, in a later paper, Bodey and Deerhake (1971) reported results showing suncillin and carbenicillin to be almost identical in activity. In these tests, both compounds were one

dilution less active than ticarcillin. Barza *et al.* (1971) also reported suncillin and carbenicillin to be almost identical in activity against *P. aeruginosa*, and the same authors showed that suncillin and gentamicin were synergistic in action against *Pseudomonas*. The activity of suncillin is unaffected by the presence of 50% human serum (Barza *et al.*, 1971), indicating that the compound is not highly bound to serum proteins. In a limited clinical trial, suncillin has been reported to be effective in the treatment of *Pseudomonas* infections when administered at a dose of 18–24 gm/day by the intravenous route (Bodey and Rodriguez, 1970).

4. *Ureidopenicillins*

Three α-substituted benzylpenicillins have been described in which the α-substituent incorporates a ureido group. The compound that has been evaluated in most detail is BL-P 1654 [6-α-(guanylureido)phenylacetamidopenicillanic acid] (Table XIII). This penicillin shows an interesting antibacterial spectrum which to some extent combines the activity of ampicillin with that of carbenicillin (Adler *et al.*, 1971; Bodey and Stewart, 1971; Price *et al.*, 1971; van Scoy *et al.*, 1971). Against *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Streptococcus faecalis*, the activity of BL-P 1654 is greater than that of carbenicillin and approaches that of ampicillin, although, against penicillin-sensitive strains of *Staphylococcus aureus*, BL-P 1654 is considerably less active. The drug is not stable to staphylococcal penicillinase and is inactive against penicillin-resistant staphylococci unless the inoculum used in the test is very small. Against *Escherichia coli* and *Proteus mirabilis* the activity of BL-P 1654 is very similar to that of ampicillin. In the authors' experience the activity of BL-P 1654 against *Haemophilus influenzae* is also similar to that of ampicillin, although Price *et al.* (1971) reported BL-P 1654 to be more active. Indole-positive *Proteus* and *Enterobacter* species have been reported to be considerably less sensitive to BL-P 1654 than to carbenicillin (Adler *et al.*, 1971; Bodey and Stewart, 1971; van Scoy *et al.*, 1971).

In tests using a small inoculum, Price *et al.* (1971) found BL-P 1654 to be considerably more active than carbenicillin against *Pseudomonas aeruginosa*, the majority of strains being inhibited at a level of 8 μg/ml, whereas a concentration of 32–64 μg/ml was required in the case of carbenicillin. With a very small inoculum, Bodey and Stewart (1971) also found BL-P 1654 to be more active than carbenicillin. The activity of BL-P 1654 against *P. aeruginosa*, however, is greatly influenced by inoculum size, and this effect is even more marked than with carbenicillin. For example, van Scoy *et al.* (1971) reported that in liquid culture the MIC values for a group of strains of *P. aeruginosa* ranged from 3.9 to 15.6 μg/ml with an inoculum of 10^3

cells/ml, from 15.6 to 62.5 μg/ml with an inoculum of 10^5 cells/ml and from 500 to 1000 μg/ml with an inoculum of 10^7 organisms/ml. In tests on solid medium, by using an inoculum of 10^5 colony-forming units, these authors found the activity of BL-P 1654 to be only equal to that of carbenicillin. Similar results have been reported by Adler *et al.* (1971), and in MIC tests on agar using a 10^{-3} dilution of overnight broth culture as inoculum, the MIC for 161 strains of *P. aeruginosa* was 25–50 μg/ml for both BL-P 1654 and carbenicillin, whereas the corresponding value for ticarcillin was 12.5 μg/ml.

The extent of binding of BL-P 1654 in human serum is marginally lower than that of carbenicillin, but in the authors' experience activity against *Pseudomonas* is, nevertheless, greatly diminished in the presence of serum. In the mouse, BL-P 1654 is poorly absorbed when given by mouth, although it is well absorbed after parenteral administration (Price *et al.*, 1971). However, according to van Scoy *et al.* (1971) and Bodey and Stewart (1971), a major limitation to the clinical use of this drug by the parenteral route is the low aqueous solubility of the compound.

In addition to BL-P 1654, evaluation data on two other α-ureidopenicillins, BL-P 1532 and BL-P 1597, have been reported by Bodey and Stewart (1971). The antibacterial spectra of these two compounds are generally similar to that of BL-P 1654 but some differences are shown in the level of activity against different pathogens. In comparative tests, Bodey and Stewart (1971) found BL-P 1597 to be slightly more active than BL-P 1654 against *P. aeruginosa*, but BL-P 1532 was significantly less active. Against *E. coli* and *P. mirabilis*, on the other hand, both BL-P 1597 and BL-P 1532 appeared less active than BL-P 1654. Data obtained in the authors' laboratory confirm that BL-P 1597 shows good activity against *P. aeruginosa*, but this activity is offset by a high level of binding to plasma proteins.

VII. Amidinopenicillanic Acids

Derivatives of 6-aminopenicillanic acid with nonacyl moieties on the amino group have generally shown little activity of interest (Price, 1969; Hoover and Stedman, 1970), but a recent report has described a number of disubstituted amidino derivatives of 6-aminopenicillanic acid with an unusual spectrum of activity (Lund and Tybring, 1972). The compound with optimum activity, 6β-[(hexahydro-1*H*-azepin-1-yl)methyleneamino]penicillanic acid (FL 1060) (Fig. 11) was reported to be relatively inactive against gram-positive cocci but was highly active against some gram-negative bacteria including *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Salmonella* and *Shigella* species. Data obtained in the authors' laboratory confirm that this compound shows a high degree

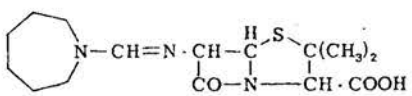


FIG. 11. Structure of FL 1060 {6β-[(hexahydro-1*H*-azepin-1-yl)methyleneamino]-penicillanic acid}.

of activity against dilute inocula of cells of certain gram-negative bacilli but that it is relatively inactive against large inocula. The significance of this difference in activity with different inocula will have to be established, but the unusual antibacterial spectrum of these amidino derivatives is of the greatest interest. Some evidence is also advanced by Lund and Tybring (1972) to suggest a difference in the mode of action of FL 1060 and ampicillin against *E. coli*. The compound is poorly absorbed in man when administered by the oral route but is well absorbed in the form of the pivaloyloxymethyl ester (Løvens Kemiske Fabrik, 1971).

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CHEMISTRY; TOXICOLOGY, PHARMACOLOGY AND MICROBIOLOGY OF A NEW ACID-STABLE PENICILLIN, RESISTANT TO PENICILLINASE (*BRL.1621**)

By

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THE use of methicillin, 2,6-dimethoxyphenyl penicillin, in the treatment of infections caused by penicillinase-producing strains of staphylococci is now well established in clinical practice. Its one disadvantage is that it must be given by injection since it is unstable in acid solution and poorly absorbed from the gastrointestinal tract. This article describes a new penicillin which does not suffer from this defect, and is, moreover, active at lower concentrations than methicillin. Clinical investigations are being reported concurrently elsewhere[1].

Chemistry

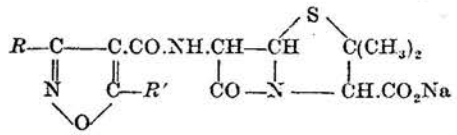
Acylation of 6-aminopenicillanic acid with reactive derivatives of several types of sterically hindered carboxylic acid gives penicillins which resist inactivation by penicillinase. These penicillins vary considerably in their chemical and antibacterial properties, and a particular class which combines resistance to both penicillinase and mineral acid with useful activity against most Gram-positive bacteria consists of 3,5-disubstituted 4-isoxazolyl penicillins (I). The preparation of two such penicillins, *BRL.*1400† ('Oxacillin') [I; $R = C_6H_5$, $R' = CH_3$] and its isomer, *BRL.*1454 [I; $R = CH_3$, $R' = C_6H_5$], has

* 'Orbenin', Beecham Research Laboratories, Limited, registered trade mark.

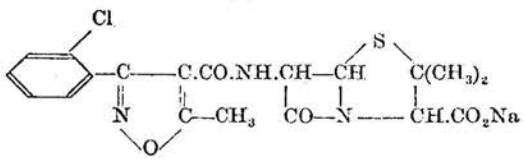
† 'Prostaphlin', Bristol Laboratories, Inc., registered trade mark.

been described in detail[2]. Many other penicillins of type (I) have been prepared[3] and examined, and one of them, 3-*o* chlorophenyl-5-methyl-4-isoxazolyl penicillin [II; *BRL*.1621], has been selected as the compound of choice.

BRL.1621 is available as the sodium salt monohydrate, which forms a colourless microcrystalline



(I)



(II) *BRL.1621**

powder. It is freely soluble in cold water to give a solution stable at 5° C for one week. A 1 per cent solution in water has $[\alpha]_D^{20}$ + 163° and *p*H of 6·0–7·5. The half-life in solution in 50 per cent aqueous alcohol at *p*H 1·3 and 35° C as determined by a previously described method[4] is 160 min, which is approximately equal to that of *BRL*.1400 and penicillin V.

Animal Studies, Toxicology and Pharmacology

Protection tests. The protective effect of *BRL*.1621 against the penicillin-resistant *Staphylococcus pyogenes* Russell (type 80) was estimated using the method of Selbie and O'Grady[5] as modified by Brown and Acred[6]. Groups of 10 mice (18–22 g) were infected by injecting into the muscle sheath of the thigh 0·2 ml. of a 1/2 dilution of an overnight growth of *Staphylococcus pyogenes* Russell. *BRL*.1621, methicillin and penicillin *G* were administered in doses of 200 and 50 mg/kg orally and subcutaneously, daily for three days, the first injection being given immediately following the infection. An infected control group received no treatment. The maximum thigh diameter of each mouse was measured with calipers on the first, second, third and sixth day after infection. The results are expressed as the percentage protection calculated in the following manner.

$$\frac{\text{mean daily thigh enlargement infected controls} - \text{mean daily thigh enlargement penicillin group}}{\text{mean daily thigh enlargement of infected controls}} \times \frac{100}{1}$$

Acute toxicities. The acute oral, subcutaneous and intravenous toxicities were determined in male mice (18–22 g), 10 mice being used at each dose level. The LD_{50} values (g/kg) for the various routes were respectively > 5·0, 3·0 and 1·2 g/kg.

Prolonged tests. (1) *Rats.* *BRL*.1621 was administered orally and subcutaneously to groups of 12 male rats over a period of 12 weeks at doses of 500 and 100 mg/kg. A control group of rats received 0·2 ml./100 g normal saline subcutaneously.

During the test, daily weights and food intake were recorded. Weekly observations were made of packed cell volume, white cell counts, and qualitative tests for proteins, sugars and occult blood were carried out on urine specimens. Spectroscopic examination of the blood of the rats in all groups and haemoglobin concentration was determined on the last week of the experiment.

All rats were killed at the end of the test and post-mortem examinations made. Weights of the spleens, livers, kidneys, adrenals and testes were recorded. In addition to determination of organ weights at autopsy, the following tissues were removed for histological examination: salivary gland, lymph node (mesenteric), thymus, thyroid, heart, lung, liver, kidney, adrenal, spleen, testes, bone marrow, stomach, duodenum, pancreas and small intestine. The tissues at the site of injection were also removed. No haematological, biochemical, histological or organ weight abnormalities were observed. Macroscopically the only untoward effect was small areas of necrosis noted at the site of injection in the rats receiving *BRL*.1621 500 mg/kg subcutaneously. These tissue reactions were only seen during the first 3 weeks of the test after which time they disappeared.

(2) *Dogs.* *BRL*.1621 was administered in doses of 2,000 and 500 mg/kg twice daily to groups of 3 dogs for a period of 4 weeks. The following biochemical and haematological estimations were made at the beginning and end of the test: ESR (mm/h), PCV (per cent), Hb G (per cent), reticulocytes (per cent), MCHC (per cent), total white cell count and differential count, blood urea (mg per cent), blood glucose

Table 1. PERCENTAGE PROTECTION BY *BRL*.1621, METHICILLIN AND PENICILLIN *G* IN MICE INFECTED WITH *Staph. pyogenes* RUSSELL (PENICILLIN-RESISTANT)

Penicillin	Dose (mg/kg)	Per cent protection	
		Oral	Subcutaneous
BRL.1621	200	98·3	97·6
BRL.1621	50	58·6	83·9
Methicillin	200	Inactive	84·7
Methicillin	50	Inactive	40·2
Penicillin *G*	200	Inactive	Inactive
Penicillin *G*	50	Inactive	Inactive

mg per cent) serum proteins—total, albumin, α_1-, α_2-, β- and γ-globulins, alkaline phosphatase KA units, SGPT units and presence of bilirubin. At autopsy the following organs were weighed: brain, heart, lungs, liver, spleen, pancreas, kidneys, thyroids, adrenals and gonads. In addition to these, the histology included examination of: stomach, ileum and colon, cervical and mesenteric lymph glands, salivary glands, skeletal muscle and bone marrow. No haematological, biochemical or histological abnormalities were noted.

Local toxic effects. There were no observable tissue reactions to intradermal and intramuscular injection of 1 per cent solutions of the penicillins in groups (3 per group) of guinea pigs and rats 3 days after the injection. The only observable reactions to administration of 10 per cent solutions were a hardening of the skin and erythema around the sites of the intradermal injections.

Following the subconjunctival injection in rabbits of 0·5 ml. of a 50 per cent solution, *BRL*.1621 penetrates into the aqueous humor but an opacity of the cornea develops which persists for at least 14 days with only a slight decrease in the intensity. It would appear, therefore, that *BRL*.1621 is contra-indicated for ophthalmological use.

General pharmacology. The effect of *BRL*.1621

Table 2. Minimum Inhibitory Concentration of *BRL*.1621 (Serial dilutions in agar, incubated at 37°C. for 24 hours)

	M.I.C. (μg/ml.)	
	Penicillin *G*	*BRL*.1621
Staph. aureus (6 strains)	0·02-0·05	0·4-0·25
Strep. β-haemolytic (5 strains)	0·002-0·005	0·05-0·1
Strep. viridans (4 strains)	0·002-0·05	0·1
Strep. pneumoniae (5 strains)	0·01-0·02	0·1-0·5
Sarcina lutea	0·005	0·25
Strep. faecalis	12·5	25
E. coli	50	> 250
Proteus vulgaris	50	> 250
Salm. typhi	2·5	> 250
Salm. typhimurium	1·25	> 250
Ps. pyocyanea	> 250	> 250
Shigella shigae	50	50
Aerobacter aerogenes	250	> 250

Table 3. Activity of *BRL*.1621 against fresh Clinical Isolates of Penicillin-resistant Staphylococci compared with that of Methicillin

	M.I.C. (μg/ml.)		
	Penicillin *G*	Methicillin	*BRL*.1621
Staph. aureus strain *C*440	500	2·5	0·5
Staph. aureus strain *C*465	500	2·5	0·5
Staph. aureus strain *C*417	250	2·5	0·5
Staph. aureus strain *C*444	250	1·25	0·5
Staph. aureus strain *C*452	250	2·5	0·5
Staph. aureus strain *C*443	250	1·25	0·25
Staph. aureus strain *C*442	125	2·5	0·5
Staph. aureus strain *C*427	25	1·25	0·25
Staph. aureus strain *C*433	25	1·25	0·25
Staph. aureus strain *C*420	5·0	1·25	0·25
Staph. aureus strain *C*421	2·5	1·25	0·25
Staph. aureus strain *C*438	0·1	2·5	0·5

Table 4. Activity of *BRL*.1621 compared with *BRL*.1400 using Staphylococci recently isolated clinically

	M.I.C. μg/mL												
	125 or greater	50	25	12·5	5·0	2·5	1·25	0·5	0·25	0·12	0·05	0·02	0·01 or less
Penicillin-sensitive strains (total 152)													
Penicillin *G*											19	63	70
BRL.1400								3	28	74	41	6	
BRL.1621							1		31	81	35		2
Penicillin-resistant strains (total 230)													
Penicillin *G*	81	20	31	49	14	14	3		2	16			
BRL.1400							36	74	87	20	12	1	
BRL.1621							2	66	116	40	5		1

on the blood pressure and respiration was determined in the anæsthetized cat.

Four cats were anæsthetized with a chloralose (1 per cent) urethane (5 per cent) mixture. Doses of up to 100 mg/kg administered intravenously had no effect on the blood pressure but doses in excess of this up to 400 mg/kg had slight hypotensive effects which usually did not exceed 10 mm mercury.

The responses to adrenaline (5·0 μg), acetylcholine (0·2–0·5 μg), histamine (0·5–5·0 μg), were unaffected by doses of 200 mg/kg of *BRL*.1621. Repeated doses of *BRL*.1621 at 5-min intervals increasing from 25, 50, 100, 200 and 400 mg/kg were given. Death occurred when the cat had received a total of 1·6 g/kg.

BRL.1621 penetrates poorly into the C.S.F. After a dose of 500 mg/kg intramuscularly in rabbits the concentration in the C.S.F. was no greater than 2·3 μg/ml. 1 h after administration, while the corresponding serum concentration was 370 μg/ml.

BRL.1621 is eliminated from the body by excretion in high concentration both in the bile and urine of rats. After the oral administration of 100 mg/kg 18·7 per cent is recovered in the urine after 24 h. while after intramuscular administration 42·1 per cent is recovered. 10·2 per cent of the orally administered antibiotic can be accounted for in the bile.

Metabolism in animals. Paper chromatographic examination of the urine collected over 24 h from rats which had received 100 mg/kg *BRL*.1621 intramuscularly, indicated the presence, in small amounts, of two metabolites. Further investigation of these substances is in progress.

Microbiology

Antibacterial activity. Minimum inhibitory concentrations (M.I.C.) for bacteria were determined as described previously[7]. It will be seen from Table 2 that *BRL*.1621 is highly active against *Staph. aureus*, *Strep. pyogenes*, *Strep. viridans* and *Strep. pneumoniae*, but against *Strep. faecalis* and the Gram-negative bacilli the level of activity is very low. Penicillin-sensitive staphylococci were usually inhibited by 0·1–0·25 μg/ml. *Strep. pneumoniae*, *Strep. viridans* and β-*haemolytic Streptococci* were usually inhibited by 0·1 μg/ml.

Penicillin *BRL*.1621 is primarily of interest for its activity against penicillin-resistant staphylococci. Results in Table 3 show that *BRL*.1621 is appreciably more active than methicillin in this respect. Results for the activity of *BRL*.1621 against a larger number of hospital strains of *Staph. aureus* are shown in Table 4. The total of 382 cultures tested

Table 5. ACTIVITY OF *BRL*.1621 COMPARED WITH *BRL*.1400 IN TESTS WITH AND WITHOUT HUMAN SERUM, USING 36 STRAINS OF STAPHYLOCOCCI RECENTLY ISOLATED CLINICALLY

	Serial dilution in agar M.I.C. μg/ml. and number of strains							
	5·0 and over	2·5	1·25	0·5	0·25	0·12	0·05	0·02 and below
Penicillin *G*	20		7		2		3	4
BRL.1621				8	21	6	1	
BRL.1621 +			23	10	3			
BRL.1400				13	17	3		
BRL.1400 +		3	28	5				

+ denotes agar containing 40 per cent human serum

comprised 152 penicillin-sensitive (penicillin G M.I.C. < 0·1 μg/ml.) and 230 penicillin-resistant (penicillin G M.I.C. 0·1 μg/ml. or greater). Taking the penicillin-sensitive strains, only one required a concentration of *BRL*.1621 as high as 1·25 μg/ml. to inhibit growth and approximately 95 per cent of the strains showed M.I.C. values in the range 0·05–0·25 μg/ml. Of the total of 230 penicillin-resistant strains only two required a concentration as high as 1·25 μg/ml. for inhibition and 97 per cent of the strains showed M.I.C. values in the range 0·125–0·5 μg/ml. The largest single group of strains were those with an M.I.C. value of 0·25 μg/ml.

Comparative results are also included in Table 4 for *BRL*.1400 (oxacillin). It will be seen that *BRL*.1621 is slightly more active than *BRL*.1400. Of the total of 230 penicillin-resistant strains, only two showed an M.I.C. as high as 1·25 μg/ml. with *BRL*.1621, whereas with *BRL*.1400 the corresponding number of strains was 36 (15 per cent).

Determination of M.I.C. values in solid media and in broth give similar results and M.I.C. values are not significantly influenced by inoculum size.

Effect of serum on activity. Activity of *BRL*.1621 is depressed by the presence of human serum to an extent somewhat greater than is the case with methicillin. Results are shown in Table 5 for 36 strains of staphylococci. In the absence of serum all were inhibited by 0·5 μg/ml. *BRL*.1621 or less and most of the strains were inhibited by 0·25 μg./ml. In the presence of human serum, however, the majority of the strains required 1·25 μg/ml. to prevent growth. It will be seen from Table 5 that *BRL*.1400 is also influenced by serum, the extent being similar to that with *BRL*.1621.

Bactericidal activity. Viable counts in broth cultures containing *BRL*.1621 were carried out in a manner similar to that of Miles, Misra and Irwin[8]. Penicillin *BRL*.1621 is highly bactericidal to penicillin-resistant staphylococci at concentrations only slightly higher than the M.I.C. values. In bactericidal tests

Table 6. Stability of *BRL*.1621 to Staphylococcal Penicillinase Compared with *BRL*.1400 and Methicillin

(a) At a concentration of 50 μg/ml.:

Time (min)	μg/ml. remaining			
	Penicillin *G*	Methicillin	*BRL*.1621	*BRL*.1400
0	50 (50)*	50	50	50
15	0 (35)	49	46	43
30	0 (17)	47	42	39
45	0 (6)	46	39	33
60	0 (0)	45	35	27
90	0 (0)	42	28	18

* Penicillinase at a dilution of 1/1,000.

(b) At a concentration of 2,000 μg/ml.:

Time (min)	μg/ml. remaining			
	Penicillin *G*	Methicillin	*BRL*.1621	*BRL*.1400
0	2,000 (2,000)*	2,000	2,000	2,000
15	0 (1,600)	1,840	1,550	1,340
30	0 (1,400)	1,720	1,350	830
60	0 (820)	1,520	800	250

* Penicillinase at a dilution of 1/200

BRL.1621 is substantially more active than methicillin and also slightly more active than *BRL*.1400. At a concentration of 2·0 μg ml. *BRL*.1621, a kill of approximately 99·9 per cent is obtained in 7 h in broth cultures of penicillin-resistant staphylococci.

Activity of BRL.1621 *by the paper disk method.* Whatman antibiotic assay disks 6 mm diameter were used with two levels of *BRL*.1621, 2 μg and 5 μg per disk. Nutrient agar plates were inoculated with the test organism to result in dense but non-confluent growth and the dishes were left at room temperature for 3–4 h with the disks in place before incubating at 37° C overnight. For a range of staphylococci of varying penicillin *G* sensitivity the zone diameters with *BRL*.1621 showed a fairly good correlation with the M.I.C. values for the strains. Using a 2 μg disk the zone diameters ranged from 19–29 mm, depending on the strain, and with a 5 μg disk the range was 22–33 mm diameter, including the diameter of the disk. Very heavy inoculum, and particularly the presence of blood in the agar, results in smaller zone diameters.

Stability to penicillinase. A penicillinase-producing strain of *Staph. aureus* was grown as described previously[7], and the whole culture, including the cells, was used as enzyme. The tests on penicillinase stability of *BRL*.1621, *BRL*.1400 and methicillin were carried out at two levels of substrate, 50 μg/ml. and 2,000 μg/ml. One volume of the enzyme was added to two volumes of phosphate buffer, *p*H 7, containing the penicillin. The reaction mixture was incubated at 37° C and assays carried out at intervals of time. Because of the extreme instability of penicillin *G* to penicillinase, a parallel test was carried out with penicillin *G* in which the penicillinase was present at a given dilution since with the penicillinase at the concentration used for the methicillin and

isoxazole tests the penicillin *G* was destroyed almost instantaneously. It will be seen from Table 6 that *BRL*.1621 is extremely stable to penicillinase, although slightly less stable than methicillin, and that *BRL*.1621 is slightly more stable than *BRL*.1400. It should be noted that the penicillinase stability of *BRL*.1621, and also that of *BRL*.1400 and methicillin, is greatly influenced by the concentration of the compound present in the test. With these compounds, the concentration of substrate is a limiting factor for penicillinase activity except at very high concentrations and the stability of these compounds is largely due to this effect of substrate concentration on rate of enzyme action. Consequently, the lower the concentration of these penicillins the more stable they become to penicillinase.

Penicillin *BRL*.1621, like methicillin, is a powerful inducer of penicillinase formation.

Emergence of resistance to BRL.1621. By repeated sub-culture of staphylococci in the presence of *BRL*.1621, resistant strains can be selected out in a step-wise manner similar to that seen with penicillin *G*, methicillin and other penicillins. Single step mutants of high resistance to *BRL*.1621 have not been encountered in laboratory experiments with staphylococci. On the other hand, naturally occurring staphylococci resistant to methicillin[9] are also resistant to *BRL*.1621, although since *BRL*.1621 is a more active compound the M.I.C. values with *BRL*.1621 against these resistant strains are lower than those for methicillin. This means that although the M.I.C. values for methicillin may be above the serum levels normally obtained, the same may not be the case with *BRL* 1621. For example, certain strains showing M.I.C. values of 12·5–25 μg/ml. with methicillin were found to be still sensitive to 1·25 μg/ml. *BRL*.1621. In general, the highly resistant strains which showed M.I.C. values of 125 μg/ml. and over with methicillin, also showed M.I.C. values with *BRL*.1621 above the concentrations obtainable in the body but one strain showing an M.I.C. value of 50 μg/ml. with methicillin was still sensitive to 1·25 μg/ml. *BRL*.1621.

Metabolite of BRL.1621. After administration of *BRL*.1621, either orally or intramuscularly, in normal human subjects a small proportion of the compound undergoes a metabolic change in the body to give rise to another penicillin. This metabolite is present in the serum as well as in the urine, and in human beings approximately 10 per cent of the penicillin excreted is in the form of the metabolite. A similar metabolite also occurs with *BRL*.1400.

The metabolite of *BRL*.1621 has been isolated and

found to have antibacterial activity very similar to *BRL*.1621 itself. Moreover, using *Sarcina lutea* and also a penicillin-resistant staphylococcus as test organism, *BRL*.1621 and the metabolite both have the same ring-diameter concentration relationship in the cup-plate assays.

In view of the very close similarity in the biological properties of the metabolite and *BRL*.1621 itself, and also in view of the small amount of the metabolite formed it seems quite certain that the latter does not in any way complicate the determinations of serum levels or the general evaluation of *BRL*.1621.

We wish to acknowledge the guidance accorded by Mr. F. P. Doyle, director of research, Brockham Park, in the preparation of the manuscript. We also thank our many colleagues who assisted in the work discussed in this article.

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Flucloxacillin, a New Isoxazolyl Penicillin, Compared with Oxacillin, Cloxacillin, and Dicloxacillin

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Summary: Flucloxacillin, a new isoxazole penicillin, is active against penicillinase-producing strains of *Staphylococcus aureus* and is well absorbed in man after oral and intramuscular administration. Compared with isoxazole penicillins in current clinical use—namely, oxacillin, cloxacillin, and dicloxacillin—flucloxacillin has proved as active against Gram-positive cocci, including penicillin-resistant staphylococci. The extent of binding of flucloxacillin to the protein of human serum was similar to that of oxacillin and cloxacillin and less than that of dicloxacillin. In man flucloxacillin given orally produced total and free serum levels higher than those obtained with oxacillin and cloxacillin; total serum levels similar to those of dicloxacillin, and free levels greater than those of dicloxacillin. Similarly, after intramuscular injection the free serum levels of flucloxacillin were higher than those of oxacillin, cloxacillin, and dicloxacillin.

Introduction

The isoxazolyl series of semisynthetic penicillins, first synthesized and evaluated in these laboratories (Doyle *et al.*, 1961;

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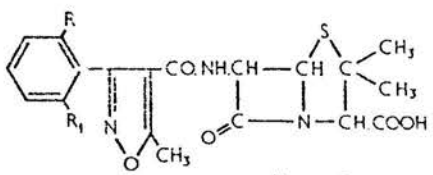
Hanson *et al.*, 1965), combines resistance to penicillinase with acid stability and activity against Gram-positive bacteria. The isoxazole penicillins in current clinical use—namely, oxacillin, cloxacillin, and dicloxacillin—are absorbed when given by mouth or by injection and show significant activity against penicillin-resistant staphylococci and other Gram-positive cocci. The therapeutic efficacy of the isoxazole penicillins in the treatment of staphylococcal and streptococcal infections is now well established.

Oxacillin, cloxacillin, and dicloxacillin have closely related chemical structures (Fig. 1), and the antibacterial activities of these compounds are also similar, though oxacillin is somewhat less active than cloxacillin and dicloxacillin against penicillin-resistant staphylococci, and cloxacillin is rather less active than oxacillin and dicloxacillin against pneumococci (Knudsen *et al.*, 1962; Bennett *et al.*, 1965; Knott *et al.*, 1965). The main differences among the isoxazole penicillins lie in the serum concentrations obtained after administration of the compounds and in the extent to which they are bound to serum protein.

Oxacillin, the first member of the series, gives rise to serum concentrations which are substantially lower than those obtained with cloxacillin both by mouth and by injection (Knudsen *et al.*, 1962; Bunn and Milicich, 1964; Gravenkemper *et al.*, 1964), while dicloxacillin results in serum levels higher than those of cloxacillin when given by mouth (Bennett *et al.*, 1965; Knott *et al.*, 1965). Dicloxacillin, however, is more highly bound to serum protein than is cloxacillin (Gloxhuber *et al.*, 1965; Kunin, 1966), and this factor may be held to detract from the superior levels of total anti-



	R	R_1
Oxacillin	H	H
Cloxacillin	Cl	H
Dicloxacillin	Cl	Cl
Flucloxacillin	Cl	F

Flucloxacillin, 3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolylpenicillin, sodium salt monohydrate

Molecular weight 493·9
(sodium salt monohydrate)

FIG. 1.—Isoxazolyl penicillins in clinical use.

biotic measured in the plasma (Gloxhuber *et al.*, 1965; Rolinson, 1967; *Medical Letter*, 1968).

In this present paper the properties of a further member of the isoxazole series is reported. This compound (3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolyl-penicillin has the generic name flucloxacillin (Floxapen) (Fig. 1) and details are given regarding antibacterial activity, protein binding, and serum levels of this new penicillin in comparison with oxacillin, cloxacillin, and dicloxacillin.

Materials

Flucloxacillin is available as the sodium salt monohydrate, in which form it is a white or off-white crystalline powder. It is freely soluble in water, and a solution of 10% w/v flucloxacillin in phosphate buffer (pH 7·0) retains 98% of its initial potency after storage for 14 days at 5° C. A 10% solution of flucloxacillin in water has a pH of 5-7.

Antibacterial Activity of Flucloxacillin

Minimum inhibitory concentrations (M.I.C.) required to prevent growth of the test organisms for 24 hours at 37°C. were determined by serial dilution in nutrient broth (Oxoid No. 2) or nutrient agar (blood agar base, Oxoid). Horse blood (5%) was incorporated in the media for tests with streptococci, pneumococci, and *Haemophilus* species. For tests in liquid medium the inoculum was normally one drop (0·03 ml.) of an overnight broth culture of the test organism (about 10^7 cells) in 5 ml. of medium. Serial dilutions in agar were poured into Petri dishes and inoculated with a multiple-inoculating device delivering a drop (0·003 ml.) of an overnight broth culture (about 10^6 cells).

Antibacterial Spectrum.—The antibacterial spectrum of flucloxacillin compared with cloxacillin and benzylpenicillin is shown in Table I, where it can be seen that flucloxacillin was active against the Gram-positive bacilli tested and against Gram-positive cocci, with the exception of *Streptococcus faecalis*. In general, the Gram-negative bacteria tested were relatively insensitive to flucloxacillin, the enterobacteriaceae requiring concentrations of 500 μg./ml. or greater for inhibition. Strains of *Haemophilus influenzae, Bordetella pertussis, Pasteurella septica,* and *Brucella abortus* were more susceptible, but were only inhibited by 5·0-12·5 μg/ml. In contrast, the gonococci and meningococci were sensitive to flucloxacillin, and were inhibited at a concentration of 0·1 μg./ml. Flucloxacillin

was as active as cloxacillin against the test organisms and was generally five to ten times less active than benzylpenicillin

TABLE I.—*Antibacterial Spectra of Flucloxacillin, Cloxacillin, and Benzylpenicillin*

Organism	M.I.C. (μg./ml.)		
	Flucloxacillin	Cloxacillin	Benzylpenicillin
*Staphylococcus aureus**	0·1–0·25	0·1–0·25	0·02
Staphylococcus aureus†	0·25–0·5	0·25–0·5	>50
β-Haemolytic streptococcus	0·05–0·1	0·1	0·01
α-Haemolytic streptococcus	0·5	0·5	0·05
Streptococcus pneumoniae	0·25	0·25–0·5	0·01
Streptococcus faecalis	25	25	5·0
Bacillus subtilis	0·5	0·5	0·1
Bacillus anthracis	0·5	0·5	0·05
Sarcina lutea	0·25	0·25	0·005
Listeria monocytogenes	1·25	1·25	0·05
Corynebacterium diphtheriae	0·25	0·1	0·02
Erysipelothrix rhusiopathiae	0·25	0·1	0·05
Clostridium tetani	0·25	0·5	0·02
Clostridium welchii	0·25	0·5	0·02
*Neisseria gonorrhoeae**	0·1	0·1	0·01
Neisseria gonorrhoeae†	2·5	2·5	0·1
Neisseria meningitidis	0·1	0·1	0·01
Haemophilus influenzae	5·0–12·5	12·5–25	0·5
Bordetella pertussis	12·5	25	0·5
Pasteurella septica	12·5	12·5	0·1
Brucella abortus	5·0	5·0	0·1
Escherichia coli	>500	>500	50
Proteus mirabilis	>500	500	5·0
Salmonella typhi	>500	500	5·0
Shigella sonnei	>500	500	12·5
Klebsiella aerogenes	>500	>500	>500
Pseudomonas aeruginosa	>500	>500	>500
Vibrio cholerae	500	250	1·25
Candida albicans	>500	>500	>500

*Penicillin-sensitive. †Penicillin-resistant.

against Gram-positive bacteria, with the exception of penicillinase-producing strains of staphylococci which were resistant to benzylpenicillin but sensitive to flucloxacillin.

Activity against Streptococci.—Flucloxacillin was inhibitory to β-haemolytic streptococci at concentrations of 0·05-0·1 μg./ml., and inhibited most of the pneumococci tested at concentrations of 0·1-0·25 μg./ml. (Table II). Against these organisms flucloxacillin was as active as dicloxacillin and oxacillin, and was slightly more active than cloxacillin.

Activity against Staphylococci.—All four isoxazole penicillins showed a similar level of activity against penicillin-sensitive strains of staphylococci compared with 0·02 μg./ml. for benzylpenicillin (Table III). In the case of the penicillin-resistant staphylococci most strains were inhibited by flucloxacillin at a concentration of 0·25-0·5 μg./ml.; both flucloxacillin and dicloxacillin were marginally more active than cloxacillin, while oxacillin was the least active of the four isoxazole

TABLE II.—*M.I.C. Values of Flucloxacillin, Cloxacillin, Dicloxacillin, and Oxacillin Against Streptococci and Pneumococci*

Organism	No. of Strains	Penicillin	M.I.C. (μg.ml.) and No. of Strains									
			>1·25	1·25	0·5	0·25	0·1	0·05	0·02	0·01	0·005	0·002
β-haemolytic streptococcus	10	Flucloxacillin					5	5				
		Cloxacillin					10					
		Dicloxacillin					6	4				
		Oxacillin					4	5	1			
		Benzylpenicillin									7	3
Str. pneumoniae	18	Flucloxacillin			1	12	3	1	1			
	18	Cloxacillin			5	9	3		1			
	18	Dicloxacillin			1	11	4	1	1			
	8	Oxacillin					6	2				
	8	Benzylpenicillin								7	1	

penicillins (Table III). The stability of flucloxacillin to staphylococcal penicillinase compared with benzylpenicillin was further illustrated by determining the effect of altering the inoculum size on the relative activities of the penicillins against a penicillinase-producing strain of *Staphylococcus aureus*. In this test, benzylpenicillin was active against an inoculum of a 1/1,000 dilution of an overnight culture at a concentration of 0·25 μg./ml., but against an undiluted culture the M.I.C. of benzylpenicillin was raised to 125 μg./ml. as a result of the penicillinase activity of the large inoculum. In contrast, the M.I.C. of flucloxacillin increased only twofold, from 0·25 to 0·5 μg./ml., under the same conditions.

Methicillin-resistant Staphylococci. — Naturally occurring strains of methicillin-resistant staphylococci were also resistant to flucloxacillin as well as to cloxacillin and dicloxacillin (Table IV). The resistance of these strains of staphylococci was not due to inactivation of the penicillin in question but to an intrinsic resistance. Such strains comprised mixed populations of cells of which only a minority was resistant while the majority was of normal sensitivity (Sutherland and Rolinson, 1964). As a result of this heterogeneity a small inoculum appeared sensitive to the isoxazole penicillins, whereas a large inoculum appeared resistant.

pH and Antibacterial Activity of Flucloxacillin.—The M.I.C.s of flucloxacillin against *Staph. aureus* were determined in nutrient broth incorporating M/20 phosphate buffer at pH 5·8, pH 7·2, and pH 8·0. The activity of flucloxacillin was not greatly influenced by the pH of the medium, the penicillin being generally two to four times more active at pH 5·8 than at pH 7·2 or above.

TABLE III.—*Relative Activities of Flucloxacillin, Cloxacillin, Dicloxacillin, and Oxacillin Against Staphylococcus aureus*

Penicillin	No. of Strains	Mean	M.I.C. (μg./ml.) and No. of Strains						
			1·25	0·5	0·25	0·1	0·05	0·02	0·01
Penicillin-sensitive Staphylococci									
Flucloxacillin	65	0·16		6	14	43	2		
Cloxacillin ..	65	0·21		10	29	26			
Dicloxacillin	65	0·17		2	27	33	3		
Oxacillin ..	43	0·15		2	13	26	2		
Benzyl-penicillin	38	0·03					12	25	1
Penicillin-resistant Staphylococci									
Flucloxacillin	72	0·31	1	22	43	4	2		
Cloxacillin ..	72	0·34	1	29	37	5			
Dicloxacillin	72	0·28	1	17	44	9	1		
Oxacillin ..	72	0·41	6	39	23	4			

7

TABLE IV.—*Relative Activities of Flucloxacillin, Cloxacillin, and Dicloxacillin Against a Naturally Occurring Methicillin-resistant Strain of Staphylococcus aureus* 1757

Inoculum (No. of Cells)	M.I.C.* (μg./ml.)					
	Flucloxacillin		Cloxacillin		Dicloxacillin	
	24 hr.	48 hr.	24 hr.	48 hr.	24 hr.	48 hr.
20×10^4	2·5	50	2·5	25	1·25	50
20	0·1	1·25	0·25	0·5	0·25	0·5

*Serial dilution in nutrient broth.

Bactericidal Activity.—In viable-count experiments both flucloxacillin and cloxacillin showed a similar degree of bactericidal activity against a penicillin-resistant strain of *Staph. aureus*, and concentrations only slightly greater than the M.I.C. resulted in a substantial reduction in the viable count during the first few hours (Fig. 2).

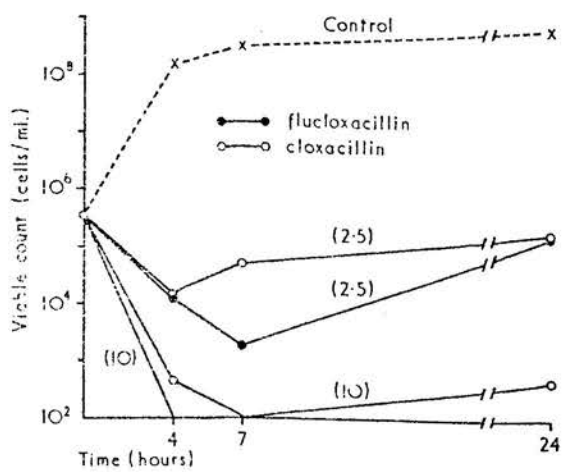


FIG. 2.—Bactericidal activities of flucloxacillin and cloxacillin against a penicillinase-producing strain of *Staphylococcus aureus* 1089. Figures in parentheses are concentrations (μg./ml.).

Effect of Serum on Antibacterial Activity.—Flucloxacillin was 10 to 20-fold less active in 95% human serum than in nutrient broth, and a similar reduction in activity was also seen with cloxacillin and dicloxacillin. Thus the minimum bactericidal concentrations of these penicillins against two penicillinase-producing strains of *Staph. aureus* increased from 0·15 μg./ml. in nutrient broth to 1·5-3·0 μg./ml. in human serum.

8

Binding of Flucloxacillin to Serum Proteins

The extent of binding of flucloxacillin and the other isoxazole penicillins to the proteins of human serum was measured by an ultrafiltration technique (Rolinson and Sutherland, 1965) in which pooled human serum, pH 7·4, containing 50 μg. of the test penicillin per ml. was filtered through Visking viscose-cellulose dialysis tubing. The penicillin content of the protein-free ultrafiltrate was measured by microbiological assay, and this quantity represented the free unbound fraction of penicillin in serum. The amount of protein-bound penicillin was derived by subtracting the level of free penicillin from the known total concentration in serum. The results of the ultrafiltration experiments are shown in Table V, where each value repre-

TABLE V.—*Extent of Binding of Isoxazolyl Penicillins in Human Serum*

Penicillin	% Bound	% Free
Oxacillin	93·1	6·9
Cloxacillin	94·0	6·0
Dicloxacillin	96·9	3·1
Flucloxacillin	94·7	5·3

sents the mean of at least three determinations. It will be seen that the unbound levels of flucloxacillin, cloxacillin, and oxacillin were very similar. Dicloxacillin, on the other hand, was bound to serum protein to a significantly greater extent, the unbound fraction of dicloxacillin being almost exactly half that of cloxacillin.

Sensitivity Disc Tests

Flucloxacillin sensitivity discs (5 μg.) produced inhibition zones in tests with penicillin-sensitive and penicillinase-producing strains of *Staph. aureus* which were of the same order as those obtained with 5 μg. cloxacillin sensitivity discs. Naturally occurring strains of *Staph. aureus*, resistant to methicillin and to the isoxazole penicillins, often appeared to be sensitive to flucloxacillin discs, as with cloxacillin discs, though resistant colonies were sometimes observed within the inhibition zones when a large inoculum of cells was examined after overnight incubation at 37° C., and were common after incubation for 48 hours at 37° C. Dilute inocula almost invariably appeared sensitive when tested at 37°C., as is the case for methicillin and cloxacillin (Sutherland and Rolinson, 1964). Resistance to flucloxacillin was generally readily demonstrated when sensitivity tests were incubated at 30°C.

(Annear, 1968) or involved the use of 5% NaCl agar (Barber, 1964; Sutherland, 1964), as is the case with other penicillinase-stable penicillins.

Absorption and Excretion in Human Volunteers

Assay in Serum and Urine

The concentrations of flucloxacillin and other isoxazole penicillins in serum and urine were measured by standard cup-plate microbiological assay with *Sarcina lutea* N.C.T.C. 8340 as assay organism (Knudsen *et al.*, 1962). For the assay of serum specimens, standard solutions of flucloxacillin, cloxacillin, or dicloxacillin were prepared in pooled human serum to give a range of 0·5-50 μg./ml.; for urine specimens the standard solutions were prepared in M/20 phosphate buffer, pH 7·0, over a range of 0·25-20 μg./ml. Oxacillin was more active than the other penicillins against *Sarcina lutea* N.C.T.C. 8340, and was diluted in serum to give a range of 0·1-10 μg./ml. or in buffer from 0·05 to 5 μg./ml. The plates were incubated overnight at 30° C., the inhibition zone diameters were measured, and the concentrations of the test specimens were derived from the standard line constructed from the standard solution.

Oral Administration

The penicillins were given in gelatin capsules as a single dose of 250 or 500 mg. to healthy fasting volunteer subjects. Blood specimens were taken at ½, 1, 2, 3, 4, or 6 hours after administration of the dose, and urine was collected over the 0 to 6-hour period. Levels of total antibiotic were measured by microbiological assay, and the serum concentrations of free, unbound antibiotic were calculated from these total levels and from the values for the extent of binding of the isoxazole penicillins in human serum.

Data in Table VI represent the total and free serum levels obtained in numerous studies with fasting subjects given single oral doses of flucloxacillin, dicloxacillin, cloxacillin, or oxacillin. It will be seen that the total serum levels of oxacillin were substantially lower than those of cloxacillin, and likewise the free levels of oxacillin were less than those of cloxacillin. With dicloxacillin, a 250-mg. dose produced total serum levels comparable to those attained with the 500-mg. dose of cloxacillin. Dicloxacillin, however, is bound to serum protein to a significantly greater extent than cloxacillin, and consequently

TABLE VI.—*Serum Concentrations in Volunteers (A Single Dose in the Form of a gelatin capsule was Taken by Volunteers in the Fasting State)*

Penicillin	Dose (mg.)	No. of Subjects	Mean Total Level (μg./ml.)					Mean Free Level* (μg./ml.)				
			½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	½ hr.	1 hr.	2 hr.	3 hr.	4 hr.
Oxacillin	500	25	5·6	4·9	1·5	0·6	0·2	0·39	0·34	0·1	0·04	0·01
Cloxacillin	500	122	8·4	9·2	4·5	<2·0	<2·0	0·5	0·55	0·27	—	—
Flucloxacillin	500	53	13·9	14·5	6·7	4·4	2·2	0·73	0·76	0·35	0·23	0·12
Lucloxacillin	250	48	6·9	8·8	5·0	2·2	<1·0	0·36	0·46	0·26	0·12	—
Flucloxacillin	125	11	1·9	5·8	2·9	1·1	<1·0	0·1	0·31	0·15	0·06	—
Dicloxacillin	250	84	6·4	9·3	6·7	3·1	1·4	0·2	0·29	0·2	0·1	0·04

*Free levels in serum samples calculated from the total levels and extent of binding. Free concentrations in human serum were found to be oxacillin 6·9%, cloxacillin 6·0%, dicloxacillin 3·1%, and flucloxacillin 5·3%.

when this factor is taken into account the peak free levels of dicloxacillin were only about half those of cloxacillin. In the case of flucloxacillin, the total levels obtained with a 250-mg. dose were similar to those obtained with 250 mg. of dicloxacillin; but because flucloxacillin is bound to serum protein to a lesser degree than dicloxacillin the free serum levels of flucloxacillin were considerably higher than those of dicloxacillin. The total levels of flucloxacillin after administration of a 250-mg. dose were almost equal to those obtained with 500 mg. of cloxacillin, and as the extent of binding of both penicillins is very similar, the unbound levels of flucloxacillin were only slightly lower than those of cloxacillin.

The mean values for flucloxacillin and cloxacillin obtained from three separate cross-over studies involving 43 healthy volunteers are shown in Fig. 3. A single dose of 500 mg. was given in capsule form to the fasting subjects. It will be seen that at all times the serum levels of total antibiotic were considerably higher in the case of flucloxacillin than with cloxacillin. Levels of free flucloxacillin were also significantly higher than those of cloxacillin, since the extent of binding of these two compounds is similar.

Similarly, data in Fig. 4 show the mean values for flucloxacillin and dicloxacillin obtained in two separate cross-over studies involving 32 subjects in the fasting state. In these studies a single dose of 250 mg. was given by mouth in capsule form. It will be seen that the serum levels of total antibiotic were similar for the two penicillins. As a result of the difference in serum binding, however, free levels of

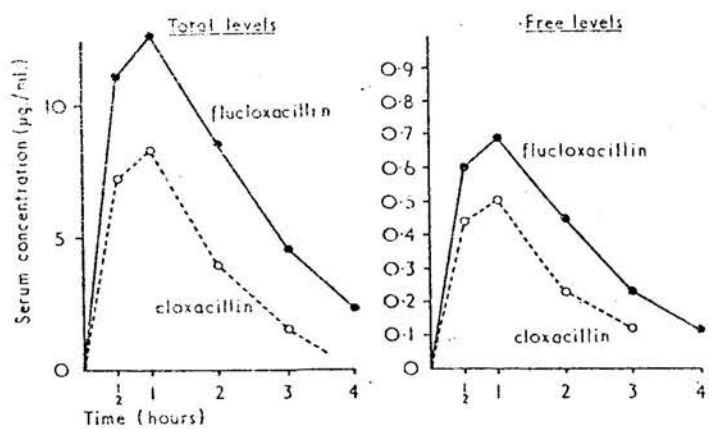


FIG. 3.—Serum concentrations of flucloxacillin and cloxacillin after a single 500-mg. oral dose to fasting human subjects. Cross-over studies in 43 subjects.

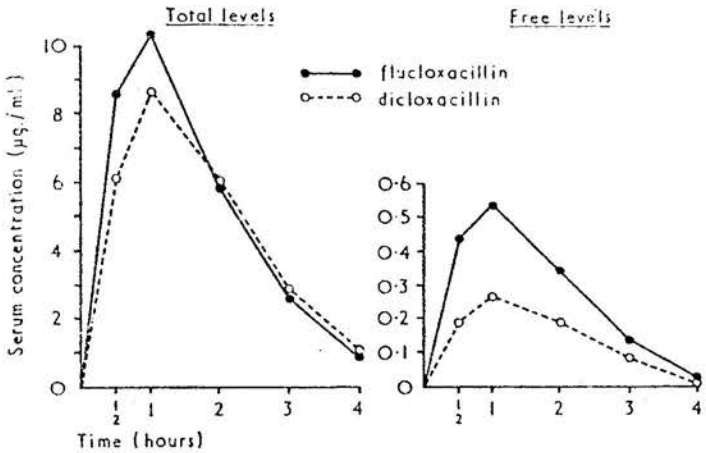


FIG. 4.—Serum concentrations of flucloxacillin and dicloxacillin after a single 250-mg. oral dose to fasting human subjects. Cross-over studies in 32 subjects.

flucloxacillin were substantially higher than those of dicloxacillin. At the peak levels this difference was about twofold.

In these studies the absorption of the isoxazole penicillins showed considerable variation between one volunteer and another, and there was also considerable variation in the time at which peak blood levels were reached, though such variation is not apparent from the mean values as shown in Figs. 3 and 4. For example, in the cross-over studies with flucloxacillin and cloxacillin, at a dose of 500 mg. the peak levels of total antibiotic for the 43 subjects involved ranged from 3·4 to 26·5 µg./ml. in the case of flucloxacillin and from 1·5 to 30·0 µg./ml. with cloxacillin. Mean peak levels, irrespective of the time the peak was reached, were 14·7 µg./ml. for flucloxacillin and 9·6 µg./ml. for cloxacillin, and as a result of a similar degree of serum binding the peak levels of unbound flucloxacillin were also significantly higher than those of cloxacillin. In the cross-over studies with flucloxacillin and dicloxacillin, at a dose of 250 mg., a wide range of peak values (from 3·0 to 26·0 µg./ml. with both penicillins) was again seen among the subjects involved. Mean peak total levels of flucloxacillin and dicloxacillin were similar (11·0 µg./ml.), but as a result of the difference in protein binding the mean peak levels of free flucloxacillin were considerably higher than those of dicloxacillin.

Urinary Excretion

In the cross-over study with flucloxacillin and cloxacillin at 500 mg. (Fig. 2), 50·5% and 39·5% of the doses respectively

were recovered in the urine collected over 0-6 hours. In the study with flucloxacillin and dicloxacillin at 250 mg. (Fig. 4) the recovery was 55% and 59% respectively over the same period.

Effect of Food on Absorption

Flucloxacillin was given at a 250-mg. or 500-mg. dose to volunteer subjects one hour after they had eaten a standard breakfast. Absorption of flucloxacillin was delayed and the peak serum concentrations were about half those obtained in fasting subjects (Table VII). Similar effects have been

TABLE VII.—*Effect of Food on Oral Absorption of Flucloxacillin*

Dose (mg.)	State	No. of Subjects	Average Serum Concentrations (μg./ml.)						Excretion 0–6 hr. (%)
			½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	6 hr.	
250	Fasting	48	6·9	8·8	5·0	2·2	<1·0	—	59
	Non-fasting	8	<1·0	2·6	5·2	4·6	2·1	—	56
500	Fasting	43	11·1	12·6	8·4	4·6	2·2	—	50
	Non-fasting	12	<1·0	3·2	7·9	—	3·6	<1·0	35

reported for cloxacillin and oxacillin (Knudsen *et al.*, 1962; Gravenkemper *et al.*, 1964).

Intramuscular Administration

Total and free serum levels are shown in Fig. 5 for cloxacillin, dicloxacillin, and flucloxacillin administered as single intramuscular injections of 250 mg. to healthy volunteers. The total levels of flucloxacillin were slightly higher than those of dicloxacillin, which in turn were higher than those of cloxacillin. Taking the extent of binding into consideration it will be seen that the free levels of flucloxacillin were appreciably higher than those of cloxacillin or dicloxacillin.

Data for oxacillin, cloxacillin, and flucloxacillin after intramuscular injection of a single 500-mg. dose to volunteers are shown in Fig. 6. Total and free levels of the three compounds were similar with regard to the peak values, but the levels of flucloxacillin were considerably more prolonged than those of oxacillin and slightly more prolonged than with cloxacillin.

Discussion

Our results show that flucloxacillin was generally similar to oxacillin, cloxacillin, and dicloxacillin, in terms of spectrum and level of activity against Gram-positive bacteria. Thus

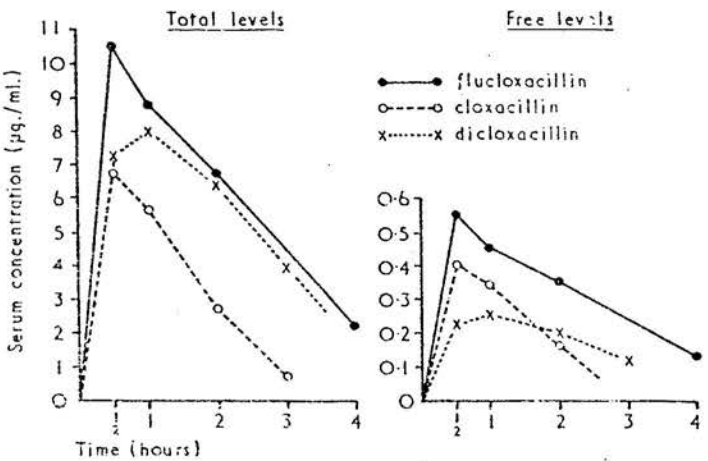


FIG. 5.—Serum concentrations after a single intramuscular injection of 250 mg. in human subjects.

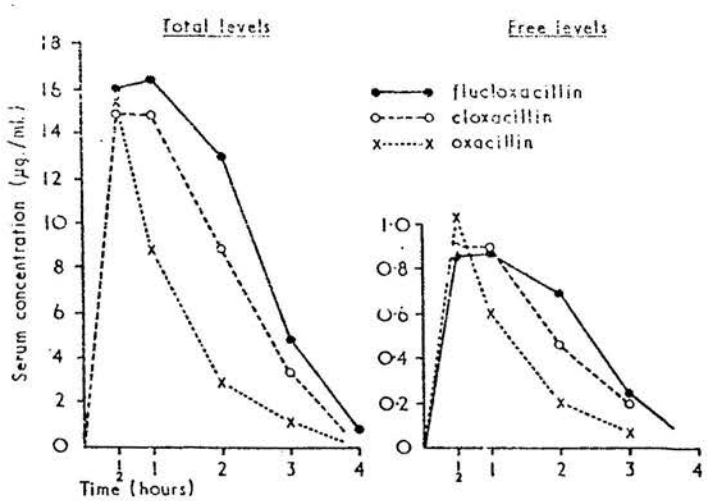


FIG. 6.—Serum concentrations after a single intramuscular injection of 500 mg. in human subjects.

flucloxacillin was as active as cloxacillin and dicloxacillin against penicillinase-producing strains of *Staph. aureus* and was more active than oxacillin in this respect. The four penicillins were equally active against penicillin-sensitive staphylococci, while cloxacillin was the least active against β-haemolytic streptococci and pneumococci.

In common with oxacillin, cloxacillin, and dicloxacillin, flucloxacillin showed a high degree of binding to serum proteins. In the tests reported here flucloxacillin was bound to serum protein to a similar extent as oxacillin and cloxacillin, but to a markedly lesser extent than dicloxacillin. Thus, for the same level of total antibiotic in serum, the unbound levels of oxacillin, cloxacillin, and flucloxacillin would be about twice as high as the unbound levels of dicloxacillin.

Flucloxacillin was well absorbed in people after administration by both oral and intramuscular routes. When given by mouth flucloxacillin gave rise to total serum levels equivalent to those obtained with dicloxacillin, and superior to those of cloxacillin and oxacillin. Moreover, when the extent of serum binding of these penicillins was considered the free levels of flucloxacillin were superior to those of dicloxacillin as well as those of cloxacillin and oxacillin. The relationship of the total serum levels for a given oral dose of oxacillin, cloxacillin, dicloxacillin, and flucloxacillin might be expressed approximately as 1:2:4:4, respectively, but the levels of unbound penicillins would be 1:2:2:4. Thus the superior absorption of dicloxacillin compared with cloxacillin is counterbalanced by the higher serum binding of the former (Kunin, 1966; *Medical Letter*, 1963) whereas the higher total levels of flucloxacillin also represent higher free levels of active penicillin. Hence the administration of oral flucloxacillin results in serum levels of unbound drug higher than those obtained with oxacillin, cloxacillin, or dicloxacillin, thus providing higher concentrations of active antibiotic for tissue distribution (Scholtan and Schmid, 1962; Kunin, 1965, 1966; Verwey *et al.*, 1966). Similarly, after administration by intramuscular injection the serum levels of total flucloxacillin were superior to those of the other isoxazole penicillins, and consequently the levels of unbound antibiotic are also greater for flucloxacillin than for oxacillin, cloxacillin, or dicloxacillin.

In addition to our data, experiments carried out in these laboratories by Mr. P. Acred and Mrs. P. Hunter have shown that flucloxacillin is highly active against experimental infections in mice with penicillin-resistant staphylococci. Consideration of the particular properties of flucloxacillin—namely, antibacterial activity, protein binding, and absorption in human subjects—shows that the compound is superior to oxacillin, cloxacillin, and dicloxacillin, and suggests that the

clinical efficacy of flucloxacillin may be expected to compare favourably with those isoxazole penicillins at present in clinical use.

We wish to thank Dr. J. H. C. Nayler and his colleagues for preparing the penicillins used in these studies, and Mrs. Eleanor Fairclough for skilled technical assistance.

Addendum

D. M. BROWN, Beecham Research Laboratories, writes: Acute toxicity determinations were carried out in the mouse and in the dog. In the mouse the LD_{50} was established at 3·8 g./kg. orally and 2·2 g./kg. subcutaneously. In the dog, doses up to 10 g./kg. were administered orally, but all dogs vomited when they received 4·5 g./kg. and a lethal effect in the species could not be established. Long-term toxicity studies were carried out in the rat and in the dog. Doses of 200, 500, and 2,000 mg./kg. orally and 250 mg./kg. subcutaneously were administered to both species, but in the dog study the high dose had to be reduced to 1,000 mg./kg. as most of the animals vomited after receiving the drug.

Laboratory studies, which included urine and blood biochemistry and haematology, were carried out at 0, 4, 12, and 25 weeks in the rat and at 0, 6, 12, 18, and 24 weeks in the dog. At termination (25th week) necropsy was carried out on all animals and the essential organs were removed for histopathology. No adverse reactions were noted except very slight irritation at the site of the subcutaneous injections.

Teratological studies were performed in the rat and the mouse, doses of 200 and 500 mg./kg. being administered orally during days 6-15 of pregnancy in the rat and days 8-17 of pregnancy in the mouse. No adverse reactions were noted in the mothers or the fetuses except in the rat at the high dose, which was greatly in excess of the therapeutic dose, where there was an increased incidence of resorptions.

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α-Amino-*p*-Hydroxybenzylpenicillin (BRL 2333), a New Semisynthetic Penicillin: In Vitro Evaluation

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α-Amino-*p*-hydroxybenzylpenicillin (BRL 2333) is a new broad-spectrum semisynthetic penicillin comparable to ampicillin in spectrum and level of activity in vitro. The new penicillin was active against penicillin-susceptible strains of gram-positive cocci, *Neisseria* species, *Haemophilus influenzae*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella* species, and *Shigella* species. Like ampicillin, BRL 2333 was not active against penicillinase-producing strains of staphylococci, *Klebsiella-Enterobacter* species, or strains of *Pseudomonas aeruginosa*. BRL 2333 was bactericidal in activity, as are other penicillins, and the activity of the compound was not significantly influenced by the presence of serum.

α-Amino-*p*-hydroxybenzylpenicillin (BRL 2333), a new semisynthetic penicillin synthesized in the chemistry laboratories of Beecham Research Laboratories (4), has a spectrum and level of activity similar to that of the broad-spectrum penicillin ampicillin (6). However, BRL 2333 differs from ampicillin in that it is absorbed to a greater extent after oral administration to human subjects and gives rise to concentrations in serum approximately twice as high as those obtained with equivalent doses of ampicillin (3, 5). The data presented in this paper were derived from experiments designed to compare the relative in vitro antibacterial activities of BRL 2333 and ampicillin.

MATERIALS AND METHODS

BRL 2333 is D(-)α-amino-*p*-hydroxybenzylpenicillin trihydrate (Fig. 1). It is an off-white, free-flowing powder with a solubility in water of 0.4 g/100 ml at room temperature. In simulated gastric juice (*p*H 1.5), a 1% solution of BRL 2333 at 37 C has a half-life of 17 hr compared with a half-life of 12 hr for ampicillin. Ampicillin used in these studies was ampicillin trihydrate (Beecham Research Laboratories; 840 μg/mg, free acid); BRL 2333 contained 830 μg/mg (free acid).

Cultures. The bacteria used in these studies were mainly clinical isolates provided by hospitals in the United Kingdom and were classified by standard fermentation and biochemical reactions.

Minimal inhibitory concentrations. Antibacterial activity was determined by serial dilution of the antibiotics in 5-ml volumes of nutrient broth (Oxoid no. 2) or 18-ml volumes of nutrient agar (Blood Agar Base, Oxoid). For tests in liquid medium, the inoculum was one drop (0.03 ml) of an undiluted overnight broth culture, and for tests with solid medium, 1 drop (0.003 ml) of an overnight broth culture delivered with a multiple inoculating device. Minimal inhibitory concentrations were determined after 18 hr of incubation at 37 C.

Bactericidal activity. The test bacteria were incubated at 37 C in nutrient broth containing known concentrations of the antibiotics. Samples were taken at intervals, and 0.02-ml volumes of suitable dilutions were pipetted onto nutrient agar plates with a standard dropping pipette. Colonies were counted after 18 hr of incubation at 37 C, and the number of viable bacteria was estimated.

Binding to serum. The extent of binding of the antibiotics to protein of human serum was measured by ultrafiltration of serum containing known concentrations of the antibiotics through a cellulose acetate (Visking) dialysis membrane (7). The amount of antibiotic present in the protein-free ultrafiltrate representing unbound antibiotic was measured by microbiological assay.

Susceptibility discs. Susceptibility discs containing selected quantities of BRL 2333 or ampicillin were prepared by pipetting 0.02-ml volumes of standard aqueous solutions of the penicillins onto the surface of Whatman antibiotic assay filter-paper discs (Whatman AA discs, 6.0-mm diameter). The discs were dried at 37 C for 30 to 60 min prior to use. The inhibition zones produced with these discs against numerous clinical

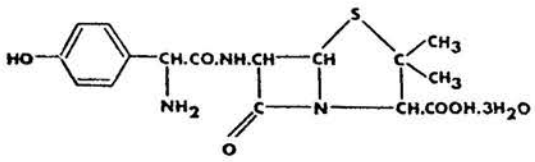

Fig. 1. *Structure of α-amino-p-hydroxybenzylpenicillin (BRL 2333).*

isolates of bacteria of known susceptibility to BRL 2333 and ampicillin were determined by two methods. (i) In the method described by Bauer et al. (2), a standardized inoculum was swabbed on the surface of Mueller-Hinton agar (Difco) plates. (ii) Nutrient agar (Blood Agar Base, Oxoid) plates were flooded with an overnight culture of the test organism, and excess culture was removed with a pasteur pipette. In both methods, inhibition zones were measured after overnight incubation at 37 C.

RESULTS

Antibacterial activity. The antibacterial spectrum of BRL 2333 against gram-positive and gram-negative bacteria is shown in Table 1. It can be seen that BRL 2333 was active at low concentrations against gram-positive cocci, including streptococci, pneumococci, and penicillin-susceptible staphylococci, and against gram-positive bacilli, including corynebacteria, listeria, and aerobic and anaerobic spore-bearing bacilli. The compound was not active against penicillinase-producing strains of *Staphylococcus aureus*, because of instability to this particular β-lactamase. BRL 2333 was also active against gram-negative bacteria, including gonococci, meningococci, *Haemophilus influenzae*, *Bordetella pertussis*, *Pasteurella* species, *Escherichia coli*, *Proteus mirabilis*, *Salmonella*, and *Shigella* species. Gram-negative organisms which were not susceptible to BRL 2333 included

Table 1. *Antibacterial spectrum of α-amino-p-hydroxybenzyl penicillin (BRL 2333)*

Organism	Minimal inhibitory concn (μg/ml)[a]
Staphylococcus aureus NCTC 6571	0.1
S. aureus[b]	250
Beta-hemolytic streptococcus	0.01
Alpha-hemolytic streptococcus	0.01
Streptococcus pneumoniae	0.05
S. faecalis	1.25
Bacillus anthracis	0.25
B. subtilis ATCC 6633	0.25
Corynebacterium diphtheriae	0.02
Erysipelothrix rhusiopathiae	0.02
Listeria monocytogenes NCTC 5348	0.1
Sarcina lutea NCTC 8340	0.005
Clostridium tetani	0.05
C. welchii	0.05
Brucella abortus NCTC 8226	0.25
B. melitensis NCTC 8223	0.1
B. suis NCTC 5061	0.25
Bordetella pertussis	0.5
Haemophilus influenzae	0.25
Neisseria gonorrhoeae	0.01
N. meningitidis	0.02
Pasteurella septica NCTC 948	0.5
Escherichia coli NCTC 10418	5.0
Salmonella typhi	1.25
Shigella sonnei	2.5
Klebsiella aerogenes	250
Pseudomonas aeruginosa	>500
Proteus mirabilis	2.5
P. morganii	250
P. rettgeri	50
P. vulgaris	250
Vibrio cholerae NCTC 8021	5.0

[a] Serial dilution in agar.
[b] Penicillinase-producing strain.

Table 2. *Activity of α-amino-p-hydroxybenzylpenicillin (BRL 2333) and ampicillin against gram-positive cocci*[a]

Organism	Penicillin	Minimal inhibitory concn[b] (μg/ml)							
		1.25	0.5	0.25	0.12	0.05	0.02	0.012	0.005
Staphylococcus aureus	BRL 2333	–	–	4	19	1	–	–	–
(24 strains)	Ampicillin	–	–	1	19	4	–	–	–
Streptococcus pyogenes	BRL 2333	–	–	–	–	–	3	17	–
(20 strains)	Ampicillin	–	–	–	–	–	7	11	2
S. pneumoniae	BRL 2333	–	–	–	–	6	5	1	–
(12 strains)	Ampicillin	–	–	–	–	4	4	3	1
S. faecalis	BRL 2333	11	5	–	–	–	–	–	–
(16 strains)	Ampicillin	16	–	–	–	–	–	–	–

[a] Results show the number of strains for which a given concentration was the minimal inhibitory concentration.
[b] Serial dilution in agar.

Klebsiella-Enterobacter species, *Pseudomonas aeruginosa*, and indole-positive *Proteus* species.

Results are given in Table 2 comparing the activities of BRL 2333 and ampicillin against gram-positive cocci. BRL 2333 was as active as ampicillin against the test strains of *Staphylococcus aureus*, *Streptococcus pyogenes*, *S. pneumoniae*, and *S. faecalis*. Likewise, BRL 2333 showed activity similar to that of ampicillin against clinical isolates of gram-negative bacteria (Table 3); strains of *E. coli*, *P. mirabilis*, *Salmonella* species, and *Shigella sonnei* were generally susceptible to both penicillins at concentrations of 5 μg or less/ml. There was complete cross-resistance between BRL 2333 and ampicillin, and strains of gram-negative bacilli which were resistant to ampicillin were resistant to BRL 2333. Strains of *Klebsiella-Enterobacter* species and *Serratia marcescens* were generally insusceptible to BRL 2333 and ampicillin. BRL 2333 inhibited most of the test strains of *H. influenzae* at concentrations of 0.25 to 0.5 μg/ml, and was similar in activity to ampicillin against this species (Table 4). BRL 2333 was as active as ampicillin against *Neisseria gonorrhoeae*. Both penicillins were slightly less active than benzylpenicillin against susceptible strains of gonococcus, but were rather more active than the latter against penicillin-insusceptible strains (Table 4).

Bactericidal activity. The relative bactericidal activities of BRL 2333 and ampicillin are compared in Fig. 2, which illustrates the results of a viable count experiment with the two penicillins. Both compounds brought about a rapid reduction in the viable count of *E. coli* NCTC 10418 at concentrations of 5 and 20 μg/ml.

Effect of serum. The bactericidal activities of

Table 3. *Activity of α-amino-p-hydroxybenzylpenicillin (BRL 2333) and ampicillin against gram-negative bacilli*[a]

Organism	Penicillin	Minimal inhibitory concn[b] (μg/ml)							
		>100	100	50	25	12.5	5.0	2.5	1.25
Escherichia coli	BRL 2333	16	1	1	2	42	90	9	5
(166 strains)	Ampicillin	12	5	1	1	23	91	29	4
Proteus mirabilis	BRL 2333	8	–	–	–	–	11	28	25
(72 strains)	Ampicillin	8	–	–	–	–	1	25	38
Shigella sonnei	BRL 2333	3	1	1	–	2	6	3	–
(16 strains)	Ampicillin	2	2	–	–	–	8	3	1
Salmonella species	BRL 2333	–	–	–	–	–	–	8	2
(10 strains)	Ampicillin	–	–	–	–	–	–	7	3
Klebsiella-Enterobacter	BRL2333	18	2	1	–	–	–	1	–
(22 strains)	Ampicillin	17	–	2	1	1	–	1	–
Serratia marcescens	BRL 2333	7	6	3	1	–	1	–	–
(18 strains)	Ampicillin	5	10	–	2	–	1	–	–

[a] Results show the number of strains for which a given concentration was the minimal inhibitory concentration.
[b] Serial dilution in agar.

Table 4. *Activity of α-amino-p-hydroxybenzylpenicillin (BRL 2333) and ampicillin against Haemophilus influenzae and Neisseria gonorrhoeae*[a]

Organism	Penicillin	Minimal inhibitory concn (μg/ml)							
		1.0	0.5	0.25	0.1	0.05	0.025	0.001	0.005
H. influenzae[b]	BRL 2333	8	27	30	14	2	1	–	–
(82 strains)	Ampicillin	5	21	40	13	3	–	–	–
N. gonorrhoeae[c]	BRL 2333	-	–	5	1	3	3	1	–
(12 strains)	Ampicillin	–	–	3	4	–	2	4	–
	Benzylpenicillin	1	3	–	1	–	2	3	3

[a] Results show the number of strains for which a given concentration was the minimal inhibitory concentration.
[b] Serial dilution in chocolate-blood-agar
[c] Serial dilution in DST agar (Oxoid) + 10% lysed horse blood.

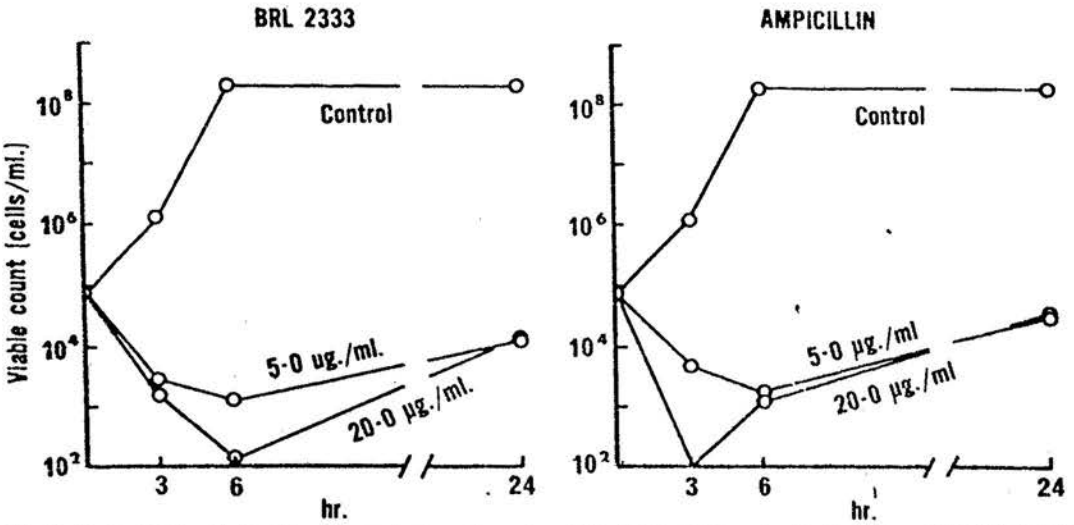

Fig. 2. Bactericidal activity of α-amino-p-hydroxybenzylpenicillin (BRL 233) against Escherichia coli NCTC 10418.

BRL 2333 and ampicillin against *S. aureus* Smith were determined in nutrient broth and in human serum. The presence of human serum did not reduce the activity of either drug. The minimal bactericidal concentration of both drugs against this strain was 0.06 µg/ml in both the presence and absence of serum.

Extent of binding to serum. BRL 2333 was not highly bound to the protein of human serum, 83% of the penicillin being present in human serum as unbound active antibiotic. The results in Table 5 show that the extent of binding to serum of BRL 2333 was similar to that of ampicillin and less than that of benzylpenicillin or carbenicillin.

Combination with penicillinase-stable penicillins. Combinations of BRL 2333 with penicillinase-stable penicillins such as methicillin or cloxacillin often showed enhanced activity against β-lactamase-producing strains of *Klebsiella-Enterobacter, Proteus* species, and *Shigella sonnei*; Table 6 shows examples of this effect. In general, the enhancement of the combination of BRL 2333 with methicillin or cloxacillin was only pronounced when either of the latter penicillins was present at a relatively high concentration (100 µg/ml). At lower concentrations of the penicillinase-stable penicillin (e.g., 10 µg/ml), synergistic effects were less frequent and less pronounced.

Susceptibility discs. The standardized single-disc method described by Bauer et al. (2) recommends the use of a 10-µg ampicillin disc

Table 5. *Extent of binding of α-amino-p-hydroxybenzylpenicillin (BRL 2333) in human serum*

Penicillin	Unbound (%)	Bound (%)
BRL 2333	83	17
Ampicillin	82	18
Benzylpenicillin	41	59
Carbenicillin	53	47

Table 6. *Antibacterial activities of combinations of α-amino-p-hydroxybenzylpenicillin (BRL 2333) and methicillin or cloxacillin*

Organism	Minimal inhibitory concn (µg/ml)[a] of BRL 2333		
	Alone	Plus methicillin[b]	Plus cloxacillin[b]
Escherichia coli			
37	125	1.25	1.25
83	>500	500	50
Klebsiella aerogenes			
A	250	1.25	25
C	500	50	125
Paracolon 232	125	2.5	1.25
Proteus morganii			
A	25	25	12.5
G	250	1.25	2.5
P. vulgaris K	250	50	12.5
Shigella sonnei			
5147	250	125	5.0
4387	250	50	2.5

[a] Serial dilution in nutrient agar.
[b] Present in a concentration of 100 µg/ml.

for determining the susceptibility of bacteria to ampicillin. In the series of tests reported here, which adhered strictly to the conditions of the method referred to above, a disc containing 15 μg of BRL 2333 was almost exactly equivalent to a 10-μg ampicillin disc, and the interpretation of zone sizes recommended for a 10-μg ampicillin disc apply equally to a 15-μg BRL 2333 disc (Table 7). Inhibition zones produced with a disc containing 10 μg of BRL 2333 were consistently smaller than those produced with a 10-μg ampicillin disc.

In the series of tests in which plates flooded with a large inoculum of cells was used, a method favored in many laboratories, a disc containing 25 μg of BRL 2333 produced inhibition zones similar to those obtained with a 25-μg ampicillin disc. In these tests, the 25-μg disc produced inhibition zones at least 15 mm in diameter with gram-negative bacilli susceptible to 5 μg or less/ml of BRL 2333 or ampicillin.

Table 7. *Correlation between antibiotic susceptibility and inhibition zones produced with α-amino-p-hydroxybenzylpenicillin (BRL 2333) and ampicillin susceptibility discs*[a]

Organism	Inhibition zone diam. (mm)		
	Resistant	Intermediate	Susceptible
S. aureus	20 or less	21-28	29 or more
All others	11 or less	12-13	14 or more

[a] Based on method of Bauer et al. (2). The results shown were obtained with 10-μg ampicillin discs and 15-μg BRL 2333 discs.

DISCUSSION

The data presented in this paper show that the new semisynthetic penicillin BRL 2333 is similar to ampicillin with respect to antibacterial activity, bactericidal activity, and extent of binding to the protein of human serum. BRL 2333 is, however, absorbed to a greater degree than ampicillin after oral administration to humans (3, 5) and produces greater levels of antibacterial activity in serum than are obtained with equivalent doses of ampicillin. Moreover, BRL 2333 has been demonstrated to be superior to ampicillin in the treatment of experimental animal infections (1). In view of these results, BRL 2333 would appear to warrant clinical trial.

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α-Amino-*p*-Hydroxybenzylpenicillin (BRL 2333), a New Semisynthetic Penicillin: Absorption and Excretion in Man

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α-Amino-*p*-hydroxybenzylpenicillin (BRL 2333) is a new broad-spectrum semisynthetic penicillin similar in activity to ampicillin. After oral administration to healthy volunteer subjects, BRL 2333 produced concentrations in serum approximately twice as high as those obtained with equivalent doses of ampicillin. BRL 2333 was excreted in urine in higher concentrations than was ampicillin, and approximately 60% of the dose of BRL 2333 administered was recovered in the urine in the first 6 hr after administration. Higher and more prolonged concentrations in serum were obtained with concomitant administration of probenecid, and the absorption of BRL 2333 was little influenced by the presence of food.

BRL 2333, α-amino-*p*-hydroxybenzylpenicillin, is a new semisynthetic penicillin derived from 6-aminopenicillanic acid. The antibacterial spectrum and level of activity of this new penicillin are similar to those of ampicillin (4), but BRL 2333 is generally more effective than ampicillin in the treatment of experimental infections in mice (1).

The present communication describes studies on the absorption and excretion of BRL 2333 in healthy volunteer subjects, and crossover absorption and excretion studies in comparison with ampicillin.

MATERIALS AND METHODS

The absorption and excretion studies described here were carried out in two panels of 12 healthy volunteer subjects, consisting of 16 men with an average age of 29 years (range, 22 to 50 years) and an average weight of 74 kg (range, 55 to 87 kg) and 8 women with an average age of 31 years (range, 22 to 38 years) and an average weight of 59 kg (range, 50 to 70 kg). Crossover studies were carried out to determine the effect of food and the effect of probenecid on the absorption and excretion of BRL 2333. BRL 2333 was also compared in crossover studies with ampicillin and pivampicillin. In these crossover studies, there was generally an interval of 1 week between the two halves of the study.

Ampicillin used in these absorption studies was ampicillin trihydrate [Penbritin, Beecham Research Laboratories (840 μg/mg, free acid)] and BRL 2333 was α-amino-*p*-hydroxybenzylpenicillin trihydrate (830 μg/mg, free acid). Pivampicillin (the pivaloyloxymethyl ester of ampicillin) was supplied by Leo Laboratories Ltd. as the ester hydrochloride.

In the studies on absorption in the fasting state, volunteers were requested to take nothing by mouth after midnight of the day prior to the study. The test penicillin, BRL 2333 or ampicillin, was administered in plain gelatin capsules with a little water at 9:30 AM on the day of the test. The subjects were allowed to eat a standard breakfast after the 1-hr blood specimen was taken. In experiments to determine the effect of food on the absorption of BRL 2333, the volunteers were instructed to partake of a normal breakfast prior to administration of BRL 2333.

Samples of venous blood were collected at 0.5, 1, 2, 4, and 6 hr after drug administration, and urine was collected in the first 6 hr after administration. Serum was separated by centrifugation and stored at -20 C until assayed on the following day. The concentrations of BRL 2333 in serum and urine specimens were determined by a conventional large-plate agar-diffusion method similar to that described for ampicillin (3) assay, with *Sarcina lutea* ATCC 9341 as assay organism.

Urine from subjects given BRL 2333 was spotted (6 μliters) on filter-paper strips (Whatman no. 1, 1 cm wide) and chromatographed with a butanol-ethyl alcohol solvent system (*n*-butanol-ethanol-water, 4:1:5, v/v, top phase). Standard solutions of BRL 2333 were prepared in 0.05 M phosphate buffer (*p*H 7.0) and chromatographed. Chromatography was carried out overnight at 5 C, and the chromatograms were dried at 40 C. The penicillins were located by placing the dried chroma-

tograms on nutrient agar (Blood Agar Base, Oxoid) seeded with a spore suspension of *Bacillus subtilis* ATCC 663[illegible] and incubating overnight at 37 C.

RESULTS

Absorption and excretion in fasting subjects. Data in Table 1 show the concentrations of BRL 2333 obtained in the serum of fasting volunteer subjects after administration of single oral doses of 125, 250, 375, 500, 625, and 1,000 mg. It can be seen that increasing the dose resulted in a corresponding increase in the serum concentrations. The mean peak concentrations at 2 hr were 2.6 μg/ml with the 125-mg dose, 5.3 μg/ml with the 250-mg dose, 10.8 μg/ml with the 500-mg dose, and 19.7 μg/ml with the 1,000-mg dose. In these studies, the concentrations in serum fell quite rapidly to relatively low levels at 6 hr, as is generally the case with penicillins. The compound was well tolerated, and no side effects were observed at any time during the tests.

In these studies in fasting subjects, the amount of BRL 2333 excreted in the urine in the 6-hr period after oral administration varied from 54% of the 125-mg dose to 66% of the 1,000-mg dose (Table 1). The average concentrations of BRL 2333 measured in the pooled urine collected over the 6-hr period after dosing were as follows: 125-mg dose, 210 μg/ml (range, 96 to 380 μg/ml); 250-mg dose, 580 μg/ml (range, 280 to 1,320 μg/ml); 500-mg dose, 850 μg/ml (range, 360 to 1,300 μg/ml).

Bioautographic examination of urine from subjects receiving BRL 2333 failed to show the presence of any biologically active metabolite.

Effect of food. Results given in Table 2 show the mean drug concentrations in serum obtained in crossover studies with 250- and 375-mg doses of BRL 2333 in volunteers in the fasting and nonfasting state. In both studies, the concentrations reached in serum were slightly lower and slightly more prolonged in the nonfasting subjects compared with fasting subjects. The amount excreted in the urine in the 6-hr period after dosing was similar in fasting and nonfasting subjects receiving the 375-mg dose, but in the study with a 250-mg dose the fasting subjects excreted 56% of the dose compared with 70% excretion for the nonfasting subjects.

Effect of probenecid. The effect of adminis-

Table 1. *Absorption and excretion of α-amino-p-hydroxybenzylpenicillin (BRL 2333) after oral administration to fasting human subjects*

Dose (mg)	No. of subjects	Mean concn in serum (μg/ml)					Urine excretion, 0-6 hr (% of dose)
		0.5 hr	1 hr	2 hr	4 hr	6 hr	
125	22	0.4[illegible] (±0.5)[a]	1.46 (±1.0)	2.62 (±0.94)	0.57 (±0.27)	0.15 (±0.07)	54 (±13)
250	22	1.73 (±1.48)	4.00 (±2.12)	5.31 (±1.09)	0.98 (±0.39)	0.30 (±0.11)	62 (±21)
375	11	2.00 (±1.53)	4.41 (±2.00)	7.10 (±1.71)	1.60 (±0.48)	0.44 (±0.13)	67 (±13)
500	12	2.32 (±2.09)	7.61 (±3.09)	10.80 (±2.92)	2.34 (±0.84)	0.84 (±0.30)	59 (±15)
625	11	2.91 (±2.78)	9.53 (±6.45)	11.40 (±3.79)	3.90 (±1.88)	1.22 (±0.58)	55 (±14)
1,000	12	4.53 (±3.9)	11.30 (±6.3)	19.70 (±5.4)	5.50 (±1.5)	1.46 (±0.46)	66 (±16)

[a] Standard deviation.

Table 2. *Effect of food on the absorption and excretion of α-amino-p-hydroxybenzylpenicillin (BRL 2333) in human subjects*[a]

Dose (mg)	State	Mean concn in serum (μg/ml)					Urine excretion, 0-6 hr (% of dose)
		0.5 hr	1 hr	2 hr	4 hr	6 hr	
250	Nonfasting	1.2	4.3	4.7	1.6	0.5	70
	Fasting	2.0	4.8	5.2	1.1	0.4	56
375	Nonfasting	2.5	5.9	5.9	2.5	0.8	53
	Fasting	2.1	5.5	7.3	1.6	0.5	53

[a] Twelve subjects were tested at each dosage.

tering probenecid (0.5 g at 11.5 hr and 0.5 g at 1 hr prior to the test) on the absorption and excretion of a 250- and 375-mg dose of BRL 2333 administered to fasting human subjects is shown in Table 3. It can be seen that the administration of probenecid resulted in a marked increase in the mean concentrations of BRL 2333 in serum, the difference being statistically significant at 2, 4, and 6 hr with the 250-mg dose and at 1, 2, 4, and 6 hr with the 375-mg dose. The administration of probenecid also resulted in a statistically significant reduction in the amount of drug excreted in the urine during the 6-hr period after dosing (Table 3).

Comparison with ampicillin. The mean concentrations in serum obtained in crossover studies to compare the absorption and excretion of BRL 2333 and ampicillin after administration of a 250- and 500-mg dose to fasting subjects are illustrated in Fig. 1 and Fig. 2. Both penicillins produced mean peak concentrations in serum at 2 hr, and in both studies the mean peak concentrations of BRL 2333 were approximately double those produced with ampicillin (250-mg dose: BRL 2333, 5.3 μg/ml, ampicillin, 2.7 μg/ml; 500 mg dose: BRL 2333, 10.8 μg/ml, ampicillin, 6.3 μg/ml). The serum concentrations of BRL 2333 were markedly higher than those of ampicillin throughout the 6-hr period and were statistically significant ($P = 0.05$) at 1, 2, and 4 hr after administration. The better absorption of BRL 2333 was reflected in the urinary excretion of the two penicillins; approximately 60% of the dose of BRL 2333 was recovered in the urine compared with an average of 40% in the case of ampicillin.

Comparison with pivampicillin. Improved concentrations of ampicillin in serum have recently been reported (2) after the oral administration of various esters of ampicillin, particularly pivampicillin (the pivaloyloxymethyl ester). After absorption, pivampicillin is hydrolyzed to ampicillin in the body.

It was, therefore, of interest to compare the concentrations of BRL 2333 in serum with the ampicillin concentrations produced in serum after oral administration of pivampicillin. The data on pivampicillin reported by Daehne et al. (2) relate to a dosage of 358 mg of the anyhydrous penicillin. Studies were therefore carried out comparing 358 mg of anhydrous pivampicillin with 358 mg of anhydrous BRL 2333.

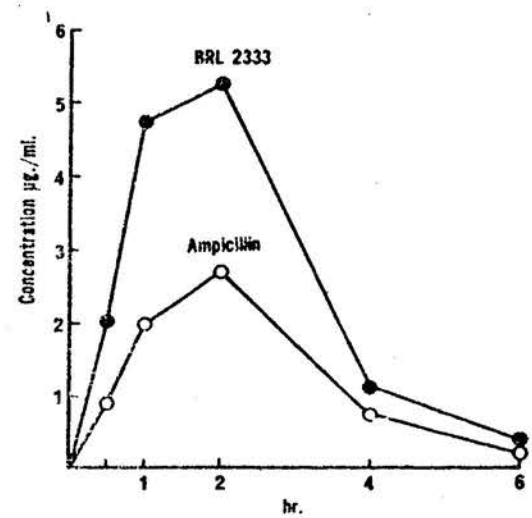

Fig. 1. *Drug concentrations in the serum of 12 fasting human subjects after a single 250-mg dose of α-amino-p-hydroxybenzylpenicillin (BRL 2333) or ampicillin.*

Table 3. *Effect of probenecid on the absorption and excretion of α-amino-p-hydroxybenzylpenicillin (BRL 2333) in fasting human subjects*

Dose (mg)	No. of subjects	Regimen	Mean concn in serum (μg/ml)					Urine excretion, 0-6 hr (% of dose)
			0.5 hr	1 hr	2 hr	4 hr	6 hr	
250	11	With probenecid	1.8	4.7	7.7	3.1	1.1	54
	12	Without probenecid	1.5	3.8	5.1	1.0	0.3	67
375	11	With probenecid	2.8	7.3	11.8	4.7	1.5	53
	11	Without probenecid	2.0	4.4	7.1	1.6	0.4	67

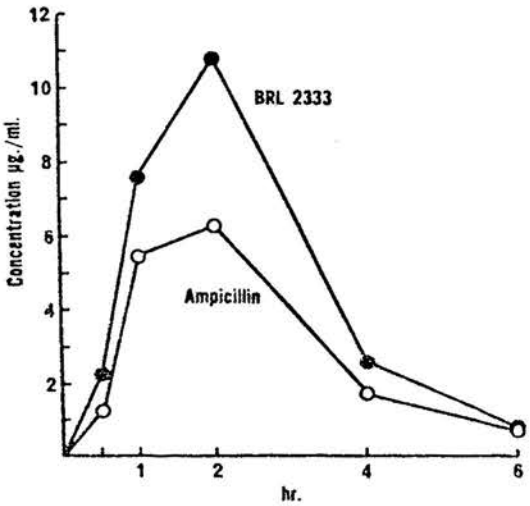

Fig. 2. *Drug concentrations in the serum of 12 fasting human subjects after a single 500-mg dose of α-amino-p-hydroxybenzylpenicillin (BRL 2333) or ampicillin.*

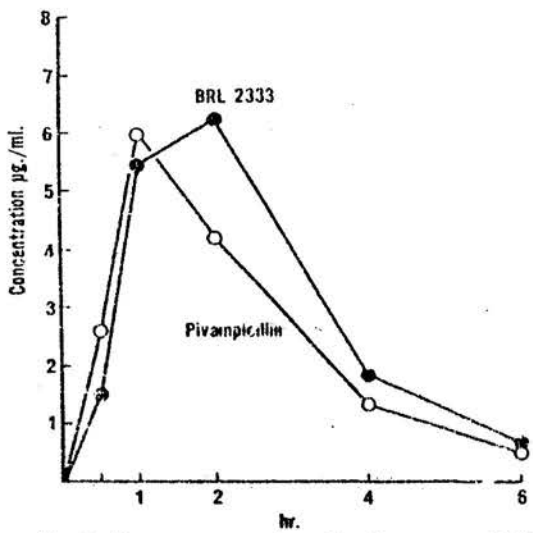

Fig. 3. *Drug concentrations in the serum of 15 fasting human subjects after a single 358-mg dose of α-amino-p-hydroxybenzylpenicillin (BRL 2333) or pivampicillin (pivaloyloxymethyl ester of ampicillin).*

Data in Fig. 3 show that pivampicillin was absorbed more rapidly than BRL 2333, and over the first hour after dosage the levels of ampicillin obtained by administration of pivampicillin were higher than the BRL 2333 concentrations in serum during the same period. Peak serum concentrations of BRL 2333 however, were reached later (2 hr after dosing) and were slightly higher than those with pivampicillin, and over the period from 2 to 6 hr the levels of BRL 2333 were higher than those of pivampicillin. The superior levels of BRL 2333 are also reflected in a greater total area under the curve compared with pivampicillin.

DISCUSSION

The data presented here show that BRL 2333 is well absorbed when given to fasting or nonfasting human subjects, and that the oral absorption of BRL 2333 is not greatly reduced after ingestion of food. As with other penicillins, administration of probenecid results in increased serum concentrations and in a decrease in the amount excreted in urine in the first 6 hr. Similarly, increasing the dose administered causes a corresponding increase in the serum concentrations attained, a characteristic of penicillin therapy.

In crossover studies to compare the absorption and excretion of BRL 2333 and ampicillin, the mean peak concentrations of BRL 2333 in serum were double those produced with the equivalent dose of ampicillin, and the serum concentrations of BRL 2333 were substantially higher than those of ampicillin over the 6-hr period after drug administration. Similarly, the excretion of BRL 2333 in urine was correspondingly greater than that of ampicillin, and high levels of BRL 2333 were reached in urine.

ACKNOWLEDGMENTS

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Amoxycillin: A new Semi-synthetic Penicillin

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Summary

Amoxycillin (α-amino-*p*-hydroxybenzylpenicillin) is a new semi-synthetic penicillin with a broad spectrum of antibacterial activity similar to that of ampicillin. Penicillin-sensitive strains of staphylococci, streptococci, and pneumococci were sensitive to concentrations of 0·1 μg or less of amoxycillin/ml. Strains of *Haemophilus influenzae* were inhibited by a level of 0·5 μg/ml, and most strains of *Escherichia coli, Proteus mirabilis, Shigella sonnei, Salmonella* species, and *Streptococcus faecalis* were sensitive to a concentration of 5 μg or less of amoxycillin/ml. Penicillinase-producing strains of *Staphylococcus aureus* and strains of *Pseudomonas aeruginosa,* indole-positive *Proteus, Klebsiella,* and *Enterobacter* were insensitive to amoxycillin. The new penicillin was bactericidal in activity, as with other penicillins, and its antibacterial activity was not reduced in the presence of serum. After oral administration to volunteer subjects amoxycillin produced serum concentrations twice as high as those obtained with similar doses of ampicillin, and the penicillin was recovered unchanged in high concentrations in the urine. The absorption of amoxycillin was not greatly influenced by food, and administration of probenecid resulted in increased and more prolonged concentrations of amoxycillin in serum.

Introduction

Amoxycillin (Amoxil; BRL 2333; α-amino-*p*-hydroxybenzylpenicillin) is a new semi-synthetic penicillin synthesized in these laboratories (Long *et al.*, 1971). The compound has a chemical structure related to that of ampicillin (Fig. 1) and its antibacterial spectrum and level of activity are similar to those of ampicillin (Neu and Winshell, 1971a; Sutherland and Rolinson, 1971). However, amoxycillin is better absorbed than ampicillin after oral administration to human subjects and produces serum concentrations considerably higher than those of ampicillin (Neu and Winshell, 1971b; Croydon and Sutherland, 1971). Results are reported here to compare the antibacterial activities and absorption and excretion in man of amoxycillin and ampicillin.

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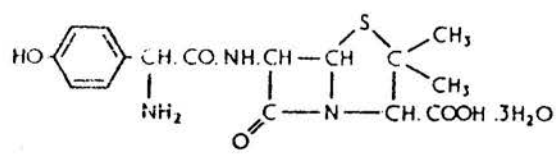
Materials and Methods

Amoxycillin is available as D(-)-α-amino-*p*-hydroxybenzylpenicillin trihydrate (Fig. 1) which, like ampicillin trihydrate, is relatively insoluble in water (0·4% v/w at room temperature),



Mol. wt. 419·46

Amoxycillin, BRL 2333

D(-)-α-amino-p-hydroxybenzylpenicillin trihydrate

FIG. 1—Structure of amoxycillin.

but aqueous solutions may be readily prepared in phosphate buffer, pH 8·0. The penicillin is relatively stable to acid and a 1% solution at 37°C has a half-life in simulated gastric juice (pH 1·5) of 17 hours compared with a half-life of 12 hours for ampicillin. Amoxycillin used in these studies had an assigned potency of 830 μg/mg, and ampicillin trihydrate (Penbritin) a potency of 840 μg/mg, both expressed in terms of anhydrous free acid.

Antibacterial Activity.—Minimum inhibitory concentrations required to inhibit growth of the test organisms for 18 hours at 37°C were measured by serial dilution in agar (Blood Agar Base, Oxoid) or nutrient broth (No. 2, Oxoid). Agar plates were inoculated with one drop (0·003 ml) of an undiluted overnight culture delivered with a multiple inoculating device. For tests in liquid medium the inoculum used was normally one drop (0·03 ml) of an overnight broth culture (about 10^7 cells) in 5 ml of medium.

Binding to Serum Protein.—The extent of binding of amoxycillin and other penicillins to protein of human serum was

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measured by ultrafiltration of serum containing known concentrations of the test penicillin through a cellulose acetate (Visking) dialysis membrane (Rolinson and Sutherland, 1965). The amount of penicillin present in the protein-free ultrafiltrate representing unbound antibiotic was measured by microbiological assay with *Bacillus subtilis* A.T.C.C. 6633 as assay organism.

Assay in Serum and Urine.—The concentrations of amoxycillin and ampicillin in serum and urine were measured by standard large plate microbiological assay with *Sarcina lutea* N.C.T.C. 8340 as assay organism (Knudsen, Rolinson, and Stevens, 1961). For the assay of serum specimens standard solutions of amoxycillin or ampicillin were prepared in pooled human serum to give a range of concentrations from 0·01 to 0·5 µg/ml; for urine specimens the standard solutions were prepared in M/20 phosphate buffer, pH 7·0, over the same range of concentrations. The specimens were diluted as required to an estimated concentration midway in the concentration range (0·05–0·1 µg/ml). The plates were incubated overnight at 30 °C, the inhibition zone diameters were measured, and the concentrations of the test specimens were derived from the standard line constructed from the standard solutions.

Absorption and Excretion Studies.—The penicillins were administered in gelatin capsules as a single dose of 125, 250, 500, or 1,000 mg to healthy male and female volunteer subjects. Venous blood was taken half an hour, one hour, and two, four, and six hours after administration of the penicillin, and urine was collected over the six-hour period. In fasting studies the subjects received the test penicillin after an overnight fast, and were allowed a standard breakfast after the one-hour specimen was taken. In studies to determine the effect of food on absorption, the penicillin was administered one hour after breakfast.

Results

ANTIBACTERIAL ACTIVITY

The antibacterial spectrum of amoxycillin against Gram-positive and Gram-negative bacteria is shown in Table I. It can be seen that the compound was active at low concentrations against Gram-positive cocci, except penicillin-resistant staphylococci, and against Gram-positive aerobic and anaerobic bacilli. Penicillinase-producing strains of *Staphylococcus aureus* were resistant to amoxycillin as a result of β-lactamase activity. Gram-negative bacteria sensitive to amoxycillin included gonococci, meningococci, *Haemophilus influenzae*, *Bordetella pertussis*, *Pasteurella septica*, *Escherichia coli*, *Proteus mirabilis*, and *Salmonella* and *Shigella* species. Strains of *Pseudomonas aeruginosa*, indole-positive *Proteus* species, *Klebsiella* and *Enterobacter* species, *Serratia marcescens*, and *Bacteroides fragilis* were relatively insensitive.

TABLE I—*Antibacterial Spectrum of Amoxycillin*

Organism	Minimum Inhibitory Concentration (µg/ml)*
Staphylococcus aureus N.C.T.C. 6571	0·1
Staph. aureus†	250
Streptococcus pyogenes	0·01
Str. viridans	0·01
Str. pneumoniae	0·02
Str. faecalis	0·5
Bacillus anthracis	0·25
B. subtilis A.T.C.C. 6633	0·25
Corynebacterium diphtheriae	0·02
Erysipelothrix rhusiopathiae	0·02
Listeria monocytogenes N.C.T.C. 5348	0·1
Sarcina lutea N.C.T.C. 8340	0·005
Clostridium tetani	0·05
Cl. welchii	0·05
Bacteroides fragilis	25
Brucella abortus N.C.T.C. 8226	0·25
B. melitensis N.C.T.C. 8223	0·1
B. suis N.C.T.C. 5061	0·25
Bordetella pertussis	0·5
Haemophilus influenzae	0·25
Neisseria gonorrhoeae	0·02
N. meningitidis	0·02
Pasteurella septica N.C.T.C. 948	0·5
Escherichia coli N.C.T.C. 10418	5·0
Salmonella typhi	1·25
Shigella sonnei	2·5
Klebsiella aerogenes	250
Proteus mirabilis	2·5
P. morganii	250
P. rettgeri	50
P. vulgaris	250
Pseudomonas aeruginosa	>500
Serratia marcescens	100
Vibrio cholerae N.C.T.C. 8021	5·0

*Serial dilution in agar. †Penicillinase-producing strain.

The activities of amoxycillin and ampicillin against Gram-positive cocci, *H. influenzae*, and gonococci are compared in Table II and against Gram-negative bacilli in Table III. Amoxycillin was as active as ampicillin against staphylococci and pneumococci and was slightly more active than the latter against β-haemolytic streptococci and enterococci. Strains of *H. influenzae* were inhibited by concentrations of both penicillins, of 0·1–0·5 µg/ml, amoxycillin being slightly less active than ampicillin. Both penicillins were somewhat less active than benzylpenicillin against sensitive strains of gonococcus, but showed slightly greater activity than the latter against penicillin-insensitive strains. Amoxycillin generally showed activity similar to that of ampicillin against Gram-negative bacilli and most strains of *E. coli*, *P. mirabilis*, *Sh. sonnei*, and *Salmonella* species were sensitive to 5 µg or less of amoxycillin/ml. Ampicillin-resistant strains of Gram-negative bacilli were found to be resistant also to amoxycillin, indicating complete cross-resistance between these two penicillins.

TABLE II—*Activity of Amoxycillin and Ampicillin against Gram-positive Cocci, H. influenzae, and Gonococci*

Organism	No. of Strains	Penicillin	M.I.C.* (µg/ml) and No. of Strains							
			1·0	0·5	0·25	0·12	0·05	0·02	0·01	0·005
Staph. aureus	29	Amoxycillin			6	20	3			
		Ampicillin			2	21	6			
β-haemolytic streptococci	28	Amoxycillin						3	25	
		Ampicillin						15	11	2
Str. pneumoniae	18	Amoxycillin					6	6	6	
		Ampicillin					7	6	4	1
Str. faecalis	53	Amoxycillin	11	39	3					
		Ampicillin	29	24						
H. influenzae	98	Amoxycillin	8	29	41	20				
		Ampicillin	5	21	46	20	6			
N. gonorrhoeae	13	Amoxycillin			5	1	3	3	1	
		Ampicillin			3	4		2	4	
		Benzylpenicillin	1	3		1		2	3	3

*Serial dilution in agar.

TABLE III—*Activity of Amoxycillin and Ampicillin against Gram-negative Bacilli*

Organism	No. of Strains	Penicillin	M.I.C.* (μg/ml) and No. of Strains							
			>100	100	50	25	12·5	5·0	2·5	1·25 or less
E. coli	206	Amoxycillin	23	1	1	2	46	115	13	5
		Ampicillin	19	5	1	1	26	113	37	4
P. mirabilis	90	Amoxycillin	13					11	28	38
		Ampicillin	13					1	26	50
Sh. sonnei	26	Amoxycillin	5	1	1		4	11	4	
		Ampicillin	2	4				11	8	1
Salm. species	20	Amoxycillin	2						8	10
		Ampicillin	2						7	11
Klebsiella-Enterobacter	29	Amoxycillin	25	2	1				1	
		Ampicillin	21	3	2	1	1		1	
S. marcescens	18	Amoxycillin	7	6	3	1		1		
		Ampicillin	5	10		2		1		

*Serial dilution in agar.

BACTERICIDAL ACTIVITY

Like ampicillin, amoxycillin was bactericidal in activity, and levels only slightly lower than the minimum inhibitory concentration resulted in a rapid reduction in the viable counts of the test organisms.

EFFECT OF SERUM

The antibacterial activity of amoxycillin was not reduced in the presence of 95% human serum. The extent of binding of amoxycillin to the protein of human serum as measured by ultrafiltration was found to be 17%, leaving 83% of the penicillin in serum as unbound active antibiotic. This degree of binding was similar to that of ampicillin (18% bound) and less than that of benzylpenicillin (59% bound).

SENSITIVITY DISCS

In general, amoxycillin sensitivity discs produced inhibition zones similar to those obtained with equivalent ampicillin discs. In tests, in which plates were flooded with a large inoculum of cells resulting in dense confluent growth, a 25-μg amoxycillin disc produced inhibition zones of 15 mm or greater in diameter with Gram-negative bacilli sensitive to 5 μg of amoxycillin/ml.

ABSORPTION AND EXCRETION IN FASTING SUBJECTS

The results in Table IV show the amoxycillin serum concentrations obtained in fasting volunteers after a single oral dose of 125, 250, 500, and 1,000 mg. The results are the summation of the mean values obtained in a number of separate studies carried out over a period of time. The number of studies and the total number of volunteers is indicated in the table. Data obtained with ampicillin, under the same experimental conditions, after a single oral dose of 250 and 500 mg are also shown in Table IV for comparison. It can be seen that increasing the dose resulted in a corresponding increase in the amoxycillin serum concentrations, and the mean peak serum concentrations, measured two hours after administration, were 2·7 μg/ml with the 125-mg dose, 5·1 μg/ml with the 250-mg dose, 10·8 μg/ml with the 500-mg dose, and 20·6 μg/ml with the 1,000-mg dose. The serum concentrations fell relatively rapidly from two hours onwards, reaching quite low levels at six hours.

In other studies, not included in Table IV, amoxycillin serum concentrations of 0·14 μg/ml at eight hours and 0·08 μg/ml at 11 hours were measured after a 250-mg dose to volunteer subjects. The amount excreted in the urine during the six-hour period after administration ranged from 58 to 68% of the dose administered, and bioautographic examination of the urine showed the presence only of unchanged amoxycillin. The average concentration of amoxycillin in pooled urine collected over the six-hour period after dosing was 580 μg/ml (range 50–1,600 μg/ml) for the 250-mg dose and 1,100 μg/ml (range 115–1,850 μg/ml) for the 500-mg dose.

The mean serum concentrations produced in these studies after a single 250-mg or 500-mg dose of amoxycillin were twice as high as those obtained with the same doses of ampicillin in fasting subjects in different studies done under the same experimental conditions, and the amounts of amoxycillin excreted in the urine during the six-hour period were significantly higher than those of ampicillin ($P < 0.001$) (Table IV). The absorption and excretion of amoxycillin and ampicillin in fasting subjects was also compared directly in cross-over studies in which the same groups of subjects were given single 250-mg and 500-mg oral doses of the penicillins. The results of these studies are

TABLE IV—*Absorption and Excretion of Amoxycillin after Oral Administration to Fasting Human Subjects*

Penicillin	Dose (mg)	No. of Studies	Total No. of Subjects	Mean Serum Concentration (μg/ml)					Excretion in Urine 0–6 hr % of dose
				½ hr	1 hr	2 hr	4 hr	6 hr	
Amoxycillin	125	4	38	0·7 (0·3–0·9)*	2·0 (1·6–2·5)	2·7 (2·4–3·0)	0·7 (0·5–0·8)	0·2 (0·16–0·24)	58 (52–64)
	250	7	76	2·1 (1·7–2·5)	4·6 (4·0–5·2)	5·1 (4·7–5·4)	1·1 (0·9–1·2)	0·4 (0·33–0·43)	64 (59–68)
	500	3	32	2·7 (1·7–3·7)	8·0 (6·4–9·7)	10·8 (9·6–12·0)	2·9 (2·4–3·4)	1·0 (0·8–1·3)	68 (63–73)
	1,000	2	22	4·2 (2·4–6·0)	11·9 (8·3–15·4)	20·6 (18·2–23·0)	5·8 (4·8–6·7)	1·7 (1·3–2·0)	67 (60–74)
Ampicillin	250	14	131	0·9 (0·7–1·0)	2·1 (1·8–2·3)	2·6 (2·4–2·8)	0·7 (0·67–0·8)	0·25 (0·22–0·28)	37 (34–40)
	500	12	123	1·6 (1·2–1·8)	4·0 (3·5–4·4)	5·2 (4·9–5·6)	1·6 (1·45–1·7)	0·56 (0·5–0·62)	48 (45–53)

*Figures in parentheses are 95% confidence intervals.

TABLE V—*Effect of Food on the Absorption of Amoxycillin in Human Subjects*

Dose (mg)	No. of Subjects	Penicillin	State	Mean Serum Concentration (μg/ml)				
				½ hr	1 hr	2 hr	4 hr	6 hr
250	12	Amoxycillin	Non-fasting	1·2	4·3	4·7	1·6	0·5
			Fasting	2·0	4·8	5·2	1·1	0·4

shown in Figs. 2 and 3, which confirm the data in Table IV—namely, that the mean serum concentrations obtained with amoxycillin were about twice as high as those with ampicillin.

Administration of 1 g of probenecid before dosing with amoxycillin resulted in increased and prolonged antibiotic serum concentrations and in a reduction in urinary excretion, which is characteristic of the effect of probenecid on the absorption and excretion of penicillins in general.

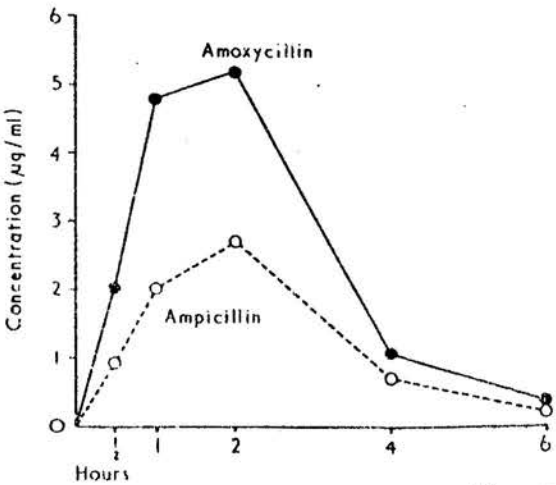

FIG. 2—Mean serum concentrations of amoxycillin and ampicillin after a single 250-mg oral dose in a cross-over study in 12 fasting subjects.

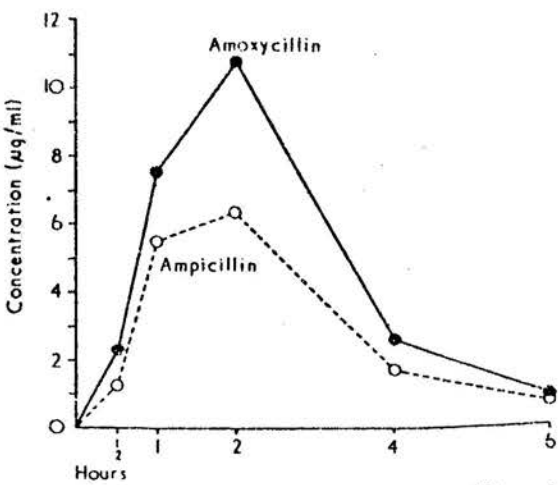

FIG. 3—Mean serum concentrations of amoxycillin and ampicillin after a single 500-mg oral dose in a cross-over study in 12 fasting subjects.

EFFECT OF FOOD

Mean serum concentrations obtained in a cross-over study after a 250-mg dose of amoxycillin in fasting and non-fasting volunteers are shown in Table V. In this study the non-fasting volunteers ate a normal breakfast one hour before taking the penicillin. It will be seen that the levels of amoxycillin achieved in serum after food were very similar to those obtained in the fasting state.

Discussion

The results presented here show that the antibacterial spectrum and level of in-vitro activity of amoxycillin is similar to that of ampicillin. Likewise, amoxycillin is not highly bound to serum protein and its activity is not reduced in the presence of serum. Amoxycillin is evidently well absorbed when given to fasting and non-fasting subjects and produces serum concentrations twice as high as those produced with equivalent doses of ampicillin. Increasing the dose of amoxycillin results in a corresponding increase in serum concentrations, which is characteristic of penicillins. The urinary excretion of amoxycillin is significantly higher than that of ampicillin and 60–70% of an oral dose of the compound appears unchanged in the urine. The taking of food does not significantly alter absorption of amoxycillin, which may be an important benefit, and the serum concentrations of amoxycillin are markedly increased after administration of probenecid.

The antibacterial activity of amoxycillin and its favourable absorption and excretion characteristics are also reflected in animal studies, and amoxycillin has been shown to be superior to ampicillin in the treatment of experimental infections (Acred *et al.*, 1971). The properties of amoxycillin suggest this new semi-synthetic penicillin may have clinical advantages over ampicillin.

We wish to thank Dr. J. H. C. Nayler and his colleagues for preparing the amoxycillin used in these studies and Mrs. Eleanor Fairclough for skilled technical assistance.

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In vitro Antibacterial Activity of Amoxicillin alone and in Combination with other Antibiotics

R. Sutherland[1])

Amoxicillin hat ein breites Spektrum antibakterieller Aktivität. Es ist ebenso aktiv wie Ampicillin gegenüber grampositiven Kokken und Stäbchen, Neisserien, Haemophilus influenzae, E. coli, Salmonellen, Shigellen und Proteus mirabilis. Ebenso wie Ampicillin ist Amoxicillin relativ unwirksam gegenüber penicillinasebildenden Staphylokokken, Klebsiella/Enterobacter und Pseudomonas aeruginosa. Amoxicillin hat einen bakteriziden Wirkungstyp, seine Wirksamkeit wird in Gegenwart von Serum nicht verhindert. Im Papierblättchentest sind die Ergebnisse mit Amoxicillin ähnlich denen von Ampicillin bei Verwendung äquivalenter Blättchen. Gegenüber Enterokokken zeigt die Kombination von Amoxicillin mit Aminoglycosidantibiotika Synergismus, während die bakterizide Wirkung von Amoxicillin durch Chloramphenicol oder Tetracyclin aufgehoben wird. Kombinationen von Amoxicillin mit Sulfonamiden ließen lediglich additive Effekte erkennen. In der Kombination mit Flucloxacillin ließ sich mit Amoxicillin gegenüber gewissen resistenten gramnegativen Bazillen als Ergebnis der Penicillinasehemmung eine gesteigerte Wirksamkeit demonstrieren.

Amoxicillin showed a broad-spectrum of antibacterial activity in vitro and was as active as ampicillin against Gram-positive cocci and bacilli, Neisseria, H. influenzae, E. coli, Salmonellae, Shigellae and P. mirabilis. Like ampicillin, amoxicillin was relatively inactive against penicillinase-producing staphylococci, Klebsiella/Enterobacter and Ps. aeruginosa. The new penicillin was bactericidal in action and its activity was not reduced in serum. In disc sensitivity tests amoxicillin discs gave results similar to those produced by equivalent ampicillin discs.
Synergism was demonstrated by combinations of amoxicillin and aminoglycoside antibiotics against enterococci, whereas the bactericidal activity of the penicillin was antagonised by chloramphenicol or tetracycline. Combinations of amoxicillin and sulphonamides showed additive effects only. In combination with flucloxacillin, amoxicillin demonstrated enhanced activity against certain resistant Gram-negative bacilli as a result of penicillinase-inhibition.

Amoxicillin, D(—)α-amino-p-hydroxybenzylpenicillin, is a new semi-synthetic penicillin with a broad-spectrum of antibacterial activity that is generally similar to that of ampicillin [7, 12,]. The new penicillin is, however, significantly more effective than ampicillin in the treatment of experimental infections in animal [1, 3,] and produces serum concentrations after oral administration to man approximately twice as high as those obtained with ampicillin [8, 11,]. Data are reported here to compare the relative in vitro antibacterial activities of amoxicillin and ampicillin, and to show the effects that may be produced when amoxicillin is combined with other antibiotics.

[1]) Sutherland, R. Dr., Beecham Research Laboratories, Chemotherapeutic Centre, Betchworth, Surrey, England.

Table 1. Antibacterial Spectrum of Amoxicillin.

Organism	MIC, µg/ml [1])
Staphylococcus aureus NCTC 6571	0.1
St. aureus (penicillinase-producing)	250
β-haemolytic streptococcus	0.01
α-haemolytic streptococcus	0.01
Streptococcus pneumoniae	0.05
S. faecalis	0.5
Bacillus anthracis	0.25
Corynebacterium diphtheriae	0.02
Erysipelothrix rhusiopathiae	0.02
Listeria monocytogenes	0.1
Clostridium tetani	0.05
Bacteroides fragilis	25
Brucella abortus	0.25
Bordetella pertussis	0.5
Haemophilus influenzae	0.25
Neisseria gonorrhoeae	0.01
N. meningitidis	0.02
Pasteurella septica	0.5
Escherichia coli NCTC 10418	5.0
Salmonella typhi	1.25
Shigella sonnei	2.5
Klebsiella aerogenes	250
Enterobacter aerogenes	250
Pseudomonas aeruginosa	>500
Proteus mirabilis	2.5
Proteus sp. (indole-positive)	250
Serratia marcescens	250
Vibrio cholerae	5.0

[1]) Serial dilution in agar

Antibacterial Spectrum

Amoxicillin is active against a wide range of Gram-positive and Gram-negative bacteria and its antibacterial spectrum is illustrated in Table 1. It can be seen that amoxicillin was inhibitory at low concentrations against Gram-positive cocci including streptococci, pneumococci and penicillin-sensitive staphylococci, and against Gram-positive aerobic and anaerobic bacilli. However, amoxicillin was not active against penicillinase-producing strains of Staphylococcus aureus as a consequence of this instability to staphylococcal penicillinase. Gram-negative bacteria sensitive to amoxicillin included gonococci, meningococci, Haemophilus influenzae, Bordetella pertussis, Pasteurella septica, Escherichia coli, Proteus mirabilis and Salmonella and Shigella species. Bacteria that were relatively insensitive to amoxicillin included Klebsiella and Enterobacter species, Pseudomonas aeruginosa, Serratia marcescens, indole-positive Proteus species and Bacteroides fragilis.

The activities of amoxicillin and ampicillin against Gram-positive cocci are compared in Fig. 1 and against Gram-negative bacilli in Fig. 2. The results show that amoxicillin was as active as ampicillin against staphylococci and pneumococci, and appeared rather more active against β-haemolytic streptococci and Streptococcus faecalis. Among the Gram-negative bacteria most of the test strains of E. coli, P. mirabilis, Shig. sonnei and Salmonella species were sensitive to 5 µg amoxicillin/ml or lower. Against these bacteria amoxicillin showed a level of activity similar to that of ampicillin. Bacteria that were resistant to amoxicillin were resistant also to ampicillin and there appeared to be complete cross-resistance between the two penicillins.

The activity of amoxicillin against gonococci was of interest as can be seen from Fig. 3 comparing the relative activities of amoxicillin, ampicillin and penicillin G against penicillin-sensitive and penicillin-insensitive strains of gonococci. The two semi-synthetic penicillins were rather less active than penicillin G against gonococci that were highly sensitive to penicillin G, but amoxicillin and ampicillin were as active as the latter compound against strains that showed a decreased sensitivity to penicillin G.

Bactericidal Activity

Like other penicillins, amoxicillin is bactericidal in activity and against most bacteria Minimum Bactericidal Concentrations (M.B.C.) were only slightly higher than the M.I.C. va-

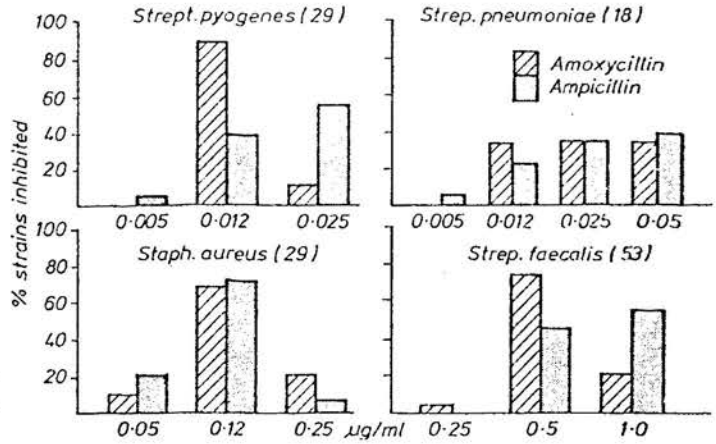

Fig. 1. Distribution of M.I.C. values of amoxicillin and ampicillin against Gram-positive cocci.

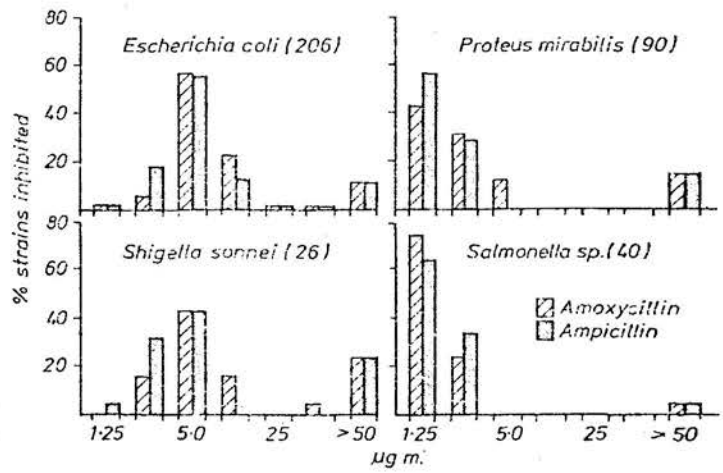

Fig. 2. Distribution of M.I.C. values of amoxicillin and ampicillin against Gram-negative bacilli.

lues (Table 2). An exception was the enterococcus and a significant proportion of cells of this organism survived in the presence of relatively high concentrations of amoxicillin, as is the case with other penicillins such as ampicillin or benzylpenicillin.

The bactericidal effects of low concentrations of amoxicillin against a sensitive strain of E. coli is illustrated in Fig. 4 where it can be seen that concentrations of 2.5 µg/ml or 10.0 µg amoxicillin/ml brought about a rapid reduction in the viable count of the organism.

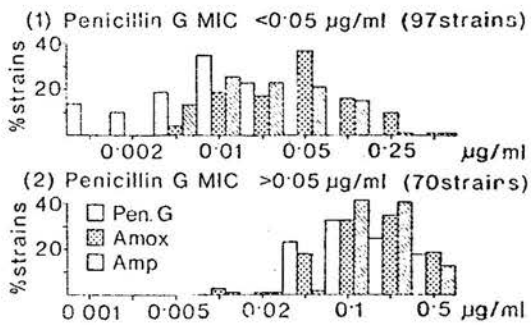

Fig. 3. Relative activities of amoxicillin, ampicillin and penicillin G against penicillin-sensitive and penicillin-insensitive gonococci.

Table 2. Bactericidal activity of amoxicillin.

Organism	M.I.C. (μg/ml)	M.B.C. (μg/ml)
Escherichia coli	2.5	5.0
Salmonella typhi	0.5	0.5
Proteus mirabilis	1.25	2.5
Staphylococcus aureus	0.05	0.1
Streptococcus pyogenes	0.01	0.01
Streptococcus faecalis	0.5	>50

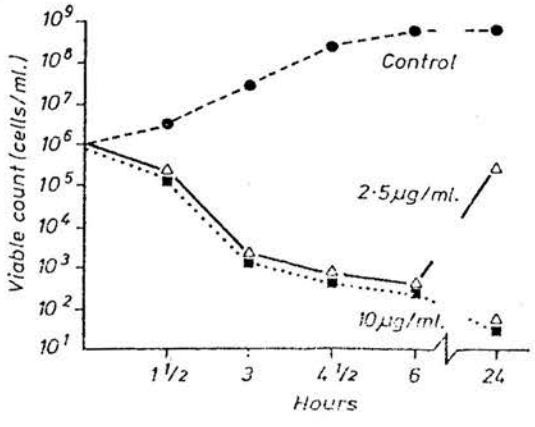

Fig. 4. Bactericidal activity of amoxicillin against *E. coli* NCTC 10418.

Effect of Serum

Amoxicillin is not highly bound to serum protein. In ultrafiltration experiments 17 % was serum-bound leaving 83 % of the penicillin as unbound active drug. This value is similar to that of ampicillin (18 % bound) and les than that of benzylpenicillin (59 % bound). As a consequence of this low level of binding to serum protein, the activity of amoxicillin is not significantly influenced by the presence of human serum. For example, the Minimum Bactericidal Concentration of amoxicillin against Staphylococcus aureus Smith was found to be 0.06 μg/ml when determined in nutrient broth or in 95 % human serum.

Sensitivity-disc testing

In antibiotic disc sensitivity tests the inhibition zones produced by amoxicillin were generally of the same order in size as those produced by equivalent strength ampicillin discs. Various strength ampicillin discs, namely 2μg, 10 μg and 25 μg ampicillin discs are available commercially, and tests were carried out to compare amoxicillin discs with ampicillin discs. The data showed that 2 μg amoxicillin discs were suitable for testing Gram-positive cocci but were not suitable for testing Gram-negative bacteria save for very sensitive organisms such as H. influenzae or Neisseria. In general, a 10 μg amoxicillin disc produced reasonably satisfactory results against Gram-negative bacteria but inhibition zones tended to be rather small when a large inoculum was used in the test. This is illustrated by the results in Table 3 which show the inhibition zones produced by amoxicillin 10 μg and 25 μg discs against large

Table 3. Effect of disc strength and inoculum size on inhibition zones produced by amoxicillin discs against 29 strains of *Escherichia coli*. (M.I.C. 5.0 μg/ml or less)

Disc (μg)	Inoculum [1])	No. of strains with inhibition zone diameter (mm)				
		6—10	11—15	16—20	21—25	26—30
10	Undiluted		25	4		
	1/1000		2	14	12	1
25	Undiluted		9	18	2	
	1/1000			1	27	1

[1]) Plates were flooded with an overnight broth culture diluted as shown.

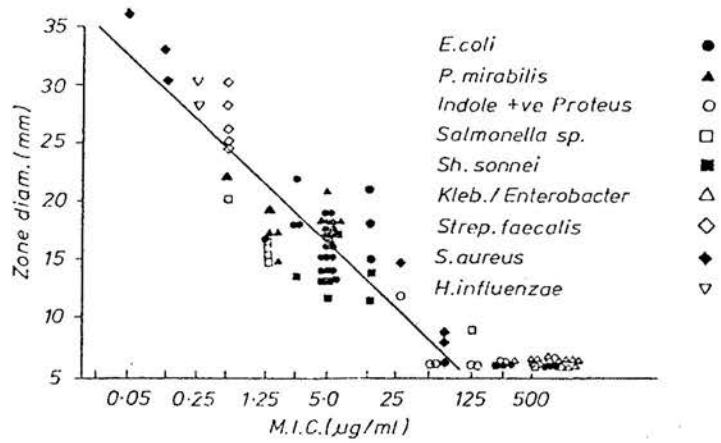

Fig. 5. Regression line relating inhibition zone diameters produced 25 µg amoxicillin disc and M.I.C. values (inoculum, an overnight undiluted broth culture).

and small inocula of sensitive strains of E. coli. It can be seen that with the undiluted inoculum nearly all of the strains gave inhibition zones of less than 15 mm in diameter against the 10 µg disc. With the dilute inoculum, or with the 25 µg disc inhibition zones were larger as might be expected. Results with a 25 µg amoxicillin disc generally showed a relatively clear distinction between sensitive bacteria and resistant bacteria as can be seen from the regression line in Fig. 5 derived from tests with a wide range of bacteria.

Antibacterial activity in combination with other antibiotics

When one antibiotic is combined with another the resulting effect may be greater than the sum of the antibacterial effects of its components (synergism), it may be smaller (antagonism) or it may be the same (additive or indifference). According to *Jawetz* and *Gunnison* [4] the combination of two bactericidal antibiotics may result in synergism, but a bacteriostatic agent may produce antagonism. Data are reported here to demonstrate the various effects arising from the combination of amoxicillin with other antibiotics.

a) Synergism

The classic example of synergism between antibiotics is that demonstrated by benzylpenicillin in combination with streptomycin against the enterococcus, Streptococcus faecalis [5]. Thus, the penicillin is bactericidal against S. faecalis but fails to sterilise cultures of the organism, streptomycin is relatively ineffective, but a combination of the two substances produces a total bactericidal effect. A similar result is obtained when amoxicillin is combined with aminoglycoside antibiotics and data in Table 4 show the bactericidal activities of amoxicillin, alone and in combination with streptomycin, kanamycin, or gentamicin against enterococci. In these bactericidal tests amoxicillin on its own was inactive but combinations of amoxicillin and aminoglycoside antibiotics were effective at low concentrations. However, the amoxicillin/aminoglycoside combination was active only when the test organism was sensitive to the aminoglycoside component of the combination, as can be seen from Table 4. Although amoxicillin failed to sterilise cul-

Table 4. Synergism between amoxicillin and aminoglycoside antibiotics against 12 strains of enterococci.

Antibiotic Combination	Minimum Bactericidal Concentration of amoxicillin (μg/ml) and number of strains									
	0.25	0.5	1.0	2.5	5.0	10	25	50	100	>100
Amoxicillin										12
Amoxicillin + Streptomycin (20 μg/ml)	1	—	2	2						7 [1])
Amoxicillin + Kanamycin (20 μg/ml)		5	4	1						2 [2])
Amoxicillin + Gentamicin (10 μg/ml)	6	3	3							

The aminoglycosides were inactive at the concentrations tested.
[1]) Streptomycin M.I.C. >5000 μg/ml; [2]) Kanamycin M.I.C. >5000 μg/ml.

Table 5. Synergism between amoxicillin and flucloxacillin against penicillinase-producing Gram-negative bacilli

Organism	MIC (μg/ml)				
	Amoxicillin	Flucloxacillin	Amoxicillin	+	Flucloxacillin
Escherichia coli	500	1000	50	+	50
Shigella sonnei	1000	1000	50	+	50
Enterobacter cloacae	500	500	25	+	25
Klebsiella aerogenes	500	1000	50	+	50
Proteus morganii	500	>1000	50	+	50

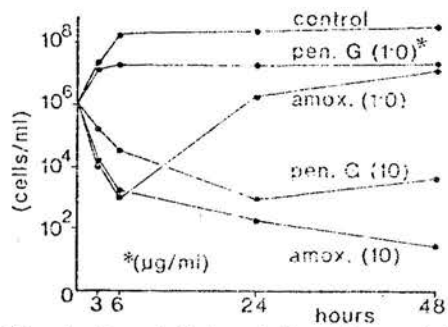

Fig. 6. Bactericidal activities of amoxicillin and benzylpenicillin against *Streptococcus faecalis*.

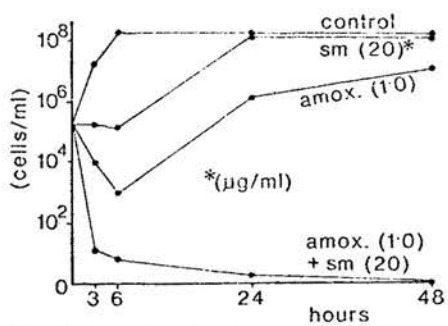

Fig. 7. Synergism between amoxicillin and streptomycin in a viable count experiment with *Streptococcus faecalis*.

tures of S. faecalis in bactericidal tests it did show pronounced bactericidal activity against the test organisms and this is illustrated in the viable count experiment in Fig. 6. Thus, amoxicillin demonstrated a rapid bactericidal effect against the test strain of enterococcus and was more effective than benzylpenicillin, but neither compound was able to sterilise that culture. However, amoxicillin was synergistic with streptomycin and a combination of subinhibitory concentrations of the two antibiotics produced a total bactericidal effect (Fig. 7).

b) Antagonism

The antagonism resulting from the combination of amoxicillin with a bacteriostatic agent is illustrated in Fig. 8 comparing the effects of amoxicillin, tetracycline and a combination of amoxicillin and tetracycline on the viable count of a sensitive strain of E. coli. Amoxicillin produced a rapid bactericidal ef-

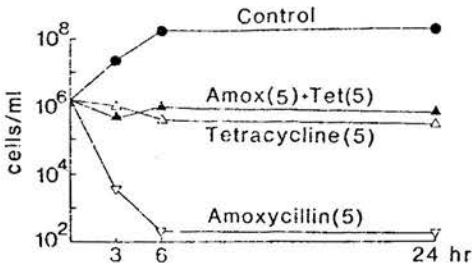

Fig. 8. Antagonistic effect of tetracycline on the bactericidal activity of amoxicillin against *E. coli* NCTC 10418.

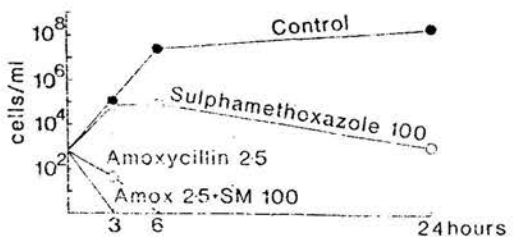

Fig. 9. Additive effect of a combination of amoxicillin and sulphamethoxazole against *E. coli* NCTC 10418.

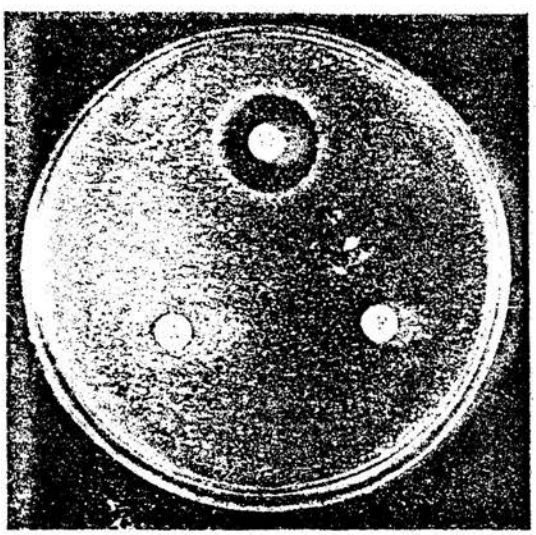

Fig. 10. Sensitivity disc test with amoxicillin, 25 µg disc (A), flucloxacillin, 25 µg disc (F), and disc containing 25 µg amoxicillin and 25 µg flucloxacillin (AF), against a penicillinase-producing strain of *E. coli*.

fect, tetracycline was bacteriostatic in action only, and the combination of amoxicillin and tetracycline was no more effective than tetracycline alone. Similar results were obtained in experiments with amoxicillin and chloramphenicol, illustrating the antagonism that may result from combining a bactericidal antibiotic with a bacteriostatic agent.

c) Indifference

According to *Manten* and *Wisse* [6] the combination of penicillin with bacteriostatic substances does not always result in antagonism and one example is the additive effects observed when penicillins are combined with sulphonamides. Results in Fig. 9 show the effects of combining amoxicillin with sulphamexothazole, and it can be seen that the combination of these compounds produced a bactericidal activity as great as that of amoxicillin alone, in agreement with the predictions of *Manten* and *Wisse* [6].

d) Synergism arising from penicillinase-inhibition

The resistance of certain Gram-negative bacilli to amoxicillin or other broad-spectrum penicillins is usually associated with penicillinase production and the ability of these bacteria to destroy the compounds. Synergism can often be demonstrated by combinations of broad-spectrum penicillins together with penicillinase-stable penicillins and this is generally ascribed to competitive inhibition of β-lactamase activity by the penicillinase-stable penicillin [2, 10]. Thus, the penicillinase-stable penicillin moiety of the combination is inactive against the test organism, but is not destroyed by its penicillinase and is able to inhibit the enzyme, thereby protecting the broad-spectrum penicillin.

Synergism of this type can be readily demon-

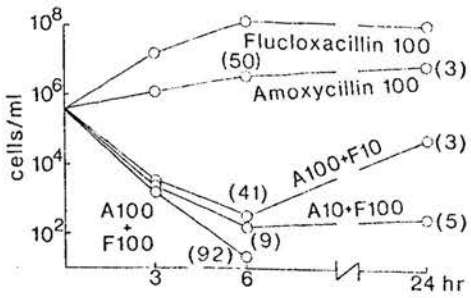

Fig. 11. Synergism between amoxicillin and flucloxacillin in a viable count experiment with a penicillinase-producing strain of a *Shigella sonnei*. (Figures in brackets show residual amoxicillin concentration in μg/ml).

strated by amoxicillin and penicillinase-stable penicillin, such as the isoxazolylpenicillins, against certain penicillinase-producing Gram-negative bacilli. For example, the results of a disc-sensitivity test with a combination of amoxicillin and flucloxacillin are illustrated in Fig. 10; a 25 μg amoxicillin disc or a 25 μg flucloxacillin disc failed to inhibit the growth of a penicillinase-producing strain of E. coli, but a large zone of inhibition was produced by the disc containing 25 μg of each penicillin.

Results in Table 5 show the enhanced activity of a combination of equal parts of amoxicillin and flucloxacillin against a number of amoxicillin-resistant bacteria. It can be seen that the amoxicillin/flucloxacillin combination was effective in inhibiting the growth of the test organisms at concentrations one-twentieth of the inhibitory concentrations of the individual penicillins. Examination of the cultures showed that the bacteria destroyed amoxicillin during the course of the tests, but that in the presence of flucloxacillin the extent of inactivation of amoxicillin was significantly reduced, indicating that the synergism observed between the penicillins was associated with inhibition of penicillinase.

This is illustrated in greater detail in the viable count experiment shown in Fig. 11 where it can be seen that amoxicillin was ineffective at a concentration of 100 μg/ml against a penicillinase-producing strain of Shigella sonnei but that combinations of amoxicillin and fluloxacillin produced bactericidal effects against the test organism. In this test it was obvious that flucloxacillin reduced the rate or extent of inactivation of amoxicillin by the test strain of Shig. sonnei, presumably as a result of competitive inhibition. It is possible however, that some mechanism of synergism other than penicillinase-inhibition was also operating, as the synergism observed during the first six hours of the test appeared to be greater than could be accounted for solely by protection of amoxicillin. For example, the combination containing 10μg amoxicillin/ml together with 100 μg flucloxacillin/ml was markedly more active than 100 μg amoxicillin/ml in the early part of the test even although there was 50 μg amoxicillin/ml present in the amoxicillin culture at the end of the six hours period. This is one of several aspects of synergism between penicillins which is currently under investigation [9].

Acknowledgements

I am indebted to Mrs. E. J. Russell and Mr. B. Slocombe for their skilled technical assistance.

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Laboratory and Pharmacological Studies in Man with Hetacillin and Ampicillin

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Hetacillin is the generic name for 6-(2,2-dimethyl-5-oxo-4-phenyl-1-imidazolidinyl)penicillanic acid, which is prepared by the reaction of ampicillin with acetone (Hardcastle *et al.*, 1966) (Fig. 1). The antibacterial spectrum of hetacillin has not been reported in detail, but Bunn *et al.* (1966) have stated that hetacillin has essentially the same spectrum of activity in vitro as ampicillin. Absorption and excretion studies with hetacillin in man have been reported by Bunn *et al.* (1966) and by Tuano *et al.* (1966). The results of clinical trials with hetacillin were reported at the Sixth Interscience Conference on Antimicrobial

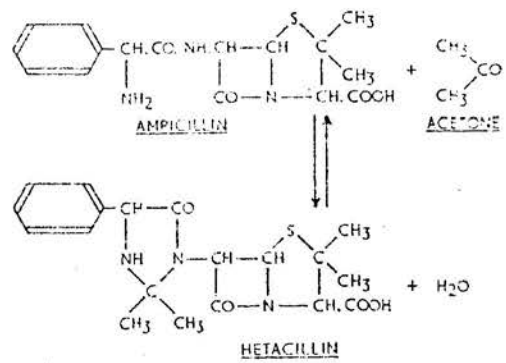

FIG. 1.—Structure of hetacillin.

Agents and Chemotherapy (Philadelphia, U.S.A., 1966—proceedings in press).

The experiments reported here were designed to investigate the hydrolysis of hetacillin to ampicillin in vitro and in man, and to compare the antibacterial activities, and the absorption and excretion in man, of hetacillin and ampicillin.

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Hydrolysis of Hetacillin to Ampicillin

In aqueous solution hetacillin hydrolyses to form ampicillin and acetone. The formation of ampicillin was demonstrated by thin-layer chromatography as described by Hardcastle *et al.* (1966). The formation of acetone was demonstrated by gas chromatography. Fig. 2 shows the rate of hydrolysis of hetacillin to ampicillin at 37° C. in aqueous solution at *p*H 7. Under these conditions hydrolysis of hetacillin was rapid, so that within 15 minutes at 37° C. more than 50% of an aqueous solution of 1 mg. of hetacillin per ml. in M/40 phosphate buffer, *p*H 7, had hydrolysed to ampicillin, and at the end of 60 minutes there was virtually no unchanged hetacillin in solution. The rate of hydrolysis under acid conditions (*p*H 2) was less rapid, but after 60 minutes there was about 50% conversion of hetacillin to ampicillin.

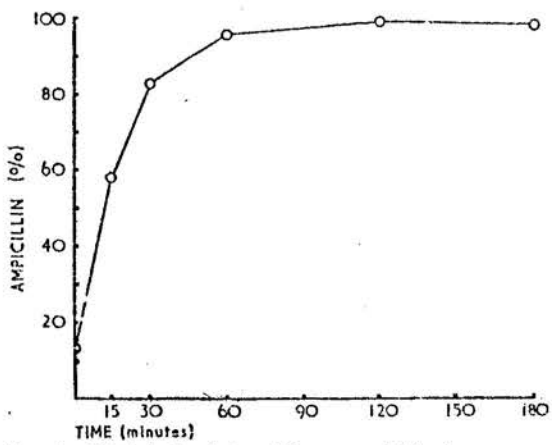

FIG. 2.—Hydrolysis of hetacillin to ampicillin in aqueous solution. The formation of ampicillin from a solution of 1 mg. hetacillin ml. in M/40 phosphate buffer at *p*H 7.0 and 37° C. was measured by hydroxylamine assay.

Antibacterial Activities of Hetacillin and Ampicillin

The minimum inhibitory concentrations of hetacillin and ampicillin required to prevent growth of bacteria for 24 hours at 37° C. were determined by serial dilution of the compounds in nutrient broth and in nutrient agar. The effect of serum on the antibacterial activities of the compounds was measured by serial dilution of the compounds in nutrient broth and in 95% human serum. After overnight incubation at 37° C., subcultures were made on to antibiotic-free agar, and the end-points were read after overnight incubation. The extent of

TABLE I.—*Relative Antibacterial Activities of Ampicillin and Hetacillin*

Organism	M.I.C. (μg./ml.)	
	Ampicillin	Hetacillin
Escherichia coli O111	2·5	2·5
Klebsiella aerogenes A*	500	500
Salmonella typhi	1·25	1·25
Salmonella typhimurium	1·25	1·25
Shigella flexneri	2·5	2·5
Proteus mirabilis	1·25	1·25
Proteus mirabilis 889*	> 500	> 500
Proteus morganii H	50	50
Pseudomonas pyocyanea	> 500	> 500
Haemophilus influenzae	0·5	0·5
Staphylococcus aureus	0·05	0·05
*Staphylococcus aureus**	250	250
Streptococcus pyogenes	0·02	0·02
Streptococcus pneumoniae	0·05	0·05
Streptococcus faecalis	1·25	1·25

* Penicillinase-producing strain.

TABLE II.—*Effect of Serum on the Antibacterial Activities of Ampicillin and Hetacillin Against Staphylococcus aureus Smith*

Compound	% Bound in Human Serum	M.I.C. (μg./ml.)	
		In Nutrient Broth	In Human Serum
Ampicillin	16	0·03	0·03
Hetacillin	16	0·03	0·03

binding of the compounds to the protein of human serum was measured by ultrafiltration of serum containing hetacillin or ampicillin (Rolinson and Sutherland, 1965).

The minimum inhibitory concentrations of hetacillin and ampicillin against a range of bacteria are given in Table I, where it can be seen that the antibacterial activities and spectra of both compounds were identical. Similarly, both compounds were bound to the protein of human serum to the same extent and showed the same activities in the presence of human serum (Table II).

Absorption and Excretion of Hetacillin and Ampicillin in Human Subjects

The subjects who took part in the studies described below were healthy men or women aged 18 to 49 years. Cross-over studies were carried out in which the same groups were given either hetacillin or ampicillin, followed by the alternative drug one week later. Blood specimens were normally taken half, one, two, four, and six hours after administration of the compound. In certain studies three-hour and eight-hour specimens were also collected. After separation of the sera the specimens were assayed on the same day, except when eight-hour specimens were collected and these were kept overnight at 4° C. before assay. During the six-hour period after administration of the

compounds urine was collected for measurement of antibiotic content.

Antibiotic serum concentrations were measured by a standard agar-diffusion assay technique with *Sarcina lutea* as assay organism (Knudsen *et al.*, 1961). Serum specimens were suitably diluted in normal human serum, or in some studies in 4% bovine plasma albumin, fraction V, and standard solutions of hetacillin and ampicillin were prepared in the same diluents. The plates were incubated overnight at 30° C., when the diameters of the inhibition zones were measured.

Serum and urine samples from subjects who received ampicillin were assayed against standard solutions prepared from ampicillin. Similarly, samples from subjects who received hetacillin were assayed against hetacillin standards, though, in fact, standard solutions prepared from hetacillin and ampicillin gave the same inhibition-zone diameters in this assay. This is not surprising in view of the speed at which hetacillin hydrolyses to ampicillin.

Conversion of Hetacillin to Ampicillin in Man

The amounts of unchanged hetacillin appearing in the blood stream of human subjects after the oral and intramuscular administration of hetacillin were measured. Hetacillin was separated from ampicillin by high-voltage agar-gel electrophoresis—essentially the technique described by Lightbown and de Rossi (1965) being used—and the compounds were demonstrated by bioautography.

After oral administration of hetacillin, ampicillin alone was detected in the blood stream for the first 30 minutes after dosing. Thereafter, unchanged hetacillin was detected in small and variable amounts in some subjects up to 90 minutes after administration, after which ampicillin alone was present in the blood stream. The amount of unchanged hetacillin present at any time represented a small fraction of the total antibiotic, and at the time of peak antibiotic serum concentrations ampicillin only was present. On intramuscular administration of hetacillin, small amounts of unchanged hetacillin were first detected after 30 minutes, declining to trace quantities 120 minutes later. Whenever hetacillin was detected this was generally only a small fraction of the total antibiotic in the blood stream.

Oral Absorption Studies

The compounds were administered as ampicillin free acid and hetacillin free acid in 250-mg. gelatin capsules. The results of these studies are summarized in Table III.

TABLE III.—*Absorption and Excretion Studies with Hetacillin and Ampicillin in Man*

Study	No. of Subjects	Dose	Route	Compound	Mean Serum Concentration (μg./ml.)							Urinary Excretion 0–6 hr. % of dose
					½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	6 hr.	8 hr.	
A	12	250 mg.	Oral	Hetacillin*	0·35	1·09	2·45	—	0·96	0·28	—	36
				Ampicillin‡	1·34	3·13	3·80	—	0·97	0·31	—	63
B	12	250 mg.	Oral	Hetacillin†	< 0·05	0·31	0·93	0·91	0·57	0·23	—	21
				Ampicillin‡	0·48	1·49	2·09	1·33	0·69	0·28	—	40
C	15	500 mg.	Oral	Hetacillin*	0·51	1·94	2·96	—	0·99	0·51	—	39
				Ampicillin‡	1·71	3·46	4·62	—	1·40	0·53	—	55
D	11	500 mg.	Oral	Hetacillin†	0·35	1·19	2·68	1·80	1·30	0·59	0·30	25
				Ampicillin‡	0·86	2·69	4·82	2·32	1·32	0·44	0·21	50
E	12	500 mg.	Oral	Hetacillin†	0·24	1·23	2·46	2·49	2·16	0·72	0·35	48
				Ampicillin‡	1·97	4·27	4·92	4·44	2·63	0·77	0·25	71
F	12	500 mg.	Oral (non-fasting)	Hetacillin†	0·12	0·70	1·06	1·07	1·20	0·68	0·46	27
				Ampicillin‡	1·17	2·56	2·98	2·36	1·26	0·38	0·17	41
G	8	250 mg.	Intramuscular	Hetacillin*	1·23	2·3	3·2	—	1·7	0·66	—	84
				Ampicillin‡	5·7	5·8	2·9	—	0·8	0·29	—	88
H	8	500 mg.	Intramuscular	Hetacillin*	1·9	3·7	5·0	—	3·6	1·9	—	75
				Ampicillin‡	7·4	9·2	6·7	—	2·4	1·2	—	86

* Material prepared in these laboratories by Dr. J. H. C. Nayler. † Versapen, Bristol Laboratories Limited. ‡ Penbritin, Beecham Research Laboratories.

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Studies A, B, C, and D (Figs. 3 and 4).—In these studies hetacillin and ampicillin were given to subjects who fasted for 12 hours before receiving the compound until after the one-hour blood specimens were taken. With both the 250-mg. dose and the 500-mg. dose the serum concentrations obtained after the administration of hetacillin rose more slowly than those obtained with ampicillin, reaching peak levels at two hours which were substantially lower than the ampicillin peak serum concentrations which also occurred two hours after administration. The serum concentrations obtained with hetacillin also fell more slowly than those of ampicillin, and from four hours onwards after administration the levels obtained with hetacillin

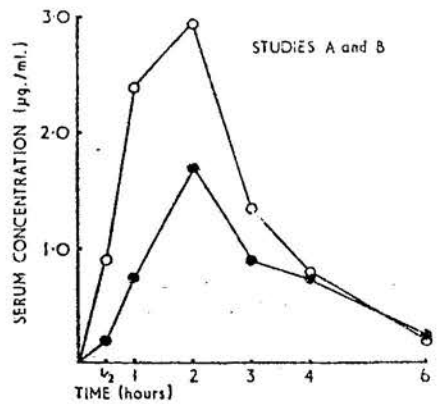

FIG. 3.—*Mean* serum concentrations in 24 fasting subjects after single 250-mg. oral dose of hetacillin and ampicillin. ●=Hetacillin. ○=Ampicillin.

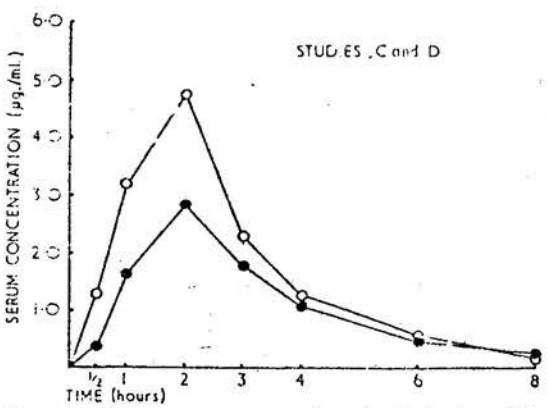

FIG. 4.—Mean serum concentrations in 26 fasting subjects after single 500-mg. oral dose of hetacillin and ampicillin. ●=Hetacillin. ○=Ampicillin.

were of the same order as those of ampicillin. The mean urinary excretion after a 250-mg. dose was 28% in the case of hetacillin and 51% with ampicillin, and after a 500-mg. dose was 32% for hetacillin and 52% for ampicillin.

Study E (Fig. 5).—This study was carried out to compare hetacillin and ampicillin under conditions similar to those described by Bunn *et al.* (1966) in their studies on the absorption and excretion of these substances. A single 500-mg. oral dose of hetacillin or ampicillin was administered to fasting subjects who abstained from food for 12 hours before dosing until three and a half hours after. In addition, the subjects

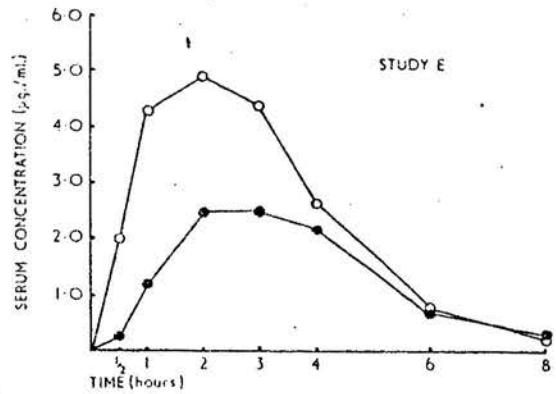

FIG. 5.—Mean serum concentrations of 12 fasting subjects after single 500-mg. oral dose of hetacillin and ampicillin. ● = Hetacillin. ○ = Ampicillin.

were given 400 ml. of water at the time of dosing as described by Bunn *et al.* (1966) and were allowed water during the experiment. Under these conditions the absorption and excretion of both compounds was greater than was observed in the fasting studies previously described (C and D), but the rise and fall of the serum concentrations followed essentially the same pattern as before. Thus the ampicillin serum concentrations were from two to eight times higher than those obtained with hetacillin in the three-hour period after dosing, and were similar for both substances four to eight hours after administration. The mean urinary excretion of ampicillin in the six-hour period after dosing was 71% and that after hetacillin 48%.

Study F—Non-fasting (Fig. 6).—A single 500-mg. dose of hetacillin and ampicillin was administered immediately before the subjects had a standard breakfast of bacon and egg, toast, and coffee. The serum concentrations obtained in the non-fasting subjects were lower than those observed in the fasting subjects, the presence of food having a particularly marked

effect on hetacillin, the serum concentrations remaining at about 1 μg./ml. from one to four hours after administration of the compound. The ampicillin serum concentrations were not depressed to anything like the same extent, and were from 2 to 10 times higher than those obtained with hetacillin for the first

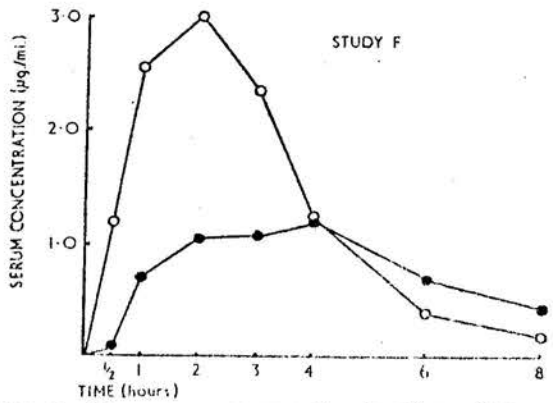

FIG. 6.—Mean serum concentrations in 12 non-fasting subjects after a single 500-mg. oral dose of hetacillin and ampicillin. ● = Hetacillin. ○ = Ampicillin.

three hours after dosing. The levels obtained with hetacillin fell more slowly than with ampicillin, and the serum concentrations obtained with hetacillin were higher than those of ampicillin from six to eight hours after administration.

Intramuscular Studies (Figs. 7 and 8)

The compounds were dissolved in 2 ml. of sterile water and injected immediately by deep intramuscular injection into the upper and outer quadrant of the buttock. Hetacillin was administered as the potassium salt and ampicillin as the sodium salt, as doses equivalent to 250 mg. and 500 mg. of ampicillin pure free acid. Ampicillin was absorbed more rapidly and the peak serum concentrations obtained one hour after administration were about twice as high as the peak serum concentrations obtained two hours after administration of hetacillin. The serum levels measured in subjects receiving hetacillin fell more slowly and were higher than those of the ampicillin group four hours and six hours after administration of the substances. After administration of hetacillin urinary recovery over a six-hour period was slightly less than that of ampicillin.

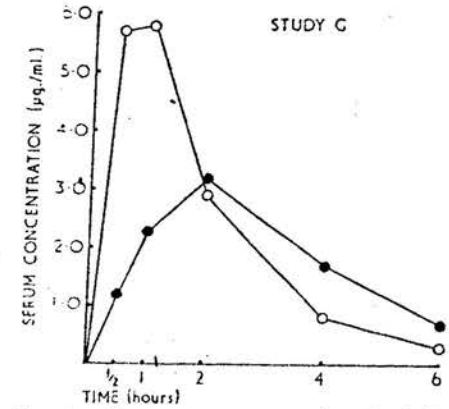

FIG. 7.—Mean serum concentrations in eight subjects after a single 250-mg. intramuscular dose of hetacillin and ampicillin. ●=Hetacillin. ○=Ampicillin.

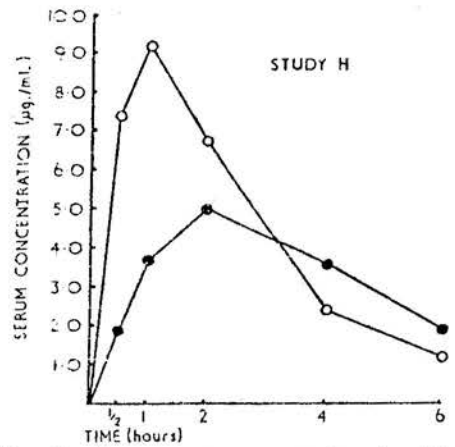

FIG. 8.—Mean serum concentrations in eight subjects after a single 500-mg. intramuscular dose of hetacillin and ampicillin. ●=Hetacillin. ○=Ampicillin.

Measurement of Antibacterial Activities of Sera

The antibacterial activities of pooled sera from fasting subjects who received single 500-mg. oral doses of hetacillin and ampicillin (Study E) were determined by serial dilution of the sera in 5% blood agar. The serum-agar mixtures were poured into petri dishes and inoculated with a drop of an overnight broth

culture of the test organisms—namely, *Staphylococcus aureus*, β-haemolytic streptococcus, and *Streptococcus pneumoniae*. After overnight incubation at 37° C. the maximum dilutions of sera inhibiting growth of the test organisms were determined. The results of this experiment are shown in Fig. 9, where it can be seen that in general the antibacterial activities of pooled sera taken half, one, two, and three hours after dosing from

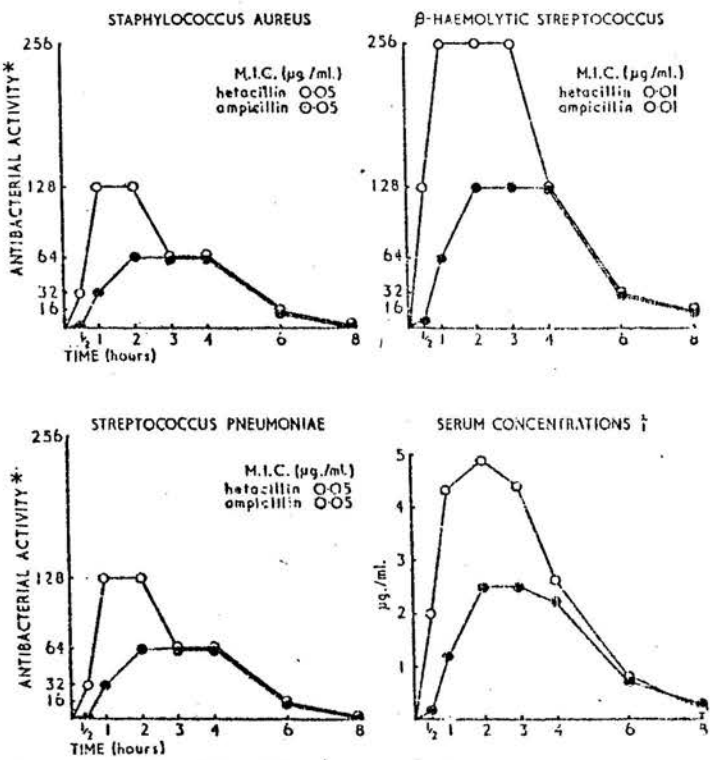

FIG. 9.—Antibacterial activity of sera of fasting human subjects after a single 500-mg. oral dose of hetacillin or ampicillin.. * Antibacterial activity of serum is expressed as the reciprocal of the highest dilution of serum inhibiting growth of the test organism. † Serum concentrations measured by agar-diffusion assay using *Sarcina lutea* as assay organism. ● = Hetacillin. ○ = Ampicillin.

subjects who received ampicillin were greater than those of sera from subjects receiving hetacillin. At later time intervals—namely, four, six, and eight hours after administration of the compounds—the antibacterial activities of sera from both groups of subjects were the same.

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Discussion

Hetacillin is prepared by the condensation of ampicillin with acetone, the reaction taking place in the presence of an excess of acetone. In the presence of water the reverse reaction takes place and hetacillin hydrolyses liberating ampicillin and acetone. The rate of hydrolysis of hetacillin in aqueous solution is rapid at neutral *p*H, and as tests for the measurement of antibacterial activity are normally performed in neutral aqueous media it is not surprising that the in-vitro antibacterial activities of hetacillin and ampicillin are identical. In tests with analogous compounds—namely, condensation products of ampicillin with formaldehyde—the activities demonstrated by these substances corresponded very closely with the rates to which these compounds hydrolysed to ampicillin. It is quite possible, therefore, that hetacillin has no intrinsic activity and that the activity demonstrated in antibacterial tests is that of its hydrolysis product, ampicillin ; but it would be difficult to confirm this supposition, as any test envisaged would involve the use of aqueous media in which there would be hydrolysis of hetacillin to ampicillin.

The conversion of hetacillin to ampicillin also takes place in the body, and the administration of hetacillin to human subjects results in the appearance of ampicillin in the blood stream, in which form it is mainly absorbed. After oral and intramuscular administration the amounts of unchanged hetacillin found in the blood are small compared with the quantities of ampicillin present, and, after oral administration in particular, represent an insignificant amount of the circulating antibiotic. The therapeutic significance of hetacillin will therefore depend upon to what extent the serum concentrations found in man after administration of the compound differ from the serum concentrations produced by equivalent doses of ampicillin.

In the sole published report in the literature comparing the absorption and excretion of ampicillin and hetacillin in man after administration by the oral and intramuscular routes, Bunn *et al.* (1966) found that the peak levels obtained with oral doses of hetacillin were as high as, or slightly higher than, those obtained with equivalent doses of ampicillin. Our findings are at variance with those of Bunn *et al.*, as we find that the serum concentrations produced by ampicillin are markedly higher than those obtained with hetacillin during the first three hours after dosing, the peak serum levels of ampicillin being generally about twice as high. In these studies the serum levels were measured by conventional agar-diffusion microbiological assay technique, but measurement of the antibacterial activity of the sera by a serial-dilution technique also showed that sera from subjects given ampicillin were more active than corresponding

sera from subjects receiving hetacillin. This would be expected if the antibiotic serum concentrations of the ampicillin group were greater than those of the hetacillin group. Like Bunn *et al.* (1966) we find that the decline in serum antibiotic concentrations from peak levels is slower in subjects receiving hetacillin, but whereas these workers thought that this might have clinical implications, we feel that at the later periods the antibiotic serum concentrations are so low that any difference in levels is unlikely to be of therapeutic significance.

The presence of food in the stomach depresses the absorption of hetacillin to a relatively greater extent than that of ampicillin. In the study reported here the mean serum concentrations produced with a 500-mg. dose of hetacillin barely exceeded 1 μg./ml. at any time after dosing.

The results of our studies of the serum concentrations obtained after intramuscular administration of hetacillin and ampicillin are in general agreement with those reported by Bunn *et al.* (1966). Thus the peak levels obtained with ampicillin occur earlier and are impressively higher than those obtained with hetacillin, and then decline to levels which are lower than those obtained with hetacillin from the fourth hour onwards. In comparing the intramuscular preparations of hetacillin and ampicillin we believe that the high serum concentrations achieved with ampicillin are likely to be of greater therapeutic significance than the maintenance of the relatively low prolonged levels observed with hetacillin.

The results of electrophoretic studies show that after oral administration trace quantities of unchanged hetacillin are present for short periods and that practically all the circulating antibiotic is in the form of ampicillin. This indicates that hetacillin is poorly or less rapidly absorbed than ampicillin, and the slow rise to relatively low serum concentrations and the relatively low urinary excretion over a six-hour period can be attributed to delay in absorption while hetacillin is hydrolysing to ampicillin in the gut. Similarly, the slow rise and delayed fall of serum concentrations after intramuscular doses of hetacillin indicates delayed absorption from the injection site. The results of electrophoretic studies show only small quantities of unchanged hetacillin persisting in the blood stream for up to 150 minutes after intramuscular administration. These facts indicate that hetacillin is poorly or slowly absorbed after injection and that there is delay in hydrolysis to ampicillin, in which form it is mainly absorbed.

There is every reason to believe that the therapeutic value of hetacillin is due to its hydrolysis product, ampicillin, and, in view of the lower blood levels obtained with hetacillin compared with ampicillin at equivalent doses, hetacillin would be expected to possess inferior therapeutic efficiency.

Summary

Hetacillin, a condensation product of ampicillin and acetone, hydrolysed rapidly in aqueous media to ampicillin and acetone. The antibacterial spectra and in vitro activities of hetacillin and ampicillin were identical and it is probable that the antibacterial activity of hetacillin is that of its hydrolysis product ampicillin. Electrophoretic studies of serum after the administration of hetacillin to volunteers indicated the majority of circulating antibiotic to be in the form of ampicillin, and cross-over studies after oral and intramuscular doses of hetacillin and ampicillin showed ampicillin to produce consistently and substantially higher peak blood levels.

The amount of antibiotic excreted over a six-hour period in the urine was considerably less in the case of hetacillin, and this together with the lower serum concentrations obtained would indicate that hetacillin is absorbed less readily than ampicillin. It is concluded from these studies that hetacillin would be expected to possess inferior therapeutic efficiency compared with ampicillin.

We are indebted to Dr. J. H. C. Nayler for providing the hetacillin used in certain of the studies, to Dr. M. Cole for the data in Fig. 2, and to Mr. P. Langley and Mr. D. Hannan for the results of the electrophoresis studies. We also thank Mrs. E. Fairclough and Miss M. Jones for skilled technical assistance.

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Metampicillin

Antibacterial Activity and Absorption and Excretion in Man

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Abstract. Metampicillin, the penicillin prepared by the reaction of ampicillin with formaldehyde, hydrolysed in aqueous solution with the formation of ampicillin. Hydrolysis was rapid under acid conditions, less rapid in neutral medium, and was incomplete in human serum. Metampicillin showed a spectrum and level of activity similar to that of ampicillin *in vitro*, and both compounds were inactive against penicillinase-producing strains of bacteria. The activity of metampicillin was markedly reduced by human serum, and the compound was less active than ampicillin in the presence of human serum. Following the oral administration of metampicillin to man, metampicillin was not detected in the blood stream nor in urine, and ampicillin alone was demonstrated in these subjects. The serum concentrations of ampicillin that were produced following the oral administration of metampicillin were somewhat lower than those obtained with equivalent doses of ampicillin. Administration of metampicillin by the intramuscular (i.m.) route to volunteers resulted in the appearance of both ampicillin and metampicillin in the blood, and of ampicillin alone in the urine of these subjects.

Key Words
Metampicillin
Ampicillin
MIC data
Human
Absorption
Excretion

Introduction

Metampicillin is the approved name for the penicillin resulting from the reaction of ampicillin with formaldehyde (fig. 1). The compound has been described as α-methyleniminobenzyl penicillin (I), [1] but GRANATECK [4] has claimed that the reaction of ampicillin with formaldehyde resulted in the formation of α-hydroxymethylaminobenzyl penicillin (II), the hydrated form of (I).

[1] We wish to thank Mrs. ELEANOR FAIRCLOUGH for skilled technical assistance, and Mr. D. F. LAWSON for producing the photographs.

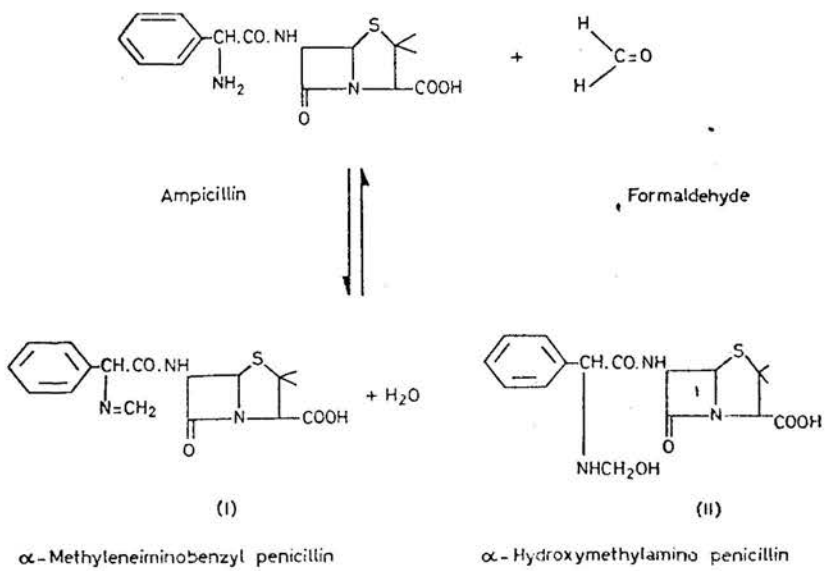

Fig. 1. The reaction of ampicillin with formaldehyde.

Metampicillin is said to have a broad spectrum of activity coupled with a marked degree of stability to bacterial penicillinase [1]. Furthermore, metampicillin is reported to be absorbed to a greater extent than ampicillin, resulting in superior blood levels in human subjects [2], and also giving high levels of antibiotic in bile following parenteral administration [5, 7]. The penicillin has been reported to have been used successfully in the treatment of clinical cases of salmonellosis [3].

The study presented here was designed to compare the antibacterial activities of ampicillin and metampicillin, and the absorption and excretion of these 2 penicillins in human subjects.

Materials and Methods

Metampicillin used in these studies was the commercial preparation that is available as sodium salt (Suvipen, Midy). Ampicillin was used in the oral absorption studies as ampicillin trihydrate and in the studies of parenteral absorption as the sodium salt of ampicillin (Penbritin, Beecham Research Laboratories).

Hydrolysis of Metampicillin

The rates of hydrolysis of metampicillin in solution, and the subsequent formation of ampicillin, were measured by incubating the compound at a concentration

of 5 μg/ml in the following media: (1) 0.05 M phosphate buffer, pH 7.0; (2) nutrient broth, pH 7.6 (Oxoid No. 2); (3) aqueous hydrochloric acid solution, pH 2.0, and (4) 90% human blood. Samples were taken from the solutions at appropriate intervals and assayed for ampicillin by electrophoretic bioassay. In this procedure 5-μl volumes of the test solutions, or of standard solutions of ampicillin prepared in the same diluent as the test solution, were spotted in duplicate onto thin-layer gel plates consisting of 1% starch (Analar grade) and 1% Ionagar (Oxoid), buffered at pH 7.5. A potential of 25 V/cm was applied across the plate, under these conditions metampicillin migrated towards the anode more rapidly than ampicillin, and a separation was achieved in 20 min. After this period the plates were overlayed with nutrient agar (Blood Agar Base, Oxoid) seeded with *Sarcina lutea* NCTC 8340 and incubated overnight (18 h) at 30 °C, after which the inhibition zones resulting from the test solutions and standard solutions were measured.

The presence of ampicillin in solutions of metampicillin of higher concentration (100–1,000 μg/ml) was demonstrated by chromatography and bioautography. Aqueous solutions of metampicillin and ampicillin were incubated at 37 °C, aliquots were taken at intervals and 6-μl volumes were spotted onto 1 cm wide chromatography strips (Whatman Chroma grade 1). The strips were run for 16 h at 4 °C in a butanol-ethanol-water (4:1:5 top phase) solvent system, dried at 40 °C and plated on nutrient agar (Blood Agar Base, Oxoid) seeded with *Bacillus subtilis* ATCC 6633. The plates were incubated at 40 °C for 8 h after which the inhibition zones produced by metampicillin and ampicillin were examined.

Antibacterial Activity

Minimum inhibitory concentrations of metampicillin and ampicillin required to inhibit growth of the test organism for 18 h at 37 °C were determined by inoculating the organism into serial dilutions of the penicillin in 5-ml volumes of nutrient broth (Oxoid, No. 2) or 18-ml volumes of nutrient agar (Blood Agar Base, Oxoid). For tests in which the penicillin was incorporated in agar the inoculum was 0.003 ml of an undiluted overnight broth culture delivered with a multiple inoculating device, and for tests in broth, 0.03 ml of an overnight broth culture.

The effect of serum on the antibacterial activity of metampicillin was measured by comparing the minimum inhibitory concentrations of the penicillin against *Escherichia coli* in nutrient agar and in nutrient agar containing 50% human serum. In addition, the effect of serum on the bactericidal activity of metampicillin and ampicillin was measured by viable count experiments in which nutrient broth (Oxoid No. 2) or whole human serum containing known concentrations of the penicillins were inoculated with *Staphylococcus aureus* (Smith) and incubated at 37 °C. Aliquots were taken from the culture at 3, 6 and 24 h after inoculation, serial dilutions prepared in sterile water, and 0.02 ml volumes of these dilutions pipetted onto antibiotic-free agar. Colonies were counted after overnight incubation at 37 °C and the viable count calculated.

Absorption and Excretion Studies

Crossover studies were carried out in groups of healthy male and female fasting volunteer subjects. In these studies the subjects refrained from taking food or

drink from midnight on the day prior to the study until after the 1-hour blood specimen was taken, after which they were allowed to eat a standard breakfast. The test penicillin, metampicillin or ampicillin, was administered in gelatine capsules with a little water, and blood specimens were taken from the antecubital vein at 30 min, 1, 2, 3, 4 and 6 h after dosing and urine was collected and pooled during this 6-hour period. The second half of the crossover was carried out 1 week later when the alternative drug was taken by the same subjects under the same conditions. In studies to determine absorption of metampicillin following i.m. administration, subjects were injected with a 250-mg dose of metampicillin dissolved in 1 ml sterile water for injection. Specimens of blood were taken as before, and after separation of the serum specimens were assayed on the same day.

Antibiotic serum concentrations were measured by a standard cup-plate agar diffusion assay as has been described for ampicillin [6] using *S. lutea* NCTC 8340 as assay organism. Rectangular glass plates ($30 \times 37^1/_2$ cm) were filled to a depth of about 4 mm with molten nutrient agar (Penassay base agar No. 1, Difco) seeded with a 0.1-percent inoculum of an overnight broth culture of *S. lutea* NCTC 8340. Holes (8 mm in diameter) were punched in the agar and the plugs of agar were removed with a broad needle. Serum specimens were diluted as required in pooled normal human serum and standard solutions were prepared in the same diluent. Urine specimens were diluted with 0.05 M phosphate buffer (pH 7.0), and assayed against standard solutions prepared in buffer. The plate was filled with the serum and urine specimens and the standard solutions, and the zones of inhibition measured after overnight incubation at 30 °C.

The relative proportions of metampicillin and ampicillin appearing in the blood stream after oral or i.m. administration of metampicillin were measured by electrophoresis and bioautography as described previously. Specimens of blood taken 30 min, 1, 2 and 3 h after administration of metampicillin were heparinised and subjected to electrophoresis without delay. Standard solutions of metampicillin and ampicillin were prepared freshly in heparinised whole human blood. Urine specimens collected during the 6-hour period after administration, were examined for metampicillin and ampicillin by paper chromatography and bioautography as described above.

Results

Hydrolysis of Metampicillin

The results in table I show the relative rates of hydrolysis of solutions of metampicillin (5 μg/ml) to ampicillin in aqueous solution, in nutrient broth, and in whole human blood. At acid pH, hydrolysis of metampicillin to ampicillin was very rapid; an aqueous solution at pH 2.0 containing 5 μg/ml metampicillin was converted completely to ampicillin in less than 5 min at 37 °C. At neutral pH the rate of hydrolysis to ampicillin was less rapid and the ampicillin concentration of a solution of metampicillin (5 μg/ml) in 0.05 M phosphate buffer (pH 7.0) increased to approximately 80% ampicillin within 3 h at 37 °C (table I

Table I. Hydrolysis of metampicillin to ampicillin in aqueous solution and in blood

Medium	Ampicillin concentration[1] (μg/ml) arising from metampicillin (5 μg/ml) after incubation at 37°C for:		
	0 h	3 h	6 h
Aqueous solution, pH 2.0	complete conversion to ampicillin within 5 min		
Aqueous solution, pH 7.0	1.0	4.6	4.2
Nutrient broth, pH 7.6	1.4	4.0	4.5
Human blood	1.6	2.0	2.9

[1] Determined by starch-agar gel electrophoresis.

Table II. Antibacterial spectrum of metampicillin

Organism	Minimum inhibitory concentration, μg/ml	
	metampicillin	ampicillin
E. coli	5.0	5.0
Salm. typhi	1.25	1.25
Sh. sonnei	5.0	2.5
K. aerogenes	250	>500
Ps. aeruginosa	>500	>500
P. mirabilis	2.5	1.25
S. aureus	0.1	0.1
S. aureus[1]	250	500
β-haemolytic streptococcus	0.02	0.01
Str. pneumoniae	0.05	0.02
Str. faecalis	1.25	1.25

[1] Penicillinase-producing strain.

and fig. 2). Metampicillin was much more stable in whole human blood and there was little change in the ampicillin content (30–40%) after 6-hour incubation at 37 °C.

Antibacterial Activity

The antibacterial spectrum of metampicillin is compared with that of ampicillin in table II, were it can be seen that both penicillins showed

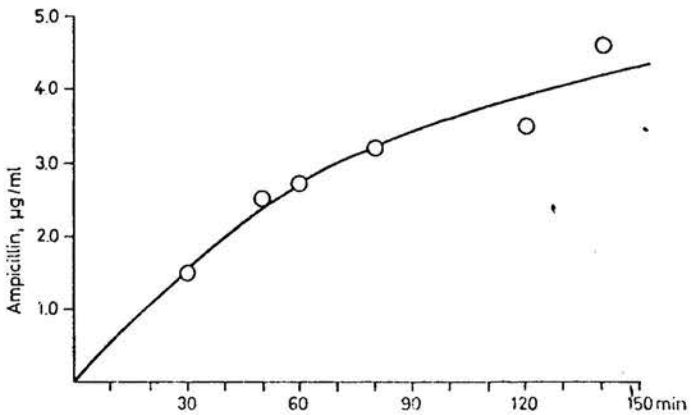

Fig. 2. Rate of formation of ampicillin from an aqueous solution of metampicillin (5 µg/ml) at pH 7.0 and 37 °C.

similar activities against a wide range of gram-positive and gram-negative bacteria, including *S. aureus*, streptococci, *E. coli*, *Salmonella typhi*, *Shigella sonnei* and *Proteus mirabilis*. The penicillins were not active against strains of *Pseudomonas aeruginosa*, *Klebsiella/Enterobacter* spp. or penicillinase-producing *S. aureus*.

A more detailed comparison of metampicillin and ampicillin made in tables III and IV shows the relative activities of the penicillins against a total of 152 clinical isolates of bacteria. In general, both penicillins were about equally active against ampicillin-sensitive bacteria, ampicillin being perhaps slightly more active (table III). Both penicillins were relatively inactive against penicillinase-producing strains of *E. coli*, *Sh. sonnei*, *Klebsiella/Enterobacter* spp., *Proteus* spp. *and S. aureus* (table IV).

Effect of serum on antibacterial activity. The antibacterial activity of metampicillin was markedly diminished in the presence of human serum, and data in table V compare the activities of metampicillin and ampicillin against *E. coli* in nutrient agar and in nutrient agar containing 50% human serum. The activity of metampicillin was reduced by 5- to 10-fold in the presence of serum, whereas the activity of ampicillin was reduced only twofold.

Likewise, in a viable count experiment to compare the bactericidal activities of metampicillin and ampicillin against *S. aureus* (Smith) in nutrient broth and in whole human serum, the activity of metampicillin

Table III. Activity of metampicillin against 85 clinical isolates of ampicillin-sensitive bacteria

Organism	Number of strains	Penicillin	MIC[1] (μg/ml) and number of strains									
			12.5	5.0	2.5	1.25	0.5	0.25	0.1	0.05	0.02	0.01
E. coli	35	metampicillin	2	28	3	2						
		ampicillin	3	23	5	4						
Salmonella spp.	8	metampicillin			1	5	1	1				
		ampicillin			2	3	2	1				
Sh. sonnei	6	metampicillin		4		2						
		ampicillin		3	1	2						
P. mirabilis	16	metampicillin	1	2	3	10						
		ampicillin	1	1		14						
S. aureus	6	metampicillin							6			
		ampicillin							6			
β-haemolytic streptococcus	7	metampicillin									4	3
		ampicillin										7
Str. pneumoniae	7	metampicillin								5	2	
		ampicillin								1	6	

[1] Minimum inhibitory concn; Serial dilution in agar.

Table IV. Activity of metampicillin against 67 clinical isolates of penicillinase-producing bacteria resistant to ampicillin

Organism	Number of strains	Penicillin	MIC[1] (μg/ml) and number of strains							
			>500	500	250	125	50	25	12.5	5.0 or less
E. coli	8	metampicillin	6	1	1					
		ampicillin	7	1						
Sh. sonnei	9	metampicillin				4	5			
		ampicillin		1	7	1				
Klebsiella/ Enterobacter	20	metampicillin	5	5	4	5	1			
		ampicillin	9	3	2	6				
Proteus spp.[2]	15	metampicillin	2	5	1	3	1	3		
		ampicillin	6	1	1	3	3	1		
S. aureus	15	metampicillin			11				4	
		ampicillin	1	5	4	1			4	

[1] Minimum inhibitory concn; Serial dilution in agar.

[2] *P. morganii, P. rettgeri and P. vulgaris.*

Table V. Effect of human serum on the antibacterial activity of metampicillin against *E. coli*

Penicillin	Medium	MIC (μg/ml) and number of strains			
		25	12.5	5.0	2.5
Metampicillin	nutrient agar			6	4
	serum agar[1]	9	1		
Ampicillin	nutrient agar			3	7
	serum agar		2	8	

[1] Nutrient agar containing 50% human serum.

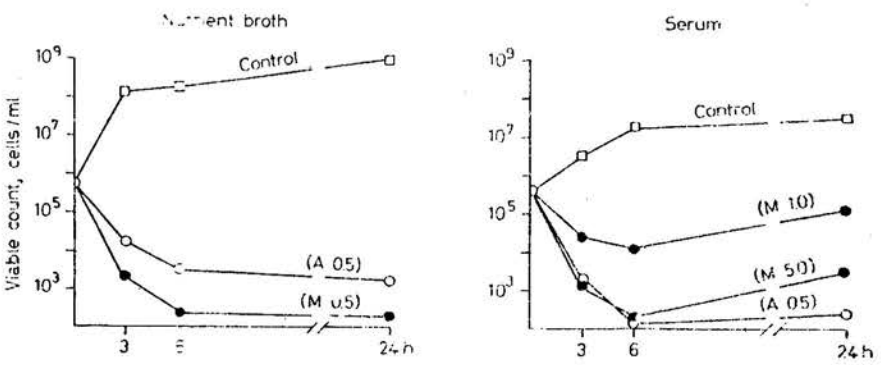

Fig. 3. Effect of human serum on the bactericidal activities of metampicillin and ampicillin against *S. aureus* Smith. ● = (M) metampicillin, ○ = (A) ampicillin. Figures in brackets are concentrations in μg/ml.

was significantly lower in serum than in broth (fig. 3). In contrast, ampicillin was as active in serum as in broth (fig. 3).

Absorption and Excretion Studies in Human Volunteers

(1) *Conversion of metampicillin to ampicillin in man.* The amount of ampicillin appearing in the blood and urine of human subjects after oral and i.m. administration of metampicillin were measured by electrophoretic or chromatographic methods. Ampicillin was separated from metampicillin in blood by electrophoresis, and in urine, containing relative-

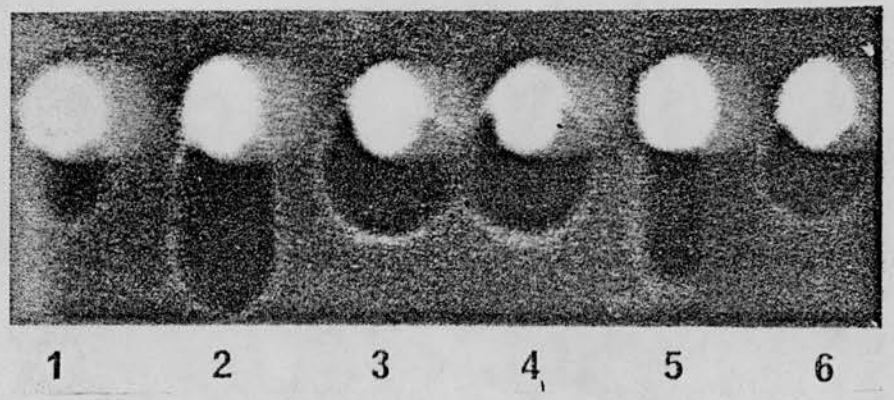

Fig. 4. Electrophoretic examination of blood specimens taken 1 h after administration of a 500-mg oral dose of metampicillin to human subjects. 1 = Blood, subject A, 2 = metampicillin standard, 2 μg/ml, 3 = ampicillin standard, 2 μg/ml, 4 = blood, subject B, 5 = metampicillin standard, 1 μg/ml, and 6 = ampicillin standard, 1 μg/ml.

ly high concentrations of ampicillin, by paper chromatography. The compounds were demonstrated by bioautography using *S. lutea* or *B. subtilis* as assay organisms.

(a) Oral administration. Following the oral administration of a single 500-mg dose of metampicillin to fasting human subjects, metampicillin was not detected and ampicillin alone was demonstrated in the blood specimens taken 1, 2, and 3 h after dosing (fig. 4). The ampicillin concentrations measured in whole blood by the electrophoretic and bioautographic method used to distinguish between ampicillin and metampicillin were in agreement with the ampicillin concentrations measured by the more sensitive standard microbiological assay (table VI) demonstrating that there was total conversion of metampicillin to ampicillin. Likewise, chromatographic examination of the urine of these subjects showed that there was no metampicillin present in the urine, and that ampicillin was the only penicillin present (fig. 5).

(b) Intramuscular administration. After administration of 250 mg metampicillin by the i.m. route to 2 human subjects, both metampicillin and ampicillin were observed in the blood of both subjects after $^1/_2$ h (fig. 6) and both penicillins were present in 1 subject 1 h after dosing. At 2 and 3 h after dosing ampicillin alone could be detected in both subjects (table VII). In a separate study in which 3 subjects were inject-

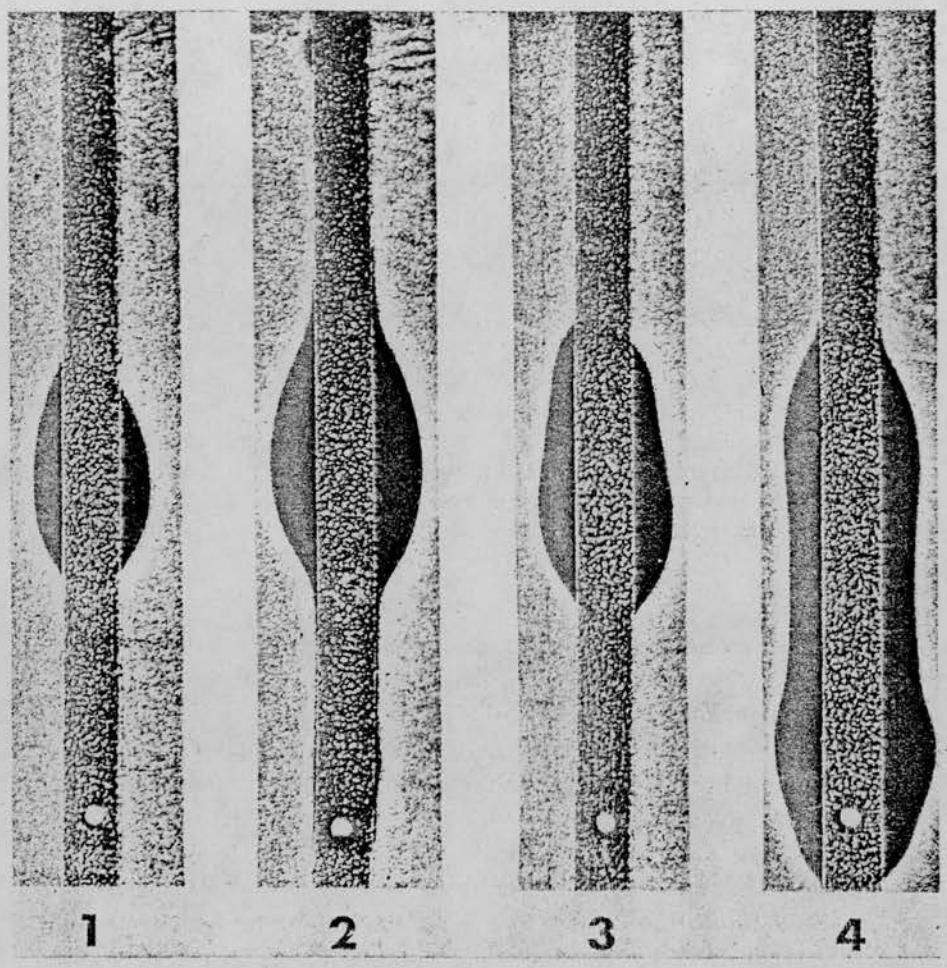

Fig. 5. Chromatographic examination of urine collected from 0–6 h after administration of a 500-mg oral dose of metampicillin to human subjects. 1 = Urine, subject A, 2 = ampicillin standard, 1,000 μg/ml, 3 = urine, subject B, and 4 = metampicillin standard, 1,000 μg/ml.

ed with a 250-mg i.m. dose of metampicillin, metampicillin and ampicillin were observed in the blood of the 3 subjects 30 min and 1 h after dosing, but ampicillin alone was present after 2 h. Chromatographic examination of pooled urine collected in both studies during the 6-hour period after dosing showed the presence of ampicillin alone (fig. 7).

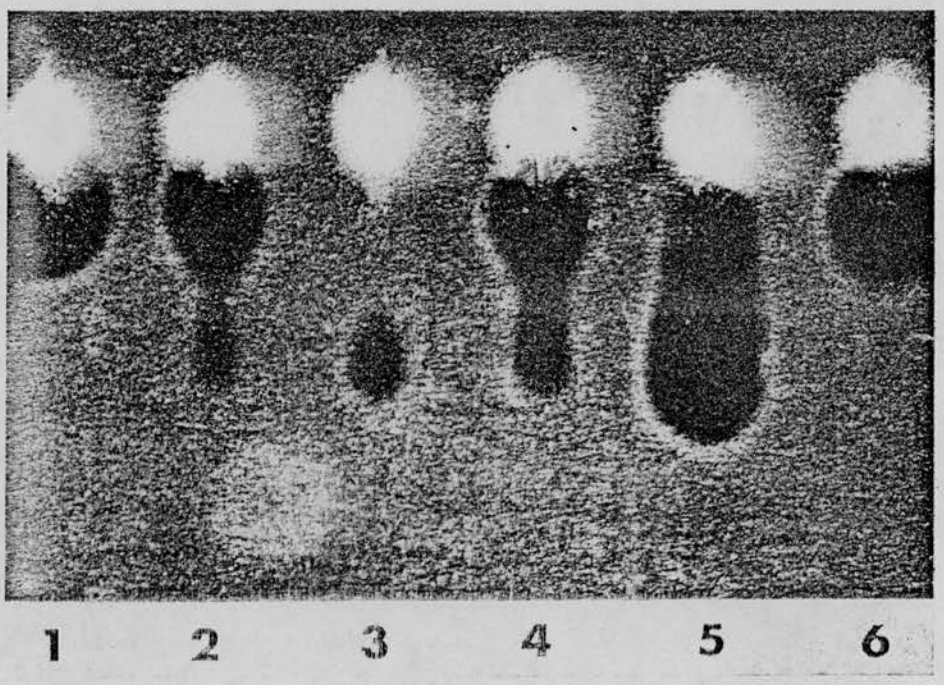

Fig. 6. Electrophoretic examination of blood taken 30 min after administration of 250 mg metampicillin by the i.m. route to human subjects. 1 = Ampicillin standard, 4 μg/ml, 2 = blood, subject A, 3 = metampicillin standard, 1 μg/ml, 4 = blood, subject B, 5 = metampicillin standard, 4 μg/ml, and 6 = ampicillin standard, 2 μg/ml.

(2) *Oral crossover studies.* The results of crossover studies in which a single 250 or 500 mg oral dose of metampicillin or ampicillin was administered to fasting human subjects are sumarised in table VIII. In these studies electrophoretic and bioautographic assay showed the presence only of ampicillin in blood and urine after oral administration of metampicillin, and consequently ampicillin serum concentrations resulting from the administration of metampicillin were measured by microbiological assay against ampicillin standard solutions. In general, both penicillins showed a similar pattern of absorption, reaching peak ampicillin serum levels 2 h after administration and falling to relatively low levels after 6 h. The ampicillin serum concentrations obtained after administration of metampicillin were rather lower than those produced with the equivalent dose of ampicillin, except for the 6-hour specimens where

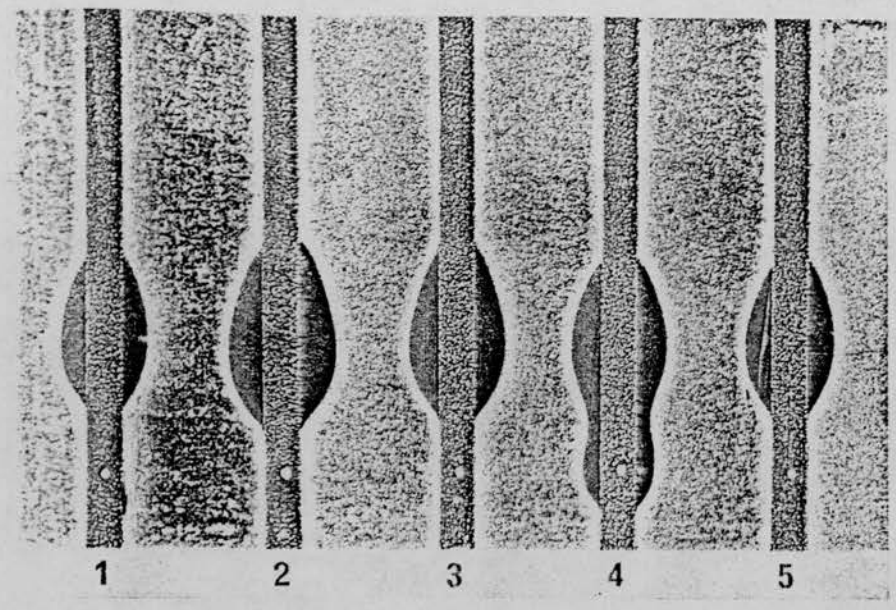

Fig. 7. Chromatographic examination of urine collected from 0-6 h after administration of 250 mg metampicillin by the i.m. route to human subjects. 1 = Urine, subject A, 2 = ampicillin standard, 1,000 μg/ml, 3 = urine, subject B, 4 = metampicillin standard 1,000 μg/ml, and 5 = urine, subject C.

Table VI. Ampicillin blood concentrations following oral administration of a single 500-mg dose of metampicillin to fasting human subjects

Subject	Assay method	Ampicillin concentration, μg/ml		
		1-hour	2-hour	3-hour
A	electrophoresis[1]	0.9	1.3	0.4
	microbiological	0.73	1.58	0.89
B	electrophoresis[1]	3.2	4.6	3.0
	microbiological	2.6	4.1	2.5

[1] Metampicillin was not detected in these blood specimens.

both compounds produced similar low levels of ampicillin. The amounts of ampicillin excreted in the urine during the 6-hour period after administration were similar for both metampicillin and ampicillin ranging from 32–37% of the dose administered.

Table VII. Ampicillin and metampicillin blood concentrations measured by electrophoresis and bioautography following a 250-mg i.m. dose of metampicillin to human subjects

Subject		Blood concentration, μg ml			
		30-min	1-hour	2-hour	3-hour
A	metampicillin	0.8	<0.5	<0.5	<0.5
	ampicillin	2.1	1.6	1.2	0.8
B	metampicillin	1.8	2.5	<0.5	<0.5
	ampicillin	3.0	2.4	2.0	1.1

Table VIII. Ampicillin serum levels in fasting human subjects following oral administration of ampicillin or metampicillin

Dose, mg	Number of subjects	Penicillin administered	Mean ampicillin serum concentration, μg/ml						Ampicillin excreted in urine in 0–6 h, %
			30-min	1-hour	2-hour	3-hour	4-hour	6-hour	
250	10	metampicillin	0.4	1.8	1.9	–	0.8	0.3	34
		ampicillin	1.2	2.5	3.1	–	1.0	0.3	37
500	10	metampicillin	0.2	1.6	4.3	2.4	1.5	0.6	32
		ampicillin	0.6	3.0	5.1	3.3	1.8	0.6	33

Discussion

Metampicillin is produced by the reaction of ampicillin with formaldehyde. In the presence of water the reverse reaction takes place leading to the hydrolysis of metampicillin with the formation of ampicillin. Under acid conditions the rate of hydrolysis of metampicillin is very rapid, so that at pH 2.0 there is complete conversion to ampicillin within a few minutes. Hydrolysis is less rapid in neutral aqueous media but, nevertheless, at therapeutic concentrations hydrolysis to ampicillin is virtually complete within 3 h at an incubation temperature of 37 °C. In contrast, metampicillin hydrolyses incompletely to ampicillin in human serum.

The antibacterial spectrum and level of activity of metampicillin is generally very similar to that of ampicillin, and this could well be due to the hydrolysis of metampicillin to ampicillin under the conditions of the *in vitro* antibacterial tests. FERRERO [1] has reported that metampicillin is more resistant than ampicillin to destruction by the bacterial flora of the rat intestine, and has suggested that this may indicate a degree of stability to penicillinase which may be of clinical significance. In contrast, the data reported here show that penicillinase-producing strains of staphylococci and of gram-negative bacilli were insensitive to both metampicillin and ampicillin, and fail to demonstrate any marked difference between metampicillin and ampicillin with regard to stability to penicillinase.

Metampicillin differs from ampicillin in showing a marked reduction in antibacterial activity in the presence of human serum and, as a consequence, metampicillin is distinctly less active than ampicillin in the presence of serum.

In considering the results to be expected following oral administration of metampicillin to human subjects, it might be predicted from the rapidity of hydrolysis of metampicillin to ampicillin under acid conditions *in vitro* that a similar conversion would take place in the acidic environment of the stomach. In the experiments reported here, metampicillin was not detected in the blood of human subjects following oral administration of the compound, and only the hydrolysis product, ampicillin, was demonstrated. Likewise, ampicillin alone was present in the urine of these subjects.

In these crossover studies the oral administration of metampicillin resulted in lower serum concentrations of ampicillin than were obtained in the same subjects following the oral administration of the equivalent dose of ampicillin, whereas FRANCHI and PERRARO [2] have reported that oral administration of metampicillin resulted in higher blood levels than ampicillin. However, FRANCHI and PERRARO assayed their serum specimens in terms of metampicillin, using metampicillin standard solutions, although the antibacterial activity of the serum specimens would be due to ampicillin alone. In the presence of serum, metampicillin is significantly less potent than ampicillin and, therefore, the use of metampicillin standard solutions to assay sera containing ampicillin would result in the reporting of misleadingly high levels of activity.

The i.m. administration of metampicillin to human subjects resulted in the appearance of both metampicillin and ampicillin in the blood

stream up to 1 h after dosing, but of ampicillin alone after 2 h. These findings mean that it is not possible to measure the serum concentrations of the early serum specimens containing both metampicillin and ampicillin by conventional microbiological assay. It would be necessary to separate the penicillins by a technique such as electrophoresis or chromatography before a satisfactory assay could be performed. In the studies reported here, the antibacterial activity of sera following i.m. administration of metampicillin was lower than that normally found with ampicillin. It is of interest that ampicillin alone was found in the urine of subjects who received metampicillin by i.m. injection.

It has been reported that metampicillin differs from ampicillin in producing high biliary antibiotic concentrations following i.v. [7] or i.m. [5] adminstration, but in these studies no attempt was made to identify the penicillin present in the bile specimens. The biliary concentrations reported in these studies were obtained by conventional microbiological assay against metampicillin standards, and this could possibly be misleading for the reasons described above.

The results presented here show that the antibacterial spectrum and level of activity of metampicillin is similar to that of ampicillin *in vitro*, but that metampicillin is less active than ampicillin in serum. After oral administration to man, metampicillin is hydrolysed to ampicillin and only ampicillin appears in the blood and urine of subjects. After i.m. administration only relatively low concetrations of metampicillin are detected in the blood accompanied by ampicillin and ampicillin alone is present in the urine. On the basis of these data metampicillin would not appear to offer any therapeutic advantage compared with ampicillin.

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Preparation, Hydrolysis, and Oral Absorption of Lactonyl Esters of Penicillins

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Lactonyl esters of ampicillin and other penicillins have been synthesized as prodrugs designed to improve the oral absorption of the parent penicillins. In general, the esters hydrolyzed rapidly in the presence of tissues including blood and certain of the esters were better absorbed than the parent penicillin. The phthalidyl ester of ampicillin [talampicillin (British Pharmacopoeia approved name), BRL 8988] was selected for extended studies. The compound was found to be well absorbed in various animal species and gave ampicillin serum concentrations in fasting human volunteers 2.5–3 times those obtained for ampicillin itself.

Esters of penicillins and, more recently, of cephalosporins continue to demand attention as a means of improving oral absorption of compounds that are not absorbed or only moderately absorbed by the oral route.[1,2] Since esters of penicillins are themselves devoid of antibacterial activity, it is recognized that the two essential requirements for an ester to be of clinical significance are that it should facilitate the absorption process and, once absorbed, the ester should hydrolyze readily to release the free penicillin into the blood and tissues. Simple alkyl and aryl esters of penicillins fail to meet this latter requirement in man as they are resistant to hydrolysis by human tissues.[3,4]

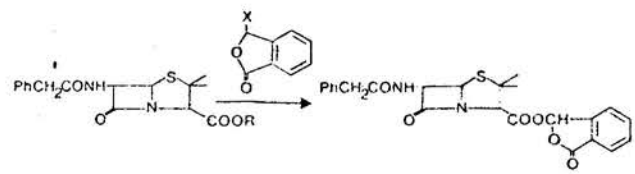
The first successful approach to the development of a penicillin ester susceptible to hydrolysis by human tissues was made by Jansen and Russell who described a series of acyloxymethyl esters of penicillin G (benzylpenicillin).[5] The acetoxymethyl ester of penicillin G, penamecillin, was investigated in some detail and was shown to be hydrolyzed by various animal tissues and by human serum.[6] Penamecillin was found to be orally absorbed to a limited extent in man resulting in serum levels of penicillin G only slightly higher than those obtained with the parent penicillin.[7]

In contrast, acyloxymethyl esters of ampicillin (D-α-aminobenzylpenicillin) were shown to be well absorbed in man, giving rise to high serum concentrations of ampicillin.[8] The ester of choice, the pivaloyloxymethyl ester, pivampicillin, was shown to produce serum concentrations of ampicillin two to three times higher than those produced by ampicillin.[9]

We now wish to describe a new class of hydrolyzable double esters of penicillins in which the second ester function is incorporated in a lactone group, preferably a phthalidyl lactone group. The preparation of these compounds, their in vitro hydrolysis rates, and oral absorption properties in man and animals are reported.

Chemistry. The penicillin lactonyl esters investigated in this study are described in Table I. It will be observed that esters 1–14 are phthalidyl or substituted phthalidyl esters, esters 15 and 16 are crotonolactonyl esters, while compounds 17 and 18 are isomeric butyrolactone esters of penicillin G. In view of the asymmetric carbon center (C-3) at the point of attachment of the thiazolidine carboxyl to the lactone ring, the esters in Table I were obtained as mixtures of epimers. In those cases examined, the proton NMR evidence suggested the epimer ratio was 1:1. The phthalidyl ester of ampicillin (4a) was examined in some detail. The NMR spectrum of its hydrochloride did not permit the epimer ratio to be calculated since proton signals overlapped. However, when the free base was prepared and its spectrum examined in the presence of the lanthanide shift reagent, Eu(fod)$_3$, the individual epimers could be readily distinguished in a ratio of 1:1. The thiazolidine C-3 proton appeared as two sharp singlets of equal intensity.

Scheme I[a]



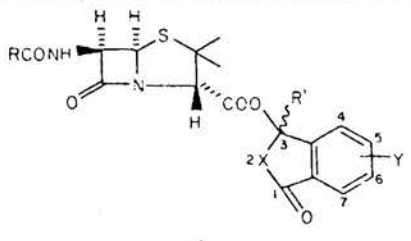
[a] Method A, R = Na; X = Br. Method B, R = Na; X = OTos. Method C, R = COOEt; X = OH.

The preparation and characterization of most of the compounds in Table I have been previously described.[10] Three general procedures were used. The preferred method—method A—was to condense an alkali or amine salt of the penicillin with the appropriate 3-halolactone. Alternatively, in place of the 3-halolactone, the corresponding arylsulfonate ester derived from the 3-hydroxylactone was used—method B. Method C consisted of preparing the penicillin mixed anhydride and reacting this with the 3-hydroxylactone. Each of these methods is illustrated in the Experimental Section and shown in Scheme I below for the phthalidyl ester of benzylpenicillin.

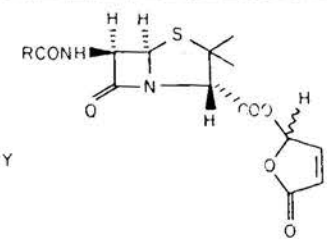
3-Bromo- or 3-chlorolactones were readily prepared by halogenation of the appropriate lactone.[10] 3-Hydroxylactones were available via the corresponding γ-keto acids with which they are in equilibrium in solution. In the preparation of the lactonyl esters of α-aminopenicillins it was desirable to protect the α-amino function prior to condensation. This was done by standard procedures, most conveniently with the enamine protecting group derived from methyl acetoacetate. This group was subsequently removed by mild acid treatment to regenerate the amine.

In order to observe if the configuration of the ester asymmetric carbon center had any effect on hydrolysis rates and oral absorption, the individual epimers of the phthalidyl esters of benzylpenicillin and ampicillin were prepared as follows. Phthalidyl benzylpenicillinate (3a) was obtained in 45% yield by condensation of the potassium salt of penicillin G with 3-bromophthalide. The crystalline product, mp 158–160 °C, was shown to consist of a 1:1 mixture of epimers by the appearance in the NMR of the thiazolidine C-3 proton as two sharp singlets, δ 4.82 and 4.87 in deuteriopyridine. Repeated fractional crystallization of this product afforded the individual epimers designated 3b, mp 179 °C, and 3c, mp 169–171 °C, with optical rotations unchanged on further crystallization. Epimer purity was confirmed by the appearance in the NMR of single sharp singlets for the C-3 proton at δ 4.82 (3c) and 4.87 (3b). These two compounds were treated separately with phosphorus pentachloride followed by methanol to cleave the phenylacetic acid side chain. Without isolation the product, phthalidyl 6-aminopenicillinate, was reacylated with D(−)-α-aminophenylacetyl chloride hydrochloride to afford the individual

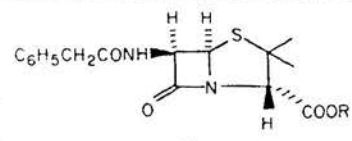
Table I. Lactonyl Esters of Penicillins[a]



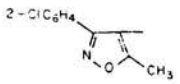
A



B



C

No.	R	R'	X	Y	% yield	Formula	Analyses
				(A) Phthalidyl Esters			
1		H	O	H	91	$C_{27}H_{24}ClN_3O_7$	H, Cl, N; C[g]
2	$C_6H_5OCH_2$	H	O	H	60	$C_{24}H_{22}N_2O_7S$	C, H, N, S
3a	$C_6H_5CH_2$	H[b]	O	H	45	$C_{24}H_{22}N_2O_6S$	C, H, N, S
3b	$C_6H_5CH_2$	H[c]	O	H		$C_{24}H_{22}N_2O_6S$	C, H, N
3c	$C_6H_5CH_2$	H[d]	O	H		$C_{24}H_{22}N_2O_6S$	C, H, N
4a	D-$C_6H_5CH(NH_2HCl)$	H	O	H	65[j]	$C_{24}H_{24}ClN_3O_6S$	H, N; C,[g] S[h]
4b	D-$C_6H_5CH(NH_2HCl)$	H[e]	O	H	63[k]	$C_{24}H_{24}ClN_3O_6S$	H, Cl, N, S; C[g]
4c	D-$C_6H_5CH(NH_2HCl)$	H[f]	O	H	72[k]	$C_{24}H_{24}ClN_3O_6S$	H, Cl, N, S; C[g]
5	$C_6H_5CH_2$	H	O	5-OMe	34	$C_{25}H_{24}N_2O_7S$	C, H, N, S
6	D-$C_6H_5CH(NH_2HCl)$	H	O	6-OMe	51[j]	$C_{25}H_{26}ClN_3O_7S$	H, Cl, N; C,[g] S[i]
7	$C_6H_5CH_2$	H	O	5,6-(OMe)$_2$	41	$C_{26}H_{26}N_2O_8S$	H, N, S; C[g]
8	D-$C_6H_5CH(NH_2HCl)$	H	O	5,6-(OMe)$_2$	29[j]	$C_{26}H_{28}ClN_3O_8S$	H, Cl, N, S; C[g]
9	D-$C_6H_5CH(NH_2HCl)$	H	O	4,5,6-(OMe)$_3$	70[j]	$C_{27}H_{30}ClN_3O_9S$	H, Cl, N, S; C[g]
10	D-$C_6H_5CH(NH_2HCl)$	H	O	6-Cl	47[j]	$C_{24}H_{21}Cl_2N_3O_6S$	H, N, S; C[g]
11	D-$C_6H_5CH(NH_2HCl)$	H	O	6-Br	44[j]	$C_{24}H_{23}BrClN_3O_6S$	H, N, S; C[g]
12	$C_6H_5OCH_2$	CH_3	O	H	32	$C_{25}H_{24}N_2O_7S$	C, H, N
13	$C_6H_5CH_2$	C_6H_5	O	H	25	$C_{30}H_{26}N_2O_6S$	
14	$C_6H_5CH_2$	H	S	H	13	$C_{24}H_{22}N_2O_5S_2$	H, N, S; C[g]
				(B) Crotonolactonyl Esters			
15	$C_6H_5CH_2$				14	$C_{20}H_{20}N_2O_6S$	H, N; C[g]
16	D-$C_6H_5CH(NH_2)$				30	$C_{20}H_{22}ClN_3O_6S$	H, Cl, N, S; C[g]
				(C) Butyrolactonyl Esters			
17					65	$C_{20}H_{22}N_2O_6S$	
18					59	$C_{20}H_{22}N_2O_6S$	C, H, N, S

[a] All penicillins were amorphous and obtained as mixtures epimeric at C_3 in the lactone function, unless stated otherwise. [b] 1:1 epimeric mixture; $[\alpha]^{20}D$ +223° (*c* 1, MeOH); mp 158–160 °C. [c] Single epimer; $[\alpha]^{20}D$ +226° (*c* 1, MeOH); mp 179 °C. [d] Single epimer; $[\alpha]^{20}D$ +179° (*c* 0.5, MeOH); mp 169–171 °C. [e] Single epimer; $[\alpha]^{20}D$ +188° (*c* 1, MeOH). [f] Single epimer; $[\alpha]^{20}D$ +109° (*c* 1, MeOH). [g] Carbon analysis 1–2% low. [h] S: calcd, 6.19; found, 7.12. [i] S: calcd, 5.85; found, 4.51. [j] Yield is esterification and deprotection. [k] Yields of 4b and 4c from 3b and 3c, respectively.

epimers of **4a**, namely, **4b** and **4c** as their hydrochlorides.[11] The epimeric purity of **4b** and **4c** was again established as greater than 95% by NMR in deuteriopyridine where singlets for the thiazolidine C-3 proton were observed at δ 4.86 (**4b**) and 4.80 (**4c**).

Hydrolysis Studies in Vitro. The rates of hydrolysis of the penicillin lactonyl esters listed in Table I were determined in 90% human blood and in 0.05 M potassium phosphate buffer at pH 7.0. Esters **2** and **4a**, **6**, **8**–**11**, **16** of the acid-stable penicillins, penicillin V (phenoxymethylpenicillin), and ampicillin, respectively, were also examined after incubation at pH 2.0.

Results in Table II show that the rates of hydrolysis of the esters were very much greater in 90% blood than in phosphate buffer at pH 7.0. The esters of the acid-stable penicillins showed no hydrolysis (<10%) at pH 2.0 over a period of 25 min. From the point of view of oral absorption, these results suggest that the lactonyl esters possess satisfactory hydrolysis characteristics in that they might be expected to be stable in the intestinal tract but to be rapidly hydrolyzed by enzymes in the blood.

As can be seen from Table II, many of the esters were very rapidly hydrolyzed in 90% blood. It should be noted that for the ester of cloxacillin (**1**), the substrate concentration was higher than for the other compounds and this could increase the time taken for 50% hydrolysis without affecting the rate of hydrolysis. The effect of substitution of methyl (**12**) and phenyl (**13**) groups at the 3 position in the phthalidyl ring was to reduce the rate of enzymic hydrolysis compared with the unsubstituted esters. This was also the case when oxygen was replaced by sulfur in the lactone ring; an appreciably slower rate of hydrolysis in blood was observed for thioester **14** compared with its oxygen analogue **3a**.

Another structural change which might be expected to influence the rate of hydrolysis was that involving differences in configuration at the epimeric center (position 3, Table I) in the lactone ring. The results in Table III

Table II. Hydrolysis of Lactonylpenicillin Esters in Phosphate Buffer and Human Blood

	Penicillin ester[a]		Time (min) taken for 50% hydrolysis	
No.	Ester group	Penicillin	In phosphate buffer, pH 7	In 90% human blood
1	Phthalidyl	Cloxacillin	>25	12[a]
2	Phthalidyl	Penicillin V	>25	<2
3a	Phthalidyl	Penicillin G	>25	<2
4a	Phthalidyl	Ampicillin	>25	<2
5	6-Methoxyphthalidyl	Penicillin G	>25	<2
6	6-Methoxyphthalidyl	Ampicillin	>25	<2
7	5,6-Dimethoxyphthalidyl	Penicillin G	>25	<2
8	5,6-Dimethoxyphthalidyl	Ampicillin	>25	5
9	4,5,6-Trimethoxyphthalidyl	Ampicillin	>25	6
10	6-Chlorophthalidyl	Ampicillin	13	2
11	6-Bromophthalidyl	Ampicillin	>25	14
12	3-Methylphthalidyl	Penicillin V	>25	>25
13	3-Phenylphthalidyl	Penicillin G	>25	10
14	2-Thiophthalidyl	Penicillin G	>25	>25
15	Crotonolactonyl	Penicillin G	22	<2
16	Crotonolactonyl	Ampicillin	>25	<2
17	3-Butyrolactonyl	Penicillin G	>25	<2
18	5-Butyrolactonyl	Penicillin G	>25	>25

[a] The concentration of all esters (not purity corrected) was such as to give 5 μg/ml of ampicillin, penicillin G, and penicillin V and 50 μg/ml of cloxacillin when completely hydrolyzed.

Table III. Hydrolysis of the Epimers of the Phthalidyl Esters of Penicillin G (3b,c) and Ampicillin (4b,c)

	Time (min) required for 50% hydrolysis[a]			
Hydrolyzing system	3b	3c	4b	4c
Phosphate buffered saline, pH 7.4	>25	>25	>25	>25
90% human blood			<2	<2
10% human blood	7	<2	3	<2
2% human blood			10	4
10% human small intestine homogenate			2	<2
0.02% human liver homogenate			25	20

[a] The concentration of ester (not purity corrected) was such as to give 5 μg/ml of parent penicillin when completely hydrolyzed.

illustrate that one epimer (3b) of the phthalide ester of penicillin G hydrolyzed at a slower rate than the other (3c) in 10% human blood. The corresponding ampicillin ester 4b, of the same relative configuration at the ester chiral center as 3b, was also observed to hydrolyze at a slightly slower rate than its epimer 4c in diluted blood and in diluted human liver and intestine homogenates. In 90% blood the hydrolysis rate was too fast for differences between 4b and 4c to be observed.

An investigation was made of the hydrolysis rates of phthalidylampicillin (4a) in homogenates of tissues from the animal species used for absorption studies. The data

Table IV. Hydrolysis of the Phthalidyl Ester of Ampicillin (4a) by Tissues of Rat, Mouse, Human, and Squirrel Monkey

Species	Tissues	Time (min) required for 50% hydrolysis[a]
Rat	90% blood	<2
	2% blood	2
	10% small intestine homogenate	3
	0.02% liver homogenate	13
Mouse	90% blood	<2
Human	90% blood	<2
	10% blood	2
	2% blood	7
	10% small intestine homogenate	3
	0.02% liver homogenate	25
Squirrel monkey	90% blood	<2
	5% small intestine homogenate	<2

[a] The concentration of ester (not purity corrected) was such as to give 5 μg/ml of ampicillin when completely hydrolyzed.

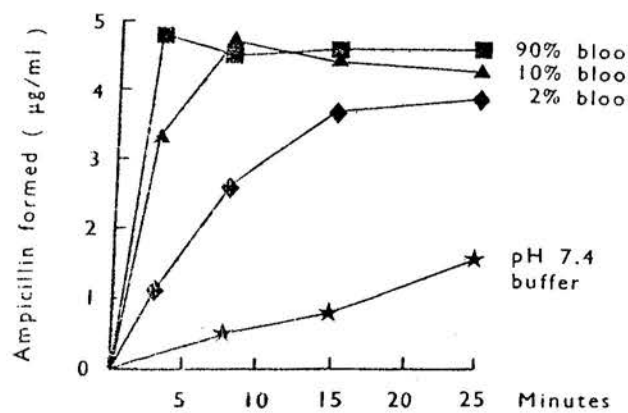


Figure 1. Rate of hydrolysis of the phthalidyl ester of ampicillin (4a) by human blood.

in Table IV show that 4a was very rapidly hydrolyzed to ampicillin by the blood and tissue homogenates of the rat, mouse, and squirrel monkey as well as human.

Figure 1 shows the effect of different dilutions of human blood on the rate of hydrolysis of the phthalidyl ester of ampicillin 4a to ampicillin. The rate was noticeably slower in 2% blood compared with 90% blood although in both cases hydrolysis was virtually complete after 25 min.

The mechanism of hydrolysis of lactonyl esters is not known with certainty. It was postulated by Agersborg that the acetoxymethyl ester of penicillin G hydrolyzed by initial cleavage of the terminal ester bond, followed by spontaneous breakdown of the unstable hydroxymethyl ester intermediate to give penicillin G and formaldehyde.[6] A similar mechanism was assumed for the acyloxymethyl esters of ampicillin and is represented in Scheme II, a.[1] In the case of the lactonyl esters it is suggested that initial cleavage of the lactone ring generates an unstable, substituted hydroxymethyl ester which decomposes to form the penicillin and an aldehydic acid (Scheme II, b). Phthaldehydic acid was indeed detected in further investigations in these laboratories[12] and also by independent workers as the initial product formed when phthalidylampicillin (4a) was incubated with various animal tissues.[13]

In support of Scheme II b was the observation that the 3-butyrolactone ester of penicillin G (17) was readily

Scheme II

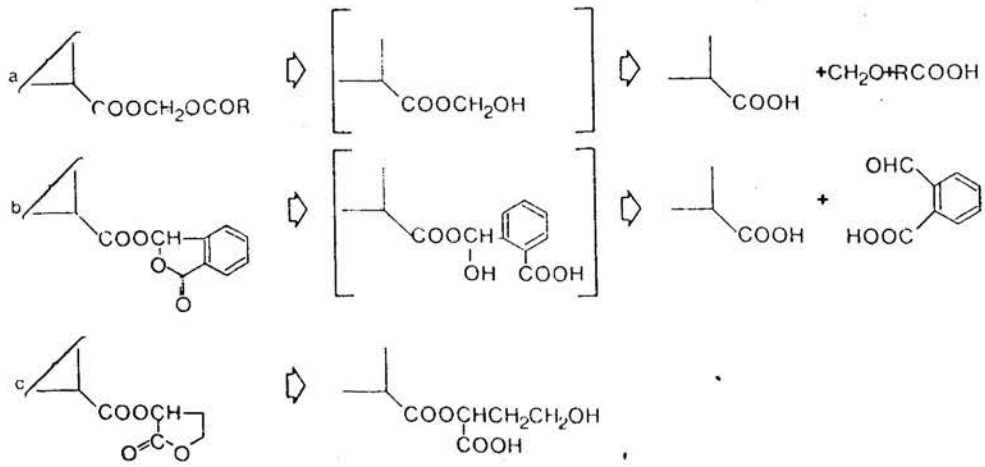


Table V. Penicillin Blood Levels in Squirrel Monkeys after Oral Administration of Lactonyl Esters[a]

Penicillin	Compd	No.	Mean blood concn of parent penicillin, μg/ml 0.5 h	1.0 h	2.0 h	4.0 h	6.0 h
Ampicillin	Phthalidyl ester	4a	7.0	8.2	7.6	1.3	0.4
	6-Methoxyphthalidyl ester	6	8.3	10.6	8.7	3.3	1.0
	5,6-Dimethoxyphthalidyl ester	8	12.7	19.5	14.8	8.0	3.0
	4,5,6-Trimethoxyphthalidyl ester	9	13.9	12.3	3.5	1.1	0.5
	6-Chlorophthalidyl ester	10	6.5	4.1	2.6	0.7	0.2
	6-Bromophthalidyl ester	11	9.9	9.0	5.5	1.5	0.2
	Crotonolactonyl ester	16	10.8	11.9	8.2	4.7	1.9
	Trihydrate		9.3	10.8	6.0	1.9	0.7
Penicillin G	Phthalidyl ester	3a	2.9	2.1	2.1	1.1	0.5
	Sodium salt		3.1	1.5	1.2	0.3	0.1
Cloxacillin	Phthalidyl ester	1	1.4	0.9	0.6	0.4	0.3
	Sodium salt		8.2	4.8	2.0	0.5	<0.5
Penicillin V	Phthalidyl ester	2	3.6	3.3	1.4	0.7	0.4
	Potassium salt		10.9	14.9	11.0	5.2	1.2

[a] Single oral dose of ester equivalent to 100 mg/kg of free acid.

hydrolyzed to penicillin G by human blood (see Table II), whereas no penicillin G was liberated when the isomeric ester 18, in which the carbonyl and oxygen functions are reversed, was incubated in blood. This is illustrated in Scheme II c where it can be seen that the initial product from 18 is an ester in which the thiazolidine carboxyl group is linked to the 2-hydroxyl of 2,4-dihydroxybutyric acid. Such an intermediate would not be expected to hydrolyze further. Additional evidence relating to the initial cleavage of 18 came from the observation that when 18 was incubated at 1 mg/ml in 10% human blood the pH of the solution fell as it did with the control compounds 4a and 17. The fall in pH suggests an initial cleavage of the lactone ring to form a stable hydroxy acid ester as depicted in the Scheme II c.

Absorption Studies. Ten of the lactonyl esters described in Table I and their parent penicillins were each administered as a single oral dose to squirrel monkeys and the blood samples assayed for antibacterial activity in terms of the parent penicillins (Table V). As hydrolysis studies had shown that all of the esters were converted within 30 min to their parent penicillins in squirrel monkey blood and in view of the likely hydrolysis in the gut wall and liver, the antibacterial material in the blood was assumed to be all in the form of the parent penicillin.

The phthalidyl (4a), 6-methoxyphthalidyl (6), and 6-bromophthalidyl (11) esters of ampicillin gave rise to ampicillin blood levels of the same order as those produced by ampicillin trihydrate, and there was some enhancement of ampicillin blood levels after dosing the trimethoxy-substituted phthalidyl ester 9 and the crotonolactonyl ester 16. The 5,6-dimethoxyphthalidyl ester 9 produced the highest levels of ampicillin, and these were about twice as high as those found after administration of the unsubstituted phthalidyl ester 4a or ampicillin itself.

The extent to which the phthalidyl ester group influenced the absorption of cloxacillin, penicillin V, and penicillin G was variable and there was no correlation between the oral absorption of the parent penicillins and the penicillin blood levels produced by their esters. For example, the phthalidyl esters of two moderately well-absorbed penicillins, penicillin V and cloxacillin, produced blood levels notably lower than those obtained with the parent penicillins. Penicillin G was poorly absorbed by the oral route and no improvement in levels was found after dosing its phthalidyl ester to squirrel monkeys.

Esterification of the thiazolidine carboxyl group of a penicillin increases its lipid solubility and the ester might be expected to demonstrate improved oral absorption properties. However, the poor oral absorption of the phthalidyl esters of penicillin G, penicillin V, and cloxacillin is presumably a consequence of their inadequate aqueous solubility in fluids within the gastrointestinal tract. On the other hand, although the phthalidyl ester of ampicillin is more lipophilic than ampicillin it retains some degree of aqueous solubility by virtue of the hydrophilic amino group.

The absorption of the phthalidyl ester of ampicillin (4a) was further compared with that of ampicillin in the mouse, rat, and fasting human volunteers in Table VI. Ester 4a was well absorbed and gave ampicillin blood levels two to three times higher than produced by ampicillin.

The serum and urinary concentrations of ampicillin produced in fasting human subjects by oral administration

Table VI. Ampicillin Blood Levels in Mouse, Rat, and Man after Oral Administration of the Phthalidyl Ester of Ampicillin (4a)[a]

Species	Dose[b]	Compd	Mean concn of ampicillin, μg/ml							
			10 min	20 min	30 min	40 min	60 min	120 min	240 min	360 min
Mouse	50 mg/kg	Ester 4a	7.6	5.0	3.2		1.2	0.4	0.1	
		Ampicillin	2.2	1.4	1.5		1.3	0.4	0.1	
Rat	100 mg/kg	Ester 4a			1.5		2.7	1.5	0.8	0.5
		Ampicillin			1.2		1.4	0.7	0.2	<0.1
Man	250 mg	Ester 4a		1.8		5.8	6.5	3.6	0.8	0.3
		Ampicillin		0.3		1.7	2.4	2.2	0.9	0.2

[a] Blood concentrations in man were measured in serum. [b] Dose is expressed in terms of ampicillin free acid.

Table VII. Absorption of Ampicillin Phthalidyl Esters in Human Volunteers

Ester	Dose, mg[a]	No. of subjects	Mean ampicillin serum concn, μg/ml							Urinary excretion of ampicillin, 0-6 h	
			20 min	40 min	60 min	90 min	120 min	240 min	360 min	Concn, μg/ml	% of dose
Phthalidyl (4a)	371	10	1.8	5.8	6.5	4.7	3.6	0.8	0.3	440	73
Phthalidyl single epimer (4b)	371	9	1.5	5.8	5.6	5.4	3.1	0.6	0.2	517	72
Phthalidyl single epimer (4c)	371	9	1.5	5.5	5.3	4.1	2.5	0.5	0.1	465	68
5,6-Dimethoxyphthalidyl (8)	413	10	1.6	6.3	7.6	4.8	4.0	1.0	0.4	494	81
Ampicillin	250	10	0.3	1.7	2.4	2.3	2.2	0.9	0.2	295	43

[a] Weight of ester hydrochloride equivalent to an oral dose of 250 mg of ampicillin free acid.

of ester 4a, a 1:1 mixture of epimers, were compared with those obtained by administration of the individual epimers 4b and 4c and with ampicillin itself (Table VII). All three esters produced very similar ampicillin concentrations in serum which were two to three times higher than those obtained with ampicillin. Likewise, the amount of ampicillin excreted in the urine in the 0–6-h period after dosing was significantly higher with the esters (68–73%) compared with ampicillin (43%). Results are also shown in Table VII for the 5,6-dimethoxyphthalidyl ester of ampicillin (8), and it can be seen that levels of ampicillin were slightly higher than those found after administration of the unsubstituted phthalidyl ester.

The phthalidyl ester of ampicillin (talampicillin, BRL 8988) was selected for further studies in animals and in man, and results obtained with this ester have been reported from these laboratories and, independently, by a separate group of workers.[13,14]

Experimental Section

Chemistry. Melting points were determined using a Büchi melting point apparatus and are recorded uncorrected. The structures of all compounds were confirmed by their ir and NMR spectra, the latter of which were determined as solutions in either $CDCl_3$ or Me_2SO-d_6. The ir spectra were obtained with a Perkin-Elmer 457 spectrophotometer and the NMR spectra with a Varian Associates spectrometer, Model A-60A. Optical rotations were measured in a Perkin-Elmer polarimeter. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within 0.4% of the theoretical values.

The three methods used for preparation of the esters in Table I are illustrated below.

Method A. (i) Phthalidyl Benzylpenicillinate (3a–c). Potassium benzylpenicillinate (2.0 g, 0.0054 mol) was dissolved in dry DMF (5 ml) and cooled to 0 °C. 3-Bromophthalide (1.15 g, 0.0054 mol) in dry DMF (2 ml) was added in one portion to the stirred penicillin solution. The reaction mixture was allowed to warm to room temperature and then stirred for a further 2 h. The mixture was then poured into ice-cold water (60 ml) and stirred vigorously. The white solid which separated was collected and washed well with H_2O and dried (1.1 g, 45%). The amorphous solid was rapidly crystallized from ethanol to give epimeric 3a: mp 158–160 °C; $[\alpha]^{20}D$ +223° (*c* 1, MeOH). Repeated fractional crystallization from 2-propanol of 3a afforded the individual epimers 3b [mp 179 °C; $[\alpha]^{20}D$ +266° (*c* 1, MeOH)] and 3c [mp 169–171 °C; $[\alpha]^{20}D$ +179° (*c* 0.5, MeOH)].

(ii) Phthalidyl D-α-Aminobenzylpenicillinate Hydrochloride (4a). A fine suspension of potassium *N*-(1-methoxycarbonylpropen-2-yl)-D-α-aminobenzylpenicillinate (2.52 g, 0.005 mol) and 3-bromophthalide (1.06 g, 0.005 mol) was stirred at ambient temperature in a 1:2 mixture of Me_2CO–EtOAc (150 ml) for 24 h. After filtration the organic layer was washed twice with 25-ml portions of aqueous $NaHCO_3$, followed by H_2O, and dried. The solution was concentrated in vacuo. Addition of a small volume of Et_2O to the solution gave phthalidyl (1-methoxycarbonylpropen-2-yl)-D-α-aminobenzylpenicillinate as a white amorphous solid (2.3 g, 81%).

The enamine-protecting group was removed from this product by dissolving it (1 g) in aqueous Me_2CO (1:1, 25 ml) and vigorously stirring this solution at pH 2.5 for 1 h. Acetone was removed in vacuo and deprotected 4a was salted out of the aqueous phase as a sticky yellow gum. The material was dissolved in EtOAc (20 ml), washed with H_2O, and dried. Careful addition of dry ether (ca. 5 ml) to the EtOAc solution of the penicillin ester yielded 4a as a fine white amorphous solid (0.7 g, 80%).

Method B. 3-Methylphthalidyl Phenoxymethylpenicillinate (12). *o*-Acetylbenzoic acid (4.92 g, 0.03 mol) was dispersed in dry EtOAc (100 ml) and cooled to 0 °C. Dry pyridine (2.37 g, 0.03 mol) was then added to the stirred suspension, followed by the dropwise addition of benzenesulfonyl chloride (5.31 g, 0.03 mol). The mixture was left overnight at 4 °C. Potassium phenoxymethylpenicillinate (11.64 g, 0.03 mol) was added with stirring to the benzenesulfonate ester solution and was allowed to react for a further 18 h at ambient temperature.

The reaction mixture was filtered and the filtrate washed with aqueous $NaHCO_3$, followed by H_2O, and dried. Removal of the solvent in vacuo yielded a yellow oil which was purified by chromatography over silica gel using EtOAc–ligroine [bp 60–80 °C (4:1 ratio)] as eluent. 3-Methylphthalidyl phenoxymethylpenicillinate (12) was obtained as a white amorphous solid (2.5 g, 17%), with identical ir and NMR spectral properties as the sample obtained by method C.

Method C. 3-Methylphthalidyl Phenoxymethylpenicillinate (12). Potassium phenoxymethylpenicillinate (11.64 g, 0.03 mol) was dispersed in dry CH_2Cl_2 (200 ml) at −5 °C. To the stirred

suspension. EtOCOCl (3.24 g, 0.03 mol) was added dropwise, together with a catalytic quantity of pyridine, and the mixture stirred at −5 °C for 0.5 h. A solution of *o*-acetylbenzoic acid (4.92 g, 0.03 mol) was added to the mixed anhydride of the penicillin and the reaction continued at ambient temperature for 18 h. Removal of the solvent in vacuo gave a yellow oil. The oily residue was taken up in EtOAc (250 ml); the resulting solution was washed with aqueous $NaHCO_3$, followed by H_2O, and dried. The product obtained after removal of EtOAc in vacuo was purified by column chromatography over silica gel using EtOAc–ligroine [bp 60–80 °C (4:1 ratio)] as eluent. 3-Methylphthalidyl phenoxymethylpenicillinate (12) was obtained as a white amorphous solid (4.7 g, 32%).

Tissue Hydrolysis Studies. Animals were killed by mechanical means and the liver and entire small intestine (pyloric junction to caecal junction) removed immediately. The small intestines were flushed with water to remove gut contents and all tissues kept frozen (−15 °C) until needed. Human liver and jejunum were removed at postmortem examination after death from bronchitis and emphysema. The tissues were immediately frozen and kept frozen until needed. Liver samples were cut into small pieces, washed with cold buffer, and blotted dry to remove excess blood before homogenizing. The entire small intestine from the rat was homogenized. The small intestine of the squirrel monkey was cut into four sections of equal length and approximately 3 cm removed from the proximal end of each length and homogenized. Tissues were homogenized at approximately 10% (w/v) in cold (about 5 °C) 0.05 M potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride, using an Ultra Turrax homogenizer and diluted to the required concentration with the same buffered saline.

Blood samples were taken from live animals and healthy human volunteers, heparinized and used immediately, neat, or diluted with 0.05 M potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride.

The ampicillin esters, being hydrochlorides, were dissolved in distilled water at a concentration equivalent to 1 mg/ml of sodium ampicillin. Esters of other penicillins were dissolved in acetone at a concentration equivalent to 5 mg/ml of the sodium salt of the parent penicillin. No correction was made for the purity of the esters which was generally estimated at 90% or more. The above solutions were immediately diluted with water to a concentration of 50 μg/ml. A one in ten dilution of this solution was then rapidly made into the blood sample, tissue homogenate, or 0.05 M potassium phosphate buffer so that the final concentration of substrate ester was equivalent to 5 μg/ml of the sodium salt of the parent penicillin. However, because of the lower antibacterial activity of cloxacillin the final concentration of its ester was equivalent to 50 μg/ml of sodium cloxacillin.

Reaction mixtures were incubated at 37 °C and sampled at 3, 8, 15, and 25 min after mixing tissue with substrate. The 5-μl samples were assayed immediately for liberated penicillin by the bioelectrophoretic method.

Bioelectrophoresis Assay. Electrophoretic separation of ester from liberated penicillin was carried out on starch–agar gel plates at pH 5.5, a pH at which further enzymatic hydrolysis is markedly reduced. Each plate was prepared by autoclaving a mixture of 1% soluble starch (Analar) + 1% agar (Oxoid Ionagar) in pH 5.5 buffer (0.87 g/l. of KH_2PO_4 + 0.047 g/l. of $Na_2HPO_4{\cdot}2H_2O$) at 121 °C for 15 min to form a molten gel. Approximately 120 ml of this was poured into a 20 × 30 cm frame on a glass sheet. When the gel was set the frame was removed and two rows of nine 1-mm holes were punched along the length of the plate using a Pasteur pipet. Four pairs of holes were then loaded with 5-μl aliquots of reaction mixtures or control solutions, the remaining five pairs of holes being filled with 5 μl of standard solutions of parent penicillin dissolved in gut homogenate, liver homogenate, blood, or buffer as appropriate. When loaded, a voltage of 15 V/cm was applied across the plate for 30 min. Under these conditions ampicillin esters migrate to the cathode at a faster rate than ampicillin which stays near the origin. Esters of the other penicillins being neutral remain at the origin, whereas their free acids migrate to the anode.

The plates were next covered with blood agar base (Oxoid) inoculated with *Sarcina lutea* NCTC 8340. This was achieved by inoculating molten blood agar base at approximately 45 °C with *S. lutea* and pouring into the frame as for the electrophoresis plates. When set, but still warm, this gel layer was slid from the glass sheet onto the electrophoresis gel, any trapped air being removed by gently running a clean glass rod over the surface. The plates were then incubated at 30 °C overnight (18 h), after which the diameters of the zones of inhibition due to liberated penicillin were measured and used to calculate the concentration in the reaction mixtures and controls.

Absorption in Experimental Animals. The esters were administered orally as aqueous suspensions by intubation to (a) squirrel monkeys (*Saimiri sciureus*) weighing 500–1000 g, (b) male albino mice, CSI strain, weighing 18–22 g, and (c) male albino rats, Sprague-Dawley OLAC strain, weighing 350–500 g. The compounds were administered at a dose of ester equivalent to 100 mg of penicillin free acid/kg to groups of six squirrel monkeys or groups of five rats. Venous blood samples were taken at 0.5, 1, 2, 4, and 6 h after dosing. Mice were dosed with ester at a dose equivalent to 50 mg of penicillin free acid/kg. Groups of five were killed at 10, 20, 60, 120, and 240 min after dosing, and blood was sampled from the cut axilla region. Blood specimens were heparinized, stored at 4 °C, and assayed on the day of sampling.

Absorption in Human Volunteers. Ampicillin esters **4a–c** and **8** were administered as their unformulated hydrochlorides in plain gelatin capsules to groups of ten fasting human volunteers as single oral doses equivalent to 250 mg of ampicillin pure free acid. The dose of the phthalidyl ester was 371 mg and of the 5,6-dimethoxyphthalidyl ester, 413 mg. Ampicillin was administered as its trihydrate. Venous blood was taken at 20 min, 40 min, 1 h, 1.5 h, 2 h, 4 h, and 6 h after administration and urine was collected over the 6-h period after dosing.

Microbiological Assay. Concentrations of penicillins in specimens of blood or urine after administration of the esters were measured by large-plate microbiological assay with *Sarcina lutea* NCTC 8340 as assay organism. Specimens of animal blood or human serum were assayed against standard solutions of the appropriate penicillin prepared in animal blood or in pooled human serum. Urine specimens of ampicillin were assayed against ampicillin standard solutions prepared in 0.05 M phosphate buffer, pH 7.0. The assay plates were incubated overnight at 30 °C, inhibition zone diameters were measured, and the penicillin concentrations of the test specimens were derived from standard lines constructed from the standard solutions.

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Carbenicillin*: A New Semisynthetic Penicillin Active against *Pseudomonas pyocyanea*

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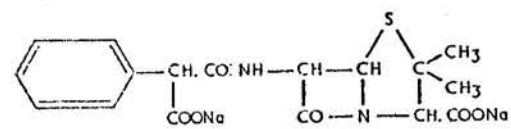
Carbenicillin (disodium α-carboxybenzylpenicillin; BRL 2064) is a new semisynthetic penicillin with a wide spectrum of activity against Gram-positive and Gram-negative bacteria. In particular, carbenicillin shows a certain level of activity against *Pseudomonas pyocyanea* (*Pseudomonas aeruginosa*), and the compound is also active against many strains of *Proteus vulgaris*, *Proteus rettgeri*, and *Proteus morganii*, which are typically resistant to ampicillin.

The structure of carbenicillin is as follows:



The compound, which should be stored in the refrigerator, is readily soluble in water, giving a clear neutral solution, which shows no significant loss of activity after 24 hours at room temperature and is stable in the refrigerator for at least seven days. In acid solution carbenicillin is relatively unstable, the half-life at *p*H 2 being approximately two hours at room temperature and approximately 30 minutes at 37° C.

Antibacterial Activity of Carbenicillin

The antibacterial spectrum of carbenicillin is shown in Table I. Minimum inhibitory concentrations were determined by serial dilution of the drug in agar, and one drop of an overnight broth culture of each organism was used as inoculum.

Carbenicillin is active against *Escherichia coli*, *Salmonella* species, *Shigella* species, *Proteus mirabilis*, and *Haemophilus*

* Pyopen is the registered trade mark of Beecham Research Laboratories for carbenicillin.

† Beecham Research Laboratories, Brentford, Middlesex.

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TABLE I.—*Antibacterial Spectrum of Carbenicillin*

	Minimum Inhibitory Concentration (μg./ml.)*
Escherichia coli	5·0
Klebsiella aerogenes	250
Salmonella typhi	12·5
Shigella flexneri	5·0
„ *sonnei*	5·0
Pseudomonas pyocyanea	50
Proteus mirabilis	2·5
„ *morganii*	5·0
„ *rettgeri*	2·5
„ *vulgaris*	5·0
Haemophilus influenzae	0·5
Staphylococcus aureus Oxford	0·5
„ *aureus*†	50
β-Haemolytic streptococcus	0·25
Streptococcus faecalis	25
„ *pneumoniae*	0·5
Bacillus subtilis	1·25
Sarcina lutea	0·5
Clostridium tetani	0·25
„ *welchii*	0·25

* Serial dilution in agar; inoculum, one drop of an overnight culture.
† Penicillinase-producing strain.

influenzae, and in this respect the spectrum of activity is similar to that of ampicillin, although carbenicillin is in general rather less active than ampicillin. Most strains of *Klebsiella aerogenes* are resistant to carbenicillin and the level of activity against enterococci (*Streptococcus faecalis*) is relatively low. Carbenicillin is active against pyogenic streptococci, pneumococci, and penicillin-sensitive strains of staphylococci, although the compound is considerably less active in this respect than penicillin G or ampicillin. Carbenicillin is not stable to staphylococcal penicillinase and is not active against penicillin-resistant strains of staphylococci. The activity of carbenicillin against several strains of *Pr. vulgaris*, *Pr. rettgeri*, and *Pr. morganii* is shown in Table II. These species of proteus are characteristically resistant to ampicillin, and in the experiment shown in Table II all the strains required an ampicillin concentration of at least 50 μg./ml. to prevent growth. In contrast, many of these strains were relatively sensitive to carbenicillin and in some cases were inhibited by concentrations as low as 1.25 μg./ml. Strains of *Pr. mirabilis*, however, which are resistant to ampicillin are also resistant to carbenicillin. The reason for the activity which carbenicillin shows against certain ampicillin-resistant proteus appears to be due to greater stability of the compound towards the penicillinases produced by these organisms. On the other hand, penicillinase-producing strains of *Pr. mirabilis*, like those of *Kl. aerogenes*, inactivate both carbenicillin and ampicillin.

The most significant feature of the antibacterial spectrum of carbenicillin is the activity against *Ps. pyocyanea*. In terms of the minimum inhibitory concentration the activity against this pathogen is relatively low, since for most strains a concentration in the order of 50 μg./ml. is required to prevent growth. How-

TABLE II.—*Activity of Carbenicillin Against Ampicillin-resistant Strains of Proteus*

		Minimum Inhibitory Concentration (μg./ml.)*	
		Carbenicillin	Ampicillin
Proteus morganii	A	1·25	50
	F	1·25	50
	G	1·25	50
rettgeri	A	1·25	50
	I	2·5	250
	B	12·5	250
vulgaris	I	2·5	250
	A	5·0	125
	E	5·0	250
	G	50	> 500
	H	250	> 500
mirabilis	8	> 500	> 500
	889	> 500	> 500

* Determined by serial dilution in agar. The inoculum was one drop of an overnight broth culture, and M.I.C. values were read after 18 hours at 37° C.

ever, from the clinical point of view activity is essentially meaningful in relation to the concentration of drug which can be reached at the site of infection. Carbenicillin itself is remarkably free from toxic effects (Acred *et al.*, 1967) and can be administered in sufficient dosage to obtain concentrations in the serum which are inhibitory to typical strains of *Ps. pyocyanea*, and the drug is also excreted in high concentration in the urine.

One characteristic of the activity of carbenicillin against *Ps. pyocyanea* is that in serial dilution tests to determine the minimum inhibitory concentration the drug does not show a sharp end-point when the inoculum is very heavy. This is illustrated in Fig. 1. Serial dilutions of carbenicillin in nutrient

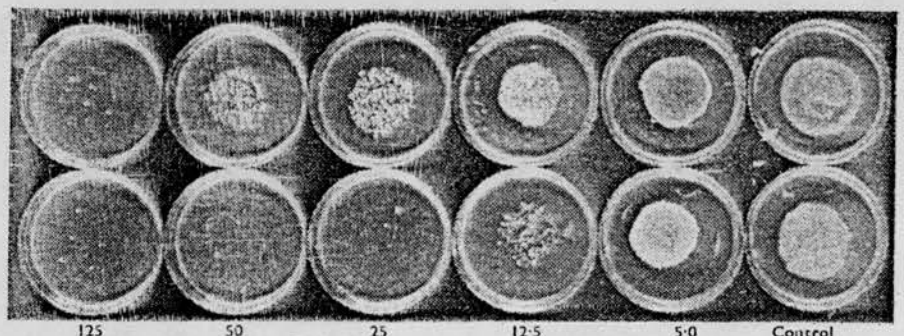

FIG. 1.—Activity of carbenicillin against a typical strain of *Ps. pyocyanea*. Serial dilutions of carbenicillin in nutrient agar were prepared in Petri dishes and an area of the agar surface of each plate was inoculated, using a cotton-wool swab. An undiluted overnight broth culture of *Ps. pyocyanea* was used to inoculate the upper row of plates and a 1/100 dilution of the same culture was used to inoculate the lower row.

agar were prepared in Petri dishes and an area of the agar surface of each plate was inoculated with an undiluted overnight broth culture of a strain of *Ps. pyocyanea* applied with a cotton-wool swab. In this way the inoculum on each plate was at least 10 million organisms. A similar series of plates was also inoculated in the same way, using the same culture of *Ps. pyocyanea* but diluted 1/100 to give an inoculum of approximately 100,000 organisms. The strain of *Ps. pyocyanea* used in this experiment was a recent clinical isolate and was typical with regard to its sensitivity to carbenicillin. With the use of the undiluted culture as inoculum growth was very markedly diminished at a concentration of 25 μg./ml. carbenicillin, but a number of colonies were present even at a concentration of 50 μg./ml. and growth could only be said to have been prevented on the plate containing 125 μg./ml. However, when the inoculum was diluted 1/100 inhibition of growth was very pronounced at a concentration of 12.5 μg./ml., and at a concentration of 25 μg./ml. only three or four colonies were seen.

Thus, in experiments of the type shown in Fig. 1, with the use of typical strains of *Ps. pyocyanea* and a very heavy inoculum level, some scanty growth may be seen with concentrations of carbenicillin as high as 125 μg./ml., and consequently if the criterion of the "minimum inhibitory concentration" is one of complete inhibition of all trace of growth the M.I.C. with a heavy inoculum may well be as high as 250 μg./ml. or more. However, the amount of growth at such concentrations is extremely meagre indeed, and with an inoculum only slightly smaller a sharp end-point is seen with complete inhibition of growth at a concentration of carbenicillin of 25–50 μg./ml.

TABLE III.—*Activity of Carbenicillin Against Pseudomonas pyocyanea*

No. of Strains	Minimum Inhibitory Concentration (μg./ml.)* and No. of Strains					
	250	125	50	25	12·5	5·0
111	5	8	61	31	6	

* Determined by serial dilution in agar. The inoculum was one drop of a 1/100 dilution of an overnight broth culture, and M.I.C. values were read after 18 hours at 37° C.

Minimum inhibitory concentrations of carbenicillin for 111 clinical isolates of *Ps. pyocyanea* are shown in Table III. These inhibitory concentrations were determined by preparing serial dilutions of carbenicillin in agar as described above, and one drop of a 1/100 dilution of an overnight broth culture of each isolate was used as inoculum. It will be seen from Table III that the majority of the strains were inhibited by a concentration of 25–50 μg./ml. carbenicillin. In comparison, the inhibitory concentrations of ampicillin and benzylpenicillin

against most strains of *Ps. pyocyanea* are of the order of 1,000–5,000 μg./ml. or greater.

The effect of the inoculum size on the determination of the minimum inhibitory concentration is not due to destruction of the drug as in the case of penicillin G and penicillin-resistant staphylococci. Carbenicillin is itself stable to the penicillinase produced by typical strains of *Ps. pyocyanea*. Furthermore, the effect of inoculum size does not appear to be due to growth of resistant mutants present in a large inoculum. When tests are carried out using as inoculum the growth which occurs in the presence of high concentrations of carbenicillin the result obtained is the same as that seen with the original culture—that is, marked suppression of growth at concentrations of 25 μg./ml., but with scanty growth nevertheless persisting up to a concentration as high as 125 μg./ml.

As might be expected, this effect of inoculum size on the minimum inhibitory concentration of carbenicillin is also seen with tests carried out in liquid medium. With a heavy inoculum—for example, one drop of undiluted broth culture—growth appears normal with formation of pigment and pellicle up to a concentration of approximately 12.5 μg./ml. cerbenicillin, but at 25 μg./ml. there is generally absence of pellicle and pigment and a markedly diminished amount of growth, although complete inhibition of growth may be obtained only with concentrations as high as 250 μg./ml. With a slightly smaller inoculum a sharp end-point is obtained, and with most strains of pseudomonas the minimum inhibitory concentration under these conditions is usually about 50 μg./ml.

Carbenicillin is bactericidal, and typical results with a strain of *Ps. pyocyanea* are shown in Fig. 2. Over the first seven hours a concentration of 50 μg./ml. usually results in a kill of at least 99% of the original inoculum, but thereafter some resumption of growth takes place which may even increase to a visible amount after 24 hours. When this growth is used as inoculum in a repeat test the same kill is again obtained over the first seven hours, followed by a certain amount of growth. The reason for this bactericidal effect followed by growth is not known, but it would not appear to be due to the selection of resistant mutants present in the original inoculum, nor is it due to inactivation of carbenicillin during the experiment.

Development of Resistance

Experiments involving repeated subculture of staphylococci and various Gram-negative bacilli, including *Ps. pyocyanea*, in the presence of carbenicillin, indicate that emergence of resistance takes place in a stepwise fashion which is characteristic of penicillins.

6

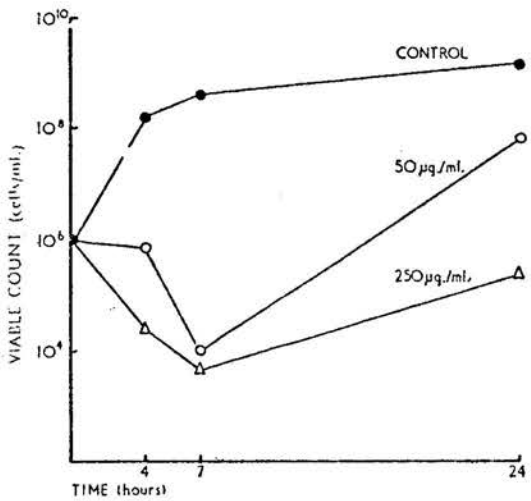

FIG. 2.—Bactericidal activity of carbenicillin against *Pseudomonas pyocyanea*.

Effect of Serum on Activity

Carbenicillin is not highly bound to serum proteins. Sensitivity tests carried out in human serum show that the activity of carbenicillin is not significantly diminished in the presence of serum. Determinations of the extent of binding by ultrafiltration indicate that in human serum approximately 53% of the drug is unbound.

Disc Sensitivity Testing with Carbenicillin

With 6-mm. paper discs containing 100 µg. carbenicillin all strains of *Ps. pyocyanea* tested in this laboratory have given zones of inhibition of 12–20 mm. with a heavy inoculum and zones of 18–30 mm. with a dilute inoculum. With a heavy inoculum a number of colonies are usually visible within the zone of inhibition, although when these colonies are picked off and retested they show the same sensitivity as the original culture and again give rise to a number of colonies within the clear zone of inhibition.

In the case of Gram-negative bacilli other than pseudomonas —for example, *E. coli* and *Proteus* species—a 25-µg. disc would be suitable for sensitivity testing. With use of a heavy inoculum inhibition zones of 20 mm. or more are usually obtained with strains that are inhibited by 5 µg./ml. carbenicillin.

7

Absorption and Excretion in Man

Assay of Carbenicillin in Serum and Urine

The concentration of carbenicillin in serum and in urine was determined by the cup-plate biological assay method. Details of the technique were as previously described (Knudsen and Rolinson, 1960) except that *Ps. pyocyanea* NCTC 10490 (Ellsworth strain, 1973) was used as test organism. *Sarcina lutea* and *Staphylococcus aureus* are unsuitable as assay organisms because carbenicillin contains a small amount of benzylpenicillin and this may appear in the serum and in the urine together with the carbenicillin. Since *S. lutea* and *Staph. aureus* are many times more sensitive to benzylpenicillin than to carbenicillin, the presence of traces of benzylpenicillin may invalidate the assay. *Ps. pyocyanea* NCTC 10490, on the other hand, is sensitive to carbenicillin but relatively resistant to benzylpenicillin. The culture is unusually sensitive to carbenicillin, showing a minimum inhibitory concentration of approximately 1.25 μg./ml., and using this strain it is possible to assay concentrations of carbenicillin as low as 2.5 μg./ml., while benzylpenicillin at concentrations of up to 500 μg./ml. fails to show any zone of inhibition. Standard solutions of carbenicillin from 1 to 100 μg./ml. were prepared in human serum, and, where necessary, serum specimens were suitably diluted in human serum to give a level within this range of concentration. For the assay of urine samples the standard solutions of carbenicillin were prepared in M/20 phosphate buffer *p*H 7, and urine samples were also diluted as required, using the same buffer.

Oral Administration

Carbenicillin in gelatin capsules was given at a dose of 500 mg. to healthy fasting volunteers. Serum samples were taken at ½, 1, 2, 4, and 6 hours after administration of the dose, and urine was collected over the period 0 to 6 hours. No carbenicillin (that is, less than 1 μg./ml.) was detected in the serum, and the quantity present in the urine amounted to less than 1% of the dose administered. It would appear, therefore, that carbenicillin is not absorbed to any appreciable extent when administered orally.

Intramuscular Administration

Carbenicillin was administered by intramuscular injection to groups of healthy adult volunteers as single doses of 250 mg.,

500 mg., and 1,000 mg. The 250-mg. dose was given in a volume of 1 ml. of water for injection, and the 500-mg. and 1,000-mg. doses were dissolved in 2 ml. of water. Blood specimens were collected ½, 1, 2, 4, and 6 hours after administration, and the urine was collected during the six-hour period after dosing. Results of these studies are summarized in Table IV.

TABLE IV.—*Mean Serum Concentrations of Carbenicillin After Intramuscular Injection in Adult Volunteers*

No. of Subjects	Dose (mg.)	Serum Concentration (μg./ml.)						% of Dose Excreted in Urine over 0–6 hrs.
		½-hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	6 hrs.	
10	250	5·9	7·9	5·7	2·6	<2·0	<2·0	71
9	500	13·7	17·7	13·2	—	2·0	<2·0	82
16	1,000	21·6	25·3	22·1	—	10·9	3·7	84

Mean serum peak levels of 7.9 μg./ml. carbenicillin were obtained one hour after administration of the 250-mg. dose, and these declined to <2 μg./ml. after four hours. Increasing the dose to 500 mg. resulted in serum concentrations twice as high as those obtained with the 250-mg. dose, but these declined to barely detectable concentrations after four hours. Impressive results were obtained with the 1,000-mg. dose, the mean serum concentrations being greater than 20 μg./ml. for the first two hours after injection, falling to 10.9 μg./ml. at four hours.

The amounts of carbenicillin excreted in the urine during the first six hours after injection were 71% of the dose with the 250-mg. dose, 82% of the 500-mg. dose, and 84% of the 1,000-mg. dose, producing average carbenicillin concentrations in the urine of 1,000 to 2,000 μg./ml. with the 500-mg. dose and 2,000 to 4,000 μg./ml. with the 1,000-mg. dose.

The effect of probenecid was investigated in a crossover study in which 10 subjects were given 1,000 mg. carbenicillin by the intramuscular route. One week later the same subjects took oral probenecid (1 g.) 10 hours and one hour before receiving 1,000 mg. intramuscular carbenicillin. The administration of probenecid resulted in a marked increase in the carbenicillin serum concentrations (Table V and Fig. 3) and in a significant reduction in the rate of elimination of carbenicillin from the serum.

Chromatographic examination of the urine after intramuscular administration of carbenicillin showed no evidence of the formation of biologically active metabolites.

Although carbenicillin is primarily of interest for its activity against *Ps. pyocyanea*, the compound is active against a wide

range of bacteria, including *E. coli* and various ampicillin-resistant proteus species, and it seems probable that intramuscular doses of 500 to 1,000 mg. carbenicillin might be successfully used to attain serum, tissue, and urine concentrations adequate for the treatment of infections caused by these organisms. On the other hand, the activity of carbenicillin against *Ps. pyocyanea* is such that inhibitory concentrations of carbenicillin with respect to this particular pathogen are not likely to be achieved in serum and tissue by intramuscular

TABLE V.—*Effect of Probenecid on Serum Levels and Urinary Excretion of Carbenicillin in Healthy Adult Volunteers. (Carbenicillin Dose 1 g. by Intramuscular Injection)*

	No. of Subjects	Mean Serum Concentration (μg./ml.)					% of Dose Excreted in Urine over 0–6 hrs.
		½-hr.	1 hr.	2 hrs.	4 hrs.	6 hrs.	
With probenecid*	10	25·7	39·9	48·0	27·4	19·4	48
Without probenecid	10	26·8	29·4	22·2	10·8	3·3	84

* 1 g. 1 hour and 10 hours before administration of carbenicillin.

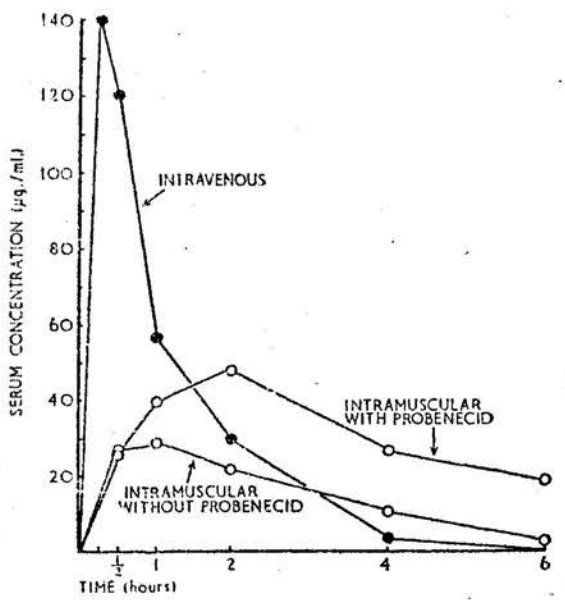

FIG. 3.—Serum concentrations in human volunteers after intramuscular and intravenous administration of 1 g. of carbenicillin.

dosing. Nevertheless, the urine concentrations attained after intramuscular administration of carbenicillin are greatly in excess of the minimum inhibitory concentrations of the drug

against *Ps. pyocyanea,* and the results of clinical trials indicate that 1,000 mg. of carbenicillin given four times daily by the intramuscular route may be adequate for the treatment of acute urinary tract infections due to this organism.

Intravenous Administration

Two subjects were given 1,000 mg. of carbenicillin by slow intravenous injection. The penicillin was dissolved in 10 ml. of sterile water for injection and administered over a period of three minutes. Serum concentrations of greater than 100 μg./ml. carbenicillin were obtained during the first half-hour after administration of the compound, although at four hours only trace amounts of carbenicillin were detected (Table VI and Fig. 3).

From the bacteriological studies, concentrations of carbenicillin in the order of 100 μg./ml. would be expected to inhibit the growth of most strains of *Ps. pyocyanea,* but in view of the

TABLE VI.—*Serum Concentration of Carbenicillin After Intravenous Administration of 1 g.*

Subject	Serum Concentration (μg./ml.)					
	¼-hr.	½-hr.	1 hr.	2 hrs.	4 hrs.	6 hrs.
A	140	127	60	28	3·7	<3·0
B	140	114	55	32	4·4	<3·0

rapid elimination of the drug from the body frequent administration would probably be necessary. In practice the results of clinical studies have shown that, for the treatment of septicaemia, tissue infections, and chronic urinary tract infections due to *Ps. pyocyanea,* carbenicillin should be given at a dose of 1 g. hourly by the intravenous route, preferably together with probenecid, in order to maintain inhibitory concentrations in the body.

Summary

Carbenicillin is a new semisynthetic penicillin active against *Pseudomonas pyocyanea.* The compound shows a broad spectrum of activity against Gram-positive and Gram-negative bacteria and is active against strains of *Proteus vulgaris, Pr. rettgeri,* and *Pr. morganii,* which are typically resistant to ampicillin. With a very heavy inoculum of *Ps. pyocyanea* marked inhibition of growth occurs with carbenicillin at 25 to 50 μg./ml., but some very scanty growth may persist even at

concentrations of 125 to 250 μg./ml. With a slightly smaller inoculum complete inhibition of growth occurs at 25 to 50 μg./ml. Carbenicillin is bactericidal and is not highly bound to serum protein. Carbenicillin is not absorbed when given by mouth, but high concentrations of the drug are obtained in serum after intramuscular or intravenous administration, and these levels are increased and are more prolonged with concomitant use of probenecid. Carbenicillin is excreted in high concentration in the urine.

We thank Dr. J. H. C. Nayler for preparing the carbenicillin used in these studies, Mr. D. Lawson for the photograph in Fig. 1, and Mrs. Eleanor Fairclough for technical assistance.

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New Semi-synthetic Penicillin active against *Pseudomonas pyocyanea*

Most of the semi-synthetic penicillins developed from 6-amino-penicillanic acid have little or no activity against *Pseudomona pyocyanea*. α-Carboxybenzylpenicillin (carbenicillin : BRL 2064), on the other hand, is active against these organisms and seems to be almost completely non-toxic to man.

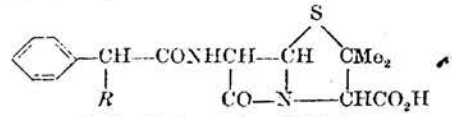
by P. ACRED, D. M. BROWN, E. T. KNUDSEN, G. N. ROLINSON and R. SUTHERLAND

Beecham Research Laboratories, Brockham Park, Betchworth, Surrey

DURING the preparation in these laboratories of many hundreds of semi-synthetic penicillins from 6-amino-penicillanic acid, activity against *Pseudomonas pyocyanea* (*Pseudomonas aeruginosa*) has been found only infrequently. This article describes investigations of the properties and pharmacology of one compound that has been selected for clinical trial. The clinical results (to be reported elsewhere) have shown that carbenicillin has effective anti-*Pseudomonas* activity when given to human subjects by intramuscular or intravenous injection. As with most penicillins, carbenicillin is virtually non-toxic and has no local irritant effects.

Chemistry

The introduction of ionized substituents into the side-chain of benzylpenicillin (I) is known to lead in some cases to enhanced activity against certain Gram-negative bacteria, as in ampicillin (II) which contains a basic α-substituent. We now wish to report on an analogue with an acidic α-substituent, namely α-carboxybenzylpenicillin (III: carbenicillin).



I : R=H (benzylpenicillin)
II : R=NH_2 (ampicillin)
III : R=CO_2H (carbenicillin)

Carbenicillin (which will shortly be available under the registered trademark of 'Pyopen' from Beecham Research Laboratories) is supplied as the disodium salt and contains 5–6 per cent water. It is a white powder which dissolves freely in cold water to give a solution with a *p*H between 6 and 8. Carbenicillin is only moderately stable to acid, the half life at 21° C and *p*H 2·0 being about 140 min and at 37° C and at *p*H 2·0 about 30 min.

On prolonged storage, carbenicillin may decompose to some extent into benzylpenicillin and carbon dioxide. The presence of benzylpenicillin can readily be detected by paper chromatography in butanol-ethanol-water followed by development on an agar plate seeded with *B. subtilis*. Carbenicillin gives a zone of inhibition of bacterial growth near the origin (R_F about 0·03) whereas benzylpenicillin has an R_F of about 0·4. The technique is sensitive because benzylpenicillin is considerably more active than carbenicillin against Gram-positive bacteria.

Microbiology

The antibacterial spectrum of carbenicillin is shown in Table 1. Serial dilutions of the drug were prepared in agar in Petri dishes and the surface of the agar was inoculated using one drop of undiluted overnight broth cultures. The minimum inhibitory concentrations were read after incubation overnight at 37° C. In some respects the antibacterial spectrum of carbenicillin is similar to that of ampicillin. For example, carbenicillin shows significant activity against penicillin-sensitive staphylococci and the streptococci, although this is substantially lower than that of ampicillin or penicillin *G*. Also, carbenicillin is not stable to staphylococcal penicillinase and is relatively inactive against penicillin-resistant strains of staphylococci. Against *Haemophilus influenzae*, *Escherichia coli*, *Salmonella* species, *Shigella* species and *Proteus mirabilis*, carbenicillin shows a similar level of activity to that of ampicillin although, in general, the activity of carbenicillin is somewhat lower. Like ampicillin, carbenicillin shows only a low level of activity against most strains of *Klebsiella aerogenes*. The spectrum of carbenicillin differs from that of ampicillin, however, with regard to *Pseudomonas pyocyanea* and certain *Proteus* species and carbenicillin is primarily interesting because it is active against these organisms.

Activity against Pseudomonas pyocyanea. Compared with the activity one is accustomed to see with most antibiotics of clinical value, the activity of carbenicillin against strains of *Ps. pyocyanea* is low. Not many antibiotics are effective against *Pseudomonas*, however, and in the case of those which are active there are certain problems of toxicity. Carbenicillin, on the other hand, is remarkably free from toxic effects and this allows a dosage to be used which can result in inhibitory con-

Table 1. ANTIBACTERIAL SPECTRUM OF CARBENICILLIN

	Minimum inhibitory concentration (μg/ml.)*
Escherichia coli	5·0
Klebsiella aerogenes	250·0
Salmonella typhi	12·5
Shigella flexneri	5·0
Shigella sonnei	5·0
Pseudomonas pyocyanea	50·0
Proteus mirabilis	2·5
Proteus morganii	5·0
Proteus rettgeri	2·5
Proteus vulgaris	5·0
Haemophilus influenzae	0·5
Staphylococcus aureus Oxford	0·5
Staphylococcus aureus †	50·0
β-Haemolytic streptococcus	0·25
Streptococcus faecalis	25·0
Streptococcus pneumoniae	0·5
Bacillus subtilis	1·25
Sarcina lutea	0·5
Clostridium tetani	0·25
Clostridium welchii	0·25

* Serial dilution in agar; inoculum, one drop of an overnight culture.
† Penicillinase-producing strain.

centrations being reached in the body even though these levels are comparatively high.

The activity of carbenicillin against seventy-four strains of *Ps. pyocyanea* is shown in Table 2. All the strains were recent clinical isolates. It will be seen that a concentration of 50 μg/ml. carbenicillin was required to inhibit the majority of the strains. Some strains were inhibited by 25 μg/ml. but other strains were only inhibited with concentrations as high as 125 μg/ml. Determination of the minimum inhibitory concentrations of carbenicillin is made difficult by the fact that typical strains of *Ps. pyocyanea* do not show a sharp end point when the inoculum is heavy (Table 3). When a heavy inoculum is used, that is, one comprising 10^8 or more cells, confluent growth occurs on agar containing concentrations of carbenicillin up to about 12·5 μg/ml., and at this concentration of drug there is no evidence of any inhibitory effect. At a concentration of 25 μg/ml. carbenicillin, however, growth is extremely scanty compared with the control and the inhibitory effect of the drug at this concentration is very marked, although growth is by no means completely inhibited. At concentrations of carbenicillin above 25 μg/ml., growth is extremely meagre, taking the form of a very thin film which may only just be discernible on the surface of the agar. Consequently, if the criterion of the "minimum inhibitory concentration" (MIC) is one of absolute inhibition of any trace of growth, the MIC with a very heavy inoculum may well be as high

Table 2. ACTIVITY OF CARBENICILLIN AGAINST *Pseudomonas pyocyanea*

	Minimum inhibitory concentration* (μg/ml.) and number of strains					
No. of strains	250	125	50	25	12·5	5·0
74		6	50	15	3	

* Determined by serial dilution of the drug in agar: inoculum one drop of overnight broth culture diluted 1/100.

Table 3. EFFECT OF CARBENICILLIN ON THE GROWTH OF A TYPICAL STRAIN OF *Ps. pyocyanea*

Inoculum	Carbenicillin (μg/ml.) 500	250	125	50	25	12·5	5·0	2·5
Undiluted (about 10^7 cells)	growth barely discernible		sparse growth with some separate colonies			confluent growth		
Diluted 1/100 (about 10^5 cells)	no growth					semi-confluent growth		

Serial dilutions of carbenicillin prepared in agar and the surface inoculated by flooding with overnight broth culture

as 250 μg/ml. or more, as a result of the scanty growth which occurs at concentrations of carbenicillin of 25 μg/ml. and greater. When a smaller inoculum is used in the sensitivity tests, a relatively sharp end point is obtained. With an inoculum of about 10^5 cells a normal, pigmented, semi-confluent growth is obtained on agar with concentrations of carbenicillin up to 12·5 μg/ml., but at 25 μg/ml. and over there is usually no growth at all.

As might be expected, this effect of inoculum size on the MIC of carbenicillin is also seen when the tests are carried out in liquid medium. With a heavy inoculum, growth appears normal with formation of pigment and pellicle up to a concentration of about 12·5 μg/ml. carbenicillin, but at 25 μg/ml. there is no pellicle or pigment and growth is markedly diminished, although complete inhibition of growth may only be obtained with concentrations as high as 250 μg/ml. With a smaller inoculum, a sharp end point is obtained and with most strains of *Pseudomonas* the MIC under these conditions is usually about 50 μg/ml.

This effect of inoculum size on the MIC is outwardly similar to that seen with penicillin *G* and penicillinase-producing staphylococci. Carbenicillin, however, is itself highly stable to the penicillinase produced by typical strains of *Ps. pyocyanea* and the inoculum effect in this case is not due to destruction of drug, nor is the effect due to the growth of resistant mutants present in the original inoculum. When sensitivity tests are carried out on the growth which occurs in the presence of high concentrations of carbenicillin the result obtained is the same as that seen with the original culture, that is, marked suppression of growth at concentrations of 25 μg/ml. and over, but with scanty growth nevertheless persisting up to a concentration as high as 250 μg/ml. when the inoculum is heavy.

Carbenicillin is bactericidal and typical results with a strain of *Pseudomonas* are shown in Fig. 1. For the first 7 h a concentration of 50 μg/ml. usually results in the killing of at least 99 per cent of the original inoculum, but some resumption of growth takes place thereafter which may even increase to a visible amount after 24 h. When this growth is used as inoculum in a repeat test, the same kill is again obtained over the first 7 h followed by a certain amount of growth. The reason for this bactericidal effect

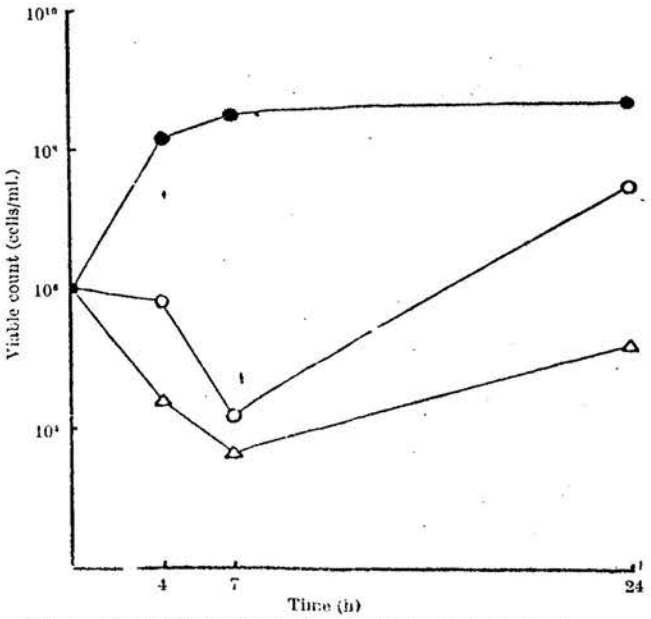


Fig. 1. Bactericidal activity of carbenicillin against *Pseudomonas pyocyanea*. ●, Control; ○, 50 μg/ml.; △, 250 μg/ml.

followed by growth is not known, but it does not seem to be due to the selection of resistant mutants present in the original inoculum.

Activity against E. coli and Proteus species. Table 4 shows the activity of carbenicillin and ampicillin against strains of *E. coli* and *Proteus* species. Against *E. coli*, carbenicillin shows activity equal to that of ampicillin when tests are carried out using dilutions of the drug in agar. An inoculum effect is also seen with *E. coli*, however, similar to that described with *Pseudomonas*. This effect is more marked with some strains of *E. coli* than others and it is also more pronounced when the tests are carried out in liquid medium. Consequently the results of sensitivity tests with *E. coli* carried out in broth frequently show carbenicillin to be markedly less active than ampicillin when the inoculum is large, but comparable with ampicillin in activity when a smaller inoculum is used.

Against *P. mirabilis*, carbenicillin shows the same pattern of activity as does ampicillin, that is, penicillinase-producing strains are resistant while non-penicillinase-producing strains are highly sensitive. Against strains of *P. morganii*, *rettgeri*, and *vulgaris* carbenicillin is interesting in that many such strains which are resistant to ampicillin are relatively sensitive to carbenicillin. This would appear to be due to the greater stability of carbenicillin to the penicillinase produced by these species of *Proteus*.

Table 4. ACTIVITY OF CARBENICILLIN AND AMPICILLIN AGAINST *E. coli* AND *Proteus* SPECIES

Organisms	No. of strains	Compound	Minimum inhibitory concentration (μg/ml.)* and number of strains								
			> 250	250	125	50	25	12·5	5·0	2·5	1·25
E. coli	51	carbenicillin	2					6	18	21	4
		ampicillin	1	1		3		3	35	7	1
P. mirabilis †	6	carbenicillin	5			1					
		ampicillin	6	1							
P. mirabilis ‡	14	carbenicillin								3	11
		ampicillin								6	8
P. morganii	5	carbenicillin								4	1
		ampicillin			2	3					
P. rettgeri	5	carbenicillin						3		2	
		ampicillin	3		1	1					
P. vulgaris	6	carbenicillin			1	1		2	2		
		ampicillin	5			1					

* Determined by serial dilution of the drug in agar; inoculum one drop of undiluted overnight broth culture.

† Penicillinase-producing strains.

‡ Non-penicillinase-producing strains.

Effect of serum on activity. Carbenicillin is not highly bound to serum proteins. Sensitivity tests carried out in broth containing 95 per cent human serum show that the activity of carbenicillin is not significantly diminished by the presence of serum, and determinations by an ultrafiltration technique show that 53 per cent of the drug is free in human serum.

Disk sensitivity testing with carbenicillin. Using 6 mm paper disks containing 100 μg carbenicillin, all strains of *Ps. pyocyanea* tested in this laboratory have given zones of inhibitions of 12–20 mm with a heavy inoculum and zones of 18–30 mm with a dilute inoculum. With a heavy inoculum a number of colonies can usually be seen within the zone of inhibition, although when these colonies are picked off and re-tested they show the same sensitivity as the original culture and again give rise to a number of colonies within the clear zone of inhibition.

In the case of Gram-negative bacilli other than *Pseudomonas*, for example, *E. coli* and *Proteus* species, a 25 μg disk would be suitable for sensitivity testing. Using a heavy inoculum, inhibition zones of 20 mm or more are usually obtained with strains which are inhibited by 5 μg/ml. carbenicillin.

Animal Studies

General pharmacology. Carbenicillin in doses up to 500 mg/kg intravenously had no marked effects on the cardiovascular and respiratory systems and the autonomic nervous systems of cats. At 500 mg/kg there was a slight transient hypotensive effect which disappeared within 10–20 min.

Absorption, distribution and metabolism. Carbenicillin is poorly absorbed orally in the rat, but after intramuscular injection the penicillin becomes distributed throughout the body in a similar manner to other penicillins. Relatively high concentrations of the penicillin appear in the alimentary tract after intramuscular administration. Chromatographic examination of intestinal contents and tissue confirms the presence of the drug in the gut. This is probably due to the excretion of bile containing carbenicillin into the intestine.

In order to determine the biliary excretion, the bile ducts of rats were cannulated with 'Polythene' tubing (0·4 mm diameter) and the animals placed in metabolism cages, after being injected with 100 mg/kg carbenicillin intramuscularly. Bile specimens were obtained at intervals of 1 h over a period of up to 6 h after dosing. A total of 15·1 per cent of the penicillin was recovered from the bile during this period and 28·8 per cent of the antibiotic was recovered from the urine over a period of 4 h.

Following intramuscular injection of carbenicillin into rabbits at a dose of 500 mg/kg, samples of cerebrospinal fluid were withdrawn from the cisterna magna at 0·5, 1, 2

and 4 h and assayed. No antibiotic was found in the cerebrospinal fluid, indicating a failure of carbenicillin to penetrate the blood-brain barrier.

When given subconjunctivally, 0·5 ml. of both 10 per cent and 25 per cent solutions of carbenicillin in xylocaine/adrenaline solution B.P. gave concentrations up to 200 μg/ml. in the aqueous humour. Detectable concentrations were present for a longer period after the injection of the 25 per cent solution.

Prolonged toxicity studies in dogs. Thirty pedigree beagle dogs were used in the study. They were housed singly and fed with weighed quantities of dry diet twice daily, the food intake being calculated. Unlimited supplies of water were provided and, in addition, one-third of a pint of milk daily was available.

The animals were divided into five groups of three males and three females and were dosed as follows: group 1, commencing at 2 g/kg/day subcutaneously; group 2, 500 mg/kg/day subcutaneously; group 3, 250 mg/kg/day subcutaneously; group 4, 250 mg/kg/day intravenously; group 5, controls dosed with saline subcutaneously.

Carbenicillin was dissolved in physiological saline so that 1 g of carbenicillin was contained in 1·5 ml. of saline; the solution was made up freshly each day. The dogs were weighed once a week and the dose to be given during the ensuing week was calculated. The intravenous injections were given into the veins of the forelimb. The duration of dosing was 3 months for the subcutaneous groups and 1 month for the intravenous group.

Clinical symptoms were recorded daily, body weight once a week, food intake twice daily and ophthalmoscopic examination was carried out just before the dogs were killed. Haematological, biochemical, urinalysis and serum glucose concentration tests were performed on all dogs, once before dosing began and at 4, 8 and 13 weeks after dosing had begun.

All animals which died during the experimental period were examined after death and all the remaining animals were killed at the end of the dosing period. The internal organs were examined macroscopically and the brain, pituitary, heart, lungs, liver, spleen, pancreas, thymus, prostate/uterus, kidneys, thyroids, adrenals and gonads were removed and weighed. Portions of these tissues, together with sections of aorta, trachea, cervical and mesenteric lymph nodes, salivary glands, various levels of the alimentary tract, gall bladder, skin from injection sites, sciatic nerve, optic nerve, eye, skeletal muscle and bone marrow, were fixed and stained with haematoxylin and eosin. Additional sections of the liver and kidney were specially stained for fat.

There were no deaths from drug toxicity and only one accidental death. There were no observable changes in general physiology, apart from slight local swelling of

tissues around the sites of intravenous injections. Severe local swelling, pain, "cyst formation", but little ulceration, occurred when the subcutaneous route was used for the highest dose level. At 500 mg/kg/day, moderate disturbances resulted, whereas at 250 mg/kg/day there was only minimal evidence of local irritation.

There was some suppression of weight gain associated with a suppression of appetite at the highest dose level by subcutaneous injection, the animals receiving 6–9 g/kg/day. Local irritation rather than the systemic absorption of carbenicillin may have been the chief cause of this loss of weight gain, because while at 250 mg/kg/day by intravenous injection there was no weight suppression, less weight was gained by the same dose level given by the subcutaneous route.

A slight reduction in the serum potassium concentration occurred after 4 weeks at the highest subcutaneous dose level but was not seen later. In two dogs dosed for 4 weeks by intravenous injection at 250 mg/kg. there was some reduction in the concentrating power of the kidney. No effect on the kidney was found in a repeat test at 500 mg/kg intravenously.

Only local tissue damage was found on macroscopic examination at post-mortems and only local irritant reactions at the injection sites were found on histological examination. There was no evidence of organ damage.

Prolonged toxicity studies in rats. Doses of 250, 500 and 1,000 mg/kg carbenicillin were given subcutaneously to groups of thirty Sprague–Dawley caesarian-derived rats from the Charles River Breeding Laboratories, Wilmington, Massachusetts, fifteen males and fifteen females to each group, over a period of 13 weeks. The rats had access to weighed quantities of autoclaved Spillers 'Laboratory Animal' diet and to tap water. The solutions of the drug were prepared freshly each day and administered in a volume of 0·4 ml./100 g. Two injection sites were chosen for each rat, each site being used on alternate days. Throughout the course of the test a record of food consumption and body weight change was kept daily and the mean weekly intake per rat calculated. Urinalysis and haematology were carried out at the beginning of dosing and at 4, 8 and 12 weeks. At the end of the test all the animals were killed by anaesthetization with ether followed by exsanguination.

After the post-mortem examination, all the essential organs were prepared for histological examination. No histological or clinical effects attributable to the drug were detected throughout the course of the dosing apart from a trend in the top group towards a slight fall in the red blood count.

Local irritancy studies. The local action of carbenicillin was determined in the rabbit eye after subconjunctival injection and after topical application. In the first

series of experiments two animals each were allocated to groups which received 0·2 ml. of a 25 per cent, 10 per cent or 1 per cent solution of carbenicillin, respectively. The 25 per cent solution of carbenicillin was prepared in xylocaine adrenaline solution B.P., in order to reduce the painful response to the hypertonic solution. All the animals were observed twice daily for one week after the injections and any inflammatory responses were recorded. At the end of the period the rabbits were killed and the eyes and surrounding tissues removed for histological examination. The 10 per cent and 1 per cent solutions produced very little reaction with only a very mild inflammatory response, and the histological examination showed no damage. The 25 per cent solution, however, caused inflammation with leucocytic infiltration within 3 h of the injection. Within 48 h, however, the reaction had subsided and by the end of the test period there was no evidence of inflammation.

0·2 ml. of a 25 per cent solution of carbenicillin was applied for 2 min to the eyes of rabbits after which the antibiotic was washed out with warm saline. The eyes of the control animals were treated with saline in the same period of time. The animals were treated twice a day for 3 days. They were then killed on the fourth day and the tissues removed for histological examination. After the first application of the 25 per cent solution there was a slight inflammation of the conjunctivæ in one animal which lasted 24 h, but on post-mortem examination no damage was observed; otherwise local application caused no ill effects.

Carbenicillin was also given by intramuscular injection. Twelve rabbits were closely shaven across the dorsum and two injection sites chosen in each of two sacro-spinalis muscles of the back. One site was injected with 1 ml. of a 50 per cent concentration of carbenicillin in saline and the other with 1 ml. of saline. 4 days later the animals were killed and the treated areas prepared for histological examination. At all the sites which received the drug there was fibrolysis and necrosis accompanied in five animals by oedema.

Effects on pregnant rats. Sprague-Dawley caesarian-derived rats from the Charles River Breeding Laboratories Wilmington, were used. The animals were allowed to mate, the day of mating being judged by the appearance of the vaginal plug. Dosing began on day 6 of pregnancy up to and including day 15. Three groups of twenty females were taken, group 1, the control group, being dosed with saline, while groups 2 and 3 received respectively 100 and 500 mg/kg carbenicillin in physiological saline, subcutaneously. The volumes of all injections were standardized to 0·2 ml./100 g body weight. The animals were given free access to Spillers 'Laboratory Animal' diet and to tap water and were closely observed

throughout pregnancy. On day 21 the animals were killed and the uterine contents examined for the number of viable young, resorption sites, litter weight and foetal abnormalities. The young were examined externally after removal from the uterus, one-third being retained for detection of visceral abnormalities and the remaining two-thirds for subsequent dissection of animals followed by clearing and staining of the skeleton by alizarin to detect skeletal abnormalities.

No obvious signs of maternal reactions were observed during the test, but treatment with carbenicillin was associated with a slight retardation in body weight gain. Conception rate, average litter weight and size were not, however, significantly affected by the treatment and there was no incidence of abnormalities due to the drug.

Effect on pregnant mice. Groups of twenty-two mice were mated and treated in a similar way to the rats in the rat pregnancy study except that the animals were killed on the seventeenth day of pregnancy when the uterine contents were similarly examined.

Carbenicillin had no effect on the body weight changes in the mouse, and conception rate, litter size and foetal loss were unaffected at either dosage. There was also no incidence of abnormalities and skeletal deformities due to the drug treatment.

Peri- and post-natal development of the rat. Tests were carried out to determine the effect of carbenicillin on male and female rats before mating and through gestation and lactation to the twenty-first day after birth. Daily doses of 100 and 500 mg/kg of carbenicillin were administered subcutaneously throughout this period.

Parent animals were unaffected, as assessed by daily observation, body weight change, conception rate, length of the gestation period, and parturition. Litter size, litter and mean pup weights and pup loss of rats receiving 500 mg/kg were unaffected at birth or after 4 or 21 days. The increased pup mortality recorded at the lower dosage of 100 mg/kg was therefore considered to be unrelated to the treatment. No abnormal pups were found.

We conclude that carbenicillin at dosages of 500 mg/kg and below does not adversely affect the peri- and post-natal development of the rat.

Absorption and Excretion in Man

The following absorption and excretion studies were undertaken in order to determine a suitable dosage and route of administration for a proposed clinical trial.

Assay methods. The concentration of carbenicillin in serum and in urine was determined by the cup-plate biological assay method. Details of the technique were as described previously[1] except that *Pseudomonas pyocyanea* NCTC 10490 (Ellsworth strain, 1973) was used as the test

organism. *Sarcina lutea* and *Staphylococcus aureus* are unsuitable as assay organisms because carbenicillin contains a small amount of benzylpenicillin and this may appear in the serum and urine together with the carbenicillin. Because *Sarcina lutea* and *Staph. aureus* are many times more sensitive to benzylpenicillin than to carbenicillin the presence of traces of benzylpenicillin may invalidate the assay. *Ps. pyocyanea* NCTC 10490, on the other hand, is sensitive to carbenicillin but is relatively resistant to benzylpenicillin. The culture is unusually sensitive to carbenicillin, showing an MIC of about 1·25 μg/ml., and it is possible to assay concentrations of carbenicillin as low as 2·5 μg/ml. using this strain, while benzylpenicillin at concentrations of up to 500 μg/ml. fails to show any zone of inhibition. Standard solutions of carbenicillin from 1–100 μg/ml. were prepared in human serum, and where necessary the serum samples were diluted with human serum to give a concentration within this range. For the assay of urine samples the standard solutions of carbenicillin were prepared in 0·05 molar phosphate buffer *p*H 7·0 and urine samples were also diluted as required using the same buffer.

Oral administration. After a dose of 500 mg carbenicillin in gelatine capsules to healthy adults in the fasting state, no carbenicillin (that is, <1·0 μg/ml.) could be detected in serum samples taken at 0·5, 1, 2, 4 and 6 h after administration.

Carbenicillin was detected in the urine, which was collected over the 6 h period after the administration of the drug, but the quantity present in the urine amounted to less than 1 per cent of the dose administered. It would appear, therefore, that carbenicillin is not absorbed to any extent when given by mouth.

Intramuscular administration. Carbenicillin was administered by intramuscular injection to healthy adults in doses of 250 mg, 500 mg and 1 g dissolved in water for injection. The 250 mg dose was dissolved in a volume of 1 ml. and the 500 mg and 1 g doses were administered in a volume of 2 ml. The serum concentrations obtained are shown in Table 5.

It will be seen that intramuscular injection resulted in a high concentration of carbenicillin in serum with a peak at about 1 h after injection. Thereafter the serum levels fell relatively quickly and about 80 per cent of the dose appeared in the urine over the first 6 h.

Table 6 shows results for the effect of probenecid on the serum levels and the urinary excretion of carbenicillin.

Table 5. MEAN SERUM CONCENTRATIONS OF CARBENICILLIN AFTER INTRAMUSCULAR INJECTION IN ADULT VOLUNTEERS

No. of subjects	Dose (mg)	Serum concentration (μg/ml.) 0·5 h	1 h	2 h	3 h	4 h	6 h
10	250	5·9	7·9	5·7	2·6		
9	500	13·7	17·7	13·2	–	2·0	
16	1000	21·6	25·3	22·1	–	10·9	3·7

Table 6. EFFECT OF PROBENECID ON SERUM LEVELS AND URINARY EXCRETION OF CARBENICILLIN IN HEALTHY ADULT VOLUNTEERS

	No. of subjects	Mean serum concentration (μg/ml.) 0·5 h	1 h	2 h	4 h	6 h	Percentage of dose excreted in urine over 0–6 h
With probenecid*	10	25·7	39·9	48·0	27·4	19·4	48
Without probenecid	10	26·8	29·4	22·2	10·8	8·3	84

Carbenicillin dose 1·0 g by intramuscular injection.

* 1 g 1 h and 10 h before administration of carbenicillin.

Probenecid was administered to healthy adults in a dose of 1 g 10 h, and again 1 h, before the intramuscular administration of 1 g carbenicillin. It will be seen that probenecid produced a substantial increase in the peak levels of carbenicillin obtained in serum and the rate of elimination of the drug from the serum was markedly diminished.

Chromatography of human urine, obtained after intramuscular administration of carbenicillin, indicated that the drug does not undergo any substantial metabolism in the body.

Intravenous administration. Results are shown in Table 7 for the serum levels obtained after intravenous injection of 1 g carbenicillin. This amount was dissolved in 10 ml. water and administered over a period of 3 min. Although the drug was eliminated rapidly from the blood it will be seen that serum levels in excess of 100 μg/ml. were obtained during the first 30 min after administration of this dose.

Table 7. SERUM CONCENTRATION OF CARBENICILLIN AFTER INTRAVENOUS ADMINISTRATION OF 1 g

Subject	Serum concentration (μg/ml.) 0·25 h	0·5 h	1 h	2 h	4 h	6 h
A	140	127	60	28	3·7	< 3·0
B	140	114	55	32	4·4	< 3·0

Carbenicillin levels in serum and in urine in relation to antibacterial activity. As a result of the extensive elimination of the drug by way of the kidneys, relatively high concentrations of carbenicillin are obtained in the urine. In the studies involving a dose of 500 mg by intramuscular injection, the concentration in the urine collected over the first 6 h was generally 1,000–2,000 μg/ml. This is considerably in excess of the concentration of drug required to inhibit the growth of *Ps. pyocyanea*. On the other hand, it is questionable whether intramuscular injection of 1 g carbenicillin at intervals of 6 h, even in conjunction with probenecid, would result in tissue concentrations which would be inhibitory towards typical strains of *Ps. pyocyanea*. The minimum inhibitory concentration of carbenicillin for most strains of *Ps. pyocyanea* appears to be 50–100 μg/ml., and although the drug is not highly bound to serum protein (about 50 per cent is bound) it would probably be desirable to achieve serum levels some-

what in excess of 100 µg/ml. This can readily be attained by intravenous administration.

In addition to the activity which carbenicillin shows against *Ps. pyocyanea*, the drug is active against other pathogens, including *E. coli*, and also strains of *Proteus morganii*, *P. rettgeri* and *P. vulgaris* which are generally resistant to ampicillin. On the basis of the *in vitro* sensitivity of these latter organisms to carbenicillin, the serum concentrations achieved by intramuscular administration of the drug might be adequate and the levels attained in urine are again greatly in excess of the minimum inhibitory concentrations.

The results obtained in these studies suggested that for clinical trials on the therapeutic effectiveness of carbenicillin in urinary tract infections, a suitable dosage might be 1·0 g by intramuscular injection at intervals of 6 h. The treatment of pseudomonas septicaemia, wound infections and tissue infection in general would require the intravenous infusion of carbenicillin (with or without probenecid) in order to attain inhibitory serum and tissue concentrations.

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Carbenicillin, a New Semisynthetic Penicillin Active Against *Pseudomonas aeruginosa*

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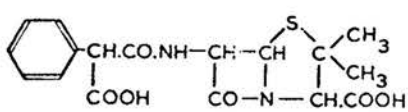
Carbenicillin, a new semisynthetic penicillin, was found to have a broad spectrum of activity against gram-positive and gram-negative bacteria, including *Pseudomonas aeruginosa*. Carbenicillin was also active against those *Proteus* species which are generally resistant to ampicillin and cephalosporins. The activity of the compound against penicillin-resistant staphylococci was limited, and strains of *Klebsiella* were generally resistant. Like other penicillins, carbenicillin is bactericidal and is not highly bound to serum proteins. Against *P. aeruginosa*, carbenicillin and gentamicin showed a synergistic effect.

The availability of 6-aminopenicillanic acid has led to the preparation of a great number of semisynthetic penicillins. Notable among the semisynthetic penicillins which have proved to be important in clinical practice are ampicillin, which shows a broad spectrum of activity, and the penicillins which are stable to staphylococcal penicillinase, e.g., methicillin and cloxacillin. The new semisynthetic penicillin carbenicillin shows a wider range of activity than ampicillin and possesses activity against *Pseudomonas aeruginosa*, as well as against strains of those species of *Proteus* typically resistant to ampicillin.

Materials and Methods

Carbenicillin (BRL 2064, produced by Beecham Research Laboratories as Pyopen) is α-carboxybenzylpenicillin (Fig. 1). The material was used as the disodium salt.

Carbenicillin is readily soluble in water, giving a clear solution with a *p*H between 6.0 and 8.0. At room temperature, solutions of carbenicillin showed no significant loss of activity after 24 hr, and such solutions could be held under refrigeration for at least 1 week without appreciable inactivation. Under acid conditions, carbenicillin was relatively unstable; the half-life at *p*H 2 was approximately 30 min at 37 C.



α-carboxybenzylpenicillin

Fig. 1. *Carbenicillin (BRL 2064).*

Determination of the antibacterial activity of carbenicillin was complicated by the fact that, as currently available, it contains a small amount of benzylpenicillin. The material used in this study contained approximately 1% benzylpenicillin, which could have contributed to the activity of the preparation.

Antibacterial activity was determined by serial dilution in nutrient agar in petri dishes which were inoculated with a replicating device delivering an inoculum of 0.003 ml. Serial dilution tests were also carried out by use of 5-ml amounts of nutrient broth which were inoculated with one drop (0.05 ml) of culture. The inoculum used in these tests was usually an undiluted overnight broth culture of the test organism, but in certain tests with *P. aeruginosa* a 1:100 dilution of this inoculum was used. Inhibition of growth was recorded after incubation overnight at 37 C.

Results and Discussion

The observed activity of carbenicillin against gram-positive bacteria is shown in Table 1. The compound was active against penicillin-susceptible staphylococci, streptococci, and pneumococci, although the level of activity shown

Table 1. *Activity of carbenicillin against gram-positive bacteria*

Organism	No. of strains	Minimal inhibitory concentration (μg/ml)[a]	
		Carbenicillin	Ampicillin
Staphylococcus aureus (penicillin-sensitive)	7	1.25-2.5	0.05-0.1
S. aureus (penicillinase-producing)	12	12.5-125	5.0->500
β-Hemolytic *Streptococcus*	6	0.5-1.25	0.02
S. pneumoniae	5	1.25-2.5	0.05
Streptococcus pneumoniae	4	50	1.25
Clostridium tetani	1	0.25	0.025
C. welchii	1	0.25	0.025
Bacillus subtilis	1	1.25	0.25
Sarcina lutea	1	0.5	0.005

[a]Serial dilution in 5% blood-agar; inoculum, one drop of an overnight broth culture.

was considerably lower than that of ampicillin. Carbenicillin was also active against *Clostridium* species, but strains of *Streptococcus faecalis* were relatively nonsusceptible.

Carbenicillin showed some degree of stability to staphylococcal penicillinase, and thus was more active against penicillin-resistant staphylococci than benzylpenicillin or ampicillin; however, this activity was considerably lower than that of cloxacillin. In Table 2, the activity of carbenicillin against 34 strains of penicillin-resistant staphylococci is compared with that of benzylpenicillin, ampicillin, and cloxacillin. The majority of the strains were inhibited by carbenicillin at a concentration of 25 μg/ml, and a substantial number were inhibited at 12.5 μg/ml. Results of these tests, in which a large inoculum was used (approximately 10^6 cells), reflect the relative stability of carbenicillin to staphylococcal penicillinase.

As shown in Table 3, the activity of carbenicillin against gram-negative

Table 2. *Activity of carbenicillin against 34 penicillinase-producing strains of Staphylococcus aureus*

Penicillin	Minimal inhibitory concn (μg/ml)[a]									
	>500	500	250	125	50	25	12.5	1.25	0.5	0.25
Carbenicillin				3[b]	3	17	11			
Benzylpenicillin		17	7		2	8				
Ampicillin	4	3	11	6		9	1			
Cloxacillin								4	21	9

[a]Serial dilution in nutrient agar; inoculum, one drop of an overnight broth culture.
[b]Number of strains for which indicated concentration was inhibitory.

Table 3. *Activity of carbenicillin against gram-negative bacteria*

Organism	No. of strains	Carbenicillin concn (μg/ml)[a]											
		>250	250	125	50	25	12.5	5.0	2.5	1.25	0.5	0.25	0.1
Escherichia coli	181	19[b]		5	4	4	25	53	59	12			
Klebsiella aerogenes	49	23	7	6	7			3	2	1			
Paracolons	10	2		1			1	3	3				
Proteus mirabilis	48	8			3	1	3	15	18				
Salmonella species	6					1	4	1					
Shigella species	2							1	1				
Haemophilus influenzae[c]	12										4	4	4

[a]Serial dilution in nutrient agar; inoculum, on drop of an overnight broth culture.
[b]Number of strains for which indicated concentration was inhibitory
[c]Tested on chocolate-blood-agar.

bacteria was similar to that of ampicillin. Carbenicillin was only slightly less active against *Haemophilus influenzae* than ampicillin. Most strains of *Escherichia coli, Proteus mirabilis, Salmonella,* and *Shigella* species, and paracolon organisms were also relatively susceptible. On the other hand, strains of *Klebsiella aerogenes* were generally resistant. Of a total of 181 strains of *E. coli* tested, 124 strains (69%) were inhibited by carbenicillin at a concentration of 5 μg/ml. This level of activity is similar to that of ampicillin against this particular pathogen. In general, *E. coli* strains and paracolon organisms resistant to ampicillin were also resistant to carbenicillin; however, some strains were encountered which were resistant to ampicillin but relatively susceptible to carbenicillin. Against *P. mirabilis,* carbenicillin was only slightly less active than ampicillin; strains which were resistant to ampicillin were also resistant to carbenicillin.

The activity of carbenicillin differed markedly from that of ampicillin with regard to strains of *P. morganii, P. rettgeri,* and *P. vulgaris.* These *Proteus* species are typically resistant to ampicillin and also to cephaloridine, but many strains of these species were susceptible to carbenicillin (Table 4). This would appear to be due to greater stability of carbenicillin to the β-lactamases produced by these particular organisms.

The activity of carbenicillin against *P. aeruginosa* is shown in Table 5. When the minimal inhibitory concentrations were determined by use of a very heavy inoculum (10^6 to 10^7 cells), very sparse growth sometimes persisted beyond the point where the major inhibition of growth occurred; when this was the case, complete inhibition of growth was obtained only with high concentrations of carbenicillin; often, as much as 250 μg/ml was required. This effect was particularly evident when the tests were carried out in liquid medium. However, when a

Table 4. *Activity of carbenicillin against ampicillin-resistant Proteus species*

Organism	Minimal inhibitory concn (μg/ml)[a]		
	Carbenicillin	Ampicillin	Cephaloridine
P. morganii			
A	1.25	50	500
F	1.25	50	500
G	12.5	125	>500
H	1.25	50	500
I	5.0	250	250
P. rettgeri			
A	1.25	50	>500
B	12.5	250	>500
C	2.5	50	>500
D	2.5	500	>500
F	1.25	50	250
I	2.5	250	>500
P. vulgaris			
A	5.0	125	250
D	12.5	500	>500
E	5.0	250	500
G	50	>500	>500
H	250	>500	>500
I	2.5	250	500
P. mirabilis			
8	>500	>500	50
889	>500	>500	12.5

[a]Serial dilution in nutrient agar; inoculum, one drop of an undiluted nutrient culture.

Table 5. *Activity of carbenicillin against 206 strains of Pseudomonas aeruginosa*

Minimal inhibitory concn (μg/ml)[a]	No. of strains
250	13
125	23
50	89
25	64
12.5	7
5.0	2
2.5	4
1.25	4

[a]Serial dilution in nutrient agar; inoculum, one drop of a 1:100 dilution of an overnight culture.

slightly smaller inoculum was used, e.g., one drop of overnight broth culture diluted 1:100, containing approximately 10^5 cells, a relatively sharp end point was obtained. Under these conditions, the majority of clinical isolates tested were inhibited by carbenicillin at a concentration of 50 μg/ml, and about 40% were inhibited by a concentration of 25 μg/ml. Approximately 18% of the strains encountered required a concentration of carbenicillin as high as 125 to 250 μg/ml for inhibition. However, concentrations of carbenicillin which failed to inhibit growth completely nevertheless at times exerted a marked influence on growth. This was apparent from the absence of pigment formation and from the grossly altered morphology.

Against strains of *P. aeruginosa*, carbenicillin frequently showed a synergistic effect in combination with gentamicin (Table 6). In experiments carried out in nutrient broth with a very heavy inoculum, an ill-defined end point occurred, as discussed above, and relatively high concentrations of carbenicillin were required for complete suppression of growth. Thus, the minimal inhibitory concentrations for carbenicillin in these experiments were generally higher than were those in experiments (Table 5) in which a slightly smaller inoculum was used. Nevertheless, it is clear (Table 6) that carbenicillin and gentamicin exerted a marked synergistic effect. With gentamicin alone, concentrations of 0.5 to 5.0 μg/ml were required for inhibition of growth, but in combination with 25 μg of carbenicillin per ml, gentamicin was inhibitory for all strains in a concentration of 0.25 μg or less per ml.

Table 6. *Activity of combinations of carbenicillin and gentamicin against Pseudomonas aeruginosa*

Strain	Minimal inhibitory concn (μg/ml)[a]			
	Carbenicillin	Gentamicin		
		Alone	With 25 μg of carbenicillin/ml	With 50 μg of carbenicillin/ml
R28	50	0.5	0.12	—
R62	50	0.5	0.25	—
R5	125	0.5	0.25	0.05
R28	250	0.5	0.12	0.12
A	250	0.5	0.12	0.12
R1	250	5.0	0.25	0.05
R52	500	1.25	0.25	0.25
R160	>500	1.25	0.25	0.25

[a]Determined in nutrient broth inoculated with one drop of an overnight broth culture.

This synergistic effect of carbenicillin and gentamicin is also illustrated in Fig. 2. Various concentrations of carbenicillin and gentamicin, alone and in

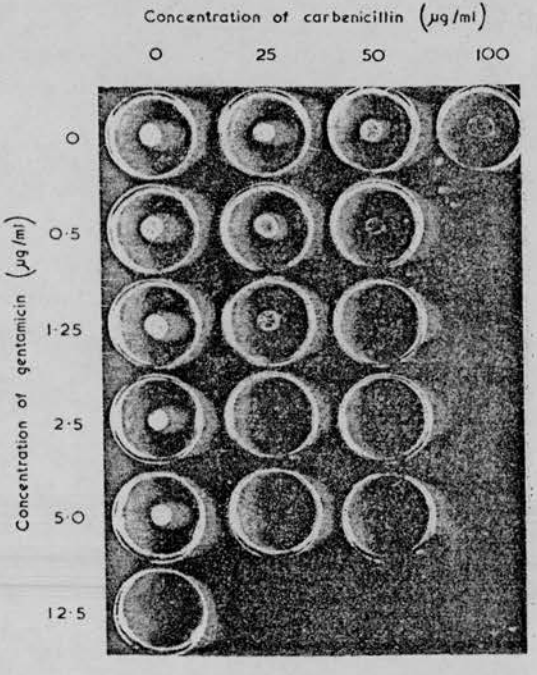


Fig. 2. *Activity of carbenicillin and gentamicin in combination against Pseudomonas aeruginosa.*

combination, were prepared in agar, and each plate was inoculated with an undiluted overnight broth culture of a typical strain of *P. aeruginosa*. With gentamicin alone, heavy growth occurred with concentrations as high as 5.0 μg/ml and complete inhibition of growth was obtained only with concentrations of 12.5 μg/ml. Similarly, with carbenicillin alone, inhibition of growth was not complete even at a concentration of 100 μg/ml. However, a concentration of 50 μg of carbenicillin per ml in combination with 1.25 μg of gentamicin per ml was completely inhibitory, and a concentration of 25 μg of carbenicillin per ml in combination with 2.5 μg of gentamicin per ml was almost completely inhibitory. Similar results were obtained with other strains of *P. aeruginosa*.

Carbenicillin is not highly bound to serum proteins. Determinations of the extent of binding by means of ultrafiltration indicated that in human serum approximately 53% of the drug was unbound, compared with 41% unbound drug in the case of benzylpenicillin. Susceptibility tests carried out in human serum showed that the activity of carbenicillin against *Pseudomonas* was not significantly diminished in the presence of serum.

The activity of carbenicillin against *P. aeruginosa* was considerably lower than that of polymyxin or gentamicin, but the compound appeared to be virtually nontoxic (1, 5). It should therefore be possible to administer carbenicillin in dosage adequate to produce inhibitory concentrations in the body (4, 5), even though these concentrations may be relatively high. Brumfitt et al. (2) and Jones and Lowbury (3) have already shown that carbenicillin is clinically effective in the treatment of *Pseudomonas* infections.

Acknowledgments

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COMPARATIVE ANTIBACTERIAL ACTIVITIES OF CARBENICILLIN AND OTHER BROAD-SPECTRUM ANTIBIOTICS

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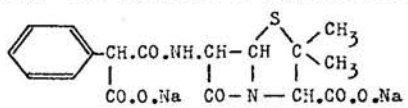
Carbenicillin is the most recent of the semi-synthetic penicillins to be introduced into clinical practice. Like ampicillin, carbenicillin is a broad-spectrum penicillin active against a wide range of Gram-positive and Gram-negative bacteria, but unlike ampicillin, carbenicillin shows significant activity against Pseudomonas aeruginosa and certain β -lactamase-producing Proteus species (Knudsen, Rolinson and Sutherland, 1967). The structure of carbenicillin is shown in Fig. 1.



Disodium α-carboxybenzylpenicillin

Carbenicillin, BRL 2064

Fig. 1. Structure of carbenicillin.

The activity of carbenicillin against various Gram-positive bacteria is compared with that of ampicillin in Table 1 where it can be seen that carbenicillin shows significant activity against pyogenic streptococci, pneumococci and penicillin-sensitive staphylococci, although the level of activity against these organisms is considerably less than that of ampicillin. Similarly, carbenicillin is less active than ampicillin against Listeria monocytogenes, Corynebacterium diphtheriae, Bacillus species and Clostridium species. The compound is also relatively inactive against Streptococcus faecalis.

Table 1 Activity of carbenicillin against Gram-positive bacteria

Organism	Minimum inhibitory concentration* (μg./ml.)	
	Carbenicillin	Ampicillin
Staph. aureus (penicillin sensitive)	1.25	0.05
Staph. aureus (penicillin resistant)	12.5 - 125	5.0 - >500
β-haemolytic streptococcus	0.5	0.02
Strep. pneumoniae	1.25	0.05
Strep. faecalis	50	1.25
Listeria monocytogenes	1.25	0.1
Corynebacterium diphtheriae	1.25	0.05
Bacillus anthracis	2.5	0.25
Bacillus subtilis	1.25	0.25
Clostridium welchii	0.25	0.025
Clostridium tetani	0.25	0.025

* Serial dilution in agar: inoculum one drop of overnight broth culture.

Carbenicillin is more stable to staphylococcal penicillinase than is ampicillin and, as a result, carbenicillin is significantly more active than ampicillin against penicillin-resistant staphylococci (Fig. 2). However, the stability of carbenicillin to staphylococcal penicillinase is only partial and it is not certain whether it is of clinical significance.

Against Gram-negative bacteria carbenicillin shows a wider spectrum of activity than ampicillin, being active against ampicillin-sensitive bacteria and also against certain ampicillin-resistant organ-

isms such as Ps. aeruginosa and some Proteus species (Table 2). The relative activities of carbenicillin and ampicillin against 84 strains of Escherichia coli are illustrated in Fig. 3. It can be seen that both penicillins show a similar level of activity, most strains being inhibited at concentrations of 5.0 μg./ml. or less. Strains of E. coli which are resistant to ampicillin are also generally resistant to carbenicillin, but a small proportion of ampicillin-resistant strains are relatively sensitive to carbenicillin (Table 3). Table 3 also shows that ampicillin-resistant strains of Shigella sonnei may

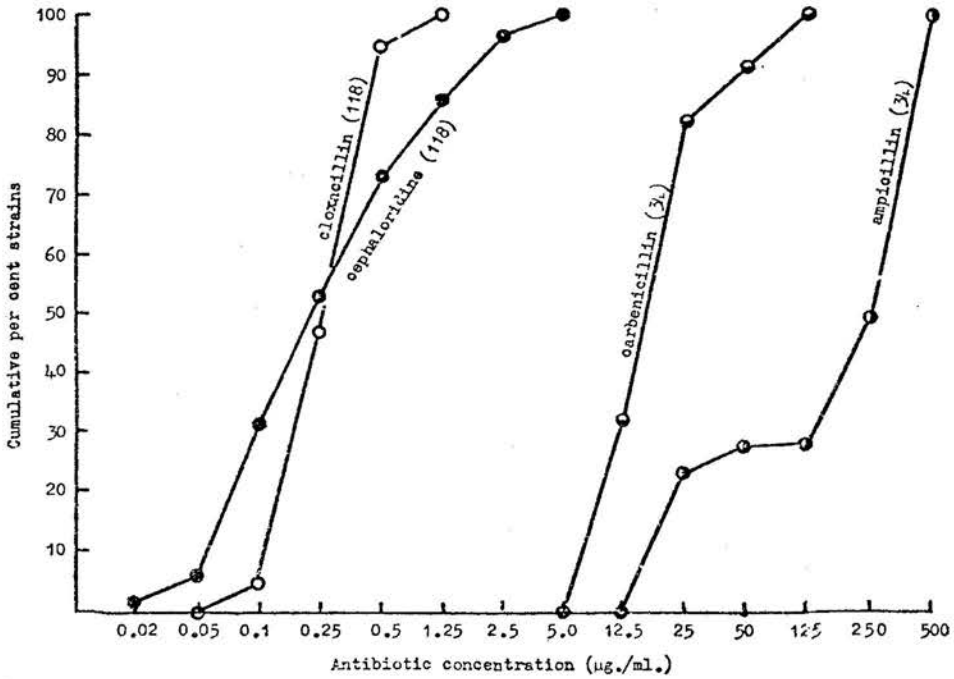


Fig. 2. Activity of carbenicillin, ampicillin, cloxacillin and cephaloridine against penicillin-resistant staphylococci (Number of strains shown in parentheses).

Table 2 Activity of carbenicillin against Gram-negative bacteria

Organism	Minimum inhibitory concentration* (µg./ml.)	
	Carbenicillin	Ampicillin
Escherichia coli	5.0	5.0
Klebsiella aerogenes	250	500
Salmonella typhi	12.5	1.25
Shigella sonnei	5.0	2.5
Proteus mirabilis	2.5	1.25
Proteus morganii	5.0	125
Proteus rettgeri	2.5	50
Proteus vulgaris	5.0	50
Pseudomonas aeruginosa	50	>500
Vibrio cholerae	2.5	2.5
Haemophilus influenzae	0.5	0.25
Neisseria gonorrhoeae	0.02	0.01
Bordetella pertussis	0.25	1.0
Pasteurella septica	1.25	0.25
Brucella abortus	2.5	0.5

* Serial dilution in agar: inoculum one drop of overnight broth culture.

be significantly more sensitive to carbenicillin than to ampicillin. This is probably due to the relative stability of carbenicillin to the β-lactamase produced by these organisms. Carbenicillin and ampicillin show a similar level of activity against Proteus mirabilis and both compounds are significantly more active than cephaloridine and tetracycline (Fig. 4). Ampicillin-resistant strains of P. mirabilis, however, are also resistant to carbenicillin. Carbenicillin shows high activity against certain β-lactamase-producing Proteus species, viz. P. morganii, P. rettgeri and P. vulgaris which bring about the destruction of ampicillin and also cephaloridine. It will be seen from Table 4 that many of these strains are inhibited by carbenicillin in the range 1-5 μg./ml. Klebsiella species are generally insensitive to carbenicillin as is the case with ampicillin and the relative activities of these penicillins together with cephaloridine and tetracycline are shown in Fig. 5.

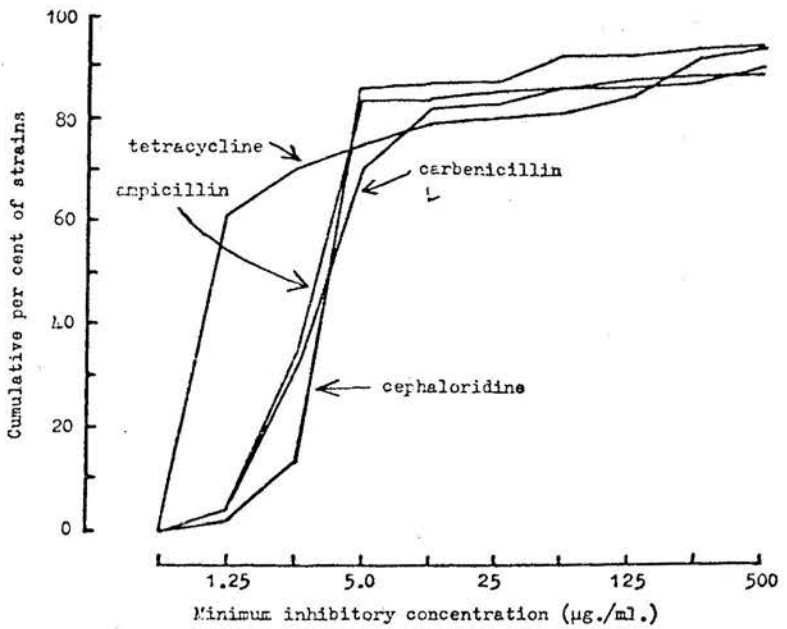


Fig. 3. Activity of carbenicillin, ampicillin, cephaloridine and tetracycline against 84 strains of Escherichia coli.

Table 3 Activity of carbenicillin against ampicillin-resistant strains of Escherichia coli and Shigella sonnei

Organism	Minimum inhibitory concentration* (μg./ml.) and number of strains								
	>500	500	250	125	50	25	12.5	5.0	2.5
Escherichia coli (18 strains)									
ampicillin	6	2	4	2	3			1	
carbenicillin	6			2	2		2	2	4
Shigella sonnei (79 strains)									
ampicillin	8	1	60	10					
cephaloridine			9	66	4				
carbenicillin	9				2	49	19		

* Serial dilution in agar: inoculum one drop of overnight broth culture.

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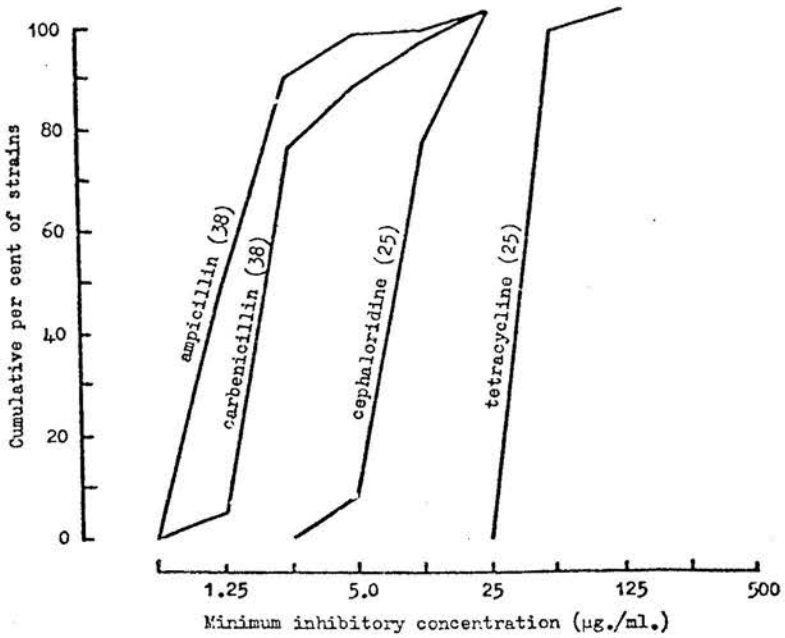

Fig. 4. Activity of carbenicillin, ampicillin, cephaloridine and tetracycline against Proteus mirabilis (Number of strains shown in parentheses).

Table 4 The relative activities of carbenicillin, ampicillin, and cephaloridine against ampicillin-resistant Proteus species

Organism		Minimum inhibitory concentration* (µg./ml.)		
		Carbenicillin	Ampicillin	Cephaloridine
Proteus morganii	A	1.25	50	500
	F	1.25	50	500
	G	1.25	50	>500
	H	1.25	50	500
	J	1.25	125	>500
	I	1.25	125	500
Proteus rettgeri	A	1.25	50	250
	I	5.0	250	>500
	B	12.5	250	>500
	C	12.5	500	>500
Proteus vulgaris	A	5.0	125	50
	I	2.5	250	250
	E	5.0	250	500
	D	5.0	500	500
	G	50	>500	500
	H	250	>500	>500
Proteus mirabilis	8	>500	>500	25
	889	>500	>500	25
	13	>500	>500	50

* Serial dilution in nutrient agar; inoculum, one drop of an overnight broth culture.

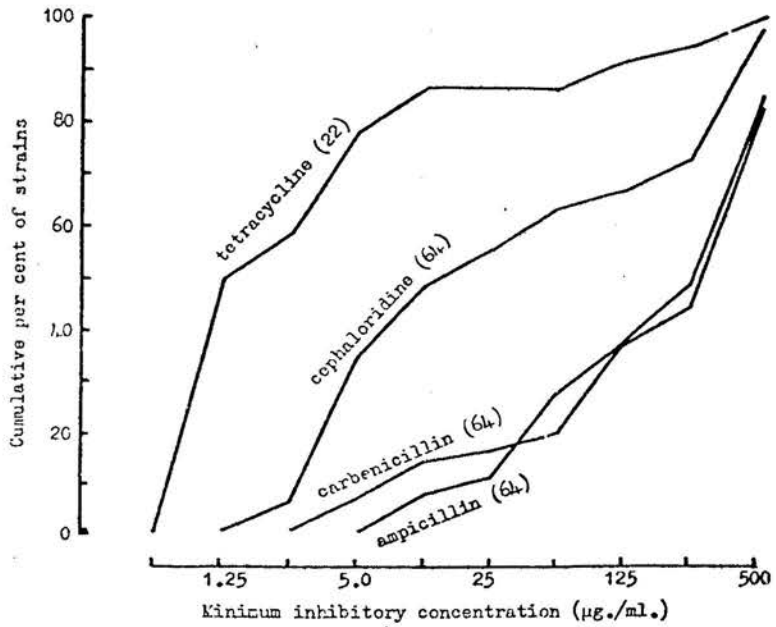

Fig. 5. Activity of carbenicillin, ampicillin, cephaloridine and tetracycline against Klebsiella species (Number of strains shown in parentheses).

The activity of carbenicillin against Ps. aeruginosa is an important feature of this broad-spectrum penicillin since only a limited number of antibiotics show useful activity against this organism. The relative activities of a number of broad-spectrum antibiotics are shown in Table 5 and it can be seen that 75% of the strains tested were inhibited by carbenicillin at a concentration of 50 μg./ml. or less in contrast to ampicillin and cephaloridine which were inactive. Tetracycline and chloramphenicol both show some activity against Pseudomonas but the clinical use of these antibiotics for the treatment of Pseudomonas infection would be severely restricted by problems of toxicity. Carbenicillin on the other hand appears to be remarkably non-toxic. Consequently, despite the relatively low level of antipseudomonas activity in vitro, carbenicillin has proved to be a useful addition to the limited number of antibiotics available for the treatment of infections due to Ps. aeruginosa (Brumfitt, Percival and Leigh, 1967; Rooyen, et al., 1967; Clifton-Bligh, Mezo and Edwards, 1968; Richardson, et al., 1968; Stratford, 1968; Cooper, Rice and Penfold, 1969; Labowitz and Holloway, 1969; Schaeper, 1969). In addition, the wide spectrum of activity of carbenicillin should prove valuable in the treatment of a number of other infections caused by Gram-negative bacteria.

Table 5 Relative activities of carbenicillin, ampicillin, cephaloridine, chloramphenicol, kanamycin, streptomycin and tetracycline against 20 strains of Pseudomonas aeruginosa

Antibiotic	M.I.C.* (μg./ml.) and number of strains								
	>500	500	250	125	50	25	12.5	5.0	2.5
Carbenicillin			1	4	11	3			1
Ampicillin	20								
Cephaloridine	20								
Chloramphenicol	3		5	11	1				
Kanamycin	8	8	2	2					
Streptomycin	12	1		2	4		1		
Tetracycline				5	15				

* Serial dilution in nutrient agar; inoculum, one drop of an overnight culture.

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Procedures for the Assay of Carbenicillin in Body Fluids

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The assay of carbenicillin in clinical specimens is complicated by the fact that carbenicillin also contains a small amount of benzylpenicillin, thereby precluding the use of conventional penicillin assay organisms. This report gives details of a microbiological assay method involving the use of a strain of *Pseudomonas aeruginosa* which is very sensitive to carbenicillin but insensitive to benzylpenicillin. The outline of a microassay method with this organism is presented, and a method for the assay of specimens containing mixtures of carbenicillin and other antibiotics is described

Carbenicillin (α-carboxybenzylpenicillin "Pyopen." Beecham Research Laboratories) is a broad-spectrum penicillin which is of interest because of its activity against gram-negative bacteria in general and against *Pseudomonas aeruginosa* in particular (1). The treatment of severe infections generally requires the administration of large doses of carbenicillin (2, 11), and it may be desirable to measure the carbenicillin content of appropriate specimens of body fluids to ensure that the required inhibitory concentrations are being achieved or maintained.

The microbiological assay of carbenicillin is complicated by the fact that the material available at present contains a small proportion of benzylpenicillin (about 1%) which is produced as a result of decarboxylation of carbenicillin during manufacture. Consequently, after the administration of carbenicillin to patients, specimens of body fluids will contain a small quantity of benzylpenicillin as well as carbenicillin; since benzylpenicillin is many times more active than carbenicillin against the assay organisms normally used in the assay of penicillins in body fluids, the presence of even small quantities of benzylpenicillin could have a significant effect on the assay of carbenicillin with these organisms. It is desirable, therefore, that carbenicillin be assayed with an organism which is sensitive to carbenicillin and resistant to benzylpenicillin. In early studies with carbenicillin (1, 7), an assay method involving a carbenicillin-sensitive strain of *P. aeruginosa* NCTC 10490 was described briefly. This report gives further details of assay methods of carbenicillin in body fluids.

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MATERIALS AND METHODS

Carbenicillin, the disodium salt of α-carboxybenzylpenicillin, is readily soluble in water (85 g/100 ml at 20 C), giving a clear neutral solution. Its structure is shown in Fig. 1. Specimens of body fluids may be kept in the refrigerator at 5 C for up to 1 week or at −20 C for several weeks without significant loss of carbenicillin activity. Carbenicillin powder should be kept in a cool place because degradation occurs more rapidly at temperatures above 15 C.

Basic assay method. Large, rectangular glass plates (30 by 30 cm) were filled to a depth of approximately 4 mm with molten nutrient agar (Oxoid no. 2) seeded with a 0.05% inoculum of an overnight broth culture of *P. aeruginosa* NCTC 10490 (ATCC 23389). When the agar was solid, holes 7 to 8 mm in diameter were punched in the seeded agar with a modified cork borer, and the plugs of agar were removed with a lancet or broad needle. The holes were filled in a random fashion in replicate with suitable dilutions of the test specimens or with standard solutions of carbenicillin. Serum specimens were diluted in pooled human serum or with 3% bovine albumin V in 0.05 M phosphate buffer (*p*H 7.0). Urine specimens were diluted in 0.05 M phosphate buffer (*p*H 7.0). Standard solutions of carbenicillin were prepared in serum or buffer as appropriate over a range of 100 to 1.0 μg of carbenicillin per ml. The plates were incubated overnight at 30 C; diameters of the zones of inhibition were measured, and the carbenicillin concentrations of the test specimens were derived from the standard line.

Microassay method. Molten nutrient agar was seeded as above with *P. aeruginosa* NCTC 10490. Assay plates were prewarmed and were filled rapidly with a minimal volume of the seeded agar to give an agar layer 1 mm or less, if possible, in depth. The test specimens, serum, lysed blood, heparinized blood, or cerebrospinal fluid were diluted, if considered necessary and 0.02 ml of specimen was pipetted onto filter-

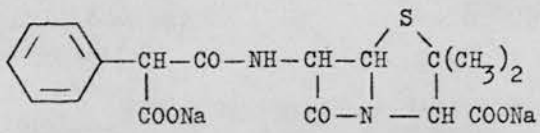

FIG. 1. *Molecular structure of carbenicillin. Molecular weight is 422.4.*

paper discs (Whatman AA discs) 6 mm in diameter with a standard dropping pipette. Standard solutions of carbenicillin (100 to 1.0 μg of carbenicillin per ml), prepared in the appropriate diluent, were pipetted onto paper discs in a similar fashion. The discs containing the test specimens and standard solutions were pressed on the surface of the seeded agar plate with sterile forceps. The plates were incubated overnight at 30 C, and the zones of inhibition were measured as described above.

Removal of benzylpenicillin from carbenicillin. Benzylpenicillin was eliminated from laboratory standard carbenicillin by treatment with a penicillin acylase enzyme preparation, which hydrolyzed benzylpenicillin to the relatively inactive 6-aminopenicillanic acid but had no effect on carbenicillin. Penicillin acylase was prepared as described by Cole and Sutherland (4). A conical flask (500 ml) containing 50 ml of a nitrogen-enriched (Yeatex) medium plus 0.15% phenylacetic acid was inoculated with 0.5 ml of an overnight nutrient broth culture of *Escherichia coli* NCIB 8473 and incubated overnight at 30 C on a rotary shaker. After incubation for 18 hr, 0.5 ml of *n*-butyl acetate was added to the culture, and shaking was continued for a further hour by which time the culture was nonviable. A 2.0-ml volume of the suspension of killed cells was added to 20 mg of carbenicillin in 18.0 ml 0.05 M phosphate buffer (*p*H 8.0). The mixture of cells and carbenicillin was incubated at 37 C for 4 hr and filtered through a Seitz filter to remove the *E. coli* cells and to eliminate penicillin acylase activity. The treated carbenicillin and standard solutions of untreated carbenicillin and benzylpenicillin were spotted (6 μliters) on filter-paper strips (Whatman no. 1, 1 cm wide) and chromatographed with a butanol-ethyl alcohol solvent system (*n*-butanol-ethanol-water, 4:1:5, v/v, top phase). Chromatography was carried out overnight at 5 C and the chromatograms were dried at 40 C. Carbenicillin and benzylpenicillin were located by placing the dried chromatograms on nutrient agar seeded with a spore suspension of *Bacillus subtilis* ATCC 6633, which was incubated overnight at 37 C. The enzyme-treated carbenicillin and standard solutions of benzylpenicillin and carbenicillin were also assayed for activity against *Sarcina lutea* NCTC 8340 and *P. aeruginosa* NCTC 10490.

Inactivation of carbenicillin with penicillinase. Inactivation of carbenicillin in specimens containing carbenicillin and other antibiotics was achieved by using a commercial preparation of *B. licheniformis* penicillinase ("Wellcome" penicillinase, Burroughs Wellcome & Co., London). The preparation, 1 ml of which was stated to inactivate 1,000,000 units of penicillin, was diluted to give a 2% solution when added to the specimen, and the mixture of penicillinase and antibiotic was incubated for 2 hr at 37 C prior to assay.

RESULTS AND DISCUSSION

Activity of carbenicillin after removal of benzylpenicillin. The removal of benzylpenicillin from carbenicillin, after treatment with *E. coli* penicillin acylase, is illustrated in Fig. 2. The bioautogram shows the presence of benzylpenicillin in untreated standard material and its absence after treatment with penicillin acylase.

The results of microbiological assays with untreated and enzyme-treated samples of carbenicillin, using *S. lutea* NCTC 8340 and *P. aeruginosa* NCTC 10490 as assay organisms, are illustrated in Fig. 3. When assayed against *S. lutea*, the zone of inhibition produced by 50 μg of standard untreated carbenicillin per ml was equivalent to that obtained with 1.0 μg of benzylpenicillin per ml. After treatment with penicillin acylase, carbenicillin was distinctly less active against sarcina, and the solution containing 50 μg of carbenicillin per ml only equalled a solution of 0.1 μg of benzylpenicillin per ml. These results suggest that, against *S. lutea* NCTC 8340, the activity shown

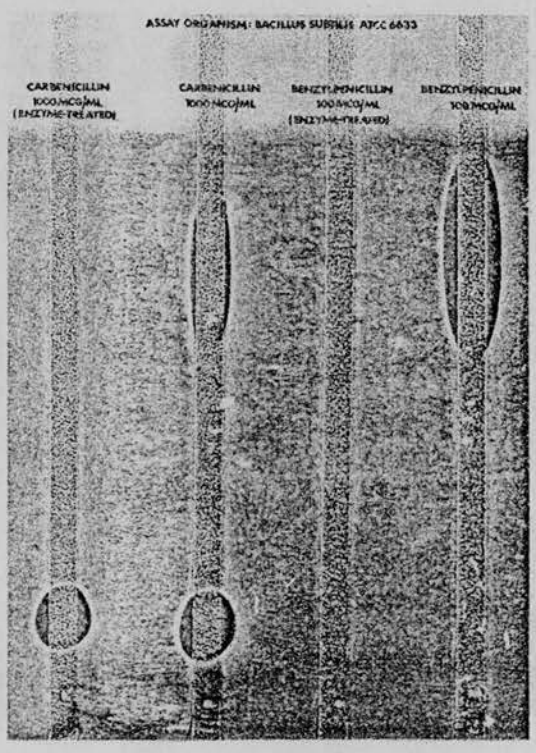

FIG. 2. *Bioautogram showing the presence of benzylpenicillin in carbenicillin and its removal after treatment with penicillinacylase.*

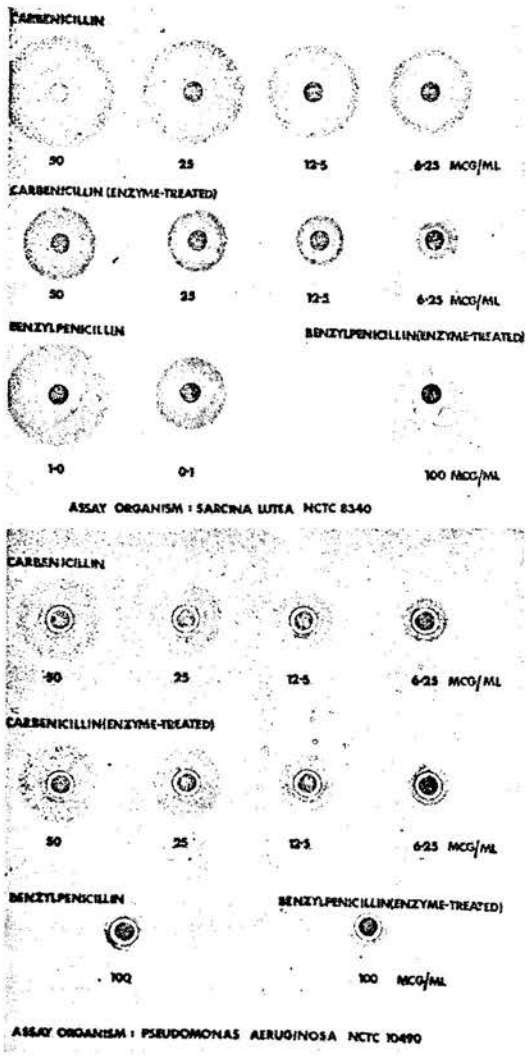

FIG. 3. *Effect of removal of benzylpenicillin from carbenicillin by treatment with penicillinacylase on the activity of carbenicillin against Sarcina lutea NCTC 8340 and P. aeruginosa NCTC 10490.*

by this particular sample of carbenicillin was largely or entirely due to that of the benzylpenicillin formed during manufacture. Treatment with penicillin acylase had no effect on the activity of carbenicillin against *P. aeruginosa* NCTC 10490.

Assay of carbenicillin with P. aeruginosa NCTC 10490. The main reason for employing *P. aeruginosa* NCTC 10490 as an assay organism for carbenicillin is that the organism is relatively sensitive to carbenicillin and relatively insensitive to benzylpenicillin (Table 1), which is invariably present in small quantities in the material available at present. The results shown here demonstrate that benzylpenicillin is much more potent against *S. lutea* NCTC 8340, a standard penicillin assay organism, than is carbenicillin; accordingly, the presence of even small amounts of benzylpenicillin in test specimens of body fluids could significantly influence the results of assays of carbenicillin with *S. lutea* (or *B. subtilis* or *Staphylococcus aureus*).

TABLE 1. *Antibiotic sensitivity of P. aeruginosa NCTC 10490*

Antibiotic	Minimal inhibitory concn[a] (μg/ml)	Minimal concn detectable in assay with *P. aeruginosa* NCTC 10490 (μg·ml)
Carbenicillin	1.0	1.0
Ampicillin	>500	>500
Benzylpenicillin	500	>500
Cephaloridine	>500	>500
Cephalothin	>500	>500
Chloramphenicol	250	>500
Colistin methane sulphonate	25	50
Gentamicin	1.25	12.5
Kanamycin	500	500
Polymyxin B sulphate	12.5	12.5
Streptomycin	50	25
Tetracycline	125	200

[a] Serial dilution in nutrient agar; inoculum, one drop of an overnight broth culture of the assay organism. Values were read after 24 hr at 37 C.

A disadvantage of using this strain of pseudomonas as an assay organism for carbenicillin is that this organism is relatively insensitive compared with the organisms conventionally employed for penicillin assays. Consequently, it is not possible to assay concentrations lower than 1 to 2 μg of carbenicillin per ml. On the other hand, in most cases of severe infections carbenicillin will be administered at a dosage which will normally produce concentrations in the body readily detectable with *P. aeruginosa* NCTC 10490.

Microassay of carbenicillin. The punch-hole assay method described above requires a volume of 1.0 to 2.0 ml of specimen, and this is not always readily available, particularly when neonates or infants are involved. The use of paper discs for microassay methods is not novel (5, 6), and the micromethod described here is based on well-tried principles. The assay of small volumes of specimens by this method is reliable, and the sensitivity of the assay is not markedly reduced provided a thin agar layer is used in the plates.

Assay of specimens containing carbenicillin and other antibiotics. The treatment of severe or un-

diagnosed infections may involve therapy with carbenicillin and one or more antibiotics. This is particularly the case with pseudomonas infections for which carbenicillin may well be used in conjunction with gentamicin or one of the polymyxins (2, 3, 8, 11). The general principle involved in the assay of such mixtures is the elimination of carbenicillin with penicillinase and the measuring of nonpenicillin antibiotic with a suitable assay organism, an established procedure for the assay of mixtures containing penicillin and nonpenicillin antibiotics (6, 9). Carbenicillin activity is measured by using an organism insensitive to the nonpenicillin antibiotic.

The use of commercial penicillinase prepared from *B. subtilis* or *B. licheniformis* is not advisable for the assay of mixtures of carbenicillin and penicillinase-stable penicillins such as methicillin or cloxacillin, as these latter penicillins are relatively unstable to the penicillinases produced by these organisms. A suitable method of assay of such mixtures involves the use of a penicillinase-producing strain of *S. aureus* sensitive to methicillin and cloxacillin but resistant to carbenicillin, as has been described for the assay of cloxacillin in specimens containing ampicillin and cloxacillin (9).

The carbenicillin content of antibiotic mixtures may normally be determined by assay with *P. aeruginosa* NCTC 10490, since most antibiotics are relatively insensitive to this organism compared with carbenicillin (Table 1). This means that antibiotic activity not due to carbenicillin may be eliminated by suitable dilution of the specimen, unless the antibiotic was present at a relatively high concentration and one which was considerably greater than that of the carbenicillin concentration in the mixture. To ensure that the activity attributed to carbenicillin after dilution of the specimen was in fact due to carbenicillin, a confirmatory test (9) could be carried out by treating the diluted specimen with penicillinase and repeating the assay on *P. aeruginosa* NCTC 10490.

As an example, specimens of serum and urine, containing carbenicillin and gentamicin, have been assayed (i) by diluting the specimen with appropriate diluent to an estimated concentration of 10 μg of gentamicin per ml or less (Table 1) and assaying the carbenicillin content with *P. aeruginosa* NCTC 10490, and (ii) by inactivating carbenicillin with a commercial preparation of penicillinase, as described, and measuring the gentamicin content with *Corynebacterium xerosis* NCTC 9455 as assay organism. With this method, it has been possible to assay specimens of serum or urine containing antibiotic concentrations as low as 1.0 μg of carbenicillin and 0.1 μg of gentamicin per ml.

ACKNOWLEDGMENT

We thank Douglas F. Lawson for taking the photographs shown in Fig. 2 and 3.

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α-Carboxy-3-Thienylmethylpenicillin (BRL 2288), a New Semisynthetic Penicillin: In Vitro Evaluation

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α-Carboxy-3-thienylmethylpenicillin (BRL 2288) is a new semisynthetic penicillin with a spectrum of antibacterial activity similar to that of carbenicillin. Studies on the antibacterial activity of BRL 2288 in vitro showed the compound to be active against a wide range of gram-positive and gram-negative bacteria, including *Pseudomonas aeruginosa*, indole-positive *Proteus* species, and *Enterobacter* species. BRL 2288 was at least twofold more active than carbenicillin against *P. aeruginosa*, and was as active as ampicillin and carbenicillin against *Escherichia coli* and *Proteus mirabilis*. However, like carbenicillin, BRL 2288 was from 10 to 20 times less active than ampicillin against streptococci, pneumococci, and penicillin-susceptible staphylococci. As with carbenicillin, synergistic activity against *P. aeruginosa* could be demonstrated with BRL 2288 in combination with gentamicin or polymyxin. Like other penicillins, BRL 2288 was found to be bactericidal, and, as a result of a low level of protein binding, the activity of the compound was not significantly reduced in the presence of serum.

The isolation of 6-aminopenicillanic acid has enabled a large number of novel semisynthetic penicillins to be prepared, some of which have found important places in the treatment of bacterial infections. For example, the introduction of methicillin and the isoxazolyl penicillins has greatly aided the management of staphylococcal infections. In recent years, however, staphylococcal infections appear to have become a less serious problem, whereas severe infections caused by gram-negative bacilli have increased. In particular, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Klebsiella* species have assumed an increasingly important role in infection, especially in debilitated patients.

The introduction of carbenicillin (3, 6) provided a new antibiotic for the treatment of infections caused by *P. aeruginosa*, indole-positive *Proteus* species, and other gram-negative bacilli (2, 4, 7, 8). The work reported here concerns a new semisynthetic penicillin, BRL 2288, which has a spectrum of activity similar to that of carbenicillin but which is significantly more active than carbenicillin against *P. aeruginosa*.

MATERIALS AND METHODS

BRL 2288, a new broad-spectrum penicillin synthesized in the chemistry laboratories of Beecham Research Laboratories (E. G. Brain and J. H. C. Nayler, British Patent 1,004, 670, 1965, U.S. Patent 3,282,926, 1966), is the disodium salt of α-carboxy-3-thienylmethylpenicillin (Fig. 1). It is a creamy-white hygroscopic noncrystalline powder, readily soluble in water (greater than 100 g per 100 ml of water), giving a clear solution with a *p*H between 6.0 and 8.0. Aqueous solutions of BRL 2288 are relatively stable; for example, a solution containing 500 mg of BRL 2288 in 1 ml of distilled water retained greater than 90% of its potency after 1 day at 25 C or 7 days at 5 C. Under acid conditions, BRL 2288 is relatively unstable; the half-life of an aqueous solution (0.1%, w/v) at *p*H 2.0 is approximately 45 min at 37 C.

Material at present available contains a small amount (about 1%, w/w) of 3-thienylmethylpenicillin. This penicillin is the decarboxylation product of BRL 2288 and arises during manufacture. The content of 3-thienylmethylpenicillin in BRL 2288 is also increased by storage under unfavorable conditions. The material used in these studies contained 790 μg/mg (free acid); carbenicillin (Beecham Research Laboratories) also contained 790 μg/mg (free acid).

Most of the cultures used in these studies were recent

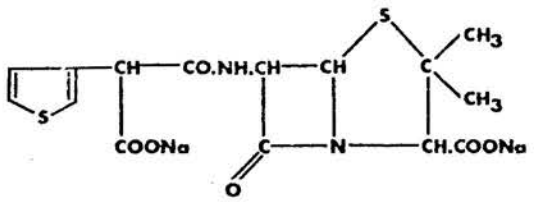

Fig. 1. *Structure of α-carboxy-3-thienylmethylpenicillin (BRL 2288), disodium salt.*

clinical isolates obtained from a number of hospitals in the United Kingdom. In addition, a number of laboratory strains were tested for comparative purposes.

Minimal inhibitory concentrations were determined by twofold serial dilution in nutrient agar (Blood Agar Base, Oxoid) in petri dishes which were inoculated with a replicating device delivering an inoculum of 0.003 ml. Serial dilution tests were also carried out in 5-ml volumes of nutrient broth (Nutrient Broth No. 2, Oxoid) which were inoculated with a drop (0.03 ml) of culture. The inoculum used in these tests was generally an undiluted overnight broth culture of the test organism, but in certain tests with *P. aeruginosa*, a 1:100 dilution of this inoculum was also used. Inhibition of growth was recorded after incubation overnight at 37 C.

Bactericidal activity was measured by determination of the reduction of the viable count of the test cultures incubated at 37 C in nutrient broth containing selected concentrations of BRL 2288. Samples were taken at intervals, and 0.02-ml volumes of suitable dilutions of the samples were pipetted onto nutrient agar plates. Colonies were counted after 18 hr of incubation at 37 C, and the count of surviving bacteria was estimated.

The effect of serum on the antibacterial activity of BRL 2288 was measured by serial dilution of the compound in 1-ml volumes of 95% human serum and 1-ml volumes of nutrient broth. The tubes were inoculated with 0.03 ml of a 10^{-3} dilution of an overnight culture of the test organism, and after overnight incubation at 37 C a loopful was taken from each tube showing no obvious visible growth and plated on nutrient agar. Minimal bactericidal concentrations were determined after overnight incubation at 37 C.

The extent of binding of BRL 2288 to the protein of human serum was determined by an ultrafiltration technique (5) in which serum containing known concentrations of BRL 2288 was filtered by positive pressure through a Visking dialysis membrane. The BRL 2288 content of the protein-free ultrafiltrate was determined by microbiological assay with *P. aeruginosa* NCTC 10701, and this represented the unbound fraction of BRL 2288 present in human serum.

RESULTS

The antibacterial spectrum of BRL 2288 against gram-positive and gram-negative bacteria is shown in Table 1. The compound was active against penicillin-susceptible strains of *Staphylococcus aureus, Streptococcus pyogenes,* and *S. pneumoniae*, but it was substantially less active than ampicillin against these organisms. BRL 2288 showed some degree of stability to staphylococcal penicillinase and was generally more active than ampicillin against penicillinase-producing strains of *S. aureus,* the majority of strains being inhibited by BRL 2288 at concentrations of 12.5 to 25 μg/ml. Aerobic and anaerobic strains of gram-positive bacilli were also susceptible to BRL 2288, but strains of *Streptococcus faecalis* were relatively insusceptible to this new penicillin.

BRL 2288 showed high activity against *Neisseria gonorrhoeae, N. meningitidis,* and *Haemophilus influenzae;* it was as active as ampicillin against these bacteria. Similarly, BRL 2288 was as active as ampicillin against *Escherichia coli, Proteus mirabilis,* and *Shigella sonnei*; it was only slightly less active than ampicillin against *Salmonella* species. Strains of *Klebsiella aerogenes* were resistant to both BRL 2288 and ampicillin. On the other hand, many strains of *Enterobacter aerogenes* were susceptible to BRL

Table 1. *Antibacterial spectrum of α-carboxy-3-thienylmethylpenicillin (BRL 2288)*

Organism	Minimal inhibitory concn[a] (μg/ml)	
	BRL 2288	Ampicillin
Staphylococcus aureus NCTC 6571	1.25	0.05
S. aureus[b]	12.5–25	>50
Streptococcus pyogenes	0.5	0.02
S. pneumoniae	1.25	0.05
S. faecalis	125	1.25
Clostridium tetani	0.5	0.05
C. welchii	0.5	0.05
Bacillus subtilis ATCC 6633	1.25	0.25
Haemophilus influenzae	0.25	0.25
Neisseria gonorrhoeae	0.02	0.02
N. meningitidis	0.02	0.02
Escherichia coli NCTC 10418	5.0	5.0
Salmonella typhi	2.5	1.25
S. typhimurium	5.0	1.25
Shigella sonnei	2.5	2.5
Klebsiella aerogenes	500	250
Enterobacter aerogenes	5.0	250
Pseudomonas aeruginosa	25	>250
Proteus mirabilis	1.25	1.25
P. morganii	2.5	125
P. rettgeri	2.5	250
P. vulgaris	2.5	500

[a] Serial dilution in 5% blood-agar; inoculum, one drop of undiluted 18-hr culture.

[b] Penicillinase-producing strain.

2288 but resistant to ampicillin. The spectrum of activity of BRL 2288 also differed from that of ampicillin in that the new penicillin demonstrated significant activity against *P. aeruginosa* and the indole-positive *Proteus* species, namely, *P. morganii, P. rettgeri,* and *P. vulgaris,* all of which are typically resistant to ampicillin.

The relative activities of BRL 2288, carbenicillin, and ampicillin against clinical isolates of *E. coli, P. mirabilis* and *Serratia marcescens* are illustrated in Fig. 2. BRL 2288 was slightly more active than ampicillin and carbenicillin against the test strains of *E. coli*, 46% of the strains being inhibited at 2.5 μg/ml and 87% at 5.0 μg/ml. Strains of *E. coli* which were resistant to ampicillin were generally resistant to BRL 2288, but a certain number of ampicillin-resistant cultures were found to be relatively susceptible to BRL 2288. The clinical isolates of *P. mirabilis* were highly susceptible to all three penicillins, and 90% of the strains were inhibited by BRL 2288 at a concentration of 12.5 μg/ml. Penicillinase-producing strains of *P. mirabilis* were resistant to all three penicillins. BRL 2288 was more active than ampicillin or carbenicillin against *S. marcescens*, but the majority of strains were relatively resistant. Thus, BRL 2288 inhibited 8 of 22 strains at a concentration of 12.5 μg/ml, but the remainder were inhibited only at concentrations greater than 125 μg/ml.

The activity shown by BRL 2288 and carbenicillin against β-lactamase-producing strains of indole-positive *Proteus* species and *Enterobacter* species, which are generally resistant to ampicillin and the cephalosporins, is shown in Fig. 3. Strains of *K. aerogenes* were almost uniformly resistant to BRL 2288, carbenicillin, and ampicillin, but were susceptible to cephaloridine. In contrast, the closely related *Enterobacter-Citrobacter* group of organisms were generally susceptible to BRL 2288 and carbenicillin but not to ampicillin and cephaloridine. Results of experiments to measure the extent of inactivation of the antibiotics used in these susceptibility tests showed that strains of *Enterobacter-Citrobacter* species inactivated cephaloridine but caused little destruction of BRL 2288, carbenicillin, or ampicillin; strains of *K. aerogenes* inactivated ampicillin but brought about relatively little destruction of BRL 2288 or carbenicillin.

Strains of the indole-positive *Proteus* species, namely, *P. morganii, P. rettgeri,* and *P. vulgaris,* destroyed ampicillin and cephaloridine as a result of β-lactamase activity and were not susceptible to these antibiotics (Fig. 3). On the other hand, BRL 2288 and carbenicillin were relatively stable to the β-lactamase activities of these organisms and inhibited growth of the test

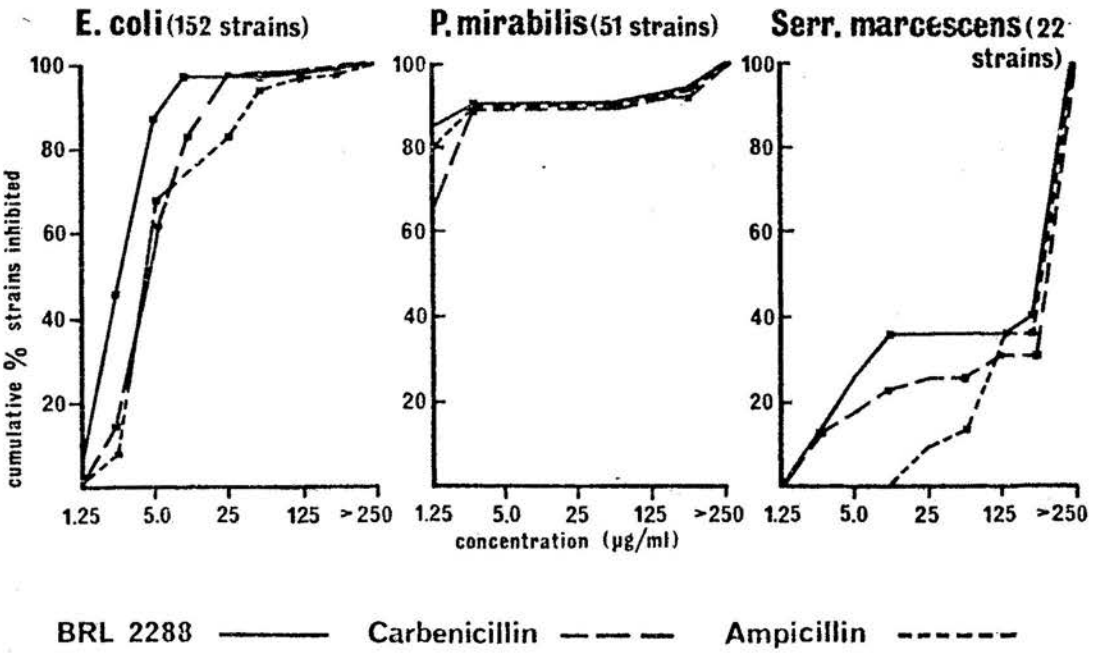

Fig. 2. *Activity of α-carboxy-3-thienylmethylpenicillin (BRL 2288) against gram-negative bacteria.*

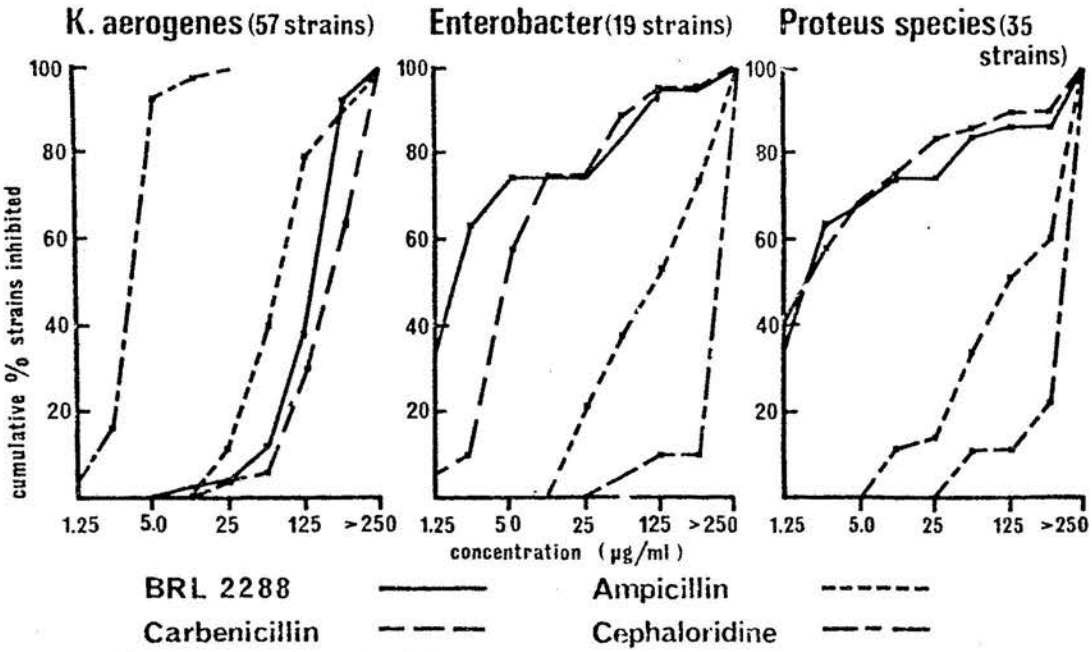

Fig. 3. *Activity of α-carboxy-3-thienylmethylpenicillin (BRL 2288) against certain β-lactamase-producing gram-negative bacteria.*

strains of indole-positive *Proteus* species at relatively low concentrations (Fig. 3). In contrast, β-lactamase-producing strains of *P. mirabilis* were relatively susceptible to cephaloridine but were resistant to the penicillins.

The antibacterial spectrum of BRL 2288 is of particular interest because of the activity of this compound against *P. aeruginosa*. In Fig. 4, the activities of BRL 2288 and carbenicillin against 74 clinical isolates of *P. aeruginosa* are compared. In general, BRL 2288 was at least twofold more active than carbenicillin against the test organisms. For example, 26% of the strains were inhibited by BRL 2288 at a concentration of 12.5 µg/ml, whereas only 8% were inhibited by the same concentration of carbenicillin. Similarly, 80% of the strains were inhibited by 25 µg of BRL 2288/ml, but only 20% of the strains were inhibited by carbenicillin at this concentration; at a level of 50 µg/ml, 95% of the strains were inhibited by BRL 2288 but only 79% were inhibited by carbenicillin.

The activity of BRL 2288 against *P. aeruginosa* is influenced by inoculum size and by the choice of solid or liquid medium; this is also the case with carbenicillin (3). Thus, when BRL 2288 was tested against a large inoculum (one

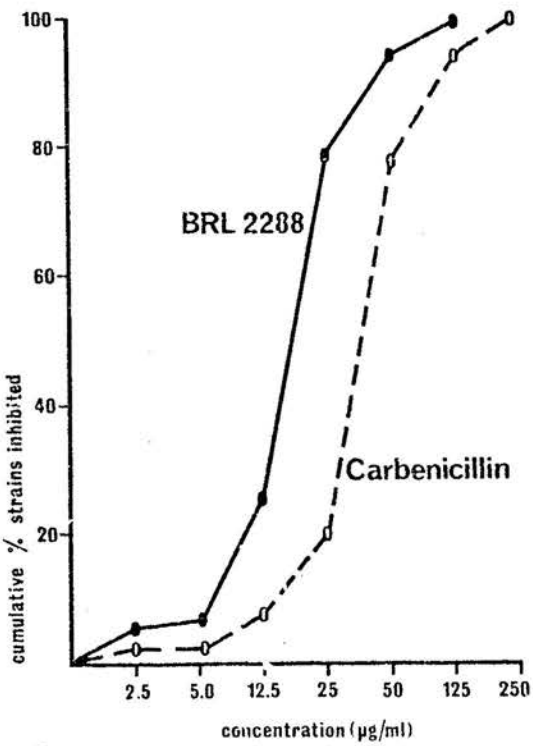

Fig. 4. *Activity of α-carboxy-3-thienylmethylpenicillin (BRL 2288) and carbenicillin against 74 strains of Pseudomonas aeruginosa.*

drop of an undiluted culture, i.e., 10^6 to 10^7 cells), sparse growth frequently persisted at concentrations greater than those which were inhibitory for 99% of growth, and complete inhibition was often achieved only at concentrations of 250 μg/ml or greater. This effect was particularly evident in the tests in liquid medium (Table 2). However, with a smaller inoculum, for example, a 1:100 dilution of an overnight culture (10^4 to 10^5 cells), complete inhibition of growth was observed at relatively low concentrations (Table 2). The growth which persisted on agar or in liquid medium when a large inoculum of cells was tested appeared not to be due to the presence of resistant variants, because subculture from this growth yielded a bacterial population which exhibited the same "tailing" phenomenon as seen with the original culture. However, Bell and Smith (1) have reported that in the case of carbenicillin, which shows the same tailing phenomenon, resistant variants could be selected from the growth which occurred in high concentrations of the drug. Further work will be required on this point with BRL 2288. As with carbenicillin, it can be shown that the persistence of growth of *Pseudomonas* in high concentrations of BRL 2288 is not due to inactivation of the drug.

Results are given in Fig. 5 showing that BRL 2288 is bactericidal in action against *P. aeruginosa*. With the particular strain of *Pseudomonas* used in this experiment, a concentration of BRL 2288 as low as 20 μg/ml exerted a marked bactericidal effect over the first 7 hr, but thereafter a resumption of growth took place. With a higher concentration of BRL 2288 (500 μg/ml), the bactericidal effect over the first 7 hr was more rapid, but again this was followed by some resumption of growth. This effect which is essentially the same as that seen with carbenicillin, may account for the inoculum effect observed in minimal inhibitory concentration tests and for the difference in susceptibility in liquid compared with solid media.

The effect of combining BRL 2288 with other anti-*Pseudomonas* antibiotics, such as gentamicin or the polymyxins, often resulted in enhanced or synergistic effects in tests with *P. aeruginosa*. This has also been reported to be the case with carbenicillin (6). Data in Table 3 show the results of experiments in which subinhibitory concentrations of BRL 2288 were added to gentamicin, polymyxin B, or colistin. The most marked effects were observed with com-

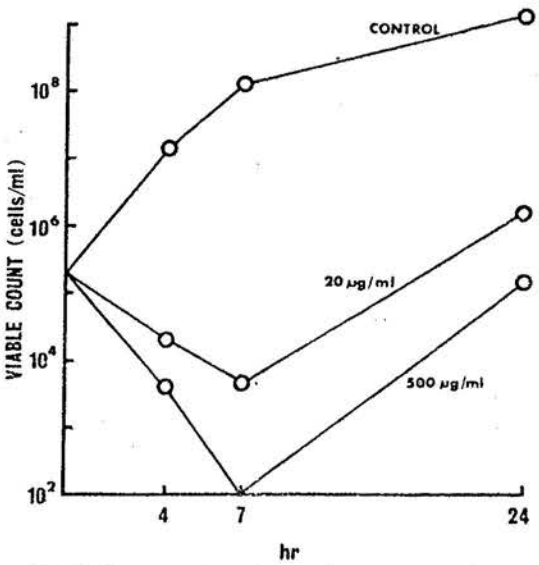

Fig. 5. *Bactericidal activity of α-carboxy-3-thienylmethylpenicillin (BRL 2288) against Pseudomonas aeruginosa R59.*

Table 2. *Effect of inoculum size on the antibacterial activity of α-carboxy-3-thienylmethylpenicillin (BRL 2288)*

Pseudomonas aeruginosa strain	Minimal inhibitory concn (μg/ml)			
	Nutrient agar		Nutrient broth	
	Undiluted[a]	1:100[b]	Undiluted	1:100
A	25	12.5	500	12.5
R1	25	12.5	250	25
R59	12.5	5.0	250	12.5

[a] Inoculum, one drop of undiluted overnight culture.
[b] Inoculum, one drop of 1:100 dilution of overnight culture.

Table 3. *Synergism between α-carboxy-3-thienylmethylpenicillin (BRL 2288) and other antibiotics against Pseudomonas aeruginosa R59[a]*

Concn of BRL 2288 (μg/ml)	Minimal inhibitory concn (μg/ml)		
	Gentamicin	Polymyxin B	Colistin
0	2.5	5.0	25.0
12.5	0.5	1.25	5.0
25	0.25	1.25	5.0

[a] Minimal inhibitory concentration of BRL 2288, 250 μg/ml.

Table 4. *Effect of serum on the antibacterial activity of α-carboxy-3-thienylmethylpenicillin (BRL 2288)*

Organism	Minimal bactericidal concn (μg/ml)	
	Nutrient broth	Human serum
Staphylococcus aureus	1.5	1.5
Pseudomonas aeruginosa	2.5	125

binations of BRL 2288 and gentamicin. It can be seen in Table 3 that inhibition of the test strain of *Pseudomonas* was achieved with a combination in which the concentration of both BRL 2288 and gentamicin was only one-tenth that required for inhibition by each drug alone. Combinations of BRL 2288 and the polymyxins (polymyxin B, colistin) were generally not as effective as combinations of BRL 2288 and gentamicin.

Measurement of the extent of binding of BRL 2288 to the protein of human serum showed that 45% of the drug was bound and 55% was present in the unbound form. In similar experiments with carbenicillin, 47% of the drug was bound and 53% was unbound. Susceptibility tests carried out in whole human serum showed that the activity of BRL 2288 was not significantly reduced compared with the activity shown in nutrient broth (Table 4).

DISCUSSION

The results presented here show that BRL 2288 has a spectrum of antibacterial activity similar to that of carbenicillin. Thus, like carbenicillin, BRL 2288 is from 10 to 20 times less active than ampicillin against gram-positive cocci, but is generally as active as ampicillin against ampicillin-susceptible gram-negative bacilli. BRL 2288 is also similar to carbenicillin in activity against *Enterobacter* and indole-positive *Proteus* species, which are generally resistant to ampicillin and the cephalosporin antibiotics. Of particular interest is the fact that BRL 2288 is about twice as active as carbenicillin against strains of *P. aeruginosa*. Like carbenicillin, BRL 2288 is bactericidal, is not highly bound to serum protein, and demonstrates synergistic effects with gentamicin.

The antibacterial activity of BRL 2288 against *P. aeruginosa* is considerably lower than that of polymyxin or gentamicin. However, the use of these latter antibiotics is limited by problems of toxicity, whereas BRL 2288 appears to be relatively nontoxic in animal studies. Carbenicillin has been shown to be effective in the treatment of severe bacterial infections including those caused by *P. aeruginosa* (2, 4, 8), but as a result of the level of activity of carbenicillin against *P. aeruginosa* high dosage is required in the treatment of such infections. In this connection, the increased activity of BRL 2288 against *P. aeruginosa*, as well as its activity against *Enterobacteriaceae* including indole-positive *Proteus* and *Enterobacter*, is of considerable interest.

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α-Carboxy-3-Thienylmethylpenicillin (BRL 2288), a New Semisynthetic Penicillin: Absorption and Excretion in Man

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The absorption and excretion of α-carboxy-3-thienylmethylpenicillin (BRL 2288), a new semisynthetic penicillin with a spectrum of activity similar to that of carbenicillin, was studied in healthy volunteer subjects. Like carbenicillin, BRL 2288 was not absorbed when administered by mouth but was well absorbed by the parenteral route. In human volunteers, peak drug concentrations in serum of approximately 35 μg/ml were obtained after a single intramuscular injection of 1,000 mg; 72% of the dose was excreted in the urine during the first 6 hr after injection. Concentrations of approximately 100 μg of BRL 2288 per ml of serum were measured after a single 1,000-mg dose given by intravenous injection, but levels fell rapidly to 2 μg/ml at 6 hr. Higher and more prolonged serum concentrations were obtained with the concomitant administration of probenecid. The antibacterial activity of serum and urine of healthy subjects after intramuscular administration of BRL 2288 was consistent with the in vitro activity of the compound and the concentrations of BRL 2288 present in the specimens of serum and urine.

BRL 2288, the disodium salt of α-carboxy-3-thienylmethylpenicillin, is a new semisynthetic penicillin derived from 6-aminopenicillanic acid. Its spectrum of antibacterial activity is similar to that of carbenicillin, but it is more active than carbenicillin against strains of *Pseudomonas aeruginosa* (4, 5). Studies in animals have demonstrated the relative lack of toxicity of this new penicillin and its effectiveness against experimental infections in mice. For example, BRL 2288 was more active than carbenicillin against *P. aeruginosa* and was as active as or more active than ampicillin or carbenicillin against infections due to *Escherichia coli* and *Proteus* species (1).

This communication describes preliminary studies of the absorption and excretion of BRL 2288 in healthy volunteer subjects.

MATERIALS AND METHODS

The absorption and excretion studies described here were carried out on healthy volunteer male and female subjects aged 20 to 50 years. When crossover studies were carried out to determine the effect of probenecid, there was usually an interval of about 1 week between the two halves of the study. BRL 2288 was used as the disodium salt [estimated potency, 790 μg/mg (free acid)].

For oral absorption studies, BRL 2288 was administered as a single 500-mg dose in gelatin capsules to fasting subjects, and for absorption by intramuscular administration, as single 250-, 500-, or 1,000-mg doses dissolved in 1 to 2 ml of water injected in nonfasting subjects. For intravenous administration, nonfasting subjects were given 1,000 mg of BRL 2288 dissolved in 10 ml of water and administered over a period of 3 min. Blood specimens were collected at 0.5, 1, 2, 4, and 6 hr after administration, and urine was collected during the 6-hr period after dosing. Specimens were normally assayed on the day of collection, but in exceptional cases were kept overnight at -20 C before being assayed.

The concentrations of BRL 2288 in serum and urine specimens were measured by a conventional agar-diffusion cup-plate technique similar to that described for carbenicillin (2, 3), with *P. aeruginosa* NCTC 10490 (or in later studies *P. aeruginosa* NCTC 10701) as assay organism. These cultures were used to assay BRL 2288 because the material used in these studies contained a small amount (about 1 to 2%) of the penicillin produced

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by decarboxylation of BRL 2288, namely, 3-thienylmethylpenicillin, which is considerably more active than BRL 2288 against conventional penicillin assay organisms, e.g., *Sarcina lutea* and *Bacillus subtilis*. *Pseudomonas* strains NCTC 10490 and NCTC 10701 are susceptible to BRL 2288 (minimal inhibitory concentration, 1 to 2 μg/ml) and relatively nonsusceptible to 3-thienylmethylpenicillin (minimal inhibitory concentration, 500 μg/ml); with either of these strains, it was possible to assay concentrations of BRL 2288 as low as 1.0 to 2.0 μg/ml. For the assay of serum specimens, standard solutions of BRL 2288 were prepared in human serum over a concentration range of 1 to 50 μg/ml, and when necessary specimens were diluted in human serum to fall within this range of concentrations. For the assay of urine specimens, standard solutions were prepared in 0.05 M phosphate buffer, *p*H 7.0, and urine samples were diluted as required in the same buffer.

The antibacterial activity of serum and urine specimens from subjects receiving a single 500-mg intramuscular dose of BRL 2288 was determined by serial dilution of the specimens in molten nutrient agar (Blood Agar Base, Oxoid). When set, the agar plates were dried and inoculated with a drop (0.003 ml) of an undiluted overnight culture of the test organism delivered with a multiple-inoculating device. The plates were incubated overnight at 37 C, and the maximal dilutions of the serum or urine specimen inhibiting growth of the test organism were measured.

Urine from subjects given BRL 2288 was spotted (6 μliters) on filter-paper strips (Whatman no. 1, 1 cm wide) and chromatographed with a butanol-ethyl alcohol solvent system (*n*-butanol-ethanol-water, 4:1:5, v/v, top phase). Standard solutions of BRL 2288 were prepared in 0.05 M phosphate buffer (*p*H 7.0) and chromatographed. Chromatography was carried out overnight at 5 C, and the chromatograms were dried at 40 C. The penicillins were located by placing the dried chromatograms on nutrient agar (Blood Agar Base, Oxoid) seeded with a spore suspension of *B. subtilis* ATCC 6633 which was incubated overnight at 37 C.

RESULTS

Oral administration. After the administration of a single 500-mg oral dose of BRL 2288 to fasting subjects, no penicillin (i.e., less than 2.0 μg of BRL 2288/ml) was detected in the serum, and the quantity present in the urine amounted to only 2.8% of the dose administered. It appeared, therefore, that BRL 2288 was not absorbed to any appreciable extent when administered by the oral route.

Intramuscular administration. The results of studies in which BRL 2288 was administered by intramuscular injection to healthy adult volunteers as single 250-, 500-, and 1,000-mg doses are summarized in Table 1, which shows the mean concentrations in serum and the amount excreted in the urine during the 6-hr period after dosing. Mean peak concentrations of 8.7 μg/ml were obtained in serum 1 hr after administration of the 250-mg dose, and this declined to less than 2.0 μg/ml after 6 hr. With the 500-mg dose, the peak concentration of 22 μg/ml was measured after 0.5 hr, but the concentration then fell to less than 2.0 μg/ml after 6 hr. Administration of the 1,000-mg dose resulted in a peak serum concentration of 35 μg/ml at 1 hr, which fell to 7 μg/ml at 6 hr. Increasing the dose

Table 1. *Concentrations of α-carboxy-3-thienylmethylpenicillin (BRL 2288) in the serum of human volunteers after a single intramuscular injection*

No. of subjects	Dose (mg)	Mean concn in serum (μg/ml)					Excretion in urine, 0-6 hr (% of dose)
		0.5 hr	1 hr	2 hr	4 hr	6 hr	
9	250	6.8 (±4.3)[a]	8.7 (±2.0)	6.8 (±1.9)	3.0 (±2.0)	<1.0	85
12	500	21.9 (±7.9)	21.4 (±6.1)	14.1 (±4.3)	4.7 (±3.2)	<1.0	99
14	1,000	22.3 (±13.7)	33.7 (±13.0)	31.2 (±4.0)	16.5 (±5.1)	6.3 (±4.3)	72
22[b]	1,000	22.3 (±14.9)	26.4 (±9.9)	22.7 (±5.0)	9.5 (±3.9)	2.7 (±2.4)	
Significance (*P*)[c]		>0.05	>0.05	<0.001	<0.001	<0.02	

[a] Standard deviation.
[b] For comparison, these subjects were given carbenicillin.
[c] BRL 2288/carbenicillin.

clearly resulted in a significant increase in serum concentrations of the drug.

The amounts of BRL 2288 excreted in the urine during the first 6 hr after injection were 85% of the dose with the 250-mg dose, 99% with the 500-mg dose, and 72% with the 1,000-mg dose, producing average BRL 2288 concentrations in the urine of 900 to 1,900 μg/ml with the 500-mg dose and 2,000 to 3,000 μg/ml with the 1,000-mg dose. Data in Table 1 also show serum concentrations obtained in human subjects after administration of a 1,000-mg intramuscular dose of carbenicillin. The mean peak serum concentrations measured after 1 hr were 35 μg/ml for BRL 2288 compared with 26 μg/ml for carbenicillin, and throughout the 6-hr period the levels of BRL 2288 were in excess of those produced by carbenicillin.

The effect of probenecid on the concentrations achieved in serum after a 1,000-mg dose of BRL 2288 was investigated in a crossover study in a group of eight subjects. Probenicid was administered by mouth, one 500-mg tablet being taken 11.5 hr before injection of BRL 2288 and a second 500-mg tablet 1 hr before injection. The administration of probenecid resulted in a marked increase in the BRL 2288 serum concentrations during the 6-hr period after dosing (Table 2), and in a reduction in the amount excreted (49% of the dose) in urine during the same period.

Bioautographic examination of the urine showed the presence of unchanged BRL 2288, and 3-thienylmethylpenicillin which was present in the material administered, and showed no evidence for the formation of biologically active metabolites.

Table 2. *Effect of probenecid on drug concentrations in the serum of human volunteers after a single intramuscular injection of 1,000 mg of α-carboxy-3-thienylmethylpenicillin (BRL 2288)*

Group[a]	Mean concn in serum (μg/ml)				
	0.5 hr	1 hr	2 hr	4 hr	6 hr
Without probenecid	21.4 (±15.5)[b]	35.0 (±15.4)	30.9 (±2.7)	16.9 (±6.16)	7.1 (±5.20)
With probenecid	30.5 (±18.2)	49.8 (±21.4)	60.3 (±15.4)	41.6 (±6.8)	27.7 (±8.4)
Significance (*P*)	<0.001	<0.001	<0.01	<0.05	<0.02

[a] Each group consisted of seven subjects.
[b] Standard deviation.

Table 3. *Effect of probenecid on drug concentrations in the serum of human volunteers after a single intravenous injection of 1,000 mg of α-carboxy-3-thienylmethylpenicillin (BRL 2288)*

Subject	Probenecid	Concn in serum (μg/ml)						Excretion in urine, 0-6 hr (% of dose)
		0.25 hr	0.5 hr	1 hr	2 hr	4 hr	6 hr	
1	–	95.0	69.5	37.0	17.7	8.0	1.5	70
	+	99.0	69.0	66.0	45.5	19.8	12.7	47
2	–	97.0	69.5	42.5	17.7	6.0	1.8	68
	+	120.0	68.0	57.0	37.0	18.0	10.5	56
3	–	99.5	49.0	34.0	17.7	3.2	2.8	83
	+	92.5	69.0	61.0	34.5	16.3	7.4	51
Mean	–	97.2	62.7	37.8	17.7	5.7	2.0	73
	+	100.5	68.7	61.3	39.0	18.0	10.2	52
Significance (*P*)		>0.05	>0.05	0.05	0.05	0.05	0.05	

Intravenous administration. Three subjects received 1,000 mg of BRL 2288 in 10 ml of water by slow intravenous injection administered over a period of 3 min. Mean concentrations of greater than 100 μg/ml were obtained in the serum during the first 15 min, but these fell to less than 40 μg/ml within 1 hr and to 2 μg/ml at 6 hr (Table 3). The amount excreted in the urine during the 6-hr period represented 75% of the dose administered.

One week later, the same subjects received 1,000 mg of BRL 2288 by injection in combination with 1,000 mg of probenecid given by mouth 2 hr before administration of the penicillin. The serum concentrations obtained in the first 0.5 hr after dosing were similar to those produced in the same subjects given BRL 2288 alone, but just over 60 μg/ml was present at 1 hr and a concentration of 10 μg/ml was measured at 6 hr (Table 3). During the 6-hr period after dosing, 52% of the dose was excreted in the urine.

Antibacterial activity. The antibacterial activity of pooled sera from a group of six subjects receiving a 500-mg intramuscular dose of BRL 2288 and of the urine of two subjects in the same study is shown in Tables 4 and 5, where it can be seen that the antibacterial activity of the serum and urine specimens corresponded to the in vitro susceptibilities of the test organisms and the BRL 2288 concentrations present in the specimens.

DISCUSSION

The data reported in this study show that BRL 2288 is not absorbed to any significant extent when given by mouth to human subjects, but produces high serum concentrations after intramuscular injection. An increase in dose results in an increase in serum concentrations, as is the case with other penicillins, and peak serum concentrations after 250-, 500-, and 1,000-mg intramuscular doses were 9, 22, and 35 μg/ml, respectively, occurring at 0.5 to 1 hr after administration. In these studies, the amount excreted in the urine in the period from 0 to 6 hr after intramuscular dosing varied, being 85% of the 250-mg dose, 99% of the 500-mg dose, and 72% of the 1,000-mg dose; concentrations of BRL 2288 in the urine reached 2,000 to 3,000 μg/ml with the 1,000-mg dose. Comparison of the serum concentrations produced with a single 1,000-mg intramuscular dose of BRL

Table 5. *Antibacterial activity of urine of volunteers after intramuscular administration of 500 mg of α-carboxy-3-thienylmethylpenicillin (BRL 2288)*

Organism	Minimal inhibitory concn (μg/ml)	Antibacterial activity[a]	
		Subject A	Subject B
Pseudomonas aeruginosa NCTC 10490	0.5	640	1,280
P. aeruginosa R1	12.5	80	80
P. aeruginosa R128	25	20	40
P. aeruginosa R160	25	20	40
Proteus morganii A	5.0	640	160
P. vulgaris A	5.0	320	320
P. rettgeri B	5.0	160	160
P. mirabilis 957	1.25	640	640
P. mirabilis 889	>500	<2	<2
Staphylococcus aureus 1555[b]	25	20	40
Escherichia coli 10418	2.5	320	320
Klebsiella aerogenes A	50	20	20
Concentration (μg/ml)		1,100	1,170

[a] Reciprocal of highest dilution of urine inhibiting growth of test organism.
[b] Penicillinase-producing strain.

Table 4. *Antibacterial activity of serum of volunteers after intramuscular administration of 500 mg of α-carboxy-3-thienylmethylpenicillin (BRL 2288)*

Organism	Minimal inhibitory concn (μg/ml)	Antibacterial activity[a]				
		0.5 hr	1 hr	2 hr	4 hr	6 hr
Escherichia coli 10418	1.25	8	8	4	<2	<2
Pseudomonas aeruginosa A	50	<2	<2	<2	<2	<2
Proteus vulgaris A	5.0	8	8	8	4	<2
P. aeruginosa NCTC 10490	1.25	16	16	8	4	2
Serum concentration (μg/ml)		20.2	21.2	16.7	6.3	<2.5

[a] Reciprocal of highest dilution of serum inhibiting growth of test organism.

2288 or carbenicillin demonstrates that BRL 2288 produces higher peak serum concentrations and higher serum concentrations throughout the 6-hr period than does carbenicillin. Serum levels observed after intramuscular and intravenous injection are increased and are more prolonged with concomitant use of probenecid, particularly after intramuscular administration of the penicillin.

The antibacterial activity of serum and urine after administration of BRL 2288 is consistent with the in vitro activity and concentrations of BRL 2288 present in the specimens. The serum levels produced by a 1,000-mg dose of BRL 2288 are in excess of the minimal inhibitory concentrations of the penicillin for the majority of strains of *P. aeruginosa* for a short period only, but the urine concentrations measured during the 6-hr period after administration are greatly in excess of the minimal inhibitory concentrations for most strains of *Pseudomonas*.

ACKNOWLEDGMENTS

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Preparation, Hydrolysis, and Oral Absorption of α-Carboxy Esters of Carbenicillin

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Twelve α-carboxy esters of carbenicillin, a parenteral broad spectrum semisynthetic penicillin, were synthesized and examined as potential oral carbenicillin derivatives. The rates at which the esters were hydrolyzed *in vitro* to carbenicillin by animal and human tissues were compared and the carbenicillin serum levels arising after oral administration of the esters were measured in squirrel monkeys and human volunteer subjects. The α-carboxyphenyl ester of carbenicillin [carfecillin (British Pharmacopoeia approved name), BRL 3475] was selected for further study and is presently undergoing clinical trial.

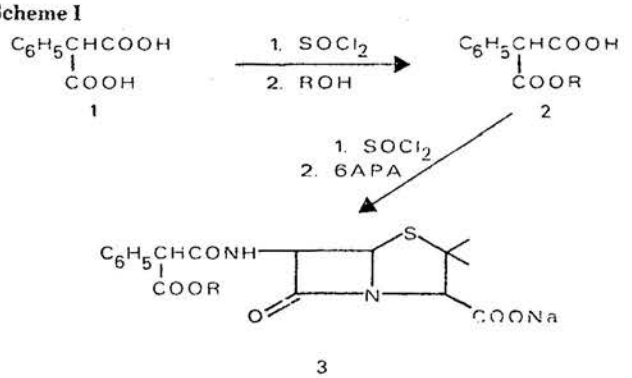
Carbenicillin (α-carboxybenzylpenicillin) has a broad spectrum of antibacterial activity and has been shown to be effective in the treatment of serious infections caused by *Pseudomonas aeruginosa* and other gram-negative bacteria. However, the compound is poorly absorbed from the gastrointestinal tract after administration by the oral route and its use is limited to parenteral administration. It has been found that certain α-carboxy esters of carbenicillin are absorbed by the oral route and undergo hydrolysis in the body to liberate carbenicillin.[1] One such ester (carbenicillin indanyl sodium) has been described in detail.[2]

Carbenicillin α-carboxy esters (that is, the ester group is in the side chain) possess a free thiazolidine carboxyl group and, hence, can be expected to demonstrate antibacterial activity *per se*. In general, we and others have found that the unhydrolyzed ester is more active than carbenicillin against gram-positive bacteria but shows a lesser degree of activity against most gram-negative bacilli.[3] However, the antibacterial spectrum shown by this class of compound will depend upon the extent of hydrolysis to carbenicillin that occurs in the course of the antibacterial test. Esters that are hydrolyzed rapidly to carbenicillin show an antibacterial spectrum similar to that of carbenicillin while esters with a slow rate of hydrolysis demonstrate activity primarily against gram-positive bacteria. In practice carbenicillin esters are of clinical interest only as a means of providing carbenicillin activity in the body after oral administration and in this context their intrinsic activity may be disregarded.

This report describes the preparation, *in vitro* hydrolysis rates, and oral absorption characteristics of a group of 12 α-carboxy esters of carbenicillin.

Chemistry. Scheme I illustrates the general route used to prepare α-carboxy esters of carbenicillin.[4] Phenylmalonic acid (1) was converted to its monoacid chloride by treatment with 1 equiv of thionyl chloride. The crude product was allowed to react with 1 equiv of alcohol or phenol (ROH) to afford the half-esters 2 described in Table I. The crystalline half-esters 2 were converted to their acid chlorides with excess thionyl chloride at 70° (higher temperatures caused degradation) and coupled directly to 6-aminopenicillanic acid (6-APA) in aqueous acetone. The penicillins 3 were isolated as their sodium salts and are described in Table II. A variation of this procedure has also been used to prepare penicillins of this type.[5]

Scheme I



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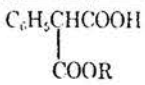
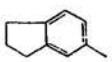
The α-carboxy esters, listed in Table II, as in the case of carbenicillin, contain an asymmetric center in their side chains and in solution their nmr spectra (Table III) suggested an approximately 1:1 mixture of epimers. However, in a number of instances (Table II) it was possible to obtain the penicillin sodium salts crystalline and these compounds were considered on nmr evidence to be single epimers in the solid state. Thus the nmr spectrum for the crystalline phenyl ester showed initially a singlet for the thiazolidine C-3 proton which rapidly changed to two singlets of equal intensity, consistent with racemization at the side-chain chiral center.†

Biological Properties. The 12 α-carboxy esters of carbenicillin, described in Table II, were examined for rates of hydrolysis to carbenicillin in aqueous solution and in the presence of isolated tissue homogenates. The 12 esters were also orally administered to both squirrel monkeys and human volunteers and levels of carbenicillin in blood and urine were measured. Details of these procedures are described in the Experimental Section. The results are discussed below.

† We thank Mr. N. Ward and Mr. A. E. Bird for these observations.

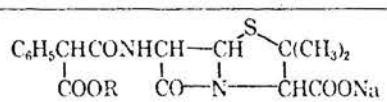
Table I. Monoesters of Phenylmalonic Acid

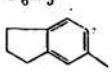


R	Yield, %	Recrystn solvents	Mp, °C	Formula	Analyses
CH_3CH_2-	57	C_6H_6–pet. ether (60–80°)	78–79	$C_{11}H_{12}O_4$	
$C_6H_5CH_2-$	70	C_6H_6–pet. ether (60–80°)	62–64	$C_{16}H_{14}O_4$	C, H
$(CH_3)_2CH-$	62	C_6H_6–pet. ether (60–80°)	64–66	$C_{12}H_{14}O_4$	C, H
$CH_3(CH_2)_3-$	64	EtOAc–pet. ether (40–60°)	47–48	$C_{13}H_{16}O_4$	C, H
$(CH_3)_2CHCH_2-$	52	EtOAc–pet. ether (40–60°)	41–49	$C_{13}H_{16}O_4$	C, H
$CH_3(CH_2)_4-$	70	EtOAc–pet. ether (40–60°)	47–48	$C_{14}H_{18}O_4$	C, H
C_6H_5-	79	C_6H_6	115–117 dec	$C_{15}H_{12}O_4 \cdot 0.5C_6H_6$	H; C[a]
	71	C_6H_6–pet. ether (60–80°)	80–82[d]	$C_{18}H_{16}O_4$	C, H
$3\text{-}CH_3C_6H_4-$	68	C_6H_6–pet. ether (60–80°)	95–96	$C_{16}H_{14}O_4$	C, H
$4\text{-}CH_3C_6H_4-$	81	C_6H_6–pet. ether (60–80°)	122–124 dec	$C_{16}H_{14}O_4 \cdot 0.5C_6H_6$	H; C[b]
$2,5\text{-}(CH_3)_2C_6H_3-$	60	C_6H_6–pet. ether (60–80°)	100–102	$C_{17}H_{16}O_4$	C, H
$3,4\text{-}(CH_3)_2C_6H_3-$	70	C_6H_6	88–90	$C_{17}H_{16}O_4$	H; C[c]

[a] C: calcd, 73.25; found, 73.79. [b] C: calcd, 73.77; found, 72.64. [c] C: calcd, 71.82; found, 72.99. [d] D. C. Hobbs, *Antimicrob. Ag. Chemother.* 2, 272 (1972), quotes mp 97–99°.

Table II. α-Carboxy Esters of Carbenicillin



R	Yield, %	R_f[a]	$[\alpha]^{20}D$, deg (c 1, H_2O)	Formula	Analyses
CH_3CH_2-[b]	78	0.52	229.8	$C_{19}H_{21}N_2O_6SNa$	C, H, N, S
$C_6H_5CH_2-$[b]	60	0.57	195.5	$C_{24}H_{23}N_2O_6SNa \cdot H_2O$	C, H, N, S
$(CH_3)_2CH-$[b]	92	0.60		$C_{20}H_{23}N_2O_6SNa \cdot H_2O$	C, H, N, S
$CH_3(CH_2)_3-$	79	0.63		$C_{21}H_{25}N_2O_6SNa$	H, N, S; C[c]
$(CH_3)_2CHCH_2-$	77	0.61			
$CH_3(CH_2)_4-$	86	0.70			
C_6H_5-[b]	70	0.59	216.2		
	53	0.70		$C_{26}H_{25}N_2O_6SNa$	C, H, N, S
$3\text{-}CH_3C_6H_4-$	72	0.65	201.7		
$4\text{-}CH_3C_6H_4-$	62	0.60	207.4		
$2,5\text{-}(CH_3)_2C_6H_3-$	49	0.75			
$3,4\text{-}(CH_3)_2C_6H_3-$	32	0.70	176.8		

[a] R_f values determined by paper chromatography in butanol–ethanol–water (4:1:5, v/v top phase), followed by development on an ag plate seeded with *B. subtilis*. [b] Sodium salts obtained crystalline from ethanol. [c] C: calcd, 55.25; found, 54.48.

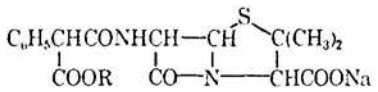
Hydrolysis by Squirrel Monkey Tissue Homogenate. The six alkyl esters (described in Table II) and the phenyl ester were examined for hydrolysis to carbenicillin by homogenates of squirrel monkey liver and small intestine. The results are presented in Table IV and represent the sum of both enzymatic and nonenzymatic hydrolysis. It will be seen that nonenzymatic hydrolysis was very small for the alkyl esters but made a significant contribution to the hydrolysis of the chemically labile phenyl ester. For all esters, the extent of hydrolysis in squirrel monkey liver homogenate was similar to that in the small intestine homogenate. The benzyl and phenyl esters were the most susceptible to hydrolysis and the isopropyl ester the least affected. The relationship of these results to carbenicillin blood levels in the squirrel monkey is discussed below.

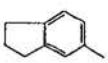
Hydrolysis by Human Liver Homogenate. Data for the conversion of the 12 carbenicillin esters to carbenicillin by aqueous human liver homogenate are presented in Table V. The most striking difference between man and the squirrel monkey was for the benzyl ester, which h drolyzed slowly in human liver homogenate but was co pletely hydrolyzed by squirrel monkey liver homogena The alkyl esters were slowly hydrolyzed by human liv homogenate as was the case with squirrel monkey liv homogenate. The aromatic esters were more susceptible hydrolysis by human liver homogenate than the alkyl ters, even allowing for the increased contribution fr aqueous hydrolysis.

In general, therefore, the aryl esters were more rapi and more completely hydrolyzed to carbenicillin th were the alkyl esters in both aqueous solution and in presence of tissue homogenates.

Further Studies on the Hydrolysis of the Phe Ester of Carbenicillin. The phenyl ester of carbenici was examined more extensively at ester concentrations 5 and 100 μg/ml. Rates of hydrolysis were measured human liver homogenate, human small intestine homo nate, and diluted human blood. The results are repor

Table III. Nmr Data (δ Values) for Penicillins



R	Solvent		$C(CH_3)_2$ (6 H)		C_3 proton (1 H)		PhCH-CO-(1 H)	β-Lactam protons (2 H)		C_6H_5- (5 H)	-CONH- (1 H)
H-	D_2O		1.51, 1.60		4.28, 4.30		4.58, 4.59	5.58		7.42	
CH_3CH_2-	DMSO	1.17 (3 H)	1.52		3.90, 3.93	4.10 (2 H)	4.98	5.38		7.35	8.83
$C_6H_5CH_2$-	D_2O		1.50		4.25, 4.29	4.93 (2 H)		5.53		7.13 (10 H)	
$(CH_3)_2CH$-	DMSO	1.18 (6 H)	1.50		3.92, 3.95	4.95 (1 H)	4.95	5.39		7.35	8.75
$CH_3(CH_2)_3$-	D_2O	0.70 (3 H) 1.33 (4 H)	1.52	4.10 (2 H)	4.27, 4.31		4.83	5.58		7.34	
$(CH_3)_2CHCH_2$-	DMSO	0.85 (6 H)	1.53	1.90 (1 H)	3.93, 3.97	3.90 (2 H)	5.06	5.11		7.36	8.40
$CH_3(CH_2)_4$-	D_2O	0.68 (3 H) 1.10 (6 H)	1.50	4.10 (2 H)	4.28, 4.32			5.61		7.35	
C_6H_5-	D_2O		1.50		4.29, 4.32			5.60		7.10 (10 H)	
	DMSO-D_2O		1.55	2.03 (2 H) 2.85 (4 H)	4.02, 4.05			5.48	6.92 (3 H)	7.45	
3-$CH_3C_6H_4$-	DMSO-D_2O		1.53	2.30 (3 H)	4.02, 4.05		5.40	5.40	7.08 (4 H)	7.41	9.10
4-$CH_3C_6H_4$-	D_2O		1.50	1.82 (3 H)	4.29, 4.31			5.63	6.70 (4 H)	7.30	
2,5-$(CH_3)_2C_6H_3$-	DMSO-D_2O		1.54	2.06, 2.28 (6 H)	4.03, 4.06			5.47	6.99 (3 H)	7.47	
3,4-$(CH_3)_2C_6H_3$-	D_2O		1.48	2.12 (6 H)	3.99, 4.02			5.17	6.50 (3 H)	7.47	

in Table VI. The phenyl ester showed marked hydrolysis in all three tissue preparations.

Absorption Studies. Results in Table VII show the mean peak carbenicillin concentrations measured in the blood of squirrel monkeys after oral administration of a single dose of 25 mg/kg of the esters. In contrast to carbenicillin, all 12 esters were absorbed by the oral route producing maximum carbenicillin concentrations ranging from 5 to 20 μg/ml in blood. The highest levels were obtained with the 3-methylphenyl ester followed by the 5-indanyl, 4-methylphenyl, phenyl, and *n*-butyl esters.

There did not appear to be any direct relationship between *in vitro* hydrolysis rates in the squirrel monkey tissue preparations (small intestine and liver) shown in Table IV and peak carbenicillin concentrations in blood. For example, although both the phenyl ester and the benzyl ester were rapidly hydrolyzed *in vitro*, the peak carbenicillin blood concentrations produced by the phenyl ester were about twice those of the benzyl ester. Similarly, although the *n*-butyl, isobutyl, and *n*-pentyl esters were all hydrolyzed relatively slowly and at about the same rates by the tissue preparations, the two former compounds produced peak carbenicillin blood levels almost twice those of the *n*-pentyl ester. The isopropyl ester was hydrolyzed at a very low rate *in vitro* and produced relatively low blood carbenicillin concentrations.

For comparison, the mean peak serum carbenicillin concentrations obtained in human volunteers are also shown in Table VII and it can be seen that there was no good correlation between the results in squirrel monkey and man. Thus, the phenyl, 3-methylphenyl, and 5-indanyl esters gave relatively high carbenicillin levels in both groups but the 4-methylphenyl ester, which produced relatively high levels in the squirrel monkey, gave low serum concentrations of carbenicillin in man. On the other hand, the isopropyl ester which produced low levels in the monkey gave relatively high levels in man. The lack of correlation between results in the squirrel monkey and in man is not entirely unexpected, since this would require not only similar absorption rates in the two species but also the rates at which the esters were hydrolyzed to carbenicillin by the body tissues would have to be the same.

The carbenicillin concentrations measured in serum and urine of human volunteers dosed with the esters are shown

Table IV. Ester Hydrolysis by Squirrel Monkey Liver and Intestine Homogenates[a]

Ester	% hydrolysis to carbenicillin after 2.5 hr in		
	Buffer, pH 7	Intestine	Liver
Ethyl	<1	5	5
Benzyl	<5	100	100
Isopropyl	<1	0.3	1.2
n-Butyl	<1	19	24
Isobutyl	<1	17	31
n-Pentyl	<1	28	33
Phenyl	23	100	100

[a]Substrate concentration 1 mg/ml, tissue concentration 2% w/v, temperature 37°.

Table V. Ester Hydrolysis by Human Liver Homogenate[a]

Ester	% hydrolysis to carbenicillin in			
	Liver at 30 min	Liver at 60 min	Liver at 180 min	Buffer, pH 7, at 180 min
Ethyl	1	2	23	<1
Benzyl	<1	1	7	<5
Isopropyl	<1	<1	<1	<1
n-Butyl	1	2	17	<1
Isobutyl	<1	1	5	<1
n-Pentyl	2	4	11	<1
Phenyl	16	46	60	23
5-Indanyl	41	71	83	18
3-Methylphenyl	58	64	85	16
4-Methylphenyl				23
2,5-Dimethylphenyl	9	16	35	7
3,4-Dimethylphenyl	57	73	84	25

[a]Substrate concentration 2 mg/ml, tissue concentration 4% w/v, temperature 37°.

in Table VIII.‡ The compounds were administered in gelatin capsules without excipients as a single dose equivalent to 500 mg of carbenicillin free acid. Table VIII also shows the amount of unhydrolyzed ester recovered from the urine of these subjects. Carbenicillin itself is poorly absorbed in human subjects after oral administration and under the conditions described here no carbenicillin was detected in the blood (<1 μg/ml) and less than 1% of the dose was found in the urine. In contrast, after administration of the esters, carbenicillin was measured in serum with all compounds, except the *n*-pentyl ester (<1.0 μg/ml), and the amount of carbenicillin excreted in the urine varied from 15 to 36% of the dose administered (*n*-pentyl ester, 6%). The highest peak carbenicillin serum concentrations (approximately 7.0 μg/ml) were obtained with the phenyl, 5-indanyl, and isobutyl esters and with these compounds approximately one-third of the dose was excreted in the urine as carbenicillin.

It was noteworthy that after administration of the alkyl esters significant amounts of unhydrolyzed ester were found in the urine along with carbenicillin, whereas unhydrolyzed ester was not detected in urine with the aryl esters. The difference between the alkyl and aryl esters is presumably a reflection of the slower rates of hydrolysis of the former as reported above.

‡ We thank Dr. K. H. Jones for supervising the studies in human volunteers.

Table VI. Hydrolysis of Carbenicillin Phenyl Ester by Human Tissue Homogenates

Hydrolyzing system	Ester concn (μg/ml) remaining after			
	0 min	30 min	60 min	180 min
Buffered saline, pH 7.4	5.0 (0)[a]	4.4 (12)	4.4 (12)	2.7 (46)
1% human liver homogenate	5.0 (0)	3.8 (24)	3.3 (34)	1.3 (74)
10% human gut homogenate	5.0 (0)	2.3 (54)	1.3 (74)	<0.5 (>90)
10% human blood	5.0 (0)	3.3 (34)	2.3 (54)	0.6 (88)
90% human blood	5.0 (0)	1.2[b] (76)		
Buffered saline, pH 7.4	100 (0)			67 (33)
1% human liver homogenate	100 (0)	83 (17)	66 (34)	10 (90)
10% human liver homogenate	100 (0)	13[b] (87)		
10% human gut homogenate	100 (0)	54 (46)	34 (66)	11 (89)

[a]Figures in parentheses are per cent hydrolysis. [b]Ester concentration remaining after 20 min.

Table VII. Carbenicillin Blood Levels in the Squirrel Monkey for Esters of Carbenicillin[a]

Ester	Mean carbenicillin blood levels (μg/ml) at					Mean peak level (μg/ml) in humans[b]
	0.5 hr	1.0 hr	2.0 hr	4.0 hr	6.0 hr	
Ethyl	8.5	7.2	2.4			3.8
Benzyl	7.8	6.8	5.5			2.5
Isopropyl	5.3	5.3	3.1	<2.5	<2.5	5.5
n-Butyl	13.9	10.9	4.1	<1.5	<1.5	3.2
Isobutyl	12.5	9.6	3.6	<1.5	<1.5	7.2
n-Pentyl	7.3	6.8	2.0			<1.0
Phenyl	11.9	14.0	10.2			7.0
5-Indanyl	17.7	13.6	5.1			6.7
3-Methylphenyl	19.7	14.2	8.8	1.7	<1.5	5.8
4-Methylphenyl	15.0	11.4	3.2			3.6
2,5-Dimethylphenyl	4.6	3.8	2.4	<1.5	<1.5	4.4
3,4-Dimethylphenyl	10.0	8.0	3.4	<1.5	<1.5	3.9

[a]Single oral dose of 25 mg of ester/kg. [b]Taken from Table VIII.

The preferred compound of the 12 esters tested appeared to be the phenyl ester, on the basis of peak carbenicillin serum concentrations, area under the blood level curve, urinary excretion of carbenicillin, and absence of unhydrolyzed ester. This compound (carfecillin, BRL 3475) was therefore selected for detailed examination. In further bioavailability studies (to be reported elsewhere) the favorable pharmacokinetic properties of the compound were confirmed and studies with selected formulations (tablets and syrups) resulted in carbenicillin serum and urine levels significantly greater than those indicated in Table VIII.

As a result of these observations the phenyl ester of carbenicillin is currently undergoing clinical trial in the treatment of urinary tract infections.

Table VIII. Carbenicillin Blood Levels in Human Volunteers for Esters of Carbenicillin[a]

Ester	Mean carbenicillin serum levels (μg/ml) at 30 min	60 min	120 min	240 min	Urinary 0–6-hr excretion, % of dose: Carbenicillin	Unhydrolyzed ester
Ethyl	1.1	2.8	3.8	1.1	21	15
Benzyl	<1.0	1.9	2.5	1.3	15	8
Isopropyl	1.2	3.6	5.5	1.8	30	25
n-Butyl	1.3	3.2	3.0	1.1	19	8
Isobutyl	<1.0	6.6	7.2	1.9	36	10
n-Pentyl	<1.0	<1.0	<1.0	<1.0	6	11
Phenyl	5.2[b]	7.0	4.6	<1.0	33[c]	<2
5-Indanyl	1.5	3.7	6.7	1.9	34	<2
3-Methylphenyl	5.5[b]	5.8	4.1	<1.5	33	<2
4-Methylphenyl	2.6	3.0	3.6	<1.0	20	<2
2,5-Dimethylphenyl	1.7	4.4	4.3	1.0	26	<2
3,4-Dimethylphenyl	<1.5[b]	2.5	3.9	<1.5	29	<2

[a] Single oral dose of ester equivalent to 500 mg of carbenicillin. [b] 40-min assay figure. [c] A further 3.8% of the dose was recovered as the penicilloic acid of carbenicillin.

Experimental Section

Chemistry. All melting points were taken on a Büchi melting point apparatus and were not corrected. Nmr spectra were obtained on a Varian A-60 spectrometer using tetramethylsilane as an internal reference when dimethyl sulfoxide was the solvent and 3-trimethylsilylpropane sulfonic acid sodium salt when water was the solvent. Where analyses are indicated in the tables only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of theoretical values.

Monoesters of Phenylmalonic Acid (2). General Method. Phenylmalonic acid (27.0 g, 0.14 mol) in dry ether (80 ml) was treated with thionyl chloride (17.85 g, 10.8 ml, 0.15 mol) and dimethylformamide (2 drops). The mixture was heated in a warm water bath at reflux for 2 hr or until a clear solution was obtained. The solution was evaporated under reduced pressure to remove any residual thionyl chloride and hydrogen chloride and the oily residue redissolved in dry ether (80 ml). The solution was treated with the hydroxy compound (0.15 mol) and the mixture refluxed for 2 hr. The reaction mixture was cooled to room temperature and washed with water (20 ml). The organic layer was extracted with saturated sodium bicarbonate solution until the extracts were alkaline. The combined extracts were washed with ether (30 ml) and the wash was discarded. The aqueous layer was acidified with 5 *N* hydrochloric acid to pH 1. The precipitated oil was extracted with methylene chloride (3 × 100 ml). The combined organic extracts were washed with water (4 × 100 ml) to remove any unreacted phenylmalonic acid, dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. The oily residue crystallized on standing and was recrystallized from appropriate solvents.

α-Carboxy Esters of Carbenicillin (3). General Method. The monoester of phenylmalonic acid (0.01 mol) mixed with thionyl chloride (5 ml) was heated in a warm water bath at 70° for 1 hr. The solution was evaporated under reduced pressure, diluted with dry benzene (5 ml), and reevaporated to remove residual thionyl chloride. The crude acid chloride, dissolved in dry acetone (50 ml), was added to a solution of 6-aminopenicillanic acid (2.16 g, 0.01 mol) in water (50 ml), 1 *N* sodium hydroxide (10 ml), 1 *N* sodium bicarbonate (15 ml), and acetone (25 ml) cooled to 12°. The mixture was stirred at room temperature for 2 hr and washed with ether (3 × 50 ml) and the wash discarded. The aqueous solution was layered with fresh ether (50 ml) and acidified, with stirring, with 1 *N* hydrochloric acid to pH 2.0. The ether layer was separated and the aqueous layer reextracted with ether (2 × 50 ml). The combined ether extracts were washed with water (20 ml) and extracted with 1 *N* sodium bicarbonate until the combined extracts were at pH 7.0. The neutral aqueous extract was freeze-dried to give the penicillin sodium salt as a noncrystalline solid. Solution of the amorphous product in ethanol and standing at room temperature gave in some instances the penicillin sodium salt as a crystalline solid.

In Vitro **Hydrolysis Studies. Hydrolysis by Squirrel Monkey Tissue Preparations.** The liver and small intestine (pyloric to caecal junction) were obtained from a freshly killed squirrel monkey (death from CO_2 asphyxiation). The gut was washed through immediately with 0.05 *M* potassium phosphate buffer, pH 7.0, containing 0.15 *M* sodium chloride to remove any contents before the mucosa degenerated. The tissues were homogenized at 20% w/v in ice-cold buffered saline and further diluted with phosphate buffer, pH 7.0, before use. Reaction mixtures consisted of ester at a final concentration of 1 mg/ml in 2% w/v homogenate. Controls to determine aqueous hydrolysis rates consisted of ester at 1 mg/ml in 0.05 *M* potassium phosphate buffer, pH 7.0. Reaction mixtures were incubated at 37° and spotted (5 μl) onto 1-cm wide chromatography tape (Whatman Grade 1) at 0 and 150 min. Standard solutions of carbenicillin were prepared in homogenate or buffer and also spotted (5 μl) onto chromatography tapes. The chromatograms were developed overnight (16 hr) by descending chromatography in a *n*-butanol–ethanol–water (4:1:5 v/v top phase) system at 4°. The tapes were then dried and laid on agar plates (Oxoid blood agar base CM 55 3.5% w/v) seeded with spores of *Bacillus subtilis* ATCC 6633. The plates were incubated at 40° for 7 hr, after which time the zones of inhibition appearing at R_f 0.05 (carbenicillin) were measured.

In this solvent system the esters had R_f values of 0.5 and above. Zone diameters for the standards were plotted against log concentrations to give a linear standard line from which the concentrations of carbenicillin in the reaction mixtures were obtained. Percentage conversions were calculated using the formula $Mc/422 \times 10$, where M = molecular weight of sodium salt ester (molecular weight of disodium carbenicillin = 422) and c = concentration (μg/ml) of carbenicillin in the reaction mixture.

Hydrolysis by Human Liver Homogenate. Reaction mixtures consisted of ester (2.0 mg/ml) in 4% (w/v) human liver homogenate in 0.05 *M* potassium phosphate buffer, pH 7.0. The liver was obtained postmortem and stored at −20° until required when it was homogenized as described below. Control reactions consisted of ester at 2.0 mg/ml in 0.05 *M* potassium phosphate buffer, pH 7.0. Tests and controls were incubated at 37° and spotted onto chromatography tapes at 0, 30, 60, and 180 min. The chromatography and assay procedures were as above.

Hydrolysis of Carbenicillin Phenyl Ester by Human Tissue Preparations. Human liver and jejunum were obtained postmortem and immediately frozen and stored at −20° until needed. Before use they were thawed and homogenized for 60 sec at 5° in 0.05 *M* potassium phosphate buffer, pH 7.4, containing 0.15 *M* sodium chloride using an Ultra Turrax homogenizer (Janke and Kunkel) to give a 10% w/v homogenate. Blood was taken from healthy volunteers, heparinized (10 units of heparin/ml of blood), and diluted with the above buffered saline. Reaction mixtures consisted of the phenyl ester at 5.0 or 100 μg/ml in 10% v/v blood, 10% w/v gut homogenate, 1% w/v liver homogenate, or buffered saline (control). Reaction mixtures were incubated at 37° and assayed at 0, 30, 60, and 180 min for residual ester by the following procedure.

Ten milliliters of a 5.0 μg/ml reaction mixture or 1.0 ml of a 100 μg/ml reaction mixture was mixed with 0.1 *M* hydrochloric acid in a centrifuge tube, using a sufficient amount to bring the sample to pH 5.5. *n*-Butyl acetate (10 ml) at 0° was added, and the tube was sealed, shaken vigorously, and then spun at 38,000*g* for 5 min in a refrigerated centrifuge (*ca.* 5°) to separate the phases.

Five milliliters of the organic phase was removed and evaporated to dryness *in vacuo*. The residue was redissolved in 0.2 ml of *n*-butyl acetate and 50 μl spotted onto each of two antibiotic assay disks (Whatman 1.3 cm). Standard solutions of the phenyl ester were made up in blood, gut homogenate, or buffered saline at 0° and immediately extracted by the same procedure. Test and standard disks were placed on an agar plate (3.5% Oxoid blood agar base CM 55) seeded with *Sarcina lutea* NCTC 8340 and incubated overnight (16 hr) at 30° and the zones of inhibition were measured. Concentration of ester remaining in the reaction mixtures was obtained by reference to a standard line of zone diameter for ester against log concentration.

Absorption Studies. Absorption in Squirrel Monkeys. The esters were administered orally by intubation as solutions of the sodium salts in water (2 ml), at a dose of 25 mg/kg, to groups of five squirrel monkeys (Saimiri sciureus) weighing 500–1000 g. Venous blood samples were removed at 0.5, 1, 2, 4, and 6 hr after dosing. The blood samples were heparinized, stored at 4°, and assayed on the day of sampling.

Absorption in Human Volunteers. The compounds were administered in plain gelatin capsules without excipients to groups of ten healthy fasting volunteer subjects as a single oral dose corresponding to 500 mg of carbenicillin. Venous blood was taken 0.5, 1, 2, 3, and 4 hr after administration of the ester and urine was collected over a 6-hr period. In order to reduce hydrolysis *in vitro* sera were kept in an ice bath until microbiological assay which was usually commenced within 1 hr of collection of the specimens. The specimens of urine were examined for carbenicillin, unhydrolyzed ester, and penicilloic acid.

Microbiological Assay. The concentrations of carbenicillin found in body fluid specimens after administration of the esters were measured by standard large plate microbiological assay with *Pseudomonas aeruginosa* NCTC 10701 as assay organism.[6] This organism is sensitive to relatively low concentrations of carbenicillin but is insensitive to unhydrolyzed carbenicillin esters. Specimens of squirrel monkey blood or human sera were assayed against standard solutions of carbenicillin prepared in squirrel monkey blood or in pooled human serum. Urine specimens were assayed against carbenicillin standard solutions prepared in 0.05 *M* phosphate buffer, pH 7.0. The assay plates were incubated overnight at 30°, inhibition zone diameters measured, and the concentrations of carbenicillin derived from the standard lines.

Penicilloic Acid in Urine. The carbenicillin penicilloic acid concentrations in urine collected in the 6-hr period after administration of the esters to human volunteers were measured by an iodometric method.[7]

Unhydrolyzed Ester in Urine. The amounts of unhydrolyzed ester found in the urine of fasting subjects were measured by chromatography and bioautography, as described above (see section on hydrolysis by squirrel monkey tissue preparations). The inhibition zones formed by the urine specimens were measured and compared with those produced by buffer standard solutions of the esters.

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MICROBIOLOGY OF CARFECILLIN, THE α-PHENYL ESTER OF CARBENICILLIN

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Carbenicillin (α-carboxybenzylpenicillin) is a broad spectrum penicillin active against a wide range of bacteria including Pseudomonas aeruginosa and is widely used in the treatment of serious infections caused by pseudomonas or other Gram-negative bacilli. Carbenicillin is poorly absorbed by the oral route and is effective only after parenteral administration, but certain α-esters of the compound are absorbed after oral administration and undergo hydrolysis in the body to liberate carbenicillin (English et al, 1972; Clayton et al, 1975). This report describes the in vitro activity of one such ester, carfecillin.

Carfecillin (Sodium α-phenoxycarbonylbenzylpenicillin) is the α-phenyl ester of carbenicillin (Fig. 1) and since the ester group is in the side chain, the compound possesses a free carboxyl group in the C_3 position of the thiazolidine ring and can be expected to demonstrate antibacterial activity per se unlike those penicillin esters where the ester group is in the thiazolidine ring. In aqueous solutions the rate of hydrolysis of carfecillin to carbenicillin is dependent on the pH value of the solution. Thus at pH 8.0, the ½ life of carfecillin at 37°C at a concentration of 100μg/ml was 90 minutes, increasing to 150 minutes at pH 7.4, and to greater than 10 hours at pH 6.5. In nutrient broth, pH 7.4, the rate of hydrolysis was similar to that in buffer, pH 7.4. In contrast, the rate of hydrolysis of carfecillin to carbenicillin in human serum was much more rapid (½ life about 10 minutes) under the same experimental conditions. As a result of the activity of non-specific esterases present in serum and tissues carfecillin is hydrolised very readily to carbenicillin and so the activity of carfecillin in the clinical situation is entirely that of carbenicillin.

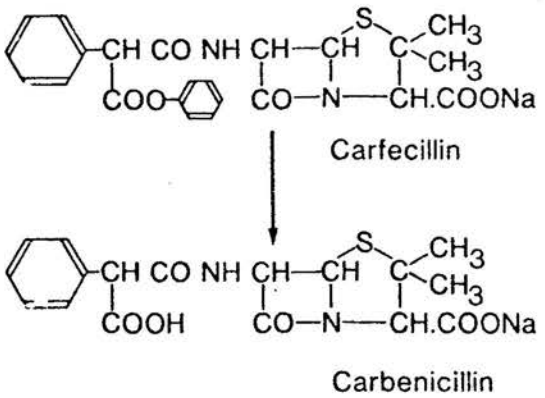

FIG. 1. Structure of carfecillin
(Sodium α-phenoxycarbonylbenzylpenicillin)

In tests in vitro for the measurement of antibacterial activity, the activity shown by carfecillin will depend, therefore, upon the extent to which hydrolysis to carbenicillin takes place. In general, conditions are more favourable for hydrolysis to carbenicillin in agar-dilution tests to measure Minimum Inhibitory Concentrations than in tests in liquid media. Against Gram-negative bacilli the Minimum Inhibitory Concentrations of carfecillin were generally about the same as, or slightly higher than, those of carbenicillin, but Klebsiella aerogenes was slightly more sensitive to carfecillin (Table 1). Against Gram-positive cocci, on the other hand, carfecillin was generally rather more active than carbenicillin. For example, carfecillin was twice as active as carbenicillin against penicillin-sensitive staphylococci and β-haemolytic streptococci, and 5 times more active against pneumococci and enterococci. However, results against the penicillin-resistant strain of Staphylococcus aureus suggest that carfecillin is less stable to staphylococcal penicillinase than is carbenicillin.

In the presence of serum the activity of carfecillin becomes that of carbenicillin as might be expected from the rapid rate of hydrolysis of carfecillin to carbenicillin in serum. This is illustrated by the results shown in Table 2 where it can be seen that in nutrient broth carfecillin was less active than carbenicillin against Ps. aeruginosa and more active than the latter against Staph. aureus, but in human serum the activities shown by the two compounds were the same.

Differences in activities of carfecillin and carbenicillin may be demonstrated more clearly in agar diffusion tests used for

TABLE 1. Minimum Inhibitory Concentrations of carfecillin and carbenicillin

Organism	MIC (μg/ml) Carfecillin	Carbenicillin
E. coli	5.0	2.5
P. mirabilis	2.5	1.25
P. morganii	5.0	5.0
Ps. aeruginosa	50	25
K. aerogenes	125	250
Enterobacter sp.	12.5	5.0
Ser. marcescens	12.5	5.0
S. aureus	0.25	0.5
S. aureus+	125	25
Str. pyogenes	0.05	0.1
Str. pneumoniae	0.05	0.25
Str. faecalis	5.0	2.5

+ β-lactamase producing strain

TABLE 2. Minimum Inhibitory Concentrations of carfecillin and carbenicillin in the presence of serum

Organism	Medium	Carfecillin	Carbenicillin
Staph.	nut. broth	0.25	1.0
aureus	serum*	1.0	1.0
Pseudomonas	nut. broth	125	50
aeruginosa	serum	50	50

* 95% human serum

microbiological assays. Against Ps. aeruginosa NCTC 10701, a strain used for the assay of carbenicillin, an aqueous solution (pH 6.0) of carfecillin was about one fifth as potent as carbenicillin (Fig. 2), but in human serum the carfecillin standard line was the same as that of the carbenicillin standard line as a result of the rapid rate of hydrolysis to carbenicillin. In contrast, against Sarcina lutea NCTC 8340, a penicillin assay organism, carfecillin was from 10 to 20 times more potent than carbenicillin.

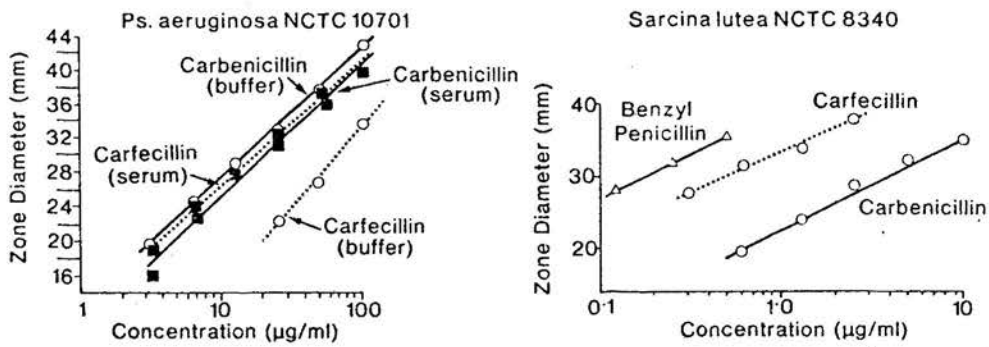

FIG. 2. Relative potencies of carfecillin and carbenicillin in microbiological assays against Ps. aeruginosa NCTC 10701 and Sarcina lutea NCTC 8340.

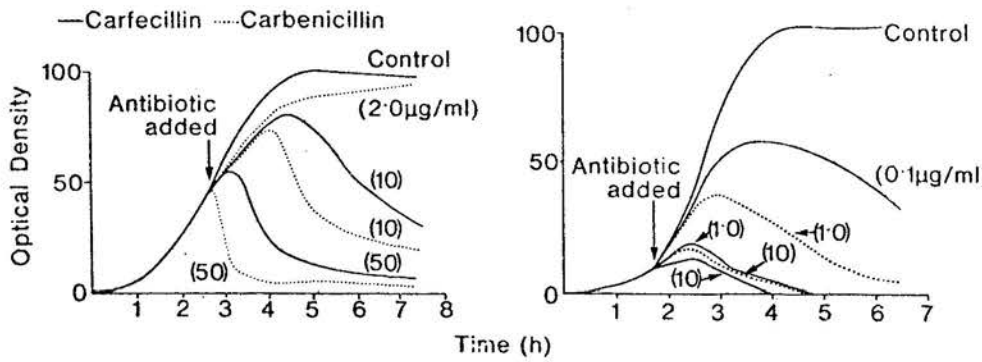

FIG. 3. Bacteriolytic activities of carfecillin and carbenicillin against E. coli (JT1) and S. aureus (Oxford)

A similar pattern emerged when the relative activities of carfecillin and carbenicillin were compared using a Bonet Maury Biophotometer continuous growth recorder. For instance, against a strain of E. coli (JT1) carbenicillin was more active than carfecillin as shown by the more rapid kill of carbenicillin compared with that of carfecillin (Fig. 3). However, against S. aureus (Oxford) the results were reversed and carfecillin produced a more rapid bacteriolytic effect than did carbenicillin (Fig. 3).

In conclusion, it is evident that carfecillin demonstrates an intrinsic antibacterial activity *in vitro* and is more active than carbenicillin against most Gram-positive cocci, but less active than the latter against Gram-negative bacilli. However, as carfecillin hydrolyses quite readily to carbenicillin in tests for antibacterial activity, the activity shown by carfecillin *in vitro* will be that of a mixture of carbenicillin and unhydrolysed ester, depending upon the extent to which hydrolysis takes place under the conditions of the test system. In the presence of serum or other body tissues, however, carfecillin is hydrolysed rapidly by non-specific esterases to carbenicillin and the activity shown in serum is entirely that of carbenicillin as is the case in the clinical condition (Wilkinson et al., 1975).

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Microbiology

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Carfecillin: Antibacterial Activity *in vitro* and *in vivo*

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Key Words. Carfecillin · Carbenicillin · Antibacterial activity · MIC data · Mouse infections

Abstract. Carfecillin, the α-phenyl ester of carbenicillin, hydrolyses rapidly in the presence of serum or body tissues to liberate carbenicillin but hydrolysis is less rapid in aqueous solution. The activity of carfecillin in antibacterial tests *in vitro* depends upon the extent of hydrolysis to carbenicillin, and in conventional serial dilution tests carfecillin shows an antibacterial spectrum generally similar to that of carbenicillin due to extensive hydrolysis. However, in tests in which the extent of hydrolysis is reduced, carfecillin displays lesser activity than carbenicillin against gram-negative bacilli and greater activity against gram-positive cocci. In the presence of serum carfecillin is hydrolysed rapidly to carbenicillin and the activity shown is solely that of carbenicillin. Unlike carbenicillin, carfecillin is well absorbed in mice after oral administration, producing significant carbenicillin blood concentrations and the compound is as effective by the oral route in the treatment of various experimental mouse infections as is parenteral carbenicillin.

Introduction

Carbenicillin is widely used in the treatment of serious infections due to *Pseudomonas aeruginosa* and other gram-negative bacilli, but it is poorly absorbed by the oral route and is effective only by parenteral administration. However, certain α-esters of carbenicillin have been shown to be well absorbed from the gastrointestinal tract after oral administration, undergoing hydrolysis in the body to liberate carbenicillin [1, 2].

[1] We wish to thank Dr. M. Cole and Miss Brenda Ridley for providing the hydrolysis data quoted here.

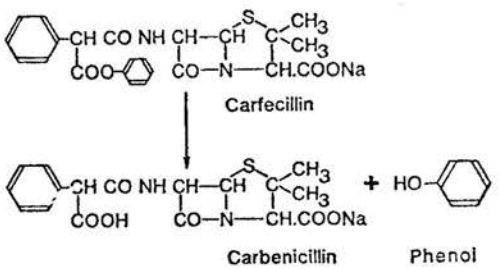

Fig. 1. Structure of carfecillin (sodium α-phenoxy carbonyl benzylpenicillin).

Carfecillin, the α-phenyl ester of carbenicillin, is one such ester which has been shown to produce relatively high concentrations of carbenicillin in man and to be effective in the treatment of urinary tract infections, including those caused by *Ps. aeruginosa* [5, 7].

Since the ester group of carfecillin is in the side chain (fig. 1) the compound is a penicillin with the free carboxyl group in the C_3 position of the thiazolidine ring essential for antibacterial activity. Consequently, carfecillin can be expected to demonstrate antibacterial activity *per se*, unlike penicillin thiazolidine esters which are devoid of intrinsic antibacterial activity.

In the presence of serum and body tissues, carfecillin hydrolyses rapidly to form carbenicillin as a result of the action of non-specific esterases [2] and so the activity shown by carfecillin in man is entirely that due to the carbenicillin liberated. In aqueous solution, hydrolysis is less rapid and the activity shown by carfecillin in antibacterial tests *in vitro* will depend not only upon the intrinsic antibacterial activity of the compound, but also upon the extent to which hydrolysis to carbenicillin takes place under the conditions of the test system. This report compares the antibacterial effects produced by carfecillin and carbenicillin *in vitro* under various experimental conditions influencing the extent of hydrolysis of carfecillin and *in vivo* in the treatment of experimental infections.

Materials and Methods

Carfecillin (Uticillin, Beecham Research Laboratories) is the sodium salt of α-(phenoxycarbonyl)benzylpenicillin (fig. 1) and the material used in these studies had an estimated potency of 960 μg/mg carfecillin sodium salt (equivalent to

760 μg/mg carbenicillin). Carbenicillin was used as the disodium salt (790 μg/mg free acid) and contained approximately 2.0% benzylpenicillin. In these tests the weights of carfecillin were adjusted to contain the equivalent amounts of carbenicillin.

Carfecillin is readily soluble in water and is comparatively stable at acid pH values, having a half-life in artificial gastric juice (pH 1.6) at 37 °C of approximately 2 h compared with 15 min for carbenicillin under the same conditions. The rate of hydrolysis of carfecillin to carbenicillin in aqueous solution is influenced greatly by the pH value of the medium, being much slower at acid pH than in alkaline conditions. Thus, the half-life of carfecillin in aqueous solution at 37 °C at a concentration of 100 μg/ml is greater than 10 h at pH 6.5, 150 min at pH 7.2 and 90 min at pH 8.0 (Beecham Research Laboratories, unpubl. data). Accordingly, in the tests described below, carfecillin solutions were prepared in 0.05 M phosphate buffer, pH 6.0, immediately prior to dilution in agar or liquid media.

Antibacterial activity in vitro. Minimum inhibitory concentrations (MIC) of carfecillin and carbenicillin required to inhibit growth of the test organism for 18 h at 37 °C were measured by serial dilution in 20-ml volumes of nutrient agar (Blood Agar Base, Oxoid; pH 7.3) or in 5-ml volumes of nutrient broth (Nutrient Broth No. 2, Oxoid; pH 7.5). Agar plates were inoculated with 0.001 ml of undiluted overnight broth culture delivered by a multiple inoculating device. For tests in liquid medium, 0.03 ml of an overnight broth culture was added to each tube, an inoculum of approximately 10^7 cells. In experiments designed to measure the effect of pH on the activity of carfecillin and carbenicillin the compounds were tested in broth buffered with 0.05 M phosphate to pH 6.0 or pH 8.0.

The effect of serum on the antibacterial activities of the compounds was measured by determination of the MIC in nutrient agar and in nutrient agar containing human serum at a final concentration of 50% serum.

Activity against experimental infections. Albino mice (Olac strain, 18–22 g) were injected by the intraperitoneal route with 0.5 ml of a suspension in hog gastric mucin (1701-W, Wilson Laboratories, Chicago) of a dilution of an overnight broth culture (Veal Infusion, Difco) of the test organism standardised to give an infective inoculum of 100–1,000 MLD. The antibiotics were administered as a single dose immediately after infection to groups of 10 mice at each dose level. Carfecillin and carbenicillin were administered as aqueous solutions while ampicillin was given in acacia suspension for oral treatment. In experiments with *Ps. aeruginosa* the compounds were administered as divided doses at 0, 2, 4 and 6 h after infection. The numbers of animals surviving 4 days after infection were recorded and the dose of penicillin required to produce 50% protection of infected animals (Median Protective Dose, PD_{50}) was calculated by the graphical method of LITCHFIELD and WILCOXON [6].

The activities of carfecillin and carbenicillin were also determined against an experimental mouse pyelonephritis produced by intravenous injection of *Ps. aeruginosa, Proteus morganii* or *Escherichia coli* [3]. Mice (Olac strain, 18–22 g) were injected intravenously with 0.2 ml of a suitable dilution in veal infusion broth (Difco) of an overnight culture of the test organism. The animals were injected with approximately 2–5 × 10^8 cells of *Ps. aeruginosa* 11, 2–5 × 10^8 cells of *P. morganii* 14, or

approximately 10^8 cells of *E. coli* JT415. The renal virulence of the bacteria was enhanced by intramuscular administration of iron sorbitol citrate (Jectofer, Astra Chemicals Ltd.), 30 mg/kg, at 18, 42, 66 and 90 h after infection. Treatment with the penicillins started 20 h after infection and continued 4 times a day at 2-hourly intervals. Groups of 10 mice were killed at each dose level, 10–15 days after infection. The kidneys were removed aseptically and examined for macroscopic abscesses, and bacterial counts were made from homogenates prepared from the kidneys.

Antibiotic blood concentrations in mice. The oral absorption of carfecillin in mice was determined by measurement of the carbenicillin blood concentrations resulting from a single oral dose of carfecillin. Healthy mice (Olac strain, 20–25 g) were dosed orally with carfecillin, carbenicillin or ampicillin and killed in groups of five at 5, 10, 20, 40, 60 and 120 min after dosing. A further group of mice were dosed with carbenicillin by subcutaneous injections. Blood was collected from the axillary region and antibiotic concentrations of heparinised specimens were measured by large plate agar diffusion assay. *Ps. aeruginosa* NCTC 10701 was used as assay organism for samples from mice dosed with carfecillin and carbenicillin, and *Sarcina lutea* NCTC 8340 was used for the assay of ampicillin. Specimens from carfecillin-treated mice were assayed against carbenicillin standard solutions.

Results

Antibacterial Activity in vitro

Results in table I show MIC obtained with carfecillin and carbenicillin in agar-dilution tests against a range of gram-negative and gram-positive bacteria. In general, carfecillin was as active as, or was twofold less active than carbenicillin against gram-negative bacteria except that *Klebsiella aerogenes* was rather less resistant to carfecillin than to carbenicillin. Gram-positive cocci were generally more sensitive to carfecillin than to carbenicillin and the compound was twice as active as the latter against penicillin-sensitive strains of *Staphylococcus aureus* and was up to five times more active against β-haemolytic streptococci, pneumococci and enterococci. However, carfecillin was less active than carbenicillin against β-lactamase-producing strains of *S. aureus* suggesting that carfecillin was less stable to staphylococcal β-lactamase.

The activity of carfecillin *in vitro* was due in part to hydrolysis to carbenicillin, as both compounds were demonstrated to be present in broth cultures during the course of serial-dilution tests. The half-life of carfecillin at a concentration of 100 μg/ml in sterile nutrient broth (pH 7.5) at 37 °C was about 3 h, and was slightly less in a broth culture of *Ps. aeruginosa* NCTC 10662 (Beecham Research Laboratories, unpubl. data). The

Table I. Minimum inhibitory concentrations of carfecillin and carbenicillin against gram-negative and gram-positive bacteria

Organism	MIC, μg/ml	
	carfecillin	carbenicillin
Escherichia coli	5.0	2.5
Salmonella typhi	2.5	2.5
Shigella sonnei	5.0	5.0
Klebsiella aerogenes	125	250
Enterobacter cloacae	5.0	5.0
Pseudomonas aeruginosa	100	50
Serratia marcescens	5.0	5.0
Proteus mirabilis	2.5	1.25
Proteus morganii	5.0	2.5
Haemophilus influenzae	0.5	0.5
Neisseria gonorrhoeae	0.02	0.02
Bacillus subtilis	0.5	0.5
Staphylococcus aureus	0.25	0.5
Staphylococcus aureus[1]	50–125	25
Streptococcus pyogenes	0.05	0.25
Streptococcus pneumoniae	0.1	0.5
Streptococcus faecalis	5.0	25

[1] β-Lactamase-producing strain.

addition of phenol at an equimolar concentration had no effect on the antibacterial activity of carbenicillin, and mixtures containing carbenicillin (4 parts) and phenol (1 part) showed the same activity as carbenicillin. The MIC of phenol against the test organisms were greater than 1,000 μg/ml in these tests, so it would be unlikely that phenol liberated as a result of hydrolysis of carfecillin would contribute to the antibacterial activity of carfecillin *in vitro*.

The effect of pH on the activity of carfecillin is illustrated in table II showing the MIC of carfecillin and carbenicillin in buffered nutrient broth at pH 6.0 and pH 8.0. In the examples shown carfecillin was rather less active against gram-negative bacilli at pH 6.0 than at pH 8.0. In the case of carbenicillin the differences in activity at the two pH values were not so great. In contrast, carfecillin was distinctly more active than carbenicillin against the penicillin-sensitive staphylococcus (strain T4) and against the enterococcus at pH 6.0, but at pH 8.0 carfecillin was less ac-

Table II. Effect of pH on minimum inhibitory concentrations of carfecillin

Organism	pH of medium	MIC, μg/ml	
		carfecillin	carbenicillin
E. coli 415	6.0	25	12.5
	8.0	6.3	6.3
Ps. aeruginosa	6.0	250	100
NCTC 10662	8.0	25	25
Ent. aerogenes	6.0	25	12.5
T764	8.0	6.3	6.3
Str. faecalis 1347	6.0	1.6	12.5
	8.0	25	25
S. aureus T4	6.0	0.4	1.6
	8.0	1.6	1.6
S. aureus T 142	6.0	< 100	25
	8.0	25	25

Table III. Effect of human serum on the antibacterial activity of carfecillin

Organism	MIC, μg/ml			
	carfecillin		carbenicillin	
	nutrient agar	agar + 50% serum	nutrient agar	agar + 50% serum
Ps. aeruginosa NCTC 10662	125	50	50	50
S. aureus NCTC 6571	0.5	1.0	1.0	1.0
Str. faecalis 1347	3.1	50	25	50

tive and showed the same activity as carbenicillin against these organisms. Against the β-lactamase-producing strain of *S. aureus* T142, carfecillin was less active in the acid medium but showed the activity of carbenicillin in the alkaline medium. Since carfecillin hydrolyses to carbenicillin more rapidly at alkaline pH values than under acid conditions, these results suggest that unhydrolysed carfecillin is generally less active than carbenicillin against gram-negative bacteria and β-lactamase-producing strains

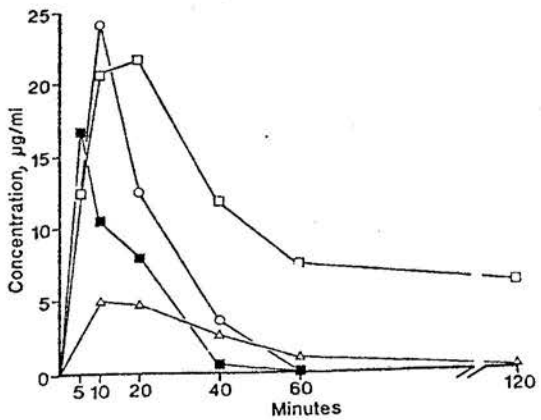

Fig. 2. Carbenicillin blood concentrations after oral administration of carfecillin to mice: □, ■ = carfecillin (oral), 400 and 100 mg/kg; △ = ampicillin (oral), 200 mg/kg; O = carbenicillin (subcutaneous), 50 mg/kg.

of *S. aureus,* but is more active than carbenicillin against sensitive strains of gram-positive cocci.

Effects of serum on antibacterial activity. Results in table III show the MIC of carfecillin and carbenicillin in nutrient agar and in agar containing 50% human serum, and it can be seen that the antibacterial activity of carfecillin became that of carbenicillin in serial dilution tests involving media containing serum. For example, in the absence of serum, carfecillin was rather less active than carbenicillin against *Ps. aeruginosa,* was rather more active against *S. aureus* and was distinctly more effective against *Str. faecalis.* In the presence of serum, however, carfecillin was as active as carbenicillin against all three organisms, showing an increase in activity against the pseudomonas and reduced activity against the gram-positive cocci. These results illustrate the rapid rate of hydrolysis of carfecillin by serum to liberate carbenicillin.

Activity in vivo

Absorption in mice. The carbenicillin blood levels produced in mice with single oral doses of carfecillin are shown in figure 2, and for comparison, values obtained with a single oral dose of ampicillin and a subcutaneous dose of carbenicillin are also presented. In general, carfecillin was well absorbed after oral administration and a single dose of 100 mg carfecillin/kg produced blood antibiotic concentrations notably higher

Table IV. Relative activities of carfecillin and carbenicillin against intraperitoneal mouse infections

Organism	MIC, μg/ml (carbenicillin)	PD_{50}, mg/kg		
		carfecillin oral	carbenicillin	
			oral	subcutaneous
E. coli 8	2.5	374	>1,000	125
E. coli 9	5.0	110	> 400	230
Salm. typhimurium 10	2.5	187	490	130
P. mirabilis 13	2.5	56	200	80
K. edwardsii 18	12.5	97	300	115
Ser. marcescens US1	12.5	53	210	100
Ps. aeruginosa 11	50	580	>4,000	400
		600	>4,000	208
Ps. aeruginosa 30	50	155	>4,000	30
		194	>4,000	155
S. aureus (Smith)	1.25	13	19	4
S. aureus 1555	50	>1,000	>1,000	600
Str. pyogenes CN10	0.25	100	120	80

[1] Median protective dose.

than those obtained with ampicillin at twice that dose (200 mg/kg). Mean peak carbenicillin concentrations of 17 μg/ml were measured 5 min after an oral dose of 100 mg carfecillin/kg and of 22 μg/ml after 20 min with a dose of 400 mg/kg to normal mice. At the lower dose, carbenicillin was not detected after 60 min but with the higher dose of carfecillin, relatively high concentrations of carbenicillin (7.8 μg/ml) were present at 120 min after administration. In contrast to these findings with carfecillin, administration of an oral dose of 400 mg carbenicillin/kg generally failed to produce detectable concentrations in the blood of mice, and variable levels of carbenicillin were measured in about 50% of animals receiving an oral dose of 1,000 mg carbenicillin/kg. Subcutaneous injection of carbenicillin (50 mg/kg) produced peak concentrations at 10 min rather higher than those produced by oral carfecillin (400 mg/kg) but the antibiotic levels fell more rapidly.

Intraperitoneal infections. The efficacy of carfecillin in the treatment of experimental mouse intraperitoneal infections produced by various gram-negative and gram-positive bacteria is shown in table IV in comparison with carbenicillin. In these tests carfecillin was given as a single oral

Table V. Relative activities of carfecillin, carbenicillin and ampicillin against mouse intraperitoneal infections due to ampicillin-resistant bacteria

Organism	MIC, μg/ml		PD_{50}, mg/kg			
	carbenicillin	ampicillin	carfecillin oral	carbenicillin oral	carbenicillin subcutaneous	ampicillin oral
E. coli 415	5.0	125	75	>1,000	70	>1,000
E. coli 487	12.5	250	240	>1,000	100	>1,000
P. morganii 14	5.0	250	31	>1,000	33	>1,000

dose immediately after infection; carbenicillin was administered orally or by subcutaneous injection. Carfecillin was markedly more active against the gram-negative bacilli than was carbenicillin when the latter was administered by the oral route, and carfecillin generally showed activity of the same order as subcutaneous carbenicillin against these organisms. However, against the sensitive strains of gram-positive cocci, namely *S. aureus* (Smith) and *Str. pyogenes* CN 10, oral carbenicillin was as effective as carfecillin and this may have been due, in part, to the presence of benzylpenicillin in the carbenicillin. Neiter carfecillin nor carbenicillin were active against the infection due to the penicillinase-producing strain of *S. aureus*.

The activities of carfecillin and carbenicillin were also determined against intraperitoneal infections due to two ampicillin-resistant strains of *E. coli* and an ampicillin-resistant strain of *P. morganii*. Ampicillin and carbenicillin were ineffective by the oral route whereas oral carfecillin was as active as subcutaneous carbenicillin in the treatment of these infections (table V).

Experimental mouse pyelonephritis. The results of treatment with carfecillin and carbenicillin of experimental mouse pyelonephritis infections due to *Ps. aeruginosa, P. morganii* and *E. coli* are illustrated in figure 3. In these infections, 90% of the kidneys of untreated mice showed macroscopic abscess formation and the bacterial counts of kidney homogenates from these animals ranged from 10^4 to 10^8 bacteria/ml of homogenate. Therapy with oral carbenicillin was ineffective against the pseudomonas infection, and the kidney bacterial counts and the macroscopic appearance of the kidneys of treated mice were the same as in the untreated animals. In contrast, treatment with parenteral carbenicillin was efficacious in protecting the mice and at the higher dose level (500 mg/kg), only one animal

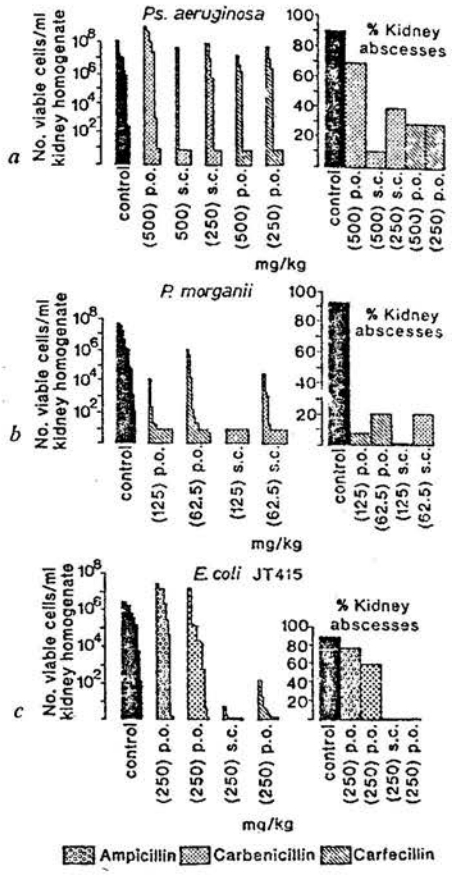

Fig. 3. Relative activities of carfecillin, carbenicillin and ampicillin in the treatment of experimental pyelonephritis in mice: (a) *Pseudomonas aeruginosa;* (b) *Proteus morganii*; (c) *Escherichia coli.*

showed visible evidence of infection due to the presence of a high bacterial kidney count, while the remainder of the group were free from infection. The dose of 250 mg carbenicillin/kg was rather less active, protecting 60% of the animals. Oral treatment with carfecillin was about as effective as subcutaneous administration of carbenicillin and with both doses only 30% of the animals showed evidence of infection Similarly, oral carfecillin was very effective in the treatment of the Proteus infection and showed activity of the same order as parenteral carbenicillin. Thus, at both dose levels, in most animals there was no evidence of macroscopic kid-

ney lesions and the kidney bacterial counts of the treated animals were of a low order compared with untreated animals. The strain of *E. coli* JT415 used to produce the experimental pyelonephritis was insensitive to ampicillin (MIC 125 μg/ml) and sensitive to carbenicillin (MIC 12.5 μg/ml) *in vitro* and this was reflected in the results of therapy. Ampicillin and oral carbenicillin showed poor activity and had little effect on the course of the infection, whereas oral carfecillin and subcutaneous carbenicillin were equally efficacious in protecting the mice and at both dose levels there was slight evidence of infection.

Discussion

The antibacterial activity of carfecillin *in vitro* is largely of academic interest, because in clinical use the compound is present in the body solely as carbenicillin, a a result of the rapid hydrolytic activity of non-specific esterases present in tissues and body fluids. However, hydrolysis of carfecillin is less rapid *in vitro* and the antibacterial activity seen in antibacterial tests *in vitro* is always a composite of carbenicillin liberated from carfecillin and unhydrolysed carfecillin. Consequently, the antibacterial activity of carfecillin *in vitro* can only be assessed by comparison with that shown by carbenicillin under the same conditions.

The results of the comparative tests reported here show that carfecillin can function as a penicillin in its own right as might be predicted from its structure. The finding that carfecillin is generally less active than carbenicillin against gram-negative bacilli, with *K. aerogenes* as an exception, and is more active against sensitive gram-positive cocci, is in agreement with results reported for carbenicillin indanyl sodium, another a-ester of carbenicillin [4]. The effect of human serum on the antibacterial activity of carfecillin illustrates the rapid hydrolysis of the ester by body fluids and is indicative that the activity to be expected *in vivo* after administration of carfecillin would be solely that of carbenicillin liberated from the ester.

The results of the chemotherapeutic tests show that carfecillin is well absorbed after oral administration to infected animals and confirm that the levels of carbenicillin liberated in the animal are adequate for the treatment of infection. In fact, it is of interest that therapy with orally-administered carfecillin was as effective as parenteral carbenicillin against the intraperitoneal infections and against the experimental pyelonephritis. These findings suggest that carfecillin should be a useful addition to the

group of antibiotics available for the oral treatment of infections due to gram-negative bacilli.

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INSTABILITY OF AZLOCILLIN AND MEZLOCILLIN TO THE β-LACTAMASES OF PSEUDOMONAS AERUGINOSA AND KLEBSIELLA AEROGENES

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R. A. Edmondson and R. Sutherland

The introduction of an acidic group in the side chain of the penicillin nucleus, resulted in the discovery of the broad-spectrum antipseudomonas penicillins, carbenicillin, sulbenicillin and ticarcillin. At a later stage certain acylated derivatives of ampicillin, ureidopenicillins, were also found to demonstrate significant activity against Pseudomonas aeruginosa. A number of these compounds have been reported and have advanced to clinical trial, including BL-P1654, pirbenicillin, apalcillin and piperacillin. Recently, two closely related ureidopenicillins, azlocillin and mezlocillin have been introduced into clinical practice in Germany, the former with claims to having a high level of activity against Pseudomonas aeruginosa, the latter as being effective against Klebsiella aeruginosa. It is the purpose of this report to compare the antibacterial properties of these two ureidopenicillins with the α-carboxypenicillin, carbenicillin.

Typical MIC values of azlocillin, mezlocillin and carbenicillin against selected bacteria are shown in Table 1 and it can be seen that azlocillin is more active than mezlocillin and carbenicillin against Ps. aeruginosa but rather less active than the other two compounds against Escherichia coli, Proteus mirabilis, indole positive Proteus and Enterobacter species. In contrast, mezlocillin has activity similar to that of carbenicillin against many of the Gram-negative bacteria, is slightly less active than the α-carboxy penicillin against Ps. aeruginosa but rather more effective against K. aerogenes.

Table 1

Antibacterial spectrum of azlocillin, mezlocillin and carbenicillin

Organism	MIC (μg/ml)		
	azlocillin	mezlocillin	carbenicillin
E. coli	12.5	2.5	2.5
K. aerogenes	50	12.5	250
Ps. aeruginosa	12.5	125	50
P. mirabilis	12.5	2.5	1.25
Indole + Proteus	50	5.0	5.0
Ent. cloacae	25	5.0	5.0
S. aureus	0.5	1.25	1.25
Str. faecalis	1.25	1.25	25

All three compounds show similar activity against penicillin-sensitive staphylococci and the ureidopenicillins are more active than carbenicillin against Streptococcus faecalis. β-Lactamase-producing strains of E. coli, P. mirabilis and Staphylococcus aureus are resistant to the penicillins.

The activity of azlocillin against Ps. aeruginosa is, however, affected greatly by inoculum size and results in Table 2 show the distribution of MIC values of azlocillin and carbenicillin measured in serial dilution tests in agar against 55 strains of Ps. aeruginosa. Against an inoculum of 10^3–10^5 c.f.u., azlocillin showed a high level of activity and inhibited 64% of strains at a concentration of 5.0 μg/ml and 85% of strains by 25 μg/ml. Carbenicillin was distinctly less active in these tests, only 30% were inhibited by a concentration of 25 μg/ml. On the other hand, when the inoculum size was increased one hundred-fold, to 10^5–10^6 c.f.u. the difference in the antibacterial activities of the two compounds was much less marked.

Table 2

Cumulative % inhibition of Ps. aeruginosa (55 strains) by azlocillin and carbenicillin

Compound	Inoculum (c.f.u.)	MIC (μg/ml)							
		≤2.5	5.0	12.5	25	50	125	250	>500
azlocillin	10^3	35	67	78	85	91	100		
carbenicillin	10^3		<1	13	38	64	85	91	100
azlocillin	10^5		<1	22	38	64	78	85	100
carbenicillin	10^5			<1	18	40	71	87	100

Thus, at a concentration of 50 μg/ml, 64% of the strains were inhibited by azlocillin compared with 40% of strains inhibited by carbenicillin. Both compounds produced comparable effects at a concentration of 125 μg/ml, inhibiting approximately 75% of the test strains.

The inoculum effect shown by azlocillin was even more marked in tests in liquid medium and the activity of the ureidopenicillin became progressively less as the inoculum was increased until at an inoculum of 10^7 cells/ml azlocillin became less active than carbenicillin.

Table 3

Effect of inoculum size on the activities of azlocillin and carbenicillin in nutrient broth against 2 strains of Ps. aeruginosa

Inoculum (cells/ml)	MIC (μg/ml)			
	NCTC 10662		1058	
	azlocillin	carbenicillin	azlocillin	carbenicillin
10^4	2.5	25	2.5	25
10^5	2.5	25	5.0	25
10^6	50	25	25	25
10^7	250	125	>500	250

In these tests, when samples from the tubes containing the highest antibiotic concentration that failed to prevent visible growth were assayed for residual penicillin it was found that most of the original azlocillin content had been destroyed, whereas the concentration of carbenicillin remained unchanged.

The variation in antibacterial activity of azlocillin in response to inoculum size might reflect instability of the test compound to the β-lactamase and the stability of azlocillin and carbenicillin were measured against a preparation of β-lactamase produced by a strain of Ps. aeruginosa. (Table 4)

Table 4

Stability of azlocillin and carbenicillin to β-lactamase of Ps. aeruginosa 1058

Incubation time (min)	Residual antibiotic (%)†			
	azlocillin		carbenicillin	
	5 BCD*	0.5 BCD	5 BCD	0.5 BCD
0	0	85	100	100
30	0	70	100	100
60	0	48	100	100
120	0	0	100	100

† Substrate concentration, 200 µg/ml

* 1 BCD = enzyme present in an overnight broth culture

In this experiment, the cells were grown in the presence of 100 µg benzylpenicillin/ml to induce β-lactamase production and an ultrasomatic preparation of an overnight culture was used as source of enzyme. It can be seen that against an enzyme concentration equivalent to five times the cell density of an overnight culture, azlocillin was

apparently so unstable that no antibacterial activity was detected in the zero time sample. A decimal dilution of the β-lactamase preparation inactivated the penicillin at a slower rate but nevertheless, 50% of the compound was destroyed in 1 hour and complete destruction occurred by 2 hours. In contrast, carbenicillin was very stable to the β-lactamase and even the concentrated enzyme concentration caused no detectable destruction of the compound during the 2 hour incubation period.

The relatively low stability of azlocillin to Ps. aeruginosa β-lactamase may afford an explanation, in part, at least, for the apparent poor bactericidal activity of the compound against Ps. aeruginosa. For instance, results in Table 5 show a much greater difference between the MIC and MBC values of azlocillin against Ps. aeruginosa compared with carbenicillin. In contrast, this effect was not observed with Escherichia coli.

Table 5

Relative bactericidal activities of azlocillin and carbenicillin

Organism	(μg/ml)			
	azlocillin		carbenicillin	
	MIC	MBC	MIC	MBC
Ps. aeruginosa 10662	5.0	>500	125	500
T1099	2.5	>500	50	250
T1044	2.5	>500	50	250
E. coli JT1	12.5	25	2.5	2.5

There was also some evidence that the activity of azlocillin against Ps. aeruginosa mouse intraperitoneal infections was more

variable than that of carbenicillin, although it was not possible to alter the size of the infective inoculum *in vivo* to the same extent as *in vitro*. In these infections, too small an inoculum was relatively avirulent, too large an inoculum produced a lethal infection which could not be treated effectively, and the difference in the size of inocula producing these effects was relatively small. Nevertheless, data in Table 6 illustrate that azlocillin was relatively more effective against the infections produced by the small inocula compared with results produced against larger inocula, whereas this effect was not as pronounced in the case of carbenicillin.

Table 6

Relative activities of azlocillin and mezlocillin against Ps. aeruginosa intraperitoneal mouse infections

Strain	No. of experiments	Mean CD_{50} (mg/kg) azlocillin		carbenicillin	
		A*	B+	A	B
31	2	24	148 (6.1)†	145	360 (2.4)
11	2	34	144 (4.2)	294	640 (2.1)
4	3	39	933 (23)	101	546 (5.4)

* A, inoculum 3-8 x 10^5 c.f.u.

\+ B, inoculum 2-4 x 10^6 c.f.u.

† ratio B/A.

In a similar fashion, the activity of mezlocillin against various bacteria, including Ps. aeruginosa and K. aerogenes was greatly influenced by inoculum size. It will be remembered that this compound was rather less active than azlocillin against Ps. aeruginosa but more

effective against other Gram-negative bacteria and, of particular interest, displayed activity against K. aerogenes. However, the activity of mezlocillin against K. aerogenes was variable, being influenced greatly by inoculum size. For instance, against an inoculum of 10^3–10^4 c.f.u., azlocillin was active against 65% of the strains at a concentration of 2.5 µg/ml and against 85% of strains by 12.5 µg/ml. However, when the inoculum was increased one hundred fold, to 10^5–10^6 c.f.u., the ureidopenicillin was only moderately effective and the majority of strains were inhibited only by concentrations of 50–125 µg azlocillin/ml. Ampicillin also showed an inoculum effect so that 75% of the strains were inhibited by a concentration of 25 µg/ml in the tests with the smaller inoculum but a concentration ten times higher was needed to produce the same effects against the larger inoculum.

Table 7

Cumulative % inhibition of K. aerogenes (20 strains) by mezlocillin, ampicillin and cefazolin

Compound	Inoculum (c.f.u.)	MIC (µg/ml)									
		<0.5	1.2	2.5	5.0	12.5	25	50	125	250	>500
mezlocillin	10^3	5	30	65	80	85	100				
ampicillin	10^3			5	10	25	75	80		100	
cefazolin	10^3	25	85	95		100					
mezlocillin	10^5				10	20	40	65	75	80	100
ampicillin	10^5					10		25	45	75	100
cefazolin	10^5	15	60	70	90	100					

In contrast, the activity of the cephalosporin antibiotic, cefazolin, was not influenced by inoculum size and the compound was active against

90% of strains at a concentration of 5.0 μg/ml in tests with the large inoculum. Carbenicillin was inactive against K. aerogenes at both inocula tested.

The variation in activity of mezlocillin against K. aerogenes at various inoculum concentrations prompted β-lactamase studies with the compound, similar to those described previously with azlocillin and pseudomonas β-lactamase. In these tests mezlocillin was no more stable than was ampicillin and both penicillins were much less stable than cefazolin to the β-lactamase produced by K. aerogenes (Table 8).

Table 8

Stability of mezlocillin, ampicillin and cefazolin to β-lactamase of K. aerogenes NCTC 8172

Incubation time (min)	Residual antibiotic (%)*					
	mezlocillin		ampicillin		cefazolin	
	1 BCD†	0.2 BCD	1 BCD	0.2 BCD	1 BCD	0.2 BCD
0	100	100	100	100	100	100
15	0	70	0	58	100	100
30	0	32	0	30	100	100
60	0	0	0	0	100	100

* Initial substrate concentration 200 μg/ml

† 1 BCD = enzyme present in an overnight broth culture.

Against an enzyme concentration equivalent to that produced by an overnight broth culture, mezlocillin at 200 μg/ml was inactivated in 15 minutes, whereas cefazolin was completely stable to this preparation for 1 hour. A slower rate of destruction of mezlocillin occurred at a fifth dilution of this enzyme although destruction of the penicillin was complete at 1 hour.

The activity of mezlocillin against Klebsiellae was measured also *in vivo* and the effective values of the compound in protecting mice infected intraperitoneally with strains of *Klebsiella* of different mouse virulence are shown in Table 9. Against *K. aerogenes* I112, which had low mouse virulence, mezlocillin was only slightly less active than cefazolin but against the other two strains with increased mouse virulence the activity of mezlocillin *in vivo* decreased markedly and against the highly virulent strain *K. pneumoniae* 62, the penicillin was ineffective. In contrast, the activity of cefazolin was relatively constant.

Table 9

Relative activities of azlocillin and cefazolin against Klebsiella mouse infections

Organism	Inoculum	No. of experiments	Mean CD_{50} (mg/kg)	
			mezlocillin	cefazolin
K. aerogenes I112	6.0×10^6*	2	94	38
K. aerogenes T767	3.0×10^6†	2	520	74
K. pneumoniae 62	6.0×10^2†	2	>1600	144

* administered in mucin

† administered in broth

In summary, the data reported show that the antibacterial activities of the ureidopenicillins, azlocillin and mezlocillin, against Ps. aeruginosa and K. aerogenes are greatly influenced by inoculum size, and this is probably associated with the relative instabilities of these compounds to the β-lactamases produced by these bacteria. The significance of the antibacterial activity expressed by these ureidopenicillins against small inocula is open to question, but will be resolved by clinical experience.

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SULPHASOMIZOLE (5-*p*-AMINOBENZENESULPHONAMIDO-3-METHYL*ISO*THIAZOLE): A NEW ANTIBACTERIAL SULPHONAMIDE

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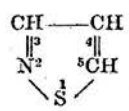
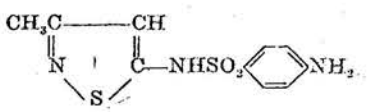
THE development of a controlled antibiotic policy, primarily to curb the spread of resistant strains of *Staphylococcus aureus*[1], has led to a renewed interest in sulphonamide therapy. Additional stimulus has also been provided by the introduction of the 'long-acting' sulphonamides, the main advantage of which is probably convenience of administration. The prolonged action of these new products may be attributable to differences in metabolic pathways brought about by differences in substitution in the heterocyclic moieties attached to the N^1 position of the classical structural types. The compounds of this class that have been used clinically during the past few years have all conformed to a traditional chemical pattern, their duration of action and rate of excretion being varied by variation of substituents in known heterocyclic nuclei.

Against this background, it is pertinent to report a new departure in sulphonamide research, in which a hitherto unknown heterocyclic nucleus—*iso*thiazole (I)—has been introduced. The full consequences of this development remain to be worked out, but sufficient work has been completed to show that this new sulphonamide, 5-*p*-aminobenzenesulphonamido-3-methyl*iso*thiazole (the name sulphasomizole has been approved by the British Pharmacopoeia Commission), is of considerable interest.



(I)

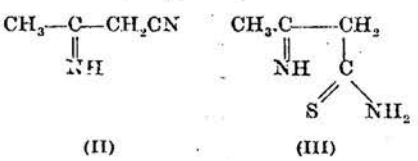


Sulphasomizole

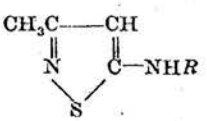
When our work began, we had no experimental evidence that the *iso*thiazole nucleus (I) could exist, but the system had long been of theoretical interest to us because of the similarity between —S— and —CH = CH— in aromatic structures. Thus, the new parent compound should resemble pyridine in many of its chemical properties[2].

We have synthesized mononuclear *iso*thiazoles by two distinct routes. The first involved the oxidation of 5-aminobenz-1,2-*iso*thiazole to *iso*thiazole-4,5-dicarboxylic acid[3], from which 4-*p*-aminobenzenesulphonamido*iso*thiazole was eventually prepared. This compound showed high activity against streptococcal and staphylococcal infections in mice, and the results stimulated us to devise a simpler synthesis of a related 5-sulphonamide.

Treatment of β-imino-*n*-butyronitrile (II) with hydrogen sulphide gave β-iminothio-*n*-butyramide (III)[4], which was oxidized by a variety of reagents to 5-amino-3-methyl*iso*thiazole (IV : R = H)[5].



(II) (III)



(IV)

The amine is a valuable intermediate for the preparation of many mononuclear *iso*thiazoles, and more than fifty derivatives of *iso*thiazole have been made[2]. One of the most interesting from the biological point of view is 5-*p*-aminobenzenesulphonamido-3-methyl-*iso*thiazole (IV : $R = SO_2.C_6H_4.NH_2$-*p*)[5], a sulphonamide of high solubility in water (*p*H 6·0, 243 mgm./100 ml.; *p*H 7·0, 2,260 mgm./100 ml.) from which almost neutral, concentrated (more than 50 per cent w/v) aqueous solutions of the corresponding sodium salt can easily be made.

The antibacterial properties of sulphasomizole have been determined by carrying out *in vitro* tests in a synthetic medium similar to that described by

Wilson[7]. Aqueous solutions of sodium sulphasomizole were used for these tests. After infection of the medium with a standardized inoculum and incubation at 37° C. for 24 hr., the minimal inhibitory concentrations were recorded.

The organisms included the following: *Staphylococcus aureus* (5 strains), *Streptococcus pyogenes* (2 strains), *Str. agalactiae*, *Str. dysgalactiae*, *Str. uberis*, *Str. pneumoniae*, *Str. equi*, *Str. viridans*, *Str. faecalis* (2 strains), *Alkaligenes faecalis*, *Aerobacter aerogenes* (2 strains), *Escherichia coli* (7 strains), *Klebsiella pneumoniae*, *Proteus vulgaris* (2 strains), *Pseudomonas pyocyanea* (2 strains), *Shigella flexneri*, *Shig. sonnei* (2 strains), *Salmonella typhi* (Rawlings), *S. paratyphi* B, *S. gallinarum*, *S. pullorum*, *S. typhi-murium*, *S. cholerae-suis*, *S. abortus-equi*, *Corynebacterium pyogenes*, *Haemophilus gallinarum*, *Pasteurella septica* and *Brucella abortus*. Sulphasomizole was active against most of this wide range of Gram-positive and -negative organisms, with the exception of one strain of each of the following organisms: *Str. faecalis*, *Aerobacter aerogenes*, *Shigella sonnei*, *Pasteurella septica*, and two strains of *Escherichia coli*; but other reference sulphonamides, tested under the same conditions, also did not show any activity against these particular strains.

Experiments were carried out in mice infected intraperitoneally with either *Str. pyogenes*, *Str. pneumoniae*, *Staph. aureus*, or *E. coli*. Sulphasomizole was given either by stomach tube at 1, 5, 24, 48 and 72 hr., or graded concentrations were mixed in the diet and fed from one day before to three days after infection. Interpretation of the results was based on two criteria: (1) determination of the median effective dose, from the survival-rates, and (2) the mean effective blood concentration (the concentration of the sulphonamide in the blood necessary for 50 per cent protection of infected mice)[8,9]. Sulphasomizole showed good activity against these experimental infections, and in general appeared as active as the sulphonamides in current use.

The absorption of sulphasomizole into the blood and cerebrospinal fluid, its distribution in the tissues and excretion in the urine were determined in several species. Estimations of the sulphonamide were made by the Bratton–Marshall method[10]. The sulphonamide was given orally or subcutaneously (as the sodium salt). After oral administration to mice, rats and

rabbits, it was well absorbed in doses up to 0·5 gm./kgm., and high concentrations of unconjugated drug were produced within half to 1 hr. (It should be emphasized that the term 'unconjugated' refers to the diazotizable material detected by the Bratton–Marshall method before hydrolysis, whereas the term 'conjugated' refers to the extra diazotizable material found after acid hydrolysis. Koechlin, Kern and Engelberg[11] prefer the terms 'directly' and 'indirectly' reacting.) The pattern of the blood concentration curves showed that the drug did not disappear rapidly from the circulation, appreciable amounts being present up to 24 hr. after administration; about 35 per cent was conjugated in the blood.

The sulphonamide was freely distributed in the tissues in rabbits. After an oral dose of 0·5 gm./kgm., maximum concentrations were found in the liver, lung, and spleen within about 2 hr., and at about 4 hr. in the kidney. In pregnant rabbits, sulphasomizole passed freely into the fœtus, and in lactating rabbits, into the milk.

Some information on the blood concentrations in man was obtained from three male volunteers and two patients. A single oral dose of 1 gm. in volunteers produced a maximum blood concentration of about 7·5 mgm./100 ml. within 4 hr., which fell only slightly by 6 hr. and had fallen to 2·0 mgm./100 ml. by 24 hr. About 40–50 per cent of the dose was recovered from the urine in 24 hr. In two patients each given 2 gm. of the sodium salt intramuscularly, the blood concentrations were 11·25 and 20·5 mgm./100 ml. (unconjugated and conjugated drug, respectively) in one patient after 9 hr., and in the other patient 15·7 and 27·2 mgm./100 ml. after 12 hr. These levels were amply maintained by repeated doses of 1 gm. at 12-hourly intervals.

In rats, after a single oral dose of 0·5 gm./kgm., about 25 per cent (of which 70 per cent was in the unconjugated form) was excreted in the urine during the first 24 hr. Excretion from rabbits was a little more rapid, about 35 per cent (of which 50 per cent was unconjugated) being found in the urine during the same period after an oral dose of 0·5 gm./kgm.

Sulphasomizole appeared in the cerebrospinal fluid of rabbits after oral administration of 0·5 gm./kgm. and the concentrations, relative to those found in the blood, were about 10 per cent. Intra-

venous injection of 0·2 gm./kgm., as a solution of the sodium salt, produced relatively high concentrations in the cerebrospinal fluid, about 85 per cent of the concentration in the blood. The difference between these two results is not fully understood.

Up to the present, we have only examined the concentration of free sulphonamide in the cerebrospinal fluid of one human patient suffering from meningococcal meningitis (James, E., Shiel, M., and Ardley, D. G., personal communication). 9 hr. after 2·0 gm. given intramuscularly as the sodium salt, the concentration in the cerebrospinal fluid was 1·85 mgm./100 ml. (16·5 per cent of the blood concentration). Two days later, after another four intramuscular injections, each of 1 gm., had been given, the concentration in the cerebrospinal fluid was 3·25 mgm./100 ml. 5 hr. after the last dose (17·2 per cent of the blood concentration).

In toxicity experiments, no deleterious effects were observed on the growth-rate of young rats or on the blood picture of guinea pigs, after daily oral doses of 0·5 gm./kgm. for several weeks. There was no significant crystalluria, even though the acetyl derivative of sulphasomizole is only sparingly soluble in water. Metabolic studies are being made to determine the nature of the urinary excretion products, and we have obtained some indication, in rats, that there is a marked increase in excretion of glycuronic acid, and a smaller increase in excretion of ethereal sulphate after continued administration of the sulphonamide.

Intramuscular injections of aqueous solutions of the sodium salt in concentrations as high as 20 per cent w/v are well tolerated in rats ; 5 per cent w/v solutions have no deleterious effect when instilled into the conjunctival sac of the rabbit's eye.

To sum up, this new sulphonamide has the attributes of a useful therapeutic agent. It possesses a good all-round antibacterial spectrum and is well absorbed and distributed in the body. It is readily excreted in the urine and animal experiments indicate that it does not produce crystalluria even in high dosage. The sodium salt is highly soluble in water and well tolerated on injection. Further experimental information will be published elsewhere.

Clinical studies of the new sulphonamide are in progress.

We wish to thank Drs. E. James and M. Shiel of

the Rush Green Hospital for the opportunity of estimating blood concentrations of this new sulphonamide in two patients.

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[5] British Provisional Patent Application No. 32259/57.
[6] British Provisional Patent Application No. 21628/56; 32302/56.
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Structure–Activity Relationships in Cephalosporins Prepared from Penicillins. 1. 7β-Acylamino Derivatives of 3-Benzyl- and 3-(3-Pyridylmethyl)ceph-3-em-4-carboxylic Acids

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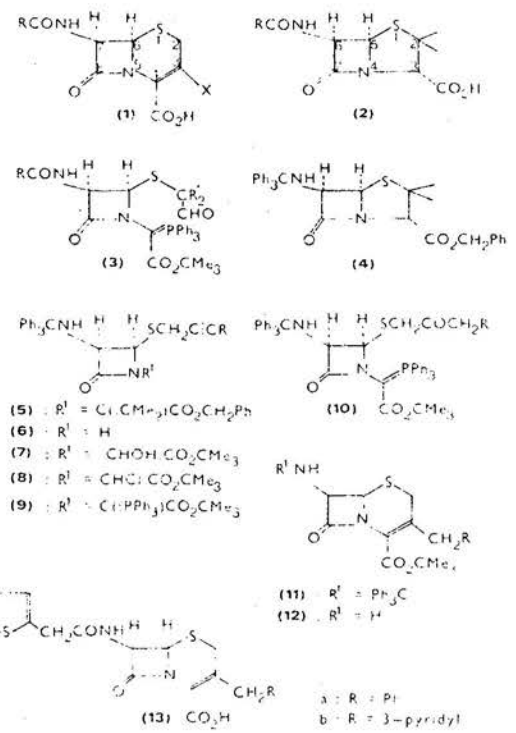
tert-Butyl 7β-aminoceph-3-em-4-carboxylates carrying either benzyl or 3-pyridylmethyl substituents at position 3 have been prepared by a multistep modification of the penicillin nucleus. Acylation of either amine, followed by deprotection, gave a range of new cephalosporins. The relationship between structure and antibacterial activity is discussed. D-Phenylglycine proved to be a preferred side chain in both series.

The very large number of cephalosporins (1) which have been described[1] includes many in which the 3-substituent has been subjected to chemical manipulation. In addition, certain cephalosporins have been prepared by chemical transformation of penicillins (2) utilizing reaction sequences which preserve the β-lactam ring and its associated stereochemistry. Thus Morin and his co-workers[2] devised an elegant route to 3-methylceph-3-ems (1, X = Me) based on a rearrangement of penicillin sulfoxides in which the 1,2 bond suffers cleavage. This route can be extended to yield ceph-3-ems in which functionality is introduced into the 3-methyl substituent, but it then becomes much more cumbersome.[3] A different multistep modification of the penicillin nucleus (2) involving initial cleavage of the 3,4 bond and subsequent cyclization of an aldehydophosphorane (3, R^1 = H or Me) provides 3-unsubstituted cephalosporins (1, X = H).[4]



We have developed a versatile route to a variety of 3-substituted cephalosporins based on the readily available penicillin derivative, benzyl 6β-(triphenylmethylamino)-penicillanate (4). Treatment of the latter with 3-substituted prop-2-ynyl bromides in the presence of strong base leads to 1,2-secopenicillanates[5] (5) which may be further modified via the stages 6, 7, 8, 9, and 10 to yield the ceph-3-ems 11 and 12. We have previously illustrated the route by describing[6] the synthesis of a 3-benzyl isostere (13a) of cephaloridine (13, R = pyridinium). In the work reported here we have used this general route to prepare a range of *N*-acyl derivatives of the 3-benzylceph-3-em nucleus and a further range of *N*-acyl derivatives of the novel 3-(3-pyridylmethyl)ceph-3-em nucleus. Acyl side chains were chosen so as to provide a variety of structural types, while including a reasonable selection of groups previously shown to confer useful antibacterial activity on semisynthetic penicillins[7] or cephalosporins.[1]

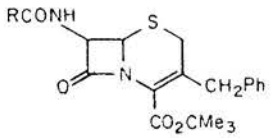
Chemistry. The amines **12a** and **12b** were treated with standard acylating agents (e.g., acid chloride, symmetrical or mixed anhydride, or carboxylic acid and dicyclohexylcarbodiimide) to give the acylamino *tert*-butyl esters listed in Tables I and II. The free cephalosporins were then liberated by treating the *tert*-butyl esters with trifluoroacetic acid. In those cases where the side chains were derived from amino acids, the amino group of the latter was protected by the *tert*-butoxycarbonyl group which was then removed at the same time as the nuclear ester group.

Biological Results and Discussion. The new cephalosporins were tested in vitro against a range of gram-positive and gram-negative bacteria in comparison with three clinically established cephalosporins, and results for representative organisms are given in Tables III and IV. All the compounds showed considerable activity against *Staphylococcus aureus* Oxford, as do the great majority of β-lactam antibiotics. Activity against the β-lactamase-producing *Staphylococcus aureus* Russell was inferior to that against the Oxford staphylococcus, sometimes considerably so. The differing ratios between the MIC values for these two strains indicate that some compounds are more susceptible than others to inactivation by staphylococcal β-lactamase.

Most of the 3-benzylcephalosporins (Table III) showed little or no activity against gram-negative bacteria, although the D-phenylglycyl compound **53** was moderately active and its *p*-hydroxy analogue **54** was about as active as cephalexin. The general level of activity against gram-negative bacteria was higher among the 3-(3-pyridylmethyl)cephalosporins (Table IV), which is in line with the general tendency among β-lactam antibiotics[1,7] for the more hydrophilic compounds to have a broader spectrum.

Compound **13b** and cephaloridine (13, R = pyridinium) are position isomers, although the former is a tertiary amine and the latter (which has better activity) a quaternary pyridinium salt. The D-phenylglycyl derivative **62** has the best broad-spectrum activity of all the new compounds, being more active than cephalexin and as active as cephalothin and cephaloridine against gram-negative bacilli. Two other compounds which are almost as active as **62** are the 2-thienyl analogue **64** and the D-mandelic acid derivative **57**.

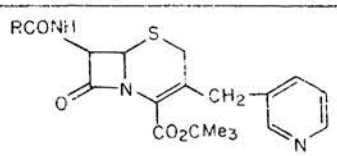
Table I. *tert*-Butyl 7β-Acylamino-3-benzylceph-3-em-4-carboxylates

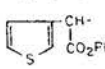
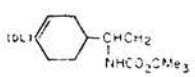


Compd	R	Acylation method	Mp, °C (solvent[a])	Formula	Analyses
14	Me	C	194–195 (E)	$C_{20}H_{22}N_2O_4S$	C, H, N
15	CF_3	C	157–158 (EA-P)	$C_{20}H_{21}F_3N_2O_4S$	M⁺
16	$NCCH_2$	B	Amorphous	$C_{21}H_{23}N_3O_4S$	C, H, N
17	$PhOCH_2$	A	Amorphous	$C_{26}H_{28}N_2O_5S$	M⁺
18	$EtSCH_2$	A	97–99 (E)	$C_{22}H_{28}N_2O_4S_2$	M⁺
19	$HO_2CCH_2SCH_2$	D	120–121 (EA-P)	$C_{22}H_{26}N_2O_6S_2$	C, H, N
20	(D)PhCHOH	E	Amorphous	$C_{26}H_{28}N_2O_5S$	M⁺
21	$PhCH(CO_2Ph)$	A	153–160 (EA-P)	$C_{33}H_{32}N_2O_6S$	C, H, N
22	C_5H_4N-*p*-CH_2	A	102 (E)	$C_{25}H_{27}N_3O_4S$	M⁺
23	C_5H_4N-*p*-SCH_2	B	207–208 (EA)	$C_{25}H_{27}N_3O_4S_2$	C, H, N
24		A	138–144 (B-P)	$C_{21}H_{24}N_6O_4S$	C, H, N, S
25	(D)MeCH($NHCO_2CMe_3$)	F	80–85 (P)	$C_{26}H_{35}N_3O_6S$	M⁺
26	(D)c-C_3H_5-CH($NHCO_2CMe_3$)	F	92–95 (P)	$C_{28}H_{37}N_3O_6S$	M⁺
27	(D)PhCH($NHCO_2CMe_3$)	F	152–153 (E-P)	$C_{31}H_{37}N_3O_6S$	C, H, N, S
28	(D)*p*-$HOC_6H_4CH(NHCO_2CMe_3)$	F	Amorphous	$C_{31}H_{37}N_3O_7S$	C, H, N

[a] B = benzene, E = ether, EA = ethyl acetate, P = petroleum ether (bp 60–80 °C). All compounds gave satisfactory NMR spectra and were homogeneous by TLC.

Table II. *tert*-Butyl 7β-Acylamino-3-(3-pyridylmethyl)ceph-3-em-4-carboxylates



Compd[a]	R	Acylation method	Formula	Analyses
29	$PhOCH_2$	A	$C_{25}H_{27}N_3O_5S$	M⁺
30	$EtSCH_2$	A	$C_{21}H_{27}N_3O_4S_2$	M⁺
31	(D)PhCHOH	E	$C_{25}H_{27}N_3O_5S$	C, H, N
32	C_4H_3S-*o*-CH_2	A	$C_{23}H_{25}N_3O_4S_2$	M⁺
33	C_5H_4N-*p*-CH_2	B	$C_{24}H_{26}N_4O_4S$	M⁺
34	C_5H_4N-*p*-SCH_2	B	$C_{24}H_{26}N_4O_4S_2$	M⁺
35		A	$C_{30}H_{29}N_3O_6S_2 \cdot H_2O$	C, H, N
36	(D)c-C_3H_5-CH($NHCO_2CMe_3$)	F	$C_{27}H_{36}N_4O_6S$	M⁺
37	(D)PhCH($NHCO_2CMe_3$)	F	$C_{30}H_{36}N_4O_6S$	M⁺
38		F	$C_{31}H_{42}N_4O_6S$	M⁺
39	(DL)C_4H_3S-*o*-CH($NHCO_2CMe_3$)	F	$C_{28}H_{34}N_4O_6S_2$	M⁺

[a] These esters were amorphous apart from 31 (mp 157–159 °C, from EtOAc–petroleum ether) and 35 (mp 105–107 °C from $CHCl_3$–Et_2O). However, all gave satisfactory NMR spectra and were homogeneous by TLC.

It is noteworthy that D-phenylglycine constitutes a preferred side chain in both the 3-benzyl series (Table III) and the 3-(3-pyridylmethyl) series (Table IV). In each series derivatives of aliphatic or cycloaliphatic amino acids are less active. D-Phenylglycine is, of course, the side chain of the leading semisynthetic penicillin, ampicillin, and of the orally effective cephalosporin, cephalexin.

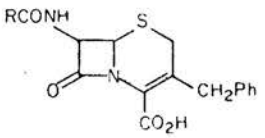
Experimental Section

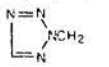
Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. ^1H NMR spectra were recorded on a Varian A-60 instrument for solutions in $CDCl_3$ with $SiMe_4$ as internal standard unless stated otherwise. Mass spectra were determined with an AEI MS9 machine. Compounds shown as having been analyzed either gave figures for the elements cited correct to within ±0.4% of the theoretical values or else gav accurate mass measurements correct to within ±5 ppm. Merc silica gel GF 254 was used for TLC and Merck silica gel H f column chromatography, with ethyl acetate–petroleum ether eluent. Petroleum ether refers to the fraction of bp 60–80 °

***tert*-Butyl 7β-Amino-3-benzylceph-3-em-4-carboxyla Toluene-*p*-sulfonic Acid Salt (12a).** This was prepared described by Nayler et al.[6]

***tert*-Butyl 7β-Amino-3-(3-pyridylmethyl)ceph-3-em- carboxylate (12b).** *tert*-Butyl glyoxylate monohydrate (1.93 13 mmol) and dry benzene (20 mL) were refluxed under nitrog in a Dean-Stark apparatus until all the water had been remove then (3*R*,4*R*)-4-[3-(pyridyl)prop-2-ynylthio]-3-(triphen methylamino)azetidin-2-one (**6b**)[5] (620 mg, 1.3 mmol) was add and the mixture refluxed under nitrogen for 3 h more, coole washed, dried, and evaporated. Chromatography gave the mix

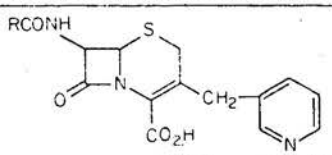
Table III. Antibacterial Activity of 7β-Acylamino-3-benzylceph-3-em-4-carboxylic Acids

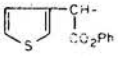
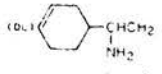


Compd	R	Minimum inhibitory concentration, μg/mL[a]						
		S. aureus Oxford	*S. aureus* Russell[b]	*E. coli*	*S. typhi*	*Sh. sonnei*	*K. aerogenes*	*P. mirabilis*
13a	C_4H_3S-*o*-CH_2	0.05	10	200	200	200	>200	>200
40	Me	0.5	2.5	>500	500	>500	>500	>500
41	CF_3	2.5	12.5	>500	>500	>500	>500	500
42	$NCCH_2$	0.12	0.5	500	250	500	>500	>500
43	$PhOCH_2$	0.02	0.12	>500	>500	>500	>500	500
44	$EtSCH_2$	0.12	1.25	>125	125	>125	>125	>125
45	$HO_2CCH_2SCH_2$	5	25	>500	250	250	>500	125
46	(D)PhCHOH	0.12	12.5	125	50	50	50	>500
47	$PhCH(CO_2Ph)$	2.5	25	>100	>100	>100	>100	50
48	C_5H_4N-*p*-CH_2	0.05	5	50	50	50	100	100
49	C_5H_4N-*p*-SCH_2	0.05	2.5	250	250	250	>500	250
50		0.12	1.25	>500	>500	125	500	125
51	(D)$MeCH(NH_2)$	5	10	>100	>100	>100	>100	>100
52	(D)c-C_3H_5-$CH(NH_2)$	5	12.5	>250	>250	>250	>250	>250
53	(D)$PhCH(NH_2)$	0.1	2.5	25	25	25	25	50
54	(D)*p*-$HOC_6H_4CH(NH_2)$	0.12	1.25	12.5	5	12.5	5	25
Cephalothin		0.12	0.5	5	12.5	5	5	5
Cephaloridine		0.02	1.25	5	1.25	1.25	2.5	5
Cephalexin		1.25	5	12.5	5	5	5	12.5

[a] Determined by serial dilution on nutrient agar using an inoculum of 0.001 mL of an undiluted overnight broth culture. MIC values were read after incubation at 37 °C for 18 h. [b] β-Lactamase-producing benzylpenicillin-resistant strain.

Table IV. Antibacterial Activity of 7β-Acylamino-3-(3-pyridylmethyl)ceph-3-em-4-carboxylic Acids



Compd	R	Minimum inhibitory concentration, μg/mL[a]						
		S. aureus Oxford	*S. aureus* Russell[b]	*E. coli*	*S. typhi*	*Sh. sonnei*	*K. aerogenes*	*P. mirabilis*
13b	C_4H_3S-*o*-CH_2	0.05	10	25	5	25	25	25
55	$PhOCH_2$	0.02	0.12	500	500	500	500	125
56	$EtSCH_2$	0.05	1.25	50	50	50	50	25
57	(D)PhCHOH	0.25	12.5	2.5	2.5	2.5	2.5	5
58	C_5H_4N-*p*-CH_2	0.12	25	25	10	25	10	10
59	C_5H_4N-*p*-SCH_2	<0.02	1	25	10	25	5	100
60		12.5		12.5	50	12.5	12.5	12.5
61	(D)c-C_3H_5-$CH(NH_2)$	5	12.5	500	250	250	250	500
62	(D)$PhCH(NH_2)$	0.25	2.5	2.5	1.25	2.5	2.5	5
63	 (DL)	0.5	1.25	125	125	125	125	250
64	C_4H_3S-*o*-$CH(NH_2)$	0.5	5	12.5	2.5	5	5	12.5
Cephalothin		0.12	0.5	5	12.5	5	5	5
Cephaloridine		0.02	1.25	5	1.25	1.25	2.5	5
Cephalexin		1.25	5	12.5	5	5	5	12.5

[a] Determined by serial dilution on nutrient agar using an inoculum of 0.001 mL of an undiluted overnight broth culture. MIC values were read after incubation at 37 °C for 18 h. [b] β-Lactamase-producing benzylpenicillin-resistant strain.

isomers of **7b** (510 mg, amorphous). This in dry dioxane (5 mL) and dry THF (5 mL) containing pyridine (202 mg) was cooled to −5 °C, treated dropwise with $SOCl_2$ (304 mg), and stirred at −5 to 0 °C for 15 min. The mixture was filtered and the filtrate and toluene washings were evaporated to give the mixed isomers of **8b** (436 mg, 0.7 mmol, amorphous). The latter in dry dioxane (10 mL) was treated with PPh_3 (368 mg, 1.4 mmol) and 2,6-lutidine (90 mg, 0.84 mmol), stirred 5 h at 50 °C under nitrogen, then cooled, diluted with ethyl acetate (100 mL), washed with brine, dried, and evaporated. Chromatography gave the phosphorane **9b** (262 mg, amorphous) which was refluxed in piperidine (5 mL) under nitrogen for 3 h and then evaporated. The gum was taken up in EtOAc, shaken with 0.1 N HCl (20 mL) for 5 min, and then basified with $NaHCO_3$ and the organic layer was separated, dried, and evaporated. Chromatography gave the ketone **10b** (197 mg, amorphous) which was refluxed in dry dioxane (5 mL) under

nitrogen for 8 h to give, after evaporation and chromatography, the cephem **11b** [95 mg (12%) from **6b**]: mp 169–171 °C (from MeOH); α^{25}_D −13.8° (*c* 1, $CHCl_3$), ν_{max} ($CHCl_3$) 1775, 1715 cm^{-1}; λ_{max} 263 nm (ε 10500) (EtOH). Anal. ($C_{36}H_{35}N_3O_3S$) C, H, N, S. Detritylation was effected by treating **11b** (95 mg, 0.16 mmol) in acetone (1 mL) at 0 °C with toluene-*p*-sulfonic acid monohydrate (68 mg, 0.36 mmol), stirring for 2 h at 0 °C and then 4 h at room temperature, and evaporating the mixture. The residue was taken up in EtOAc, washed with $NaHCO_3$ solution, dried, and chromatographed to give *tert*-butyl 7β-amino-3(3-pyridylmethyl)ceph-3-em-4-carboxylate (**12b**) as a foam (72%): ν_{max} ($CHCl_3$) 1775, 1715 cm^{-1}; δ ($CDCl_3$) 1.55 (9 H, s), 2.7 br (2 H, exch), 3.02 and 3.48 (2 H, AB q, *J* = 18 Hz), 3.44 and 4.08 (2 H, AB q, *J* = 15 Hz), 4.76 (1 H, d, *J* = 5 Hz), 4.99 (1 H, d, *J* = 5 Hz), 7.0–8.8 (Ar multiplet); M^+ 347.1292 ($C_{17}H_{21}N_3O_3S$ requires M 347.1303).

Acylation of *tert*-Butyl 7β-Aminoceph-3-em-4-carboxylates. Acylation of the amines **12a** and **12b** gave the amides listed in Tables I and II, all of which showed the spectroscopic properties typical of Δ^3-cephalosporin esters including λ_{max} (EtOH) 263–270 nm and ν_{max} ($CHCl_3$) 1780–1790 cm^{-1} (β-lactam CO) and 1710–1720 cm^{-1} (ester CO). The following acylation procedures are representative.

Method A (Acid Chloride). The toluene-*p*-sulfonic acid salt of **12a** (0.5 g, 0.96 mmol) and $NaHCO_3$ (0.4 g) were suspended in dry acetone (30 mL) and stirred at 0 °C while ethylthioacetyl chloride[8] (0.4 g, 2.9 mmol) was added dropwise. The mixture was stirred for 1 h at 0 °C, followed by 3 h at room temperature, and then evaporated. The residue, dissolved in benzene, was washed successively with cold 5% HCl, saturated $NaHCO_3$, and brine, then dried, and evaporated. Chromatography gave the ester **18** (0.23 g, 66.5%).

Method B (Carbodiimide). The amine **12a** (115 mg, 0.33 mmol) in CH_2Cl_2 (1.5 mL) was cooled in an ice bath and treated with *N,N'*-dicyclohexylcarbodiimide (65 mg, 0.31 mmol) in CH_2Cl_2 (1.0 mL). Cyanoacetic acid (30 mg, 0.35 mmol) in DMF (0.5 mL) was added dropwise. The mixture was stirred for 2 h, then diluted with CH_2Cl_2, washed with 0.5 N HCl, dried ($MgSO_4$), and evaporated. The residue was chromatographed on silica gel to give the cyanoacetamido derivative **16** (125 mg, 91%).

Method C (Symmetrical Anhydride). The toluene-*p*-sulfonic acid salt of the cephem **12a** (800 mg, 1.54 mmol) in dry CH_2Cl_2 (5 mL) was cooled in an ice bath and NEt_3 (0.30 mL) was added. Ac_2O (0.25 mL, 2.5 mmol) in $CHCl_3$ (15 mL) was added dropwise over 30 min, maintaining the temperature at 0–5 °C. The mixture was then allowed to warm to room temperature and washed successively with aqueous $NaHCO_3$ and brine, then dried ($MgSO_4$), and evaporated to leave a partially crystalline residue. Chromatography, followed by crystallization from ether, gave the acetyl derivative **14** (547 mg, 91%).

Method D (Cyclic Anhydride). 12a (520 mg) in CH_2Cl_2 at 0 °C was treated with a solution of thiodiglycolic anhydride[9] (198 mg, 1.5 mmol) in CH_2Cl_2. After 2 h at room temperature the solvent was removed by evaporation to give a foam (562 mg) which on trituration with ether gave the ester **19** as a solid.

Method E (*O*-Carboxyanhydride). D-Mandelic *O*-carboxyanhydride[10] (196 mg, 1.1 mmol) was added over 5 min to a stirred solution of the amine **12b** (347 mg, 1 mmol) in dry CH_2Cl_2 (20 mL) at −20 °C; then the mixture was stirred for 2 h at −20 °C and evaporated. Chromatography of the residue gave the cephem ester **31** (318 mg, 66%).

Method F (Mixed Anhydride). Redistilled $ClCO_2Me$ (100 mg, 1.05 mmol) in dry THF (15 mL) was stirred at −10 °C while a solution of *N*-(*tert*-butoxycarbonyl)-D-α-phenylglycine (261 mg, 1.05 mmol), NEt_3 (105 mg), and $PhCH_2NMe_2$ (1 drop) in dry THF (10 mL) was added over 5 min. The mixture was stirred at −10 °C for a further 25 min to complete formation of the mixed anhydride and then treated dropwise over 5 min with the amine **12a** (330 mg, 0.95 mmol, regenerated from toluene-*p*-sulfonate salt) in dry THF (5 mL). The mixture was stirred for 2 h at −10 °C, then NEt_3HCl was removed, and the filtrate evaporated. The residue was taken up in EtOAc and washed successively with water, dilute HCl, 5% $NaHCO_3$, and water, then dried, and evaporated. Chromatography gave the ester **27** (461 mg, 77%).

Deesterification of *tert*-Butyl Esters (12). (a) The ester **15** (359 mg) was dissolved in THF (6 mL) and, after 5 min at room temperature, the solution was evaporated in vacuo. Recrystallization of the residue from EtOAc–petroleum ether gave 3-benzyl-7β-trifluoroacetamidoceph-3-em-4-carboxylic acid (**41**): mp 181–182 °C; α^{23}_D −9.4° (*c* 1, $CHCl_3$); λ_{max} (EtOH) 260 nm (ε 9650); ν_{max} 1785 and 1725 cm^{-1}. Anal. ($C_{16}H_{13}F_3N_2O_4S$) C, H, N.

(b) Similar treatment of **22**, but with a reaction time of 40 min, gave the TFA salt of 3-benzyl-7β-(4-pyridylacetamido)ceph-3-em-4-carboxylic acid. This dissolved readily in water, but after some time the free cephalosporin **48** crystallized: mp 207–210 °C dec. Anal. ($C_{21}H_{19}N_3O_4S$) C, H, N.

(c) The ester **28** (662 mg) was dissolved in anhydrous TFA (10 mL), set aside at room temperature for 35 min, and evaporated in vacuo. After addition of toluene and repetition of the evaporation (three times), the residue was triturated with ether to give the TFA salt of 7β-(D-α-amino-*p*-hydroxyphenylacetamido)-3-benzylceph-3-em-4-carboxylic acid (**54**) (585 mg): mp 158–165 °C dec; α^{22}_D +47.1° (*c* 0.828, H_2O); λ_{max} (EtOH) 264 nm (ε 11100). Anal. ($C_{24}H_{22}F_3N_3O_7S$) C, H, N.

(d) The other esters in Tables I and II were similarly treated to give the free cephalosporins. Due to the small quantities available the end products were not fully characterized, but spectroscopic data indicated them to be sufficiently pure for antibacterial testing (see Tables III and IV).

Supplementary Material Available: High-resolution mass spectral data, NMR data, and elemental composition data (3 pages). Ordering information is given on any current masthead page.

References and Notes

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Structure–Activity Relationships in Cephalosporins Prepared from Penicillins. 2. Analogues of Cephalexin Substituted in the 3-Methyl Group

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A previously outlined general procedure for preparing various 3-substituted cephalosporins from the penicillin nucleus has been used, with modifications where required, to prepare a series of analogues of cephalexin with various substituents in the 3-methyl group. The 3-substituents most conducive to broad-spectrum antibacterial activity were 3-pyridylmethyl and *m*- or *p*-carboxybenzyl. The compounds were only poorly absorbed by the oral route in mice, but the 3-(carboxybenzyl) compounds gave more prolonged useful serum levels than the usual cephalosporins.

We have previously reported[1,2] a multistep procedure for converting the penicillin nucleus into cephalosporins. In the preceding paper[3] we described various 7β-acylamino derivatives of 3-benzyl- and 3-(3-pyridylmethyl)ceph-3-em-4-carboxylic acids. In both the benzyl and the 3-pyridylmethyl series the D-phenylglycyl derivative showed relatively good antibacterial activity against both gram-positive and gram-negative bacteria, so we decided to employ this acyl group as our derivative of choice for evaluating the potential of other cephem nuclei. Hence in this paper we report a series of analogues of cephalexin (12, X = H) with various substituents in the 3-methyl group.

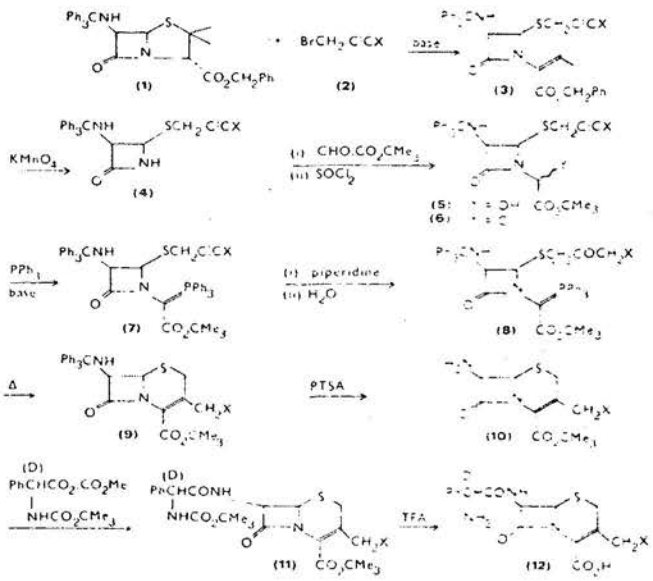
Chemistry. The various cephalexin analogues (12) were prepared as previously described[3] for the 3-benzyl and 3-(3-pyridylmethyl) analogues, i.e., by treating the appropriate *tert*-butyl 7β-aminoceph-3-em-4-carboxylate (10) with a mixed anhydride derived from *N*-(*tert*-butoxycarbonyl)-D-phenylglycine and then removing both acid-labile protecting groups by means of TFA. In those cases where the final cephalosporin contained a second carboxyl group as part of the 3-substituent, this function was also protected during the earlier part of the synthesis as a *tert*-butyl or *p*-methoxybenzyl ester and the final TFA treatment removed three protecting groups.

The requisite *tert*-butyl 3-substituted 7β-aminoceph-3-em-4-carboxylates (10) were synthesized from the penicillin derivative, benzyl 6β-(triphenylmethylamino)-penicillanate (1). In most cases the synthesis involved a precisely similar reaction sequence (Scheme I) to that previously used[1,2] to prepare *tert*-butyl 7β-amino-3-benzylceph-3-em-4-carboxylate (10, X = Ph).

The only case encountered of a 3-substituted prop-2-ynyl bromide (2) which failed to give a 1,2-secopenicillanate 3 on reaction with benzyl 6β-tritylaminopenicillanate (1) in the presence of base was the methoxycarbonyl compound 2 (X = CO_2Me). Hence, in order to synthesize 25 we prepared the unsubstituted prop-2-ynyl sulfide 7 (X = H) according to Scheme I, treated it with butyllithium followed by carbon dioxide to give the crude acid 7 (X = CO_2H), esterified the latter with diazomethane, and then again proceeded according to Scheme I.

A modified sequence was also used to prepare the 1-methyl-2-pyrrolyl derivative 20. For this purpose the prop-2-ynyl sulfide 3 (X = H) was treated with iodine nitrate to give the iodopropyne 3 (X = I), which was converted according to Scheme I into 7 (X = I). Treatment of the latter with 1-methyl-2-pyrrolylcopper(I) then gave 7 (X = 1-methyl-2-pyrrolyl). The remaining steps then followed Scheme I except that, because of the sensitivity of pyrroles to acids, we used pyridine hydrochloride instead of *p*-toluenesulfonic acid to detritylate 9 (X = 1-methyl-2-pyrrolyl). The route via 7 (X = I) should be capable of extension to other 3-aralkylcephems and would obviate the need to start with a different 3-substituted prop-2-ynyl bromide (2) in each case.

Scheme I



In one case the 3-substituent was modified after completion of the cephem ring system and introduction of the protected D-phenylglycine side chain. Thus, treatment of the cephem 45 with *p*-toluenesulfonic acid and isopropenyl acetate removed the tetrahydropyranyl group and replaced it by acetyl to give the 3-(β-acetoxyethyl)ceph-3-em 46.

Biological Results and Discussion. Table I shows the activity of the new cephalosporins against two gram-positive and five gram-negative bacteria in vitro, corresponding data for two clinically useful compounds with the same D-phenylglycyl side chain (cephalexin and cephaloglycin) being included for comparison.

Staphylococcus aureus Oxford typifies a gram-positive organism which is normally sensitive to β-lactam antibiotics and, as expected, all the compounds in Table I were reasonably active against it. They were rather less active against the β-lactamase-producing *Staphylococcus aureus* Russell, and the MIC figures show that a number of the new cephalosporins are less stable than cephalexin or cephaloglycin toward staphylococcal β-lactamase.

Activity against the gram-negative organisms varied considerably. That of the 3-benzyl compound 13 described in paper 1[3] was not improved by introduction of neutral substituents into the benzene ring (compounds 14 and 15) but was enhanced by a carboxyl substituent. When this carboxyl group occupied the ortho position (compound 16) activity was only slightly improved, but the meta and para isomers (17 and 18) were about as active as cephalexin, cephaloglycin, or the 3-(3-pyridyl)methyl compound 22 described in paper 1.[3] In the pyridyl series introduction

Table I. Antibacterial Activity of Cephalexin Analogues (12)

Compd	X	Minimum inhibitory concentration, μg/mL[a]						
		S. aureus Oxford	*S. aureus* Russell[b]	*E. coli*	*S. typhi*	*Sh. sonnei*	*K. aerogenes*	*P. mirabilis*
Cephalexin	H	1.25	5	12.5	5	5	5	12.5
Cephaloglycin	OCOMe	1.25	2.5	5	1.25	2.5	5	5
13	Ph	0.1	2.5	25	25	25	25	50
14	C_6H_4-*o*-CF_3	0.5	5	>100	>100	>100	>100	>100
15	C_6H_4-*p*-SO_2NMe_2	0.5	500	25	25	25	50	125
16	C_6H_4-*o*-CO_2H	5	12.5	12.5	50	12.5	5	5
17	C_6H_4-*m*-CO_2H	0.25	1.25	12.5	2.5	25	2.5	2.5
18	C_6H_4-*p*-CO_2H	0.5	2.5	12.5	0.5	5	2.5	1.25
19	2-c-$C_5H_{10}O$	5	5	>50	25	25	10	>50
20	2-C_4H_3N-1-Me	1.25	50	500	50	500	500	500
21	2-C_5H_4N	1.25	12.5	50	12.5	25	>500	50
22	3-C_5H_4N	0.25	2.5	2.5	1.25	2.5	2.5	5
23	3-C_5H_3N-4-Me	0.12	12.5	12.5	5	12.5	12.5	25
24	3-C_5H_3N-5-CO_2H	1.25	12.5	12.5	2.5	12.5	1.25	1.25
25	CO_2Me	0.5	16	31	8	16	63	63
26	CH_2OCOMe	0.5	10	>50	25	50	25	>50

[a] Determined by serial dilution on nutrient agar using an inoculum of 0.001 mL of an undiluted overnight broth culture. MIC values were read after incubation at 37 °C for 18 h. [b] β-Lactamase-producing benzylpenicillin-resistant strain.

Table II. Blood Levels in Mice

Compd	Route	μg/mL following 50 mg/kg dose[a]					
		10 min	20 min	30 min	1 h	2 h	4 h
Cephalexin	po	(23.9)[b]		21.7	8.3	2.5	0.56
Cephaloglycin	po	1.7	2.6	2.0	0.6	0.7	0.07
13	po	0.11	0.18	0.21	0.19	0.34	0.15
16	po	1.1	1.4	2.4	3.2	2.8	3.2
17	po	0.87	1.05	1.8	1.7	1.6	0.55
18	po	<1	<1	1.6	2.4	2.3	1.9
22	po	2.85	1.44	1.25	0.62	0.65	0.10
24	po	0.55	0.85	1.7	1.5	0.6	<0.4
Cephalexin	sc	27.0	20.5	13.3	3.0	0.42	<0.15
Cephaloglycin	sc	37.1	30.2	21.7	3.1	0.20	<0.1
Cephaloridine	sc	32.8	24.4	18.5	5.6	0.71	<0.2
16	sc	63.2	66.0	58.0	49.2	40.6	31.2
17	sc	84.2	64.2	49.6	26.2	13.7	1.6
18	sc	75.2	87.4	68.8	48.0	22.0	9.3
24	sc	70.4	54.0	35.8	12.2	2.0	<0.4
Cephaloglycin	iv	62.4	40.2	11.0	1.5	<0.1	<0.1
Cephaloridine	iv	48.8	23.2	14.3	2.9	0.32	<0.05
16	iv	102	83.6	75.4	64.4	29.1	18.1
18	iv	70.3	48.2	39.2	25.4	4.5	2.4

[a] Groups of five mice were killed at each time interval. Blood was collected from the cut axilla region, heparinized, stored at 4 °C, and subjected on the day of sampling to large plate microbiological assay using *Sarcina lutea* NCTC 8340 with overnight incubation at 30 °C. Standard solutions of the appropriate cephalosporins were prepared in mouse blood. Phosphate buffered saline, pH 7.2, was used as diluent when required. [b] At 15 min.

of a carboxyl group (24) did not improve activity further, in contrast to the previously noted effect in the benzyl series. The 3-(2-pyridyl)methyl- (21) and 3-(4-methyl-3-pyridyl)methyl- (23) cephalosporins were rather less active than 22, and the compounds with nonbasic heterocyclic substituents (19 and 20) were considerably less active. Finally, two compounds structurally related to cephaloglycin, the "reversed ester" 25 and the higher homologue 26, showed only moderate activity.

In summary, the most active of the new cephalosporins (17, 18, and 22) were about as active as the cephalosporins currently in clinical use. The breadth of the antibacterial spectrum was also similar to that of the established compounds, normally cephalosporin-resistant gram-negative organisms such as *Pseudomonas aeruginosa*, *Proteus morganii*, and *Enterobacter cloacae* being resistant to the new compounds also.

The distinctive property of cephalexin is that, in contrast to the great majority of cephalosporins, it is well absorbed when given by mouth. Compounds 13, 16–18, 22, and 24 were accordingly administered to groups of mice at 50 mg/kg po and blood levels of the antibiotic were determined by microbiological assay at intervals thereafter. Table II shows that 13 gave only very low levels, while the other compounds gave peak levels of the same order as those given by cephaloglycin. All the peak levels were much lower than that achieved with cephalexin, but levels of the carboxybenzylcephalosporins 16–18 appeared to be relatively well maintained 2 and 4 h after administration.

Blood levels resulting from parenteral administration of the carboxy compounds 16–18 and 24 were also determined, and in all cases the peak levels were found to exceed those of the reference cephalosporins. As in the oral experiments, marked prolongation of significant blood levels was seen with the carboxybenzyl compounds 16–18 but not with the carboxypyridyl compound 24.

Urine from mice which had received 17 and 18 was subjected to paper strip chromatography using a butanol–ethanol–water (4:1:5) solvent system. In the case of 18, but not 17, visualization on agar plates seeded with *Sarcina lutea* revealed a second zone of antibacterial activity due to an unidentified active metabolite. No active metabolite could, however, be detected by similar chromatography of blood samples, so the figures in Table II

Table III. Comparison of Activity of Cephalexin and Compound 18 in Infected Mice

		CD_{50}, mg/kg, total dose			
		Cephalexin		18	
Organism	Dose	sc	po	sc	po
S. aureus Smith	Single[a]	0.46	0.25	1.25	17
S. aureus Smith	Divided[b]	1.0	0.38	1.8	28
E. coli 8	Single[a]	7.4	8.5	180	>1000
E. coli 8	Divided[b]	17	17	60	2000
P. mirabilis 13	Single[a]	64		14	
P. mirabilis 13	Divided[b]	84		19	

[a] 1 h postinfection. [b] 1 and 5 h postinfection.

should represent unchanged antibiotic.

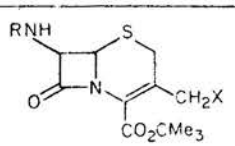
Taking into account both in vitro activity (Table I) and the blood level data (Table II), the *p*-carboxybenzylcephalosporin (18) appeared to be the most promising of the new cephalexin analogues. It was therefore tested against intraperitoneal infection in mice in comparison with cephalexin, and the results are given in Table III. By the subcutaneous route, compound 18 proved to be slightly less active than cephalexin against *S. aureus* Smith and significantly less active against *Escherichia coli*, but it was more active than cephalexin against *Proteus mirabilis*. As expected, 18 showed only poor activity by the oral route.

Experimental Section

Melting points were determined using a Kofler hot-stage apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian A-60 instrument for solutions in $CDCl_3$ with $SiMe_4$ as internal standard unless stated otherwise. Mass spectra were determined with a AEI MS9 machine. Compounds shown as having been analyzed either gave figures for the elements cited correct to within ±0.4 of the theoretical values or else gave accurate mass measurements correct to within ±5 ppm. Merck silica gel GF 254 was used for TLC and Merck silica gel H for column chromatography, with ethyl acetate–petroleum ether as eluent. Petroleum ether refers to the fraction of bp 60–80 °C.

Since most of the syntheses followed a lengthy but now standard[1-3] route (Scheme I), no attempt was made to characterize all the intermediates fully, but normal spectroscopic data were satisfactory. Several typical examples of the first two stages of Scheme I, namely, preparation of the secopenicillanates (3) and their oxidative conversion into 4-alkynylthioazetidin-2-ones (4), have been described.[4] In every sequence, at least one cephem ester (9, 10, or 11) was fully characterized as summarized in Table IV. All *tert*-butyl ceph-3-em-4-carboxylates showed λ_{max} (EtOH) 263–273 nm and ν_{max} ($CHCl_3$) 1770–1790 cm^{-1} (β-lactam CO) and 1700–1720 cm^{-1} (ester CO). In the final preparative step TFA treatment[3] removed both the *tert*-butyl ester and the *N*-*tert*-butoxycarbonyl-protecting groups to give cephalexin analogues 12 as their TFA salts, which showed one zone of antibacterial activity on biochromatograms (BuOH–EtOH–H_2O and/or BuOH–AcOH–H_2O) and gave the expected IR and UV spectra.

Table IV. Intermediate *tert*-Butyl Ceph-3-em-4-carboxylates



Compd	Precursor for	R[a]	X[a]	Mp, °C (solvent)[b]	Formula	Analyses
27	14	Ph_3C	C_6H_4-*o*-CF_3	Amorphous	$C_{38}H_{35}F_3N_2O_3S$	M$^+$
28	14	PhCHCO(NH(Boc))	C_6H_4-*o*-CF_3	110-111 (EA-P)	$C_{32}H_{36}F_3N_3O_6S$	M$^+$
29	15	Ph_3C	C_6H_4-*p*-SO_2NMe_2	185-186 (M)	$C_{39}H_{41}N_3O_5S_2$	C, H, N, S
30	15	PhCHCO(NH(Boc))	C_6H_4-*p*-SO_2NMe_2	156-157 (EA-P)	$C_{33}H_{42}N_4O_8S_2$	C, H, N, S
31	16	PhCHCO(NH(Boc))	C_6H_4-*o*-CO_2An	157-159 (Tr E)	$C_{40}H_{45}N_3O_9S$	C, H, N, S
32	17	Ph_3C	C_6H_4-*m*-CO_2An	171-172 (C-E)	$C_{46}H_{44}N_2O_6S$	C, H, N, S
33	18	Ph_3C	C_6H_4-*p*-CO_2An	101-102 (M)	$C_{46}H_{44}N_2O_6S$	C, H, N, S
34	18	PhCHCO(NH(Boc))	C_6H_4-*p*-CO_2An	152-154 (Tr E)	$C_{40}H_{45}N_3O_9S$	C, H, N, S
35	19	H	THP	Amorphous	$C_{17}H_{26}N_2O_4S$	M$^+$
36	19	PhCHCO(NH(Boc))	THP	Amorphous	$C_{30}H_{41}N_3O_7S$	M$^+$
37	20	Ph_3C	1-Me-2-pyrrolyl	167-170 (M)	$C_{36}H_{37}N_3O_3S$	M$^+$
38	20	H	1-Me-2-pyrrolyl	Amorphous	$C_{17}H_{23}N_3O_3S$	M$^+$
39	21	H	2-Pyridyl	Amorphous	$C_{17}H_{21}N_3O_3S$	M$^+$
40	21	PhCHCO(NH(Boc))	2-Pyridyl	Amorphous	$C_{30}H_{36}N_4O_6S$	M$^+$
41	23	H	4-Me-3-pyridyl	155-157 (C-E)	$C_{18}H_{23}N_3O_3S$	C, H, N, S
42	23	PhCHCO(NH(Boc))	4-Me-3-pyridyl	Amorphous	$C_{31}H_{38}N_4O_6S$	M$^+$
43	24	H	5(CO_2CMe_3)-3-pyridyl	156-158 (C-P)	$C_{22}H_{29}N_3O_5S$	M$^+$
44	25	Ph_3C	CO_2Me	109-110 (Tr E)	$C_{33}H_{34}N_2O_5S$	M$^+$
45	26	PhCHCO(NH(Boc))	CH_2OTHP	Amorphous	$C_{31}H_{43}N_3O_6S$	M$^+$
46	26	PhCHCO(NH(Boc))	CH_2OCOMe	Amorphous	$C_{28}H_{37}N_3O_8S$	M$^+$

[a] Abbreviations: An = *p*-methoxybenzyl, Boc = *tert*-butoxycarbonyl, THP = 2-tetrahydropyranyl. [b] Crystallized from C = chloroform, E = ethanol, EA = ethyl acetate, M = methanol, P = petroleum ether, Tr E = trituration with ether. All compounds gave satisfactory NMR spectra and were homogeneous by TLC.

The exact sequence (Scheme I) previously described[3] for preparation of 13 and 22 was used to prepare 14, 15, 19, 21, and 23.

3-(Carboxybenzyl)-7-β-(D-phenylglycylamino)ceph-3-em-4-carboxylic Acids (16–18). The following route to 18 is typical.

3-*p*-(*p*-Methoxybenzyloxycarbonyl)phenylprop-2-ynol. This was prepared from copper(I) 3-(tetrahydropyran-2-yloxy)prop-1-ynide (33.2 g, 0.164 mol) and *p*-methoxybenzyl 4-iodobenzoate (60.5 g, 0.164 mol) by the general method of Harris et al.[4] The product (35.7 g, 74%) had mp 104.5–105.5 °C; ν_{max} 3590, 3400, 1705, 1609, 1520 cm^{-1}.

2-*p*-(*p*-Methoxybenzyloxycarbonyl)phenylprop-2-ynyl Bromide. To a suspension of the above alcohol (33 g, 0.111 mol) and PPh_3 (33 g, 0.126 mol) in dry benzene (150 mL) was added CBr_4 (45 g, 0.136 mol). The mixture was stirred and the exothermic reaction controlled by cooling so that the internal temperature did not exceed 40–45 °C. After 10 min the clear solution was evaporated and the residual oil chromatographed to give the bromide (26 g, 90%): needles (from AcOEt–petroleum ether); mp 60 °C; ν_{max} ($CHCl_3$) 1720, 1610, 1590, 1520 cm^{-1}. Anal. ($C_{18}H_{15}BrO_3$) C, H, Br.

1-(1-Benzyloxycarbonyl-2-methylprop-1-enyl)-4-[3-*p*-(*p*-methoxybenzyloxycarbonyl)phenylprop-2-ynylthio]-3-(triphenylmethylamino)azetidin-2-one. The above bromide (20 g) and benzyl 6β-(triphenylmethylamino)penicillanate (1, 28 g, 51 mmol) in dry THF (250 mL) were treated with freshly powdered NaOH (2.5 g, 62.5 mmol) and stirred at room temperature for 24 h. The mixture was then diluted with EtOAc, washed, dried, and evaporated. Chromatography of the residue gave the 1,2-secopenicillanate (26.7 g, 63%) as a foam: ν_{max} ($CHCl_3$) 1765, 1720 cm^{-1}. Anal. ($C_{52}H_{46}N_2O_6S$) C, H, N, S.

4-[3-*p*-(*p*-Methoxybenzyloxycarbonyl)phenylprop-2-ynylthio]-3-(triphenylmethylamino)azetidin-2-one. The above secopenicillanate (5 g, 6.05 mmol) in DMF (15.1 mL) and pyridine (15.1 mL) containing water (3.05 mL) was cooled to −20 °C and stirred while freshly powdered $KMnO_4$ (1.43 g, 9 mmol) was added portionwise at such a rate that the temperature did not exceed 0 °C. The mixture was stirred for 1 h at −20 °C, then diluted with EtOAc (40 mL) and water (40 mL), and treated with SO_2 until the MnO_2 had dissolved. The organic phase was separated, washed with 1 N HCl followed by $NaHCO_3$ solution, dried, and evaporated. Chromatography of the residue gave unchanged secopenicillanate (0.61 g) and the azetidinone (1.1 g, 29%) as a foam: ν_{max} ($CHCl_3$) 3400, 1762, 1710, 1610 cm^{-1}.

1-(1-Hydroxy-1-*tert*-butoxycarbonylmethyl)-4-[3-*p*-(*p*-methoxybenzyloxycarbonyl)phenylprop-2-ynylthio]-3-(triphenylmethylamino)azetidin-2-one. A solution of the above azetidinone (13.2 g, 0.021 mol) and *tert*-butyl glyoxylate (21 g, 0.16 mol) in benzene (250 mL) was boiled under reflux with provision for the removal of water. After 5 h the solution was cooled, washed, dried, and evaporated. Chromatography of the residue gave the α-hydroxy ester as a mixture of epimers (11.68 g, 74%): a foam; ν_{max} ($CHCl_3$) 3500, 1770, 1730, 1610 cm^{-1}.

1-(1-*tert*-Butoxycarbonyl-1-triphenylphosphoranylidenemethyl)-4-[3-*p*-(*p*-methoxybenzyloxycarbonyl)phenylprop-2-ynylthio]-3-(triphenylmethylamino)azetidin-2-one. The above α-hydroxy ester (5 g, 6.5 mmol) in dry THF (50 mL) containing 2,6-lutidine (2.38 g, 22 mmol) was cooled to −10 °C and treated dropwise with $SOCl_2$ (2.32 g, 19.5 mmol) during 15 min. After a further 3 min the precipitated solid was removed by filtration and the filtrate was evaporated to leave the crude α-chloro ester (4.0 g) as a foam: ν_{max} ($CHCl_3$) 1770, 1730 cm^{-1}. This was dissolved in dry dioxane (50 mL) containing 2,6-lutidine (3.4 g, 32 mmol) and triphenylphosphine (3.4 g, 13 mmol) and stirred under N_2 at 50 °C for 16 h and then filtered and the filtrate was evaporated. Chromatography of the residue gave the phosphorane (4.25 g, 64%) as a foam: ν_{max} ($CHCl_3$) 1755, 1715, 1635, 1610 cm^{-1}.

1-(1-*tert*-Butoxycarbonyl-1-triphenylphosphoranylidenemethyl)-4-[3-*p*-(*p*-methoxybenzyloxycarbonyl)phenyl-2-oxopropylthio]-3-(triphenylmethylamino)azetidin-2-one. The above acetylene (7.86 g) in piperidine (50 mL) was stirred at 50 °C for 4 h and then evaporated in vacuo. The residue was dissolved in EtOAc, washed successively with 0.1 N HCl, H_2O, and $NaHCO_3$ solution, dried, and evaporated. Chromatography gave the ketone (6.5 g, 82%) as a foam: ν_{max} ($CHCl_3$) 1750, 1710, 1630, 1610 cm^{-1}.

***tert*-Butyl 3-[*p*-(*p*-Methoxybenzyloxycarbonyl)benzyl]-7β-(triphenylmethylamino)ceph-3-em-4-carboxylate (33).** This was obtained (82%) by heating the above ketophosphorane in refluxing dioxane under N_2 for 24 h.

***tert*-Butyl 7β-(*N*-*tert*-Butoxycarbonyl-D-α-phenylglycyl)amino-3-[*p*-(*p*-methoxybenzyloxycarbonyl)benzyl]ceph-3-em-4-carboxylate (34).** The cephem 33 (3.8 g, 5 mmol) in CH_2Cl_2 (10 mL) was detritylated by treatment at −20 °C with toluene-*p*-sulfonic acid (1 g, 6 mmol) in MeOH (5 mL). The solution was left at 0 °C for 16 h and then washed with $NaHCO_3$ solution. Evaporation of the organic layer gave the primary amine (1.75 g, 68%): mp 134–135 °C; ν_{max} ($CHCl_3$) 1775, 1710 cm^{-1}. This amine (250 mg, 0.5 mmol) in THF (2 mL) was added over 5 min to a cooled (−20 °C) THF solution of mixed anhydride prepared[3] from *N*-*tert*-butoxycarbonyl-D-α-phenylglycine (135 mg, 0.54 mmol) and $ClCO_2Me$ (50 mg, 0.53 mmol). The mixture was stirred for 2 h at −20 °C and then evaporated in vacuo. The residue was taken up in EtOAc and washed successively with 0.25 N HCl (2 × 10 mL), water, and $NaHCO_3$ solution. Evaporation of the dried solution followed by chromatography gave the protected cephalosporin 34 (193 mg, 53%): ν_{max} ($CHCl_3$) 3400, 1785, 1710 cm^{-1}.

3-(*p*-Carboxybenzyl)-7β-(D-α-phenylglycyl)aminoceph-3-em-4-carboxylic Acid (18). The triply protected compound 34 (86 mg) was dissolved in TFA (0.5 mL) and kept at room temperature for 10 min. The solution was then evaporated in vacuo and the evaporation thrice repeated after successive additions of dry toluene (5-mL portions) to leave the TFA salt of 18 as an amorphous solid (63 mg): ν_{max} (Nujol) 2300–2700, 1770, 1690 cm^{-1}.

3-(5-Carboxy-3-pyridylmethyl)-7β-(D-α-phenylglycyl)amino)ceph-3-em-4-carboxylic Acid (24). This was prepared by a similar reaction sequence to 18, except that the final TFA treatment of 11 (X = 5-*tert*-butoxycarbonyl-3-pyridyl) required 3 h for complete deprotection [checked by running biochromatograms in BuOH–AcOH–H_2O (12:3:5) until only one zone of antibacterial activity (R_f 0.37) remained].

***tert*-Butyl 3-Methoxycarbonylmethyl-7β-(triphenylmethylamino)ceph-3-em-4-carboxylate (44).** (3*R*,4*R*)-Prop-2-ynylthio-3-(triphenylmethylamino)azetidin-2-one[4] (4, = H; 9.3 g) was converted by the method of Scheme I into the phosphorane (7, X = H; 11.4 g, 65%) and the latter in THF was treated at −70 °C with *n*-butyllithium (2 equiv). After 5 min the mixture was saturated with CO_2, diluted with EtOAc, washed with dilute HCl, dried, and evaporated to small volume. Treatment with diazomethane in ether gave, after chromatography, the methyl ester 7 (X = CO_2Me) as a foam (30%): ν_{max} ($CHCl_3$) 228, 1760, 1720, 1635 cm^{-1}. Treatment with piperidine at room temperature for 6 h, followed by usual work-up with aqueous acid gave the ketone 8 (X = CO_2Me) as a foam (67%), ν_{max} ($CHCl_3$) 1755, 1720, 1635 cm^{-1}, which when refluxed in dioxane for 24 h gave the cephem 44 (Table IV) in 70% yield.

1-(1-Benzyloxycarbonyl-2-methylprop-1-enyl)-4-(3-iodoprop-2-ynylthio)-3-(triphenylmethylamino)azetidin-2-one (3, X = I). A solution of $AgNO_3$ (12.6 g, 74 mmol) in $CHCl_3$ (1 mL) and pyridine (50 mL) was stirred and cooled in an ice bath while iodine monochloride (4.2 mL, 83 mmol) in $CHCl_3$ (50 mL) was added over 5 min. AgCl was removed by centrifugation and washed with pyridine (50 mL). The combined solutions, containing iodonium nitrate, were treated with the 1,2-seco penicillanate[4] (3, X = H, 28 g, 47.8 mmol) in $CHCl_3$ (50 mL) and stirred at room temperature for 4 h. The mixture was evaporated in vacuo and the residue taken up in EtOAc, washed, dried, and again evaporated. Chromatographic purification gave the diacetylene 3 (X = I, 25.9 g, 76%) as a yellow foam: ν_{max} ($CHCl_3$) 2980, 1765, 1715, and 1630 cm^{-1} (found M$^+$ 712.1291; $C_{37}H_{33}IN_2O_4$ requires M 712.1258).

1-(1-*tert*-Butoxycarbonyl-1-triphenylphosphoranylidenemethyl)-4-(3-iodoprop-2-ynylthio)-3-(triphenylmethylamino)azetidin-2-one (7, X = I). This was prepared (10% overall) from 3 (X = I) according to Scheme

***tert*-Butyl 3-(1-Methyl-2-pyrrolylmethyl)-7β-(triphenylmethylamino)ceph-3-em-4-carboxylate (37).** Methylpyrrole (0.5 g, 6.2 mmol) and tetramethylethylenedia

(0.87 g, 5.7 mmol) in dry ether were treated with *n*-butyllithium (1 equiv) at reflux for 1 h. The solution of 2-lithio-*N*-methylpyrrole was stirred with copper(I) bromide (1.2 g) for 90 min at room temperature to give a brown suspension of 2-cuprio-*N*-methylpyrrole. This was treated with the iodoacetylene 7 (X = I, 5.0 g, 6.5 mmol) in THF (20 mL) and stirred at room temperature overnight. The mixture was diluted with benzene, the solids were removed by centrifugation, and the supernatant solution was concentrated and then chromatographed to give the pyrrolylacetylene 7 (X = 1-methyl-2-pyrrolyl) (28%): ν_{max} ($CHCl_3$) 2980, 1750, and 1630 cm^{-1}. Refluxing in piperidine for 12 h, followed by the usual aqueous work-up, gave the ketone 8 (X = 1-methyl-2-pyrrolyl, 59%): ν_{max} ($CHCl_3$) 2980, 1760, and 1635 cm^{-1}. Cyclization by refluxing in dioxane for 30 h gave the cephem 37 (Table IV) in 83% yield.

***tert*-Butyl 7β-Amino-3-(1-methyl-2-pyrrolylmethyl)-ceph-3-em-4-carboxylate (38).** For this acid-sensitive compound the following exceptionally mild detritylation procedure was preferred. The trityl derivative 37 in redistilled MeOH was stirred with an equal weight of pyridine hydrochloride for 4 days; then the solvent was evaporated and the residue dissolved in EtOAc. The solution was washed with aqueous $NaHCO_3$, dried, and evaporated, and the residue was chromatographed to give the primary amine 38 (81%).

***tert*-Butyl 3-(2-Acetoxyethyl)-7β-(*N*-*tert*-butoxycarbonyl-D-α-phenylglycyl)aminoceph-3-em-4-carboxylate (46).** The *O*-tetrahydropyranyl derivative 45 (106 mg, 0.17 mmol, synthesized according to Scheme I) was treated with toluene-*p*-sulfonic acid (35 mg, 0.2 mmol) in isopropenyl acetate (4 mL) at 0 °C for 4 h and then at room temperature for a further 4 h. EtOAc was added and the solution washed with dilute aqueous $NaHCO_3$, followed by brine. Evaporation of the dried solution, followed by chromatography, gave the cephem 46 (Table IV) in 25% yield.

Supplementary Material Available: High-resolution mass spectral data, NMR data, and elemental composition data (4 pages). Ordering information is given on any current masthead page.

References and Notes

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7

SYNERGIC ACTIVITIES OF PENICILLIN COMBINATIONS

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The use of combinations of antibiotics is commonplace in clinical practice despite the disapprobation of many authorities. Combined therapy is usually based on a desire to extend the antibacterial spectrum but in a number of cases it is also hoped that the individual agents may interact to produce synergistic effects. Penicillins are often combined with other antibiotics and this report describes examples where antibacterial synergy may arise from penicillin combinations.

Synergy between Pencillins and Aminoglycosides against Enterococci

Perhaps the best known example of synergism between antibiotics is that demonstrated by penicillins and aminoglycosides against enterococci. Enhancement of the bactericidal activity of benzylpenicillin against *Streptococcus faecalis* by streptomycin was studied by Jawetz and his collaborators in their early studies on antibiotic interactions (Jawetz, Gunnison and Coleman, 1950) and the value of combined penicillin/aminoglycoside therapy in the treatment of enterococcal endocarditis is well established.

Enterococci are moderately sensitive to benzylpenicillin, amoxycillin and ampicillin and the growth of the organisms is inhibited by relatively low concentrations of the penicillins (*Table 7.1*). Aminoglycoside antibiotics are notably

Table 7.1 Distribution of minimum inhibitory concentrations (MICs) of penicillins and aminoglycosides against 55 strains of enterococci (after Russell and Sutherland, 1975)

	Number of strains with an MIC (μg ml⁻¹) of											
Antibiotic	*0.25*	*0.5*	*1.25*	*2.5*	*5.0*	*12.5*	*25*	*50*	*125*	*250*	*500*	*>500*
Amoxycillin	5	49	1	0	0	0	0	0	0	0	0	0
Ampicillin	0	25	29	1	0	0	0	0	0	0	0	0
Benzylpenicillin	0	2	49	4	0	0	0	0	0	0	0	0
Gentamicin	0	0	1	19	26	7	2	0	0	0	0	0
Kanamycin	0	0	0	0	5	19	22	5	2	0	0	2*
Streptomycin	0	0	0	0	0	7	9	21	2	0	1	15*

*MIC >5000 μg ml⁻¹

less active than the penicillins against these organisms, but newer compounds such as gentamicin and tobramycin show a marked improvement in activity compared with streptomycin (*Table 7.1*). The penicillins are bactericidal in action against *S. faecalis*, but less so than against most other bacteria, and a significant number of survivors persist in cultures exposed to penicillins. However, when the penicillin is combined with an aminoglycoside, at a concentration of the latter which is itself ineffective, a marked synergistic action occurs with many strains of enterococci resulting in a striking increase in the bactericidal activity of the penicillin (Sonne and Jawetz, 1968; Watanakunakorn, 1971; Russell and Sutherland, 1975).

Thus, results in *Table 7.2* show the Minimum Bactericidal Concentrations (MBCs) of amoxycillin against enterococci alone and in combination with sublethal concentrations of streptomycin, kanamycin and gentamicin. The bactericidal activities of the combinations were much greater than those of amoxycillin

Table 7.2 Minimum bactericidal concentrations (MBCs) of amoxycillin and aminoglycosides alone, and in combination, for 12 strains of enterococci (after Russell and Sutherland, 1975)

Penicillin	*Aminoglycoside and its concentration* ($\mu g\ ml^{-1}$)	*Number of strains with an MBC* ($\mu g\ ml^{-1}$)* *of*									
		0.25	*0.5*	*1.0*	*2.5*	*5.0*	*10*	*25*	*50*	*100*	*>100*
Amoxycillin	None	0	0	0	0	0	0	0	0	0	12
	Streptomycin (20)	1	0	2	2	0	0	0	0	0	7†
	Kanamycin (20)	0	5	4	1	0	0	0	0	0	2‡
	Gentamicin (10)	6	3	3	0	0	0	0	0	0	0

*For an inoculum size *ca* 10^6 cells ml^{-1} of medium.
†Streptomycin MIC >5000 $\mu g\ ml^{-1}$.
‡Kanamycin MIC >5000 $\mu g\ ml^{-1}$.

alone provided that the test organism was sensitive to the aminoglycoside. In these tests, streptomycin-resistant strains were resistant to amoxycillin + streptomycin but were killed by combinations containing kanamycin or gentamicin and, similarly, kanamycin-resistant strains were not killed by amoxycillin + kanamycin but were sensitive to the combination with gentamicin. It should be noted that although amoxycillin appeared to show poor bactericidal activity against enterococci in these tests to measure MBCs, this was because the test was particularly stringent, requiring the viable count to fall from the initial inoculum of 10^6 cells ml^{-1} to less than 10^2 cells ml^{-1}, i.e. 99.99% kill. When the inoculum was reduced to 10^4 cells ml^{-1}, amoxycillin readily reduced the bacterial count to less than 10^2 cells ml^{-1} (99.0% kill), and the MBCs were of the same order as the Minimum Inhibitory Concentrations (MICs).

The bactericidal activity of amoxycillin against *S. faecalis* and its enhancement in combinations with aminoglycosides is illustrated in greater detail in *Figure 7.1*, which shows the effects of combinations of amoxycillin + streptomycin and amoxycillin + kanamycin against a streptomycin-resistant strain, *S. faecalis* 816 (MIC >5000 μg streptomycin ml^{-1}). Amoxycillin was bactericidal at a concentration of 1.0 $\mu g\ ml^{-1}$ over the first 6 h and reduced the viable count

by 99.9% but this was followed by a resumption of growth to visible levels. Streptomycin (20 $\mu g\ ml^{-1}$) had no effect on the growth of the organisms while kanamycin (20 $\mu g\ ml^{-1}$) was bactericidal for 6 h but at 24 h growth equalled that of the control antibiotic-free culture. The combination of streptomycin and

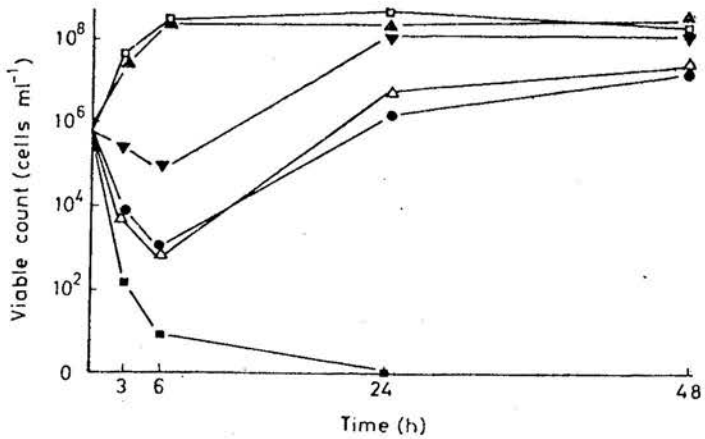

Figure 7.1 Bactericidal activity of amoxycillin, alone and in combination with streptomycin and kanamycin, against Streptococcus faecalis *816.* □ *= Control;* ▲ *= streptomycin 20 $\mu g\ ml^{-1}$;* ▼ *= kanamycin, 20 $\mu g\ ml^{-1}$;* △ *= amoxycillin + streptomycin, 1.0 + 20 $\mu g\ ml^{-1}$;* ● *= amoxycillin, 1.0 $\mu g\ ml^{-1}$;* ■ *= amoxycillin + kanamycin, 1.0 + 20 $\mu g\ ml^{-1}$*

amoxycillin was no more effective than amoxycillin alone, which is not unexpected in view of the high level of resistance of the organism to streptomycin, whereas amoxycillin + kanamycin caused a 99.99% kill by 6 h and had sterilized the culture after 24 h incubation.

It has been suggested that the mechanism of synergism between penicillin and streptomycin against enterococci is that the penicillin induces the formation of enterococcal L-forms that are more sensitive to the aminoglycoside than the parent strains (Hewitt, Seligman and Deigh, 1966). An alternative mechanism is proposed from the results of studies with radio-labelled streptomycin which have shown that enterococci are relatively impermeable to the compound, but that uptake of the aminoglycoside is greatly enhanced by the presence of penicillin (Moellering and Weinberg, 1971). Uptake of streptomycin was also enhanced in cells with a high level of resistance to streptomycin and it appears that the failure of penicillin + streptomycin to produce synergy against these strains is due to the resistance of the ribosomes to the aminoglycoside (Zimmerman, Moellering and Weinberg, 1971).

Combined therapy with benzylpenicillin and streptomycin has long been regarded as the treatment of choice for severe enterococcal infection on the basis of the bactericidal synergy that can be demonstrated *in vitro*, and the use of this antibiotic combination is the most widely accepted example of clinical synergism (Jawetz, 1968). However, a significant proportion of strains of enterococci are resistant to the combined effects of penicillin + streptomycin (Standiford, de

Maine and Kirby, 1970; Moellering, Wennersten and Weinberg, 1971) and there is increasing interest in the activity of combinations of penicillins with newer aminoglycosides such as gentamicin, tobramycin and amikacin (Watanakunakorn, 1971; Moellering, Wennersten and Weinstein, 1973; Iannini, Ehret and Eickhoff, 1976). Nevertheless, the treatment of choice for enterococcal infection remains an appropriate penicillin/aminoglycoside combination.

Penicillin/Aminoglycoside Combinations against *Pseudomonas aeruginosa*

Serious infections due to Gram-negative bacilli now constitute a major problem in hospital practice and infections due to *Pseudomonas aeruginosa* are particularly difficult to treat because of the resistance of this opportunistic pathogen to most antibiotics. Carbenicillin was the first penicillin to show clinically useful anti-pseudomonas activity and gentamicin has been the aminoglycoside of choice for the treatment of pseudomonas infections for a number of years. Early studies showed that combinations of carbenicillin and gentamicin provided synergistic antibacterial effects against *Ps. aeruginosa in vitro* (Brumfitt, Percival and Leigh, 1967; Rolinson and Sutherland, 1968) and synergism has been demonstrated against experimental infections in laboratory animals (Andriole, 1971). Combinations of the compounds have been used with significant success in the treatment of serious infections (Klastersky, Cappel and Debusscher, 1971; Schimpff *et al.*, 1971; Yuce and Van Rooyen, 1971), although there are few controlled studies to show that combined therapy is distinctly better than the use of either drug alone.

More recently, a number of penicillins active against *Ps. aeruginosa* and other Gram-negative bacteria have been introduced, for example sulbenicillin and ticarcillin, and there has been a proliferation of new, active aminoglycosides, namely, amikacin, sisomicin and tobramycin. Synergy, similar to that originally reported with carbenicillin and gentamicin has also been described in laboratory studies *in vitro* and *in vivo* with various combinations of these newer penicillins and aminoglycosides (Klastersky, Nyamubeya and Vandenborre, 1974; Klastersky, Hensgens and Debusscher, 1975; Wald *et al.*, 1975; Kelly and Matsen, 1976).

Table 7.3 Comparative activities of penicillin/aminoglycoside combinations against 3 strains of *Ps. aeruginosa*

	Minimum Inhibitory Concentration ($\mu g\ ml^{-1}$)		
Antibiotic	*R2*	*986*	*HT1*
Ticarcillin	31	31	62
Carbenicillin	62	125	250
Tobramycin	0.12	0.25	0.12
Gentamicin	0.5	1.0	1.0
Ticarcillin/tobramycin	8 + 0.03	8 + 0.06	16 + 0.03
Ticarcillin/gentamicin	8 + 0.12	16 + 0.12	16 + 0.06
Carbenicillin/tobramycin	8 + 0.12	31 + 0.06	62 + 0.03
Carbenicillin/gentamicin	31 + 0.12	62 + 0.25	31 + 0.50

Results in *Table 7.3* show the effects demonstrated by a number of penicillin/aminoglycoside combinations against three strains of *Ps. aeruginosa*. The antibacterial activities were determined by a modified chess-board system using the microtiter technique as shown in *Figure 7.2*. The action of the test combination

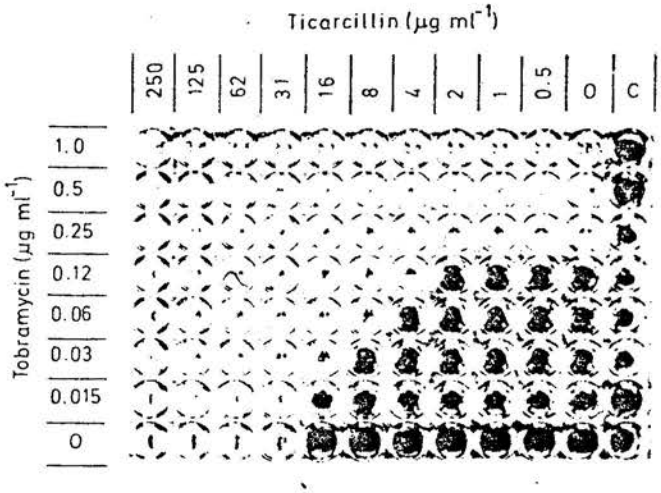

Figure 7.2 Microtiter chess-board technique to demonstrate synergism between ticarcillin and tobramycin against Pseudomonas aeruginosa

of antibiotics was considered synergistic when the MIC of each compound was at least four times as effective in combination as the MIC of that drug alone. It can be seen that in most cases synergy was demonstrated and that combinations of ticarcillin and tobramycin produced the greatest activity against the test strains, which might be expected since these compounds were the most active of the penicillins and aminoglycosides tested. The synergistic bactericidal action of a combination of ticarcillin and tobramycin against *Ps. aeruginosa* is illustrated in *Figure 7.3*, which shows that neither compound produced significant bacteri-

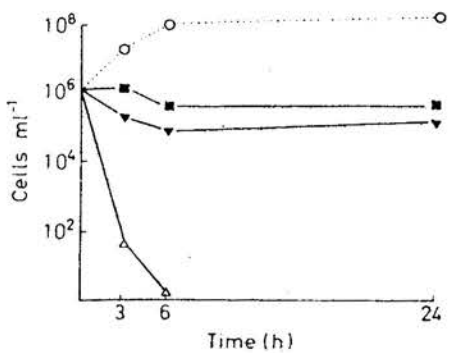

Figure 7.3 Bactericidal synergy between ticarcillin and tobramycin against Pseudomonas aeruginosa. *O–O = Control; ■–■ = ticarcillin (100); ▼ – ▼ = tobramycin (1.0); △–△ = ticarcillin (100) + tobramycin (1.0)*

cidal effects on its own but that a combination of the two antibiotics reduced the viable count to zero within 6 h.

Although the combination of carbenicillin and gentamicin is used successfully in the treatment of clinical infection, inactivation of gentamicin by carbenicillin which is readily demonstrable *in vitro* has also been reported to occur during therapy (McLaughlin and Reeves, 1971) and fears have been expressed that the value of this combination might be limited because of the interaction between the two drugs. However, it is now generally accepted that the combination of drugs should not be withheld from patients with normal renal function (Riff and Jackson, 1972).

The mechanism of the synergy between penicillins and aminoglycosides is not known, but may involve an enhancement of the uptake of the aminoglycoside into the bacterial cell in the presence of the penicillin, which acts by inhibiting cell-wall synthesis, as has been suggested for the effects produced by penicillin–aminoglycosides against enterococci. Whatever the mechanism, the evidence does suggest that combined therapy may well produce enhanced clinical effects in the treatment of infections due to *Ps. aeruginosa.*

Mecillinam Combinations

Penicillins lacking an acyl group in the 6-position of the nucleus are normally devoid of significant antibacterial activity but mecillinam is an example of a non-acyl penicillin with unusual antibacterial properties *in vitro* (Lund and Tybring, 1972; Neu, 1976). For example, the antibacterial spectrum of mecillinam differs from that of conventional penicillins in that the compound shows a high level of activity against certain Gram-negative bacilli but is quite inactive against Gram-positive bacteria. In addition, mecillinam has been shown to have a different mode of action which is readily evident from the morphological responses provoked by mecillinam against sensitive bacteria. Bacterial cells are converted to large spherical forms which are relatively stable in normal media compared with the spheroplasts induced by penicillins (Greenwood and O'Grady, 1973; Melchior *et al.*, 1973).

It has been suggested that, because mecillinam and penicillins have different binding sites, combinations of the compounds would exert synergistic effects (Park and Burman, 1973; Matsuhashi *et al.*, 1974; Spratt, 1975). In practice, synergy has been reported when mecillinam was combined with ampicillin but the extent of synergism appeared to be variable and was greatly influenced by inoculum size and the osmolality of the medium (Tybring and Melchior, 1975). According to these workers synergy arises because the spherical bacterial forms induced by mecillinam are particularly sensitive to ampicillin.

The morphological effects produced by mecillinam were studied in some detail by Greenwood and O'Grady (1973), who reported interesting results when mecillinam was combined with the cephalosporin antibiotic, cephalexin. In a turbidimetric system, neither mecillinam nor cephalexin produced any obvious effect on the growth of *Escherichia coli,* but when the two agents were combined, rapid lysis ensued (*Figure 7.4*) similar to the effects produced by penicillins in this system. Microscopic examination of the various cultures showed the round cell-forms produced by mecillinam and the filament formation

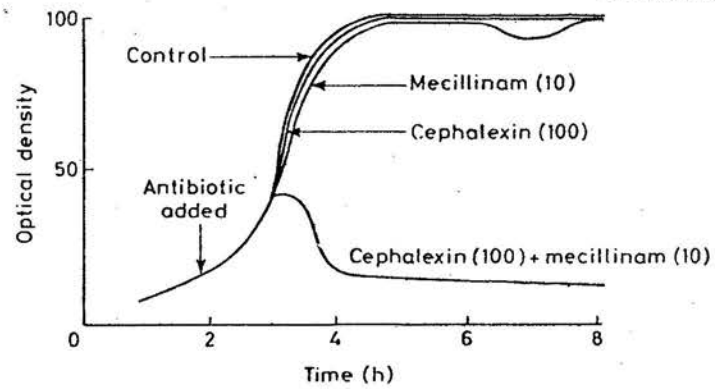

Figure 7.4 Bacteriolytic activities of cephalexin and mecillinam against E. coli, *alone and in combination*

associated with cephalexin, but in the presence of the combination of compounds, spheroplasts similar to those induced by penicillins were observed (*Figure 7.5*).

These results show that two β-lactam antibiotics, each of which produces different morphological effects in sensitive bacteria, presumably by functioning against different sites in the bacterial cell, can interact to produce the classical lytic effects associated with penicillins. The clinical significance of the interaction of mecillinam with other β-lactam antibiotics is uncertain as yet but the phenomenon observed in the laboratory is of considerable interest.

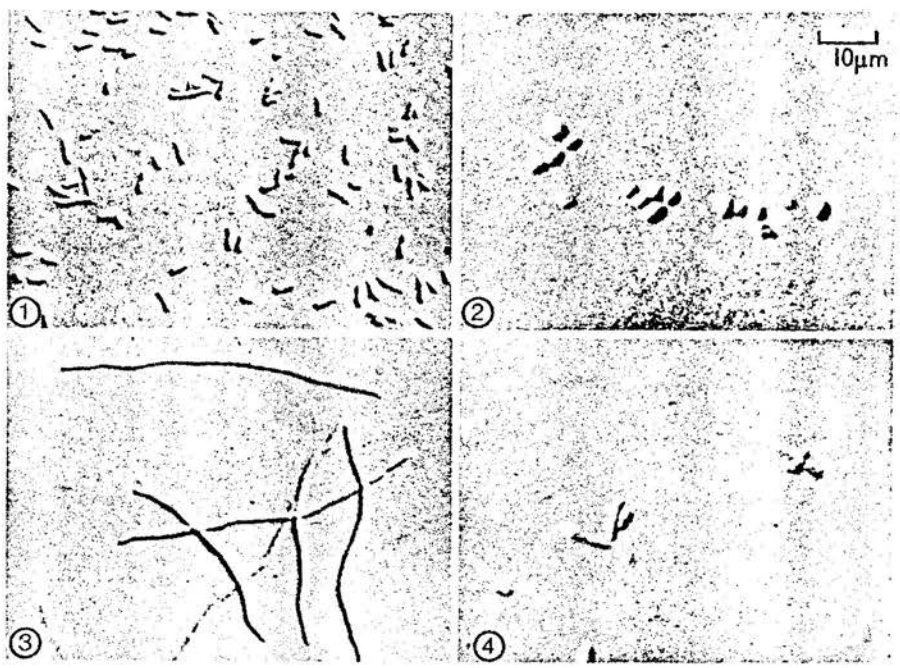

Figure 7.5 Photomicrographs showing the morphological effects produced by cephalexin and mecillinam against E. coli. *(1) Control; (2) mecillinam, 10 μg ml⁻¹; (3) cephalexin 100 μg ml⁻¹; (4) mecillinam 10 μg ml⁻¹ + cephalexin 100 μg ml⁻¹*

Synergy arising by Inhibition of β-Lactamase

An entirely different example of synergy involving penicillins, for which the mechanism appears to be clearly established, is that depending upon competitive inhibition of β-lactamase whereby synergism may be demonstrated between pairs of β-lactam antibiotics. The resistance of Gram-negative bacilli to broad-spectrum penicillins or cephalosporins is often associated with β-lactamase activity and the ability of these bacteria to destroy the β-lactam antibiotics (Smith, Hamilton-Miller and Knox, 1969; Richmond and Curtis, 1974). Certain combinations of broad-spectrum penicillins, e.g. ampicillin or amoxycillin, and β-lactamase-stable penicillins, e.g. methicillin or cloxacillin, exhibit synergistic antibacterial effects which are due to inhibition of the β-lactamase by the stable penicillin (Hamilton-Miller and Smith, 1964; Sutherland and Batchelor, 1964). The β-lactamase-stable compounds have little antibacterial activity against Gram-negative bacilli but have a relatively high affinity for their β-lactamases and are able to protect the labile broad-spectrum penicillin by inhibition of the enzyme.

Table 7.4 Synergistic activity of a combination of ampicillin against ampicillin-resistant bacteria, *in vitro* and *in vivo*

	MIC (μg ml^{-1})			CD_{50}* (mg kg^{-1}) *(oral)*		
Organism	*Ampicillin*	*Cloxacillin*	*Ampicillin + cloxacillin*	*Ampicillin*	*Cloxacillin*	*Ampicillin + cloxacillin*
Escherichia coli 487	250	500	25 + 25	>5000	>1000	320 + 320
Shigella sonnei 3606	250	500	12.5 + 12.5	>5000	>1000	90 + 90
Proteus morganii 718	125	1000	12.5 + 12.5	>5000	>1000	80 + 80

*Mice infected by the intraperitoneal route were dosed immediately after infection with a single dose of the compound or combination.

The results in *Table 7.4* illustrate examples of synergy between β-lactam antibiotics and show the antibacterial activities *in vitro* and *in vivo* of a combination of equal parts of ampicillin and cloxacillin against three cultures of β-lactamase-producing bacteria. Both penicillins were relatively inactive against the organisms (MIC 125–1000 μg ml^{-1}) but the combination of penicillins was notably more active (MIC 25–50 μg ml^{-1}). The synergistic antibacterial activity of ampicillin + cloxacillin was also exhibited against experimental infections caused by these β-lactamase-producing bacteria. Ampicillin and cloxacillin were ineffective in preventing death of infected mice whereas the combination of the two penicillins demonstrated marked protective effects.

Inhibition of β-lactamase activity is illustrated in *Figure 7.6* which shows the bactericidal activities of ampicillin and ampicillin + cloxacillin against a β-lactamase-producing strain of *E. coli*. Ampicillin (10 μg ml^{-1}) produced a slight inhibition of growth for the first 3 h of the test but by 6 h the concentration of ampicillin had fallen to 2 μg ml^{-1} and the viable count was similar to that of the control culture. At a higher concentration (100 μg ml^{-1}), ampicillin was active

during the early part of the experiment but at 24 h the concentration had fallen to 4 μg ml^{-1} and the viable count had increased to greater than 10^6 cells ml^{-1}. In the presence of cloxacillin, however, which was itself relatively ineffective, the bactericidal effects of ampicillin were greatly increased and a combination of 10 μg ampicillin ml^{-1} + 10 μg cloxacillin ml^{-1} was significantly more active than 100 μg ampicillin ml^{-1} during the first 6 h of the test. A higher concentration of cloxacillin (100 μg ml^{-1}) almost completely prevented destruction of ampicillin (10 μg ml^{-1}) and caused a total bactericidal effect at 24 h. The combination containing 100 μg ml^{-1} of each penicillin sterilized the culture and there was no obvious destruction of ampicillin at the end of 24 h.

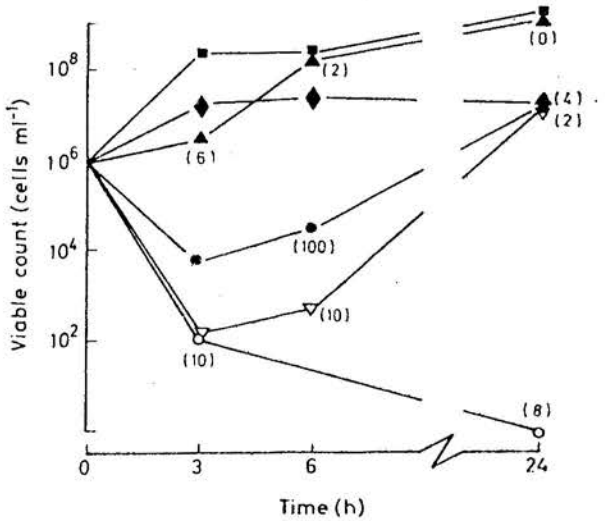

Figure 7.6 Bactericidal activities of ampicillin and ampicillin/cloxacillin combinations against E. coli *425.* ■ *= Control;* ◆ *= cloxacillin, 100 μg ml^{-1};* ▲ *= ampicillin, 10 μg ml^{-1};* ● *– ampicillin, 100 μg ml^{-1};* ▽ *= ampicillin, 10 μg ml^{-1} + cloxacillin, 10 μg ml^{-1};* ○ *= ampicillin, 10 μg ml^{-1} + cloxacillin, 100 μg ml^{-1}*

Although there has been considerable interest in applying the principle of β-lactamase inhibition as a means of treating resistant bacteria, clinical application of combinations of pencillins for this purpose has been limited (Sabath *et al.*, 1967; Riff, Olexy and Jackson, 1970; Klastersky, Debusscher and Ruhl, 1972). This is largely because no one compound has been found to be an effective and potent inhibitor of the more important of the wide range of β-lactamases produced by Gram-negative bacilli. Nevertheless, penicillins which are much more effective inhibitors of β-lactamases than the compounds clinically available have been described (Cole, Elson and Fullbrook, 1972) and one compound studied in detail, BRL 1437, an analogue of nafcillin, demonstrated impressive synergy in combination with other β-lactam antibiotics (Greenwood and O'Grady, 1975). Moreover, reports appearing in the literature describing potent mould-metabolite β-lactamase inhibitors (Ohno *et al.*, 1973; Umezawa

et al., 1973; Brown *et al.*, 1976) suggest the possibility of clinically useful substances being introduced to potentiate the efficacy of existing penicillins and cephalosporins against infections due to β-lactamase-producing bacteria.

Conclusion

Although the casual use of combinations of antibiotics is to be deprecated, there are numerous clinical settings which justify combined therapy with penicillins. The bactericidal synergy demonstrated *in vitro* between penicillins and aminoglycosides against enterococci is considered to have clinical significance and is generally accepted to provide an example of clinical synergism. The use of combinations of carbenicillin and aminoglycosides for the treatment of infections due to Gram-negative bacilli, particularly *Ps. aeruginosa*, is also widely accepted, largely on the grounds of the synergy that can be demonstrated *in vitro*, although clinical evidence is less readily available. The application of β-lactamase inhibitors to clinical therapy is limited as yet but there is evidence that proper use of this principle to produce synergy with β-lactam antibiotics has significant clinical potential.

Acknowledgments

I wish to thank my colleagues, D.F. Lawson and D.A. Wilson, for providing the photomicrographs illustrated in *Figure 7.5.*

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ACTIVITY OF AMOXYCILLIN AGAINST ENTEROCOCCI AND SYNERGISM WITH AMINOGLYCOSIDE ANTIBIOTICS

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ENHANCEMENT of the bactericidal activity of benzylpenicillin against enterococci by streptomycin is one of the earliest and best known examples of antibiotic synergism (Jawetz, Gunnison and Coleman, 1950). Enterococci are moderately sensitive *in vitro* to benzylpenicillin, which may also exert marked bactericidal activity though it generally fails to bring about complete sterilisation of cultures of these organisms. However, when benzylpenicillin is combined with streptomycin, which by itself is relatively ineffective, a marked synergistic action occurs with many strains of *Streptococcus faecalis*, resulting in a striking increase in the bactericidal activity of the penicillin. Treatment of enterococcal endocarditis with benzylpenicillin alone has often failed, whereas treatment with combinations of penicillin and streptomycin has achieved a large number of cures (Robbins and Tompsett, 1951; Geraci and Martin, 1954; Mandell *et al.*, 1970). The use of penicillin+streptomycin combinations for this purpose is probably the most widely accepted example of clinical synergism (Jawetz, 1968).

However, a number of strains of *S. faecalis* show a high level of resistance to streptomycin and in such cases synergism is not observed *in vitro* with combinations of penicillin and streptomycin (Harvard, Garrod and Waterworth, 1959; Standiford, de Maine and Kirby, 1970; Moellering *et al.*, 1971*a*). Other aminoglycoside antibiotics, namely kanamycin and gentamicin, are generally more active than streptomycin against enterococci (Toala *et al.*, 1970) and streptomycin-resistant strains are often sensitive to kanamycin and are almost always sensitive to gentamicin (Moellering, Wennersten and Weinberg, 1971*b*; Watanakunakorn, 1971). Combinations of benzylpenicillin with kanamycin or gentamicin are usually synergistic against *S. faecalis* (Watanakunakorn, 1971; Wilkowske *et al.*, 1971) provided that the strain is sensitive to the aminoglycoside (Standiford *et al.*, 1970; Moellering *et al.*, 1971*b*). Ampicillin has been shown to be more active than benzylpenicillin against *S. faecalis* (Sonne and Jawetz, 1968) and synergism has been demonstrated with ampicillin in combination with streptomycin or kanamycin (Fekety and Weiss, 1967; Sonne and

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Jawetz, 1968; Wilkowske *et al.*, 1971). Combinations of ampicillin and streptomycin have been used successfully in the treatment of enterococcal endocarditis (Mandell *et al.*, 1970).

Amoxycillin (D-α-amino-*p*-hydroxybenzylpenicillin) is a new semi-synthetic penicillin with an antibacterial spectrum and level of activity generally similar to that of ampicillin (Neu and Winshell, 1971*a*; Sutherland and Rolinson, 1971); it is particularly well absorbed after oral administration to human subjects (Croydon and Sutherland, 1971; Neu and Winshell, 1971*b*; Kosmidis *et al.*, 1972; Sutherland, Croydon and Rolinson, 1972). This new penicillin has been shown to be slightly more active than ampicillin against *S. faecalis* (Sutherland *et al.*, 1972) and this report is concerned with a study of the extent of in-vitro bactericidal activity of amoxycillin against the enterococcus, alone and in combination with aminoglycoside antibiotics.

Materials and methods

Enterococcal strains. The 55 strains of enterococci used in this study had been isolated from a variety of clinical sources (blood, urine and wounds). They were identified according to the criteria of Wilson and Miles (1964) and comprised *S. faecalis* (25 strains), *S. faecalis* subsp. *zymogenes* (15 strains), *S. faecalis* subsp. *liquefaciens* (13 strains) and *S. faecium* (2 strains).

Minimum inhibitory concentrations. Serial dilutions of the test antibiotics were added to 18-ml volumes of molten 5% blood agar (Blood Agar Base, Oxoid CM55, and Horse Blood, defibrinated, Wellcome) at 50°C and poured into petri dishes. The plates were dried at 37°C and inoculated with a drop (0·001 ml) of an undiluted overnight broth culture (Nutrient Broth no. 2, Oxoid CM67) of each strain of enterococcus by means of a replicating device delivering 20 cultures to each plate. Minimum inhibitory concentrations (MIC) were determined after incubation for 18 h at 37°C, the MIC being taken as the lowest concentration of antibiotic completely preventing visible growth.

Minimum bactericidal concentrations. Minimum concentrations of the antibiotics or of combinations of them inhibitory to large inocula were determined. Serial dilutions of drugs were made in tubes containing 5-ml volumes of nutrient broth. The tubes were inoculated with 0·03 ml of an overnight culture of the test organism, giving an inoculum of about 10^6 cells per ml, and the MICs were read after overnight incubation at 37°C. A loopful of culture was taken (loop diameter 5 mm) from each tube showing no visible growth after overnight incubation and was streaked on antibiotic-free blood agar containing a 1 in 100

Table I

Distribution of minimum inhibitory concentrations (MICs) of penicillins and aminoglycosides against 55 strains of enterococci

Antibiotic	Number of strains with an MIC (μg per ml) of											
	0·25	0·5	1·25	2·5	5·0	12·5	25	50	125	250	500	>500
Amoxycillin	5	49	1	0	0	0	0	0	0	0	0	0
Ampicillin	0	25	29	1	0	0	0	0	0	0	0	0
Benzylpenicillin	0	2	49	4	0	0	0	0	0	0	0	0
Gentamicin	0	0	1	19	26	7	2	0	0	0	0	0
Kanamycin	0	0	0	0	5	19	22	5	2	0	0	2*
Streptomycin	0	0	0	0	0	7	9	21	2	0	1	15*

* MIC>5000 μg per ml.

dilution of Wellcome penicillinase (Burroughs Wellcome Limited). The plates were incubated for 18 h at 37°C and the minimum bactericidal concentration (MBC) was determined as the lowest concentration of antibiotic from which subculture failed. Absence of growth corresponded to a viable count of 10^2 cells per ml or less. In a number of tests the inoculum was reduced to 0·03 ml of a 1 in 100 dilution of an overnight culture to determine the effect of a reduced inoculum size on the MBC of the compounds.

Bactericidal activity. The bactericidal activities of the penicillins and aminoglycosides, alone and in combination, were determined by measurement of the reduction in the viable count of an organism after exposure to the antibiotics. Tubes of nutrient broth (20-ml volumes) containing known concentrations of the antibiotics were inoculated with a drop (0·03 ml) of an overnight broth culture and incubated at 37°C. Samples taken after incubation for 3, 6, 24 and 48 h were suitably diluted in sterile distilled water and 0·02-ml volumes were pipetted on to the surface of blood-agar plates containing penicillinase. In tests with combinations of penicillins and aminoglycosides or where it was thought that the viable counts had fallen to very low levels, subcultures were made by spreading 1·0-ml volumes of the undiluted cultures over the plates. Colonies were counted after overnight incubation at 37°C and the viable counts were calculated.

RESULTS

Minimum inhibitory concentrations

The MICs of the penicillins and aminoglycosides for 55 strains of enterococci are shown in table I. All three penicillins were inhibitory at relatively low concentrations; amoxycillin was the most active, being about twice as active as benzylpenicillin which was itself rather less active than ampicillin. The aminoglycosides were less active than the penicillins against the enterococci, and gentamicin, the most active aminoglycoside, was two to four times less active than benzylpenicillin. Kanamycin was four times less active than gentamicin and was at least twice as active as streptomycin. All the strains of enterococci were sensitive to the penicillins, but 15 showed a high level of resistance to streptomycin (MIC>5000 μg per ml) and two of these were also insensitive to kanamycin (MIC>5000 μg per ml). None of the enterococci tested was resistant to gentamicin. There were no marked differences in the

TABLE II

Effect of inoculum size on the minimum bactericidal concentrations (MBCs) of amoxycillin, ampicillin and benzylpenicillin for 10 strains of enterococci

Inoculum	Penicillin	Number of strains with an MBC (μg per ml) of									
		0·25	0·5	1·25	2·5	5·0	12·5	25	50	100	>100
Large (*c.* 10^8 cells per ml)	Amoxycillin	0	0	0	0	0	0	0	0	0	10
	Ampicillin	0	0	0	0	0	0	0	0	0	10
	Benzylpenicillin	0	0	0	0	0	0	0	0	0	10
Small (*c.* 10^4 cells per ml)	Amoxycillin	1	0	5	4	0	0	0	0	0	0
	Ampicillin	0	0	1	8	0	1	0	0	0	0
	Benzylpenicillin	0	0	1	1	6	1	1	0	0	0

sensitivities of the various sub-species of *S. faecalis* to the penicillins or aminoglycosides, but the two strains of *S. faecium* were rather more resistant to the aminoglycosides than were the rest. The streptomycin-resistant enterococci included strains of *S. faecalis*, *S. faecalis* subsp. *liquefaciens* and *S. faecalis* subsp. *zymogenes*.

Minimum bactericidal concentrations

In tests in which a large inoculum (10^6 cells per ml) was used the MBCs of the penicillins exceeded 100 μg per ml for all 10 strains tested (table II) but with a smaller inoculum (10^4 cells per ml) MBCs were much lower and were quite close to the MICs of the compounds. In tests on 12 strains (10^6 cells per ml) the aminoglycosides showed some evidence of bactericidal activity against aminoglycoside-sensitive enterococci, but not against resistant strains (table III). When sub-lethal concentrations of the aminoglycosides were combined with amoxycillin, ampicillin or benzylpenicillin, the bactericidal activities of all combinations were very much greater than those of the compounds alone against aminoglycoside-sensitive strains of enterococci. However, bactericidal activity was not detected when the strain was resistant to the aminoglycoside in the combination under test. Thus the streptomycin-resistant

TABLE III

Minimum bactericidal concentrations (MBCs) of penicillins and aminoglycosides alone, and in combination, for 12 strains of enterococci

Penicillin	Aminoglycoside and its concentration (μg per ml)	Number of strains with an MBC (μg per ml)* of									
		0·25	0·5	1·0	2·5	5·0	10	25	50	100	>100
Amoxycillin	None	0	0	0	0	0	0	0	0	0	12
	Streptomycin (20)	1	0	2	2	0	0	0	0	0	7†
	Kanamycin (20)	0	5	4	1	0	0	0	0	0	2‡
	Gentamicin (10)	6	3	3	0	0	0	0	0	0	0
Ampicillin	None	0	0	0	0	0	0	0	0	0	12
	Streptomycin (20)	1	1	2	1	0	0	0	0	0	7†
	Kanamycin (20)	0	5	4	1	0	0	0	0	0	2‡
	Gentamicin (10)	1	3	7	0	1	0	0	0	0	0
Benzylpenicillin	None	0	0	0	0	0	0	0	0	0	12
	Streptomycin (20)	1	0	0	0	4	0	0	0	0	7†
	Kanamycin (20)	1	0	0	3	5	1	0	0	0	2‡
	Gentamicin (10)	3	2	2	4	1	0	0	0	0	0
None	Streptomycin	0	0	0	0	0	0	0	1	3	8
None	Kanamycin	0	0	0	0	0	0	0	2	5	5
None	Gentamicin	0	0	0	0	0	1	1	4	4	2

* For an inoculum size *c.* 10^6 cells per ml of medium.
† Streptomycin MIC>5000 μg per ml.
‡ Kanamycin MIC>5000 μg per ml.

strains were not killed by penicillin+streptomycin but were susceptible to the combinations that included kanamycin or gentamicin, except for two strains that were also resistant to kanamycin and these were not killed by the penicillin +kanamycin combinations.

Bactericidal activity

The bactericidal effects of the penicillins, aminoglycosides and penicillin+ aminoglycoside combinations are shown in greater detail in figs. 1–4. Thus, in a comparison of the bactericidal activities of amoxycillin and benzylpenicillin, amoxycillin was more effective than the latter in killing *S. faecalis* subsp. *zymogenes* strain no 816 (fig. 1). At a concentration of 1·0 μg per ml, benzylpenicillin was largely ineffective whereas amoxycillin reduced the viable count by greater than 99% after 6 h although this was followed by resumption of growth to a visible level at 48 h. At a higher concentration (10 μg per ml) benzylpenicillin showed greater bactericidal activity and brought about a 99% reduction in the viable count after 24 h, but was distinctly less active than amoxycillin and produced a slower rate of killing than did amoxycillin during the first 6 h. Amoxycillin reduced the viable count at 48 h by 99·99% but failed to sterilise the culture.

The results of a viable count experiment with combinations of amoxycillin with streptomycin or with kanamycin against the streptomycin-sensitive

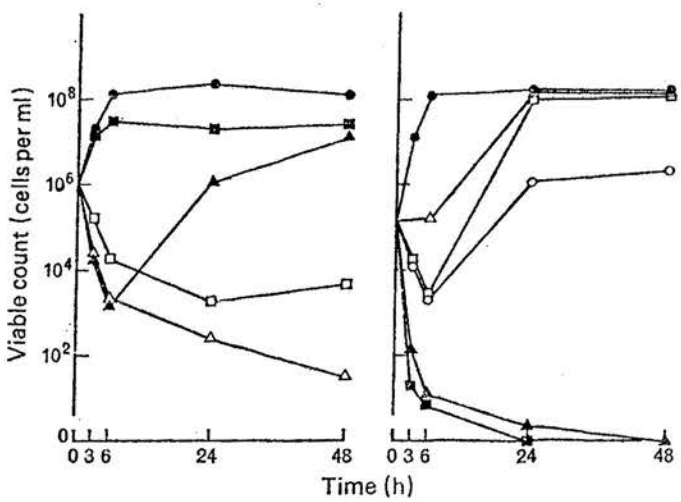

FIG. 1 (left).—The bactericidal activities of amoxycillin and benzylpenicillin against *Streptococcus faecalis* subsp. *zymogenes* strain no. 816; ●——● control; ▲——▲ amoxycillin 1·0 μg per ml; △——△ amoxycillin 10 μg per ml; ■——■ benzylpenicillin 1·0 μg per ml; □——□ benzylpenicillin 10 μg per ml.

FIG. 2 (right).—Bactericidal activities of amoxycillin, streptomycin and kanamycin alone and in combination against the streptomycin-sensitive *S. faecalis* strain no. 14; ●——● control; ○——○ amoxycillin 1·0 μg per ml; △——△ streptomycin 20 μg per ml; □——□ kanamycin 20 μg per ml; ▲——▲ amoxycillin 1·0 μg per ml + streptomycin 20 μg per ml; ■——■ amoxycillin 1·0 μg per ml +kanamycin 20 μg per ml.

S. faecalis strain no. 14 are shown in fig. 2. Streptomycin (20 μg per ml) inhibited growth of the organism for 6 h but by 24 h the viable count was the same as that in the control culture. Kanamycin (20 μg per ml) was bactericidal for 6 h but thereafter growth increased to equal that of the control. Similarly, amoxycillin (1·0 μg per ml) was bactericidal over the first 6 h but this was followed by a resumption of growth to a level greater than the initial count after 24 h. In contrast, a combination of amoxycillin (1 μg per ml) with streptomycin (20 μg per ml) or kanamycin (20 μg per ml) brought about a 99·99% reduction in viable count within 6 h, and the combinations sterilised the culture within 24 h in the case of kanamycin and within 48 h with streptomycin.

On the other hand a combination of amoxycillin and streptomycin failed to show synergistic effects against the streptomycin-resistant *S. faecalis* subsp. *zymogenes* strain no. 816 (MIC>5000 μg streptomycin per ml), the bactericidal activity of amoxycillin with streptomycin being no greater than that of amoxycillin alone (fig. 3). This strain was sensitive to kanamycin (MIC 25 μg per ml) and to gentamicin (MIC 25 μg per ml) and combinations of amoxycillin with kanamycin (20 μg per ml) or with gentamicin (10 μg per ml) showed significant bactericidal effects, sterilising the culture within 6 h with gentamicin or within 24 h with kanamycin. Similarly, combinations of amoxycillin with streptomycin or kanamycin were no more effective than amoxycillin alone in reducing the viable count of a culture of *S. faecalis* subsp. *zymogenes* strain no. 830 which was resistant to both streptomycin and kanamycin (MIC>5000 μg per ml) (fig. 4). However, the organism was sensitive to gentamicin and a combination of the latter with amoxycillin was synergistic, reducing the viable count to zero after 48 h (fig. 4).

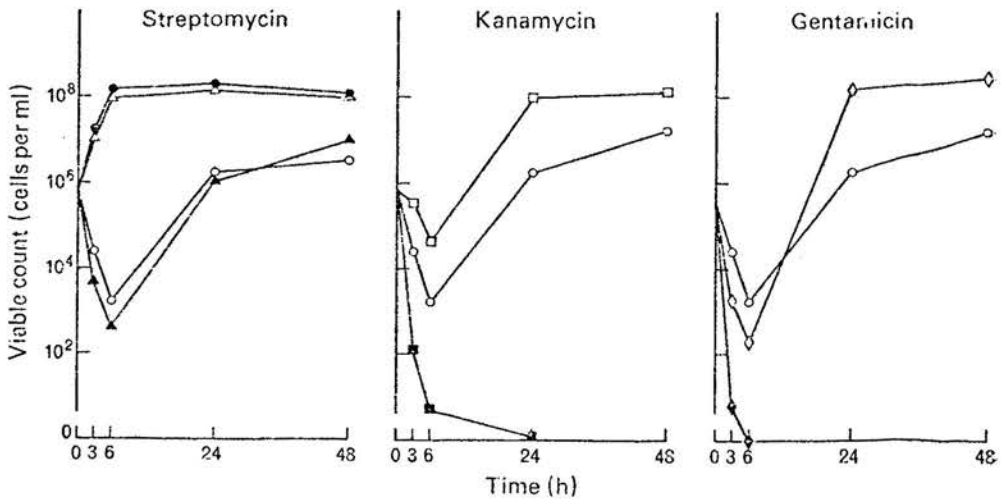

FIG. 3.—Bactericidal activities of amoxycillin, streptomycin, kanamycin and gentamicin alone and in combination against the streptomycin-resistant *S. faecalis* subsp. *zymogenes* strain no. 816: ●——● control; ○——○ amoxycillin 1·0 μg per ml; △——△ streptomycin 20 μg per ml, ▲——▲ amoxycillin 1·0 μg per ml + streptomycin 20 μg per ml; □——□ kanamycin 20 μg per ml; ■——■ amoxycillin 1·0 μg per ml + kanamycin 20 μg per ml; ◇——◇ gentamicin 10 μg per ml; ◆——◆ amoxycillin 1·0 μg per ml + gentamicin 10 μg per ml.

Discussion

The results reported here confirm that *in vitro* amoxycillin is more active than ampicillin (Sutherland *et al.*, 1972) and that ampicillin is more active than benzylpenicillin (Sonne and Jawetz, 1968) against enterococci, although the differences in the activities of the three penicillins are not pronounced. The enterococci tested were all sensitive to moderately low concentrations of the penicillins, but a number of strains showed a high level of resistance to streptomycin or kanamycin. Toala *et al.* (1970) state that there has been no apparent decrease in recent years in the sensitivity of enterococci to benzylpenicillin but that the contrary is true for other antibiotics including aminoglycosides. A relatively high incidence of resistance to streptomycin and kanamycin among enterococci was reported by Standiford *et al.* (1970) and Moellering *et al.* (1971a and *b*), but all strains were sensitive to gentamicin. In those studies it was found that streptomycin-resistant strains of enterococci were often sensitive to kanamycin but kanamycin-resistant strains were almost invariably resistant to streptomycin. A similar pattern of cross-resistance to the aminoglycoside antibiotics was observed among the enterococci described here.

The penicillins appeared to show poor bactericidal activity against a large inoculum of enterococci in terms of the minimum bactericidal concentrations (MBC) of the compounds. However, determination of the MBC was a particularly stringent test, requiring a fall in viable count to 10^2 cells per ml or less, which corresponded to a 99·99% kill of a large inoculum (10^6 cells per ml), for a bactericidal effect to be recorded. Under these conditions the MBCs of the penicillins were above 100 μg per ml. With a smaller inoculum (10^4 cells

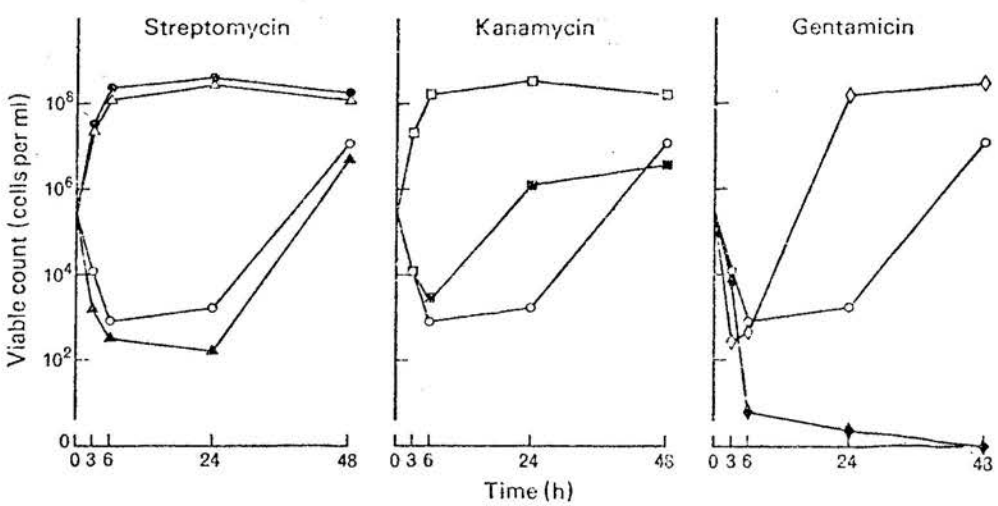

FIG. 4.—Bactericidal activities of amoxycillin, streptomycin, kanamycin and gentamicin alone and in combination against *S. faecalis* subsp. *zymogenes* strain no. 830, highly resistant to streptomycin and kanamycin; ●——● control; ○——○ amoxycillin 1·0 μg per ml; △——△ streptomycin 20 μg per ml; ▲——▲ amoxycillin 1·0 μg per ml + streptomycin 20 μg per ml; □——□ kanamycin 20 μg per ml; ■——■ amoxycillin 1·0 μg per ml + kanamycin 20 μg per ml; ◇——◇ gentamicin 10 μg per ml; ◆——◆ amoxycillin 1·0 μg per ml + gentamicin 10 μg per ml.

per ml), the penicillins readily reduced the viable count to less than 10^2 cells per ml and the MBCs were quite close to the MICs. In fact, the bactericidal activity experiments showed that moderately low concentrations of the penicillins rapidly produced marked bactericidal effects against a large inoculum of enterococci although the compounds failed to sterilise the cultures within the period of the test. In these tests amoxycillin was more effective than benzylpenicillin.

The synergism against enterococci shown by amoxycillin when combined with streptomycin, kanamycin or gentamicin is homologous with that of combinations of benzylpenicillin or ampicillin with aminoglycoside antibiotics. However, in the studies reported here, not all strains of enterococci showed increased susceptibility to penicillin+aminoglycoside combinations since synergism occurred only when the organism was sensitive to (inhibited by) the aminoglycoside being investigated. This finding is in agreement with data reported by Standiford *et al.* (1970) and Moellering *et al.* (1971*a*) who found that there was a good correlation between failure of penicillin+aminoglycoside combinations to produce synergism and resistance of the test strain of enterococcus to the bacteristatic action of aminoglycoside antibiotics. The relevance of the in-vitro synergism between penicillins and aminoglycosides against enterococci to the clinical situation has been questioned (Tompsett and Pizette, 1962) and clinical cures have been obtained with penicillins alone (Hein and Berg, 1949; Geraci and Martin, 1954), but the weight of evidence suggests that more favourable results are obtained with combinations of antibiotics (Garrod and Waterworth, 1962; Jawetz and Sonne, 1966; Mandell *et al.*, 1970). The treatment of choice for enterococcal endocarditis is generally considered to be a combination of benzylpenicillin with streptomycin but the fairly frequent occurrence of highly streptomycin-resistant strains of enterococci throws some doubt upon this. It is evident that there is a relatively high incidence of resistance to streptomycin and kanamycin among enterococci and that appropriate laboratory tests need to be carried out to assist in the choice of therapy. Many hospital laboratories find tests for synergism difficult and time-consuming, and it has been suggested that knowledge of the aminoglycoside sensitivity of the strain of enterococcus can be used to predict bactericidal synergism because of the good correlation already mentioned between these two characteristics (Standiford *et al*, 1970; Moellering *et al.*, 1971*a*). The results of this limited study are in accord with this suggestion.

Our results suggest that amoxycillin may be superior to ampicillin or benzylpenicillin in the treatment of enterococcal infections. In addition to its greater antibacterial activity, amoxycillin is particularly well absorbed after oral administration to man and produces penicillin serum-concentrations that are higher than those obtained with oral ampicillin and of about the same order as those produced by intramuscular benzylpenicillin. These considerations seem to justify clinical trial of amoxycillin in the treatment of enterococcal infection.

SUMMARY

Amoxycillin was more active *in vitro* than ampicillin or benzylpenicillin against clinical isolates of enterococci. All 55 strains tested were sensitive to the three penicillins but 15 strains showed a high level of resistance to streptomycin and two of these were also insensitive to kanamycin. All strains were sensitive to gentamicin, which was the most active of the aminoglycoside antibiotics.

The penicillins showed pronounced bactericidal activity against the enterococci but failed to sterilise cultures of these organisms. Combinations of penicillins and aminoglycosides invariably produced synergistic bactericidal effects which resulted in sterilisation of cultures of enterococci provided that the strain was sensitive to the aminoglycoside moiety of the antibiotic combination. Synergism was not observed with a combination of a penicillin and an aminoglycoside when the enterococcus was resistant to the aminoglycoside.

The data reported suggest that amoxycillin may have certain advantages, compared with ampicillin or benzylpenicillin, for the treatment of enterococcal infections.

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Activity of amoxycillin, alone, and in combination with aminoglycoside antibiotics against streptococci associated with bacterial endocarditis

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Clinical isolates of *Streptococcus viridans*, *Streptococcus bovis* and enterococci (*Streptococcus faecalis*) were tested for sensitivity to amoxycillin, ampicillin and benzylpenicillin and to streptomycin, kanamycin, gentamicin, tobramycin, amikacin and dibekacin. Amoxycillin was more active than ampicillin and benzylpenicillin against enterococci and was as active as benzylpenicillin and more active than ampicillin against *S. bovis* and viridans streptococci. The strains of *S. bovis* and *S. viridans* were more sensitive to the penicillins than were the enterococci, and amoxycillin demonstrated greater bactericidal activity against the first two species than against the last. Combinations of amoxycillin and aminoglycosides generally produced greater bactericidal effects than did amoxycillin alone against all three species of streptococci unless the organism was resistant to the aminoglycoside. Kanamycin-resistant strains of *S. faecalis* showed an increased resistance to amikacin in tests of bactericidal activity.

Introduction

Infection with streptococci is the commonest cause of classical bacterial endocarditis with the viridans group accounting for up to 70% of cases (Tompsett & Skripka, 1967; Gray, 1975). Other streptococci, particularly Group D streptococci, are also associated with the disease and these organisms are more common in the elderly patient or in infective endocarditis following cardiac surgery (Gray, 1975). Group D streptococci implicated in this condition can be classified broadly into enterococci, represented by *Streptococcus faecalis* and *Streptococcus faecium*, and the non-enterococcal species, *Streptococcus bovis*. Viridans streptococci are typically very sensitive to penicillins, and endocarditis caused by these organisms often responds to treatment with a penicillin alone, although an aminoglycoside, usually streptomycin, is frequently included in the treatment regimen. In contrast, because enterococci are less sensitive to penicillin, enterococcal endocarditis requires intensive combined therapy with a penicillin and an aminoglycoside. Strains of *S. bovis* are more like viridans streptococci than enterococci in their sensitivity to penicillins (Wilkowske, Facklam, Washington & Geraci, 1971; Thornsberry, Baker & Facklam, 1974) so that endocarditis caused by *S. bovis* may not require concomitant aminoglycoside therapy.

Treatment of endocarditis often involves parenteral administration of a penicillin, particularly in the initial stages, but compounds such as phenoxymethylpenicillin, phenethicillin and ampicillin have been used for oral therapy of suitable infections (Gray, Tai, Wallace & Calder, 1964; Tan, Terhune, Kaplan & Hamburger, 1971). Streptomycin has often been recommended as the aminoglycoside of choice for combined therapy with penicillins but awareness of the increasing incidence of streptomycin-resistant strains of enterococci has led to interest in the activity of newer aminoglycosides, particularly gentamicin (Hook, Roberts & Sande, 1975).

Amoxycillin is a new penicillin which has good activity against streptococci, including enterococci (Russell & Sutherland, 1975) and is well absorbed by the oral route (Sutherland, Croydon & Robinson, 1972). This report describes the relative activities of amoxycillin, ampicillin and benzylpenicillin and of streptomycin, kanamycin, gentamicin, tobramycin, amikacin and dibekacin against streptococci associated with endocarditis, and illustrates the bactericidal effects produced by combinations of these aminoglycosides with amoxycillin.

Materials and methods

Antibiotics

The penicillins used were amoxycillin trihydrate, ampicillin trihydrate and sodium benzylpenicillin (Beecham Pharmaceuticals), and the aminoglycosides were streptomycin sulphate (Glaxo Laboratories Ltd.) kanamycin sulphate (Winthrop Laboratories), gentamicin sulphate (Roussell Laboratories Ltd), tobramycin sulphate (Eli Lilly and Co. Ltd.), amikacin base (Bristol Laboratories) and dibekacin sulphate [(3′,4′-dideoxykanamycin B; Umezawa, Umezawa, Tsuchiya & Okazaki, 1971) Meiji Seika Kaisha Ltd.].

Cultures

The strains of streptococci tested were recent clinical isolates and mainly from patients with endocarditis. The cultures of *S. bovis* and most of the viridans streptococci were kindly donated by Dr M. T. Parker and Mrs L. C. Ball of the Central Public Health Laboratory, Colindale. Classification of the Group D strains as enterococcal or non-enterococcal species was confirmed using tests proposed by Deibel (1964) and by Facklam (1972).

Minimum Inhibitory Concentrations (MIC)

Serial dilutions of the test antibiotics were added to 18 ml volumes of molten 5% blood agar (Blood Agar Base, Oxoid; Defibrinated horse blood, Wellcome) at 50°C and poured into Petri dishes. The plates were dried at 55°C and inoculated with 0·001 ml of an undiluted 18 h broth culture of the test strain by means of a replicating device (Dynatech Laboratories) delivering 20 cultures per plate, an inoculum of 10^5 cells. MICs were determined after incubation for 18 h at 37°C, the MIC value being taken as the lowest concentration of antibiotic preventing visible growth.

Minimum Bactericidal Concentrations (MBC)

Serial dilutions of the antibiotics were made in 5 ml volumes of nutrient broth (Nutrient broth No. 2, Oxoid, for enterococci; Todd-Hewitt broth, Oxoid, for other streptococci) which were inoculated with 0·03 ml of an 18-h broth culture of the test organism, at

inoculum of about 5×10^6 cells. MIC values were recorded after 18 h incubation at 37°C. A loopful of culture (internal loop diameter 4 mm, volume about 0·01 ml) was taken from each tube not showing visible growth and streaked onto antibiotic-free blood agar containing penicillinase (Difco). The plates were incubated for 18-h at 37°C and the MBC was recorded as the lowest concentration of antibiotic from which a loop subculture failed to yield viable colonies. By this criterion the MBC was equal to that concentration of antibiotic which reduced the viable count of the culture to less than 10^2 cells/ml.

Bactericidal activity

Nutrient broth (20 ml) containing selected concentrations of the test antibiotics was inoculated with 0·03 ml of an 18-h broth culture of the test organism and incubated at 37°C. Samples taken after 0, 3, 6 or 24 h were suitably diluted in sterile distilled water and 0·02 ml vol. were dropped with a standard dropping pipette onto blood agar containing penicillinase. In tests where it was thought that only small numbers of viable cells remained, 1·0 ml vol. of the cultures were spread undiluted over the surface of blood agar plates containing penicillinase. Colonies were counted after 18 h incubation at 37°C and the viable counts were calculated.

Results

MIC

All three penicillins were very effective against viridans streptococci, amoxycillin and benzylpenicillin being of similar activity and both compounds were slightly more active than ampicillin. For example, amoxycillin and benzylpenicillin inhibited 50% of the strains at 0·01 μg/ml, whereas ampicillin caused 50% inhibition at 0·02 μg/ml (Figure 1). A number of strains were rather less sensitive to the penicillins and required 0·05 to 0·2 μg benzylpenicillin/ml for inhibition, compared with 0·01 to 0·1 μg amoxycillin/ml or 0·05 to 0·5 μg ampicillin/ml. The aminoglycosides were notably less effective than the penicillins. Gentamicin and tobramycin were the most active of these compounds and inhibited 50% of the strains at 1·0 μg/ml. Dibeckacin and streptomycin were slightly less active than tobramycin and inhibited 50% of strains at 2·5 μg/ml. The least active aminoglycosides were kanamycin and amikacin, which showed similar activity and were two- to fourfold less effective than dibekacin.

The strains of *S. bovis* were rather less sensitive to the penicillins than were the viridans streptococci. Amoxycillin and benzylpenicillin were again very similar in activity and both compounds were slightly more effective than ampicillin. Results in Figure 1 show that benzylpenicillin inhibited the growth of 90% of the strains at 0·05 μg/ml and that a similar concentration of amoxycillin or of ampicillin inhibited 70 and 50% of the strains, respectively. One strain showed a slight increase in resistance to benzylpenicillin and required 0·2 μg/ml for inhibition, but this strain was not more resistant to amoxycillin or to ampicillin. The aminoglycosides were less active than the penicillins against *S. bovis*, as was the case with the viridans streptococci. Gentamicin and tobramycin were again the most effective agents and both compounds inhibited 50% of the strains at 5·0 μg/ml. Dibekacin was slightly less active than tobramycin and amikacin and kanamycin were respectively two and fourfold less active than dibekacin. Streptomycin, like amikacin, inhibited 50% of the strains at 10 μg/ml, but one strain was very resistant to streptomycin (MIC 1000 μg/ml).

The enterococci were distinctly less susceptible to the penicillins than was the non-enterococcal Group D species (*S. bovis*) and showed a different pattern of sensitivity. Amoxycillin was the most active compound and was slightly more effective than ampicillin and twofold more active than benzylpenicillin. It can be seen from Figure 1 that 95% of strains were inhibited by 0·5 μg amoxycillin/ml and 60% by the same concentration of ampicillin. None of the strains was inhibited by 0·5 μg benzylpenicillin/ml but the compound inhibited 85% of strains at 1·0 μg/ml. The aminoglycosides were

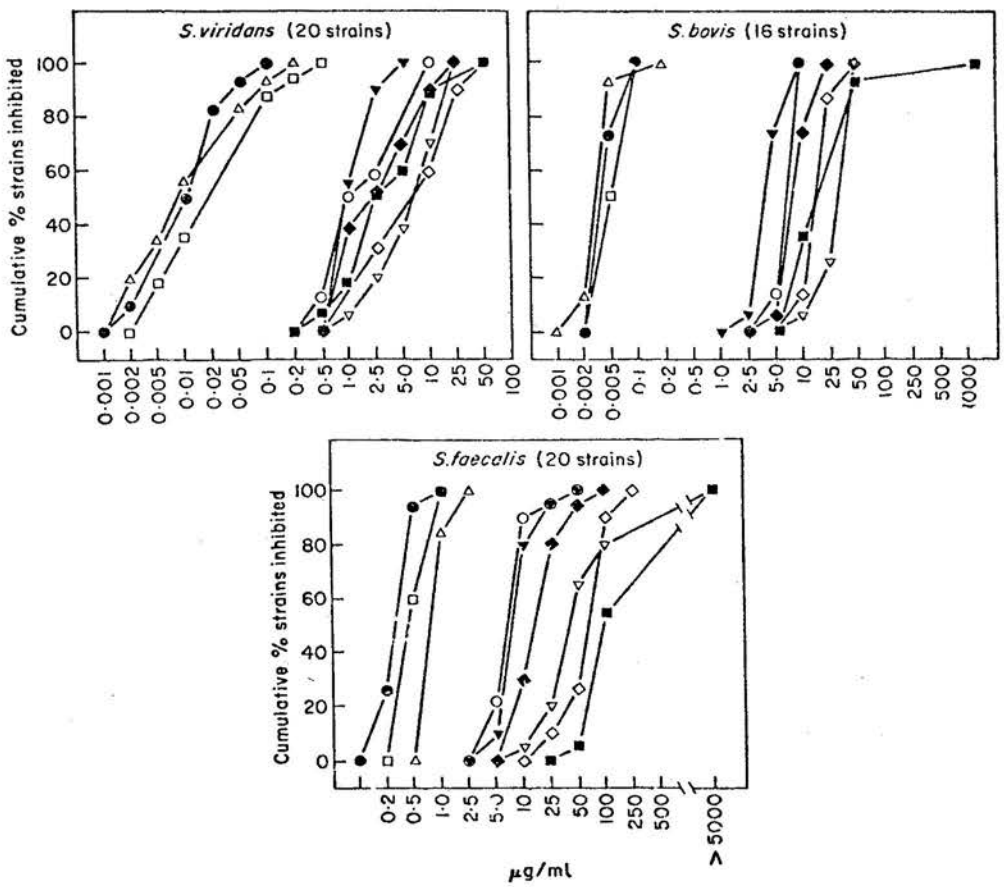

Figure 1. Activity of penicillins and aminoglycosides against streptococci. △, Benzyl penicillin; ⊖, amoxycillin; □, ampicillin; ▼, gentamicin; ○, tobramycin; ◆, dibekacin; ■, streptomycin; ◇, amikacin; ▽, kanamycin.

less active than the penicillins but gentamicin, tobramycin and dibekacin were as active against enterococci as against the non-enterococcal Group D streptococci. Gentamicin and tobramycin inhibited 50% of the strains of *S. faecalis* at 5·0 μg/ml and dibekacin at 10·0 μg/ml. Amikacin was rather less active than dibekacin and 50 μg/ml was required for inhibition of 50% of the strains. None of the enterococci was highly resistant to any of these four aminoglycosides but a number of strains were resistant to streptomycin and kanamycin. For example, streptomycin inhibited 55% of the strains at 100 μg/ml but the others were not inhibited by 5000 μg streptomycin/ml. Similarly,

kanamycin was as active or slightly more active than amikacin against 85% of the strains but the remainder were highly resistant with MIC values in excess of 5000 µg kanamycin/ml. Strains which were resistant to kanamycin were always resistant also to streptomycin but not all streptomycin-resistant strains were resistant to kananiycin.

MBC

In these tests, the penicillins showed MBC values only slightly higher than their MIC values against viridans streptococci or against *S. bovis* but against enterococci the compounds appeared to demonstrate poor bactericidal activity with MBC values greater than 100 µg/ml (Table I). The MBC values of amoxycillin against enterococci were

Table I. MIC and MBC values of amoxycillin, ampicillin and benzylpenicillin against *S. viridans* *S. bovis* and *S. faecalis*

Streptococcus species	Strain No.	Amoxycillin MIC (µg/ml)	Amoxycillin MBC (µg/ml)	Ampicillin MIC (µg/ml)	Ampicillin MBC (µg/ml)	Benzylpenicillin MIC (µg/ml)	Benzylpenicillin MBC (µg/ml)
S. viridans	V1	0·02	0·1	0·02	0·1	0·02	0·1
	V2	0·02	0·05	0·05	0·05	0·02	0·05
	V3	0·02	0·2	0·02	0·2	0·02	0·1
	V4	0·05	0·1	0·05	0·2	0·05	0·2
	V5	0·1	0·2	0·1	0·2	0·05	0·2
	V6	0·1	0·2	0·1	0·2	0·1	0·1
S. bovis	S1	0·1	0·5	0·1	0·5	0·1	0·5
	S2	0·1	0·5	0·1	0·5	0·1	0·5
	S3	0·1	0·2	0·1	0·5	0·1	0·2
	S4	0·2	0·2	0·5	0·5	0·1	0·5
	S5	0·2	0·5	0·2	0·5	0·1	0·2
S. faecalis	F1	0·5	>100	0·5	>100	1·0	<100
	F2	0·5	>100	0·5	>100	1·0	<100
	F3	0·5	>100	0·5	>100	1·0	<100
	F4	0·5	>100	1·0	>100	1·0	<100
	F5	0·5	>100	0·5	>100	1·0	<100
	F6	0·5	>100	1·0	>100	2·5	<100

Table II. Minimum bactericidal concentrations of amoxycillin alone and in combination with various aminoglycosides against nine strains of *S. faecalis*

Aminoglycoside	Concentration (µg/ml)	MBC (µg amoxycillin/ml) 0·5	1·0	2·5	>100
None					9
Streptomycin	20		1	2	6*
Kanamycin	20	2	2	2	3†
Gentamicin	5	6	2	1	
Tobramycin	5	6	1	2	
Amikacin	20		4	2	3†
Dibekacin	5	5	2	2	

*Streptomycin MIC >5000 µg/ml.
†Kanamycin MIC >5000 µg/ml.

improved when the penicillin was combined with sub-inhibitory concentrations of an aminoglycoside (Table II), but no synergism was detected with combinations of amoxycillin and streptomycin or kanamycin against enterococci which were highly resistant to these aminoglycosides. Combinations of amoxycillin with gentamicin, tobramycin or dibekacin were equally effective against all the strains tested. Interestingly, the kanamycin-resistant strains appeared to be resistant also to the amoxycillin/amikacin combination in these bactericidal tests, even though the strains were not highly resistant to amikacin in the growth inhibition (MIC) tests.

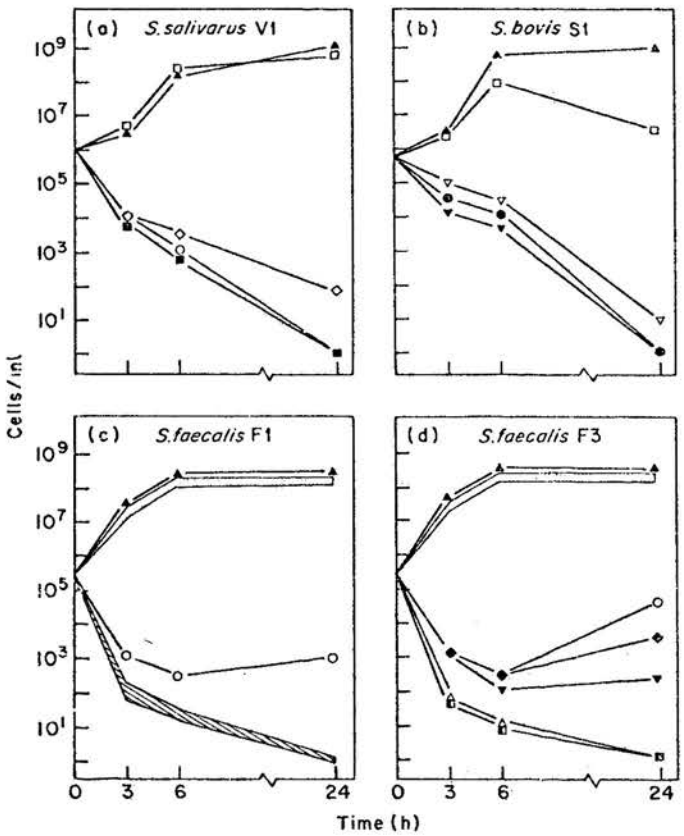

Figure 2. (a) Bactericidal activity of amoxycillin and gentamicin against *S. salivarius* V1. ▲, Control; ◇, amoxycillin 0·2 μg/ml; ○, amoxycillin 1·0 μg/ml; □, gentamicin 1·0 μg/ml; ■, amoxycillin + gentamicin 0·2 μg/ml + 1·0 μg/ml.

(b) Bactericidal activity of amoxycillin and gentamicin against *S. bovis* S1. ▲, Control; ▽, amoxycillin 0·5 μg/ml; ●, amoxycillin 2·5 μg/ml; □, gentamicin 1·0 μg/ml; ▼, amoxycillin + gentamicin 0·5 μg/ml + 1·0 μg/ml.

(c) Bactericidal activity of amoxycillin and aminoglycosides against *S. faecalis* F1. ▲, Control; ○, amoxycillin 1·0 μg/ml; ⁞⁞⁞⁞, aminoglycosides, amikacin 20 μg/ml, dibekacin 5·0 μg/ml, gentamicin 5·0 μg/ml, kanamycin 20 μg/ml and tobramycin 5·0 μg/ml; ⧵⧵⧵⧵, amoxycillin + aminoglycosides, 1·0 μg/ml + 5·0 or 20·0 μg/ml.

(d) Bactericidal activity of amoxycillin and aminoglycosides against *S. faecalis* F3. ▲, Control; ○, amoxycillin 1·0 μg/ml; ⁞⁞⁞⁞, as Figure 2(c); ▼, amoxycillin + amikacin 1·0 μg/ml + 20 μg/ml; △, amoxycillin + dibekacin 1·0 μg/ml + 5·0 μg/ml; ■□, amoxycillin + gentamicin or tobramycin 1·0 μg/ml + 5·0 μg/ml; ◆, amoxcillin + kanamycin 1·0 μg/ml + 20 μg/ml.

Bactericidal activity in viable count experiments

The bactericidal effects of amoxycillin alone and in combination with aminoglycosides against the various streptococcal species were measured also by viable count experiments and the results are shown in Figure 2. For example, low concentrations of amoxycillin (0·2 or 1·0 μg/ml) reduced the viable count of a viridans streptococcus (*Streptococcus salivarius* V1) by 99·0 to 99·9% within 6 h and at 1·0 μg/ml reduced the 24 h count to zero [Figure 2(a)]. The rate of kill was enhanced by the addition of an ineffective concentration of gentamicin and a combination of 0·2 μg amoxycillin/ml plus 1·0 μg gentamicin/ml was slightly more effective than was 1·0 μg amoxycillin/ml alone. Amoxycillin produced marked reductions in the viable counts of *S. bovis* S1 similar to those seen with the strain of *S. salivarius* and as shown in Figure 2(b) a concentration of 2·5 μg amoxycillin/ml reduced the counts by up to 95% in 6 h and sterilized the culture within 24 h. Again, an increased bactericidal effect was obtained by combination with gentamicin, as amoxycillin at 0·5 μg/ml plus gentamicin at 1·0 μg/ml was rather more effective than 2·5 μg amoxycillin/ml alone.

Amoxycillin produced marked bactericidal effects against *S. faecalis* (strains F1 and F3) and caused a 99·9% fall in the viable count by 6 h [Figure 2(c) and (d)] but at 24 h about 10^3 bacteria/ml were still viable. No improvement in bactericidal effect was observed when the concentration of amoxycillin was increased from 1·0 to 100 μg amoxycillin/ml but a marked enhancement of activity was obtained when the penicillin was combined with an aminoglycoside, provided the strain of enterococcus was sensitive to the aminoglycoside. In the case of the aminoglycoside-sensitive strain, *S. faecalis* F1, combinations of amoxycillin with any of the aminoglycosides examined produced marked bactericidal synergism such that by 24 h the culture was completely sterile [Figure 2(c)]. In contrast, with the kanamycin-resistant strain *S. faecalis* F3, combinations of amoxycillin and kanamycin or amoxycillin and amikacin were little better than amoxycillin alone [Figure 2(d)]. However, combinations of amoxycillin and gentamicin, tobramycin or dibekacin were synergistic and reduced the viable count of this strain to zero within 24 h.

Discussion

The viridans streptococci were the most sensitive of the streptococci tested to the penicillins and the compounds showed good bactericidal activity against these organisms. The aminoglycosides were much less active than the penicillins but the addition of a sub-inhibitory concentration of gentamicin enhanced the bactericidal activity of amoxycillin against a culture of *S. salivarius*. The bactericidal synergism demonstrated between benzylpenicillin and streptomycin against viridans streptococci *in vitro* is well-known (Hunter, 1952) and more recently, this combination was demonstrated to be superior to penicillin alone in preventing experimental endocarditis in rabbits infected with *S. viridans* (Durack & Petersdorf, 1973) and in treating established infections in this animal model (Durack, Pelletier & Petersdorf, 1974). On the other hand in clinical practice, many cases of endocarditis caused by viridans streptococci are treated with a penicillin, alone because of the extreme sensitivity of this group of organisms to penicillins (Gray, 1975), although streptomycin has often been included in the treatment regimen to enhance bactericidal activity (Wolfe & Johnson, 1974).

The Group D streptococci differed greatly in their sensitivities to the penicillins, the non-enterococcal species (*S. bovis*) being much more sensitive than were the enterococci

and being more like the viridans streptococci in this respect. Amoxycillin demonstrated pronounced bactericidal activity against *S. bovis* with MBC values of the same order as MIC values and low concentrations of amoxycillin sterilized a culture of *S. bovis* within 24 h. However, a combination of amoxycillin and gentamicin enhanced the bactericidal activity of amoxycillin against the test strain of *S. bovis* as was the case with the viridans streptococcus.

In contrast, the enterococci were distinctly less sensitive to the penicillins and the concentrations required to inhibit these organisms were up to ten times greater than those inhibitory for the strains of *S. bovis*. In addition the penicillins appeared to show poor bactericidal activity in MBC tests and amoxycillin failed to sterilize cultures of *S. faecalis*. There was usually no difference between the enterococci and *S. bovis* in their sensitivities to the aminoglycosides, gentamicin, tobramycin and dibekacin being the most active compounds. However, about half of the strains of enterococci were highly resistant to streptomycin and one-third of these were resistant also to kanamycin whereas only one strain of *S. bovis* was streptomycin-resistant. None of the strains of enterococci was highly resistant to gentamicin, tobramycin, amikacin or dibekacin in the growth inhibition (MIC) tests but it is noteworthy that amikacin was relatively ineffective against kanamycin-resistant strains of enterococci in the tests of bactericidal activity. Amikacin is a semi-synthetic derivative of kanamycin (Kawaguchi, Naito, Nakagawa & Fujisawa, 1972) and these findings indicate some degree of cross-resistance between the compounds against this organism.

Strains of non-enterococcal Group D streptococci (*S. bovis*) are being implicated more frequently in bacterial endocarditis (Ravreby, Bottone & Keusch, 1973; Watanakunakorn, 1974), and so the differences in the penicillin sensitivities of *S. bovis* and enterococci could well be of clinical importance and emphasize the need for correct identification of Group D streptococci associated with this condition. For example, as strains of *S. bovis* resemble viridans streptococci rather than enterococci in their sensitivity to penicillins it is reasonable to suppose that *S. bovis* endocarditis might respond to treatment with penicillin alone, and Thornsberry *et al.* (1974) have reported the successful bacteriological cure of 12 cases treated in this manner.

The desirability of using a penicillin/aminoglycoside combination for the treatment of enterococcal endocarditis is well established but in view of the incidence of streptomycin- and kanamycin-resistant strains of *S. faecalis* (Moellering, Wennersten, Medrek & Weinberg, 1971; Watanakunakorn, 1971) it is essential that the aminoglycoside sensitivity of the organism is known. It appears clear that bactericidal synergism will only be observed with a penicillin/aminoglycoside combination when the strain of enterococcus is sensitive to the aminoglycoside in question. On this basis, it has been suggested by Standiford, de Maine & Kirby (1970) and by Moellering *et al.* (1971) that synergism can be predicted from the results of MIC tests, and the findings reported here are in general agreement with this. However, the results with amikacin against kanamycin-resistant strains of enterococci indicate the need for tests for synergism in this particular case. Another example where synergism could not be predicted on the basis of the results of MIC tests was reported by Moellering, Wennersten & Weinstein (1973) who found that combinations of penicillin and tobramycin failed to show bactericidal synergism against apparently sensitive strains of *S. faecium*. Unfortunately it was not possible to confirm this report as none of the strains of enterococci tested here belonged to this species.

Amoxycillin might well be expected to be effective in the treatment of streptococcal endocarditis as the compound is highly active against streptococci associated with the disease and is bactericidal, alone, or in combination with established or newer aminoglycosides. Oral therapy with ampicillin has been successful in the treatment of many cases of streptococcal endocarditis (Gray, 1975) and amoxycillin is more active than ampicillin against streptococci and produces serum antibiotic concentrations in man twice those of ampicillin (Sutherland *et al.*, 1972). Amoxycillin has been reported to be effective in the treatment of a limited number of cases of streptococcal endocarditis, either alone or in combination with gentamicin (Mashimo, 1973; Seligman, 1974) so that further trials would appear to be justified.

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Aminoglycoside-resistant enterococci

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SUMMARY Thirty-four recent clinical isolates of *Streptococcus faecalis* were tested for sensitivity to amoxycillin, benzylpenicillin, streptomycin, kanamycin, gentamicin, tobramycin, and amikacin. Amoxycillin was two- to four-fold more active than benzylpenicillin and all strains were inhibited by low concentrations of the penicillins. The aminoglycosides were less active against the enterococci than were the penicillins and a significant number of strains were insensitive or relatively insensitive to one or more of the aminoglycosides. Thus, eight (23%) strains showed a high level of resistance to streptomycin and kanamycin (MIC > 5000 μg/ml) but were sensitive to gentamicin, tobramycin, and amikacin. In addition, two strains of *Strep. faecalis*, isolated at different hospitals from patients who had received topical gentamicin therapy, were relatively resistant to gentamicin (MIC 250 to 500 μg/ml) and were less sensitive also to the other aminoglycosides. Bactericidal synergy was demonstrated by amoxycillin/aminoglycoside combinations against the enterococci, provided that the test strain of *Strep. faecalis* was sensitive to the aminoglycoside in the combination. An exception to this was the combination of amoxycillin plus amikacin which was not synergistic against kanamycin-resistant strains of *Strep. faecalis* although these organisms were sensitive to amikacin in the growth inhibition tests. The gentamicin-resistant strains showed variable responses to amoxycillin/aminoglycoside combinations in tests for bactericidal synergy and were generally less sensitive than typical strains of *Strep. faecalis*.

The aminoglycoside group of antibiotics is relatively inactive against enterococci and these compounds are not usually considered for the treatment of enterococcal infections. However, combinations of aminoglycosides with penicillins can be shown to produce bactericidal synergy against many strains of enterococci, and combined penicillin/aminoglycoside therapy is recommended for the treatment of severe infections, notably enterococcal endocarditis (Garrod *et al.*, 1973). Synergism is not always demonstrated *in vitro* by combinations of penicillins and aminoglycosides and is dependent upon the level of resistance of the organism to the aminoglycoside. Thus a number of strains of enterococci are highly resistant to streptomycin or kanamycin, and combinations of penicillin and streptomycin or penicillin and kanamycin are not synergistic against these strains (Standiford *et al.*, 1970; Moellering *et al.*, 1971; Russell and Sutherland, 1975).

The newer aminoglycosides, gentamicin and tobramycin, are more active *in vitro* than streptomycin and kanamycin against enterococci (Moellering *et al.*, 1973; Russell and Sutherland, 1975; Iannini *et al.*, 1976), and amikacin, a semisynthetic derivative of kanamycin, is as active as kanamycin against these organisms (Iannini *et al.*, 1976). Enterococci that are resistant to high levels of streptomycin or kanamycin are sensitive to these new aminoglycosides, and combined therapy with the new compounds and penicillins has been proposed for enterococcal infections (Moellering *et al.*, 1971; Moellering *et al.*, 1973; Ruhen and Darrell, 1973; Russell and Sutherland, 1975; Iannini *et al.*, 1976).

There have been no reports in the literature of enterococci resistant to gentamicin, tobramycin or amikacin, but during a study in this laboratory of bacteria isolated from various hospitals during December 1975 it was observed that three cultures of *Strep. faecalis* were relatively resistant to gentamicin and tobramycin. It was also noted that amikacin failed to produce bactericidal synergy in combination with penicillins against kanamycin-resistant strains of *Strep. faecalis*. This report describes the sensitivities of recent isolates of *Strep. faecalis* to penicillins, aminoglycosides, and combinations of the compounds.

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Material and methods

ANTIBIOTICS

The penicillins tested were amoxycillin trihydrate and sodium benzylpenicillin (Beecham Pharmaceuticals) and the aminoglycosides were streptomycin sulphate (Glaxo Laboratories Ltd), kanamycin sulphate (Winthrop Laboratories), gentamicin sulphate (Roussell Laboratories Ltd), tobramycin sulphate (Eli Lilly and Co Ltd), and amikacin base (Bristol Laboratories).

CULTURES

The strains of enterococci tested were clinical isolates collected from three hospitals during December 1975 which had been cultured from a variety of sources (blood, urine, and wounds). The organisms were identified as group D streptococci by serotyping and were classified as strains of *Strep. faecalis* according to the criteria of Deibel (1964).

MINIMUM INHIBITORY CONCENTRATIONS

Antibacterial activity was measured by serial dilution of the antibiotics in 18 ml volumes of 5% blood agar (Blood Agar Base, Oxoid; Defibrinated Horse Blood, Wellcome). Agar plates were inoculated with 0·001 ml of an undiluted overnight culture of the test strain delivered with a multiple inoculating device (Dynatech Laboratories). Minimum inhibitory concentrations (MIC) were measured after incubation at 37°C for 18 hours.

MINIMUM BACTERICIDAL CONCENTRATIONS OF AMOXYCILLIN/AMINOGLYCOSIDE COMBINATIONS

Serial dilutions of amoxycillin were made in 4·5 ml volumes of nutrient broth (Nutrient broth No. 2, Oxoid), and 0·5 ml volumes of selected concentrations of the aminoglycosides were added to each tube. The aminoglycoside concentrations were selected as being levels attainable in the blood after usual dosage, and at these concentrations the compounds failed to demonstrate bactericidal activity against the test organisms. The tubes were inoculated with 0·03 ml of an overnight broth culture (approximately 10^7 cells) and incubated at 37°C for 18 hours. A loopful of culture was taken from each tube not showing visible growth and streaked onto blood agar containing penicillinase (Difco). The plates were incubated overnight at 37°C and the minimum bactericidal concentration (MBC) was recorded as the lowest concentration of antibiotic from which subculture failed to yield viable colonies.

BACTERICIDAL ACTIVITY

Tubes of nutrient broth (10 ml) containing known concentrations of the antibiotics were inoculated with 0·03 ml of an 18-hour broth culture and incubated at 37°C. Samples were taken at intervals and 0·02 ml volumes of suitable dilutions were pipetted onto blood agar containing penicillinase. Colonies were counted after incubation at 37°C for 24 hours and the number of viable bacteria was estimated.

Results

MINIMUM INHIBITORY CONCENTRATIONS

Results in Table 1 show the distribution of the minimum inhibitory concentrations of amoxycillin, benzylpenicillin, and various aminoglycosides against 34 recent clinical isolates of *Strep. faecalis*. Amoxycillin was the most active of the compounds and was two- to four-fold more active than benzylpenicillin. All cultures were sensitive to low concentrations of the penicillins. The aminoglycosides were notably less active than the penicillins, and the descending order of activity was tobramycin, gentamicin, kanamycin, amikacin, and streptomycin. Eight strains were highly resistant to both streptomycin and kanamycin (MIC values > 5000 μg/ml) but these strains showed no increase in resistance to gentamicin, tobramycin, or amikacin. Another strain was highly resistant to streptomycin but was sensitive to kanamycin and the other compounds.

Three strains of *Strep. faecalis* were notably less sensitive to gentamicin than were the majority of strains, and the sensitivities of these organisms to the

Table 1 *Distribution of minimum inhibitory concentrations of amoxycillin, benzylpenicillin, and aminoglycosides against 34 strains of* Streptococcus faecalis

Antibiotic	*Minimum inhibitory concentration (μg/ml) and number of strains*												
	0·25	*0·5*	*1·25*	*2·5*	*5·0*	*12·5*	*25*	*50*	*125*	*250*	*500*	*1250*	*>5000*
Amoxycillin	30	4											
Benzylpenicillin		17	13	4									
Streptomycin								3	16	2	3	1	9
Kanamycin						3	6	11	3	—	—	3	8
Gentamicin				5	12	11	2	1	—	2	1		
Tobramycin			5	3	8	12	2	1	2	1			
Amikacin						1	2	17	10	—	3	1	

Table 2 *Minimum inhibitory concentrations of amoxycillin, benzylpenicillin, and aminoglycosides against clinical isolates and laboratory-selected strains of* Strep. faecalis *resistant to gentamicin or streptomycin*

Antibiotic	*Minimum inhibitory concentration (μg/ml)*						
	Clinical isolates				*Laboratory-selected strains*		
	C134	*C135*	*W464*	*Control[1] C36*	*C90 parent*	*C90 S res[2]*	*C90 G res[3]*
Amoxycillin	0·25	0·25	0·25	0·25	0·25	0·25	0·25
Benzylpenicillin	0·5	0·5	1·25	0·5	1·0	0·5	0·25
Streptomycin	500	500	1250	>5000	50	>5000	125
Kanamycin	1250	1250	1250	>5000	50	50	500
Gentamicin	250	250	500	5·0	10	10	250
Tobramycin	125	125	250	5·0	10	10	100
Amikacin	500	500	1250	50	125	125	2500

[1]Streptomycin, kanamycin resistant.
[2]Streptomycin resistant, selected after three subcultures *in vitro* in the presence of streptomycin.
[3]Gentamicin resistant, selected after three subcultures *in vitro* in the presence of gentamicin.

aminoglycosides are shown in Table 2. Two of the strains, C134 and C135 isolated from different sites in the same patient, showed the same characteristics and were regarded as being the same organism. The third strain, W464, was isolated from a patient at a different hospital. Both patients had been treated with topical gentamicin for the treatment of leg infections. The three strains were 50- to 100-fold less sensitive to gentamicin than was a control sensitive strain and required 250 to 500 μg gentamicin/ml for inhibition of growth in these tests. These strains also showed a reduced sensitivity to the other aminoglycosides, but the reduction in sensitivity to streptomycin, kanamycin, and amikacin was lower than that seen with gentamicin or tobramycin.

The extent of the cross-resistance observed among the aminoglycosides against the clinical isolates resistant to gentamicin was similar to that seen with gentamicin-resistant strains selected *in vitro* by cultivation in the presence of gentamicin. Thus results in Table 2 show the sensitivities of a strain of *Strep. faecalis* C90, sensitive to streptomycin and gentamicin, and of cultures of this strain selected after three subcultures in the presence of streptomycin or gentamicin. The cultures isolated after exposure to streptomycin showed a high level of resistance to streptomycin (MIC >5000 μg/ml) but displayed no increase in resistance to the other aminoglycosides. In contrast, the strain cultured in the presence of gentamicin showed a moderate level of resistance to gentamic in (MIC250 μg/ml) and also demonstrated an increase in resistance to the other aminoglycosides.

Attempts to demonstrate transfer of gentamicin-resistance from *Strep. faecalis* C134 and *Strep. faecalis* W464 to a plasmid-free recipient strain of *Strep. faecalis* (JH2-2) (Jacob and Hobbs, 1974) were unsuccessful, although haemolysin production, which is known to be plasmid-mediated (Jacob *et al.*, 1975) was transferred from *Strep. faecalis* C134 to the recipient strain in these tests. Also no loss of gentamicin resistance was observed after growth of the cultures in the presence of ethidium bromide, acridine-orange or incubation at 44°C, under which conditions haemolysin production was eliminated from *Strep. faecalis* C134. In contrast, transfer of streptomycin and kanamycin resistance was demonstrated from strains of *Strep. faecalis* with high level resistance (MIC >5000 μg/ml) to the recipient strain of *Strep. faecalis* JH2-2 under the same conditions.

BACTERICIDAL SYNERGISM STUDIES

The results of tests to determine the minimum bactericidal concentrations (MBC) of combinations of amoxycillin and the aminoglycosides against a number of clinical isolates of *Strep. faecalis* of different aminoglycoside sensitivities are shown in Table 3. Amoxycillin was ineffective in these tests (MBC >100 μg/ml), but in combination with sub-lethal concentrations of the aminoglycosides, amoxycillin MBC values (5·0 μg/ml) against the aminoglycoside sensitive strain of *Strep. faecalis* (T1089) were not greatly in excess of inhibitory concentrations (*ca* 1·0 μg/ml). Against the streptomycin-resistant strain C119, the amoxycillin/streptomycin combination was not bactericidal but the other combinations were as effective against this strain as against the sensitive strain, T1089. Similarly, the amoxycillin/streptomycin and amoxycillin/kanamycin combinations failed to demonstrate bactericidal activity against the streptomycin/kanamycin resistant strain of *Strep. faecalis* C36. Unexpectedly, the MBC of the amoxycillin/amikacin combination was in excess of 100 μg amoxycillin/ml + 20 μg amikacin/ml, although the organism showed typical sensitivity to amikacin in the growth inhibition tests (MIC 50 μg/ml). Similar results were obtained in tests with the other seven kanamycin-resistant strains isolated in this study, and amoxycillin/amikacin and benzyl-

Table 3 *Minimum bactericidal concentrations of amoxycillin, alone, and in combination with aminoglycosides, against aminoglycoside-resistant strains of* Streptococcus faecalis

Amoxycillin/aminoglycoside combination	*Minimum bactericidal concentration (μg/amoxycillin/ml)*			
	T1089[1] *(sens)*	*C119 (S res)*	*C36 (S K res)*	*C134/W464 (G res)*
Amoxycillin alone	>100	>100	>100	>100
+ streptomycin (20)[2]	5·0	>100	>100	5·0[3]
+ kanamycin (20)[2]	5·0	5·0	>100	5·0[3]
+ gentamicin (5·0)[2]	5·0	1·0	5·0	5·0[3]
+ tobramycin (5·0)[2]	5·0	1·0	1·0	5·0[3]
+ amikacin (20)[2]	5·0	5·0	>100	5·0[3]

[1]Strain number.
[2]Aminoglycoside concentration (μg/ml); the aminoglycosides were ineffective at these concentrations.
S, streptomycin; K, kanamycin; G, gentamicin.
[3]Growth often occurred on subculture from tubes containing higher concentrations.

Table 4 *Bactericidal activities of amoxycillin alone and in combination with aminoglycosides in duplicate tests against gentamicin-resistant strains of* Streptococcus faecalis

Amoxycillin/aminoglycoside combination	*Viable count after 24 h at 37°C (cells/ml)*							
	Strain C134		*Strain C135*		*Strain W464*		*Control strains*	
	1	*2*	*1*	*2*	*1*	*2*	*T1089*[2]	*T1101*[3]
Amoxycillin (2·5)[1] alone	>100	350	>100	200	>100	250	>100	200
+ streptomycin (20)[1]	40	200	2	0	70	750	0	200
+ kanamycin (20)[1]	0	50	>100	0	0	15	0	>100
+ gentamicin (5·0)	0	40	>100	100	0	11	0	0
+ tobramycin (5·0)	1	80	80	13	1	36	0	0
+ amikacin (20)	0	35	75	0	10	70	0	450

[1]Concentration (μg/ml); the aminoglycosides were ineffective at these concentrations.
[2]Aminoglycoside-sensitive.
[3]Resistant to streptomycin and kanamycin.

penicillin/amikacin combinations failed to produce synergistic bactericidal effects against any of the kanamycin-resistant (amikacin-sensitive) strains of *Strep. faecalis*. There was no evidence of cross-resistance with the amoxycillin/gentamicin and amoxycillin/tobramycin combinations against the kanamycin-resistant strain *Strep. faecalis* C36, and both combinations produced marked bactericidal activity against this strain and other kanamycin-resistant enterococci.

The gentamicin-resistant isolates responded in a more variable fashion to amoxycillin/aminoglycoside combinations in the bactericidal tests compared with gentamicin-sensitive strains. In the MBC tests, sensitive strains gave a sharp end-point so that no growth was observed on subculture from combinations containing amoxycillin at concentrations of 5·0 μg/ml or more. With the gentamicin-resistant strains there was no growth from cultures containing 5·0 μg amoxycillin/ml or 10 μg amoxycillin/ml in combination with the aminoglycosides but growth sometimes occurred in subcultures from tubes containing higher concentrations of amoxycillin. In the viable count tests, gentamicin-sensitive strains of *Strep. faecalis* were always sterilised by combinations of 2·5 μg amoxycillin plus 5·0 μg gentamicin/ml whereas, with the gentamicin-resistant strains, on some occasions the cultures were made sterile, but on repeat tests viable bacteria were often recovered (Table 4). Similar variable responses were observed with combinations containing other aminoglycosides.

Discussion

The results reported here for a limited number of recent clinical isolates of *Strep. faecalis* show the prevalence of a relatively high proportion of strains with reduced sensitivity to aminoglycoside antibiotics. Strains resistant to streptomycin or kanamycin demonstrated a high level of resistance to the compounds but were sensitive to gentamicin, tobramycin or amikacin. In contrast, the strains with reduced sensitivity to gentamicin showed a moderate level of resistance to gentamicin and to the other aminoglycosides.

Enterococci with a high level of resistance to streptomycin have been known for some time (Havard *et al.*, 1959), and more recent reports have described a relatively high incidence of resistance to streptomycin and kanamycin (Standiford *et al.*,

1970; Moellering *et al.*, 1971; Ruhen and Darrell, 1973; Iannini *et al.*, 1976). In this study, the streptomycin-resistant strains of *Strep. faecalis* isolated in 1975 were almost always resistant to kanamycin which contrasts with the results of earlier studies reported from this laboratory. For example, none of 10 streptomycin-resistant strains of *Strep. faecalis* isolated in 1961 and only two of 15 streptomycin-resistant strains isolated in 1967 were resistant also to kanamycin (Sutherland and Robinson, 1964; Russell and Sutherland, 1975). The apparent increase in the proportion of kanamycin-resistant strains relative to streptomycin-resistant strains observed here may be a reflection of increased usage of kanamycin, but the number of strains tested was very small.

Enterococci with a high level of resistance to streptomycin or kanamycin have been shown to be sensitive to gentamicin, tobramycin, and amikacin (Iannini *et al.*, 1976), and bactericidal synergy has been demonstrated by a combination of penicillins and gentamicin or tobramycin against these resistant strains (Moellering *et al.*, 1971; Moellering *et al.*, 1973; Ruhen and Darrell, 1973; Russell and Sutherland, 1975). The results of this study are in agreement with these reports but the failure to demonstrate synergism with penicillin/amikacin combinations against kanamycin-resistant strains of *Strep. faecalis* was unexpected in view of the apparent sensitivity of these organisms to amikacin. Amikacin/penicillin combinations produce synergistic effects against kanamycin-sensitive strains, and the lack of synergy against kanamycin-resistant strains is evidence of cross-resistance between kanamycin and amikacin which is not observed in growth-inhibition tests.

Gentamicin-resistant enterococci have not been reported previously in the literature, and the strains described here do not show the high level of resistance which is characteristic of streptomycin and kanamycin-resistant enterococci. Nevertheless the concentrations of gentamicin required to inhibit growth of the two cultures of *Strep. faecalis* (250 to 500 μg/ml) were notably higher than the levels required to inhibit the majority of strains (5·0-12·5 μg/ml). Moreover, the variable results obtained in the bactericidal tests with these two strains contrasted with the uniform bactericidal synergy observed with combinations of amoxycillin and gentamicin against sensitive strains of *Strep. faecalis*. The rationale for the use of penicillin/aminoglycoside combinations in the treatment of enterococcal infections has been largely based upon the fact that such combinations can sterilise cultures of enterococci *in vitro* (Garrod *et al.*, 1973), but with these gentamicin-resistant strains this effect was not always observed.

The characteristics of the gentamicin-resistant clinical isolates, namely, the moderate level of resistance and degree of cross-resistance with other aminoglycosides, were markedly similar to those of the resistant strains selected *in vitro* after subculture in the presence of gentamicin. These findings, coupled with the failure to demonstrate conjugal transfer of resistance or to eliminate resistance with curing agents, suggest that the strains might have arisen *in vivo* by selection as a result of therapy with topical gentamicin, as has been reported for *Pseudomonas aeruginosa* (Snelling *et al.*, 1971; Holmes *et al.*, 1974) and, more recently, for *Staphylococcus aureus* (Porthouse *et al.*, 1976: Warren and Roberts, 1976).

Therapy with benzylpenicillin and streptomycin has often been recommended as the treatment of choice for severe enterococcal infections but awareness of the increasing incidence of streptomycin and kanamycin-resistant strains has led to an increased interest in the activity of combinations of penicillins with newer aminoglycosides, namely, gentamicin, tobramycin, and amikacin. The finding of strains that are apparently less sensitive to these compounds emphasises the need for appropriate laboratory tests for the selection of the most suitable penicillin/aminoglycoside combination for therapy of enterococcal infections.

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Synergy Between Ticarcillin and Tobramycin Against *Pseudomonas aeruginosa* and *Enterobacteriaceae* In Vitro and In Vivo

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The antibacterial activities of ticarcillin, carbenicillin, tobramycin, and gentamicin and of combinations of these antibiotics were measured against *Pseudomonas aeruginosa* and other gram-negative bacilli in vitro and in experimental mouse infections. Synergistic effects were produced by the penicillin/aminoglycoside combinations in growth inhibition tests and in bactericidal tests against many of the bacteria tested. Combinations of ticarcillin + tobramycin were more active in vitro than carbenicillin + gentamicin against *P. aeruginosa* but were no more active than the latter against other gram-negative bacilli. Ticarcillin + tobramycin and carbenicillin + gentamicin also demonstrated synergistic activities against *P. aeruginosa*, *Escherichia coli*, and *Enterobacter cloacae* in experimental mouse infection models. Thus, the penicillin/aminoglycoside combinations produced greater protective effects than the individual antibiotics against lethal intraperitoneal infections and also were more effective in reducing kidney counts of viable bacteria and kidney abscess formation in experimental pyelonephritis infections. As was the case in vitro, ticarcillin + tobramycin was more effective than carbenicillin + gentamicin against the experimental *P. aeruginosa* infections. The results of these in vitro and in vivo studies suggest that combined therapy with ticarcillin and tobramycin may be warranted in the treatment of serious infections due to *P. aeruginosa* and *Enterobacteriaceae*.

Combined therapy with carbenicillin and gentamicin is often recommended for the treatment of serious infections caused by gram-negative bacilli because of the synergy that can be demonstrated in vitro and in vivo against *Pseudomonas aeruginosa* and other gram-negative bacilli (2, 3, 10, 12, 18). A number of newer aminoglycosides with claims to being superior to gentamicin in some respect have become available, and combinations of carbenicillin with tobramycin (2, 7, 9, 21), amikacin (9, 11, 14), sisomicin (9, 14), and netilmicin (14) have been reported to be synergistic against *P. aeruginosa*. Similarly, ticarcillin, a semisynthetic penicillin analogue of carbenicillin, has been shown to produce synergy when combined with gentamicin (1, 15, 20, 21), but no experimental data have been reported for combinations of ticarcillin with the newer aminoglycosides.

The present study was designed to investigate the antibacterial effects produced by combining ticarcillin with tobramycin against *P aeruginosa* and other gram-negative bacilli. The combinations of antibiotics were examined for synergy in growth inhibition and bactericidal tests in vitro and against two experimental mouse infection models. In these tests the activity of ticarcillin + tobramycin was compared with that of carbenicillin + gentamicin.

MATERIALS AND METHODS

Bacteria. Most of the cultures tested in vitro and in vivo were clinical isolates from specimens of blood, urine, and wounds.

Mice. Male and female albino mice, 18 to 22 g, were used for the intraperitoneal infection studies, and female mice, 18 to 20 g, were used for the experimental pyelonephritis. The OLAC strain of mouse (Oxford Laboratory Animal Colony) was employed throughout.

Antibiotics. Dry powder preparations of ticarcillin disodium, 815 μg/mg (Beecham Pharmaceuticals); carbenicillin disodium, 790 μg/mg (Beecham Pharmaceuticals); tobramycin sulfate, 910 μg/mg (Eli Lilly and Co. Ltd., Basingstoke, England); and gentamicin sulfate, 587 μg/mg (Nicholas Laboratories Ltd., Slough, England) were used in the in vitro and in vivo studies. All concentrations were adjusted to penicillin free acid or aminoglycoside free base.

MICs. The activities of the penicillins and aminoglycosides, alone and in combination, were measured by a chess board test using a microtiter broth

dilution technique (3). Nutrient broth (Oxoid no. 2) containing the penicillin, the aminoglycoside, or a combination of the penicillin + aminoglycoside was serially diluted by twofold steps in 0.05-ml volumes per well to produce the desired range of antibiotic concentrations. Each well was inoculated with a replicating device (Dr. Tidley-Tech Ltd., Billingshurst, Sussex, England), using stainless-steel pins delivering 0.001 ml of a 1/10 dilution of an 18-h broth culture of the test organism, an inoculum of approximately 5×10^4 cells per well, equivalent to approximately 10^6 cells per ml. Minimum inhibitory concentrations (MICs) were read after 18 h of incubation at 37°C.

MBCs. Samples of culture (0.001 ml) were removed from each well of the microtiter plates by means of the inoculating device described above, after the MIC values had been read, and subcultured in 0.05-ml volumes of sterile, antibiotic-free nutrient broth in each well of a microtiter plate. The wells were examined for growth after overnight incubation at 37°C, and the minimum bactericidal concentration (MBC) was taken as the concentration of antibiotic or combination of antibiotics from which there was no visible growth.

Viable count experiments. Nutrient broth (10 ml) containing selected concentrations of the antibiotics, alone and in combination, was inoculated with 0.02 ml of an overnight culture of the test strain of *P. aeruginosa*, an inoculum of about 10^6 cells per ml. In one test, the compounds were added to 10-ml volumes of pooled human serum that were inoculated with the test strain of pseudomonas, and the cultures were incubated in a GasPak jar (Baltimore Biological Laboratory, Cockeysville, Md.) in an atmosphere of 10% CO_2/90% air to maintain the pH value of the serum at about 7.4. The cultures were incubated at 37°C, samples were taken at 3, 6, and 24 h, and 0.02-ml volumes of suitable dilutions were pipetted onto the surface of antibiotic-free agar plates. In tests where it was thought that the viable counts had fallen to low levels, 0.1-ml volumes of undiluted culture were spread over the surfaces of the plates. Colonies were counted after overnight incubation at 37°C and the numbers of viable bacteria were calculated.

Intraperitoneal infections. Mice were injected by the intraperitoneal route with 0.5 ml of a suspension in 5% hog gastric mucin (1701-W, Wilson Laboratories, Chicago, Ill.) of a dilution of an overnight broth culture of the test organism, standardized to give an infective inoculum of approximately 100 median lethal doses. Three clinical isolates of *P. aeruginosa* and one strain of *Enterobacter cloacae* were tested in this model. The antibiotics were administered by the subcutaneous route to groups of 10 mice 1, 3, 5, 7, and 9 h after infection, 0.2 ml/20 g for each dose, as solutions in phosphate-buffered saline. The combinations of a penicillin plus an aminoglycoside were mixed immediately before dosing. The numbers of animals surviving 4 days after infection were recorded, and the percentage of survivors per treatment group was calculated. The data were subjected to a binomial test of proportions for the assessment of statistically significant differences (19).

Bactericidal activity in vivo. Mice were infected intraperitoneally with *P. aeruginosa* 4 and dosed with either ticarcillin or tobramycin alone or with a combination of ticarcillin + tobramycin 1, 3, 5, 7, and 9 h after infection. Groups of three mice were killed by dislocation of the neck at intervals during the 24-h period after infection. The abdomen was swabbed with 70% alcohol, and 4.5 ml of sterile phosphate-buffered saline was injected forcefully into the peritoneum. The abdomen was carefully massaged to ensure adequate mixing, a small incision was made into the peritoneum, and a sample of the washings was collected with a sterile Pasteur pipette. The samples were diluted in 0.2% yeast extract (Difco Laboratories, Detroit, Mich.), 0.02-ml volumes were plated on predried nutrient agar plates, and viable colonies were counted after overnight incubation at 37°C.

Pyelonephritis infections. Mice were infected intravenously with 0.2 ml of a culture of the test organism suitably diluted in double-strength veal infusion broth (Difco). The mice were injected with approximately 10^8 cells of *Escherichia coli* JT415 or 2×10^8 to 5×10^8 cells of *P. aeruginosa* 11. Iron sorbitol citrate (Jectofer, Astra Chemicals Ltd., Watford, England) was administered by the intramuscular route 18, 42, 66, and 90 h after infection to promote the growth of the organism in the kidney (4). Treatment with subcutaneous injection of the ticarcillin and tobramycin was started 20 h after infection and continued four times a day at 2-h intervals for 4 days. Groups of five mice were killed between days 7 and 11 for the pseudomonas infection, and on days 7 and 15 for the *E. coli* infection. After infection, kidneys were examined for macroscopic abscesses, and bacterial counts were performed on homogenates of the infected kidneys.

RESULTS

In vitro studies. Combinations of penicillins and aminoglycosides were considered to be synergistic when one-quarter the MIC or MBC of each antibiotic inhibited growth or killed the test organism and to be partially synergistic when the effective combination contained one-quarter the MIC or MBC of one antibiotic combined with half the MIC or MBC of the other. An indifferent effect was recorded when the concentration of both compounds was only twice as effective in combination as each alone or when the MIC or MBC of one drug did not change when in combination with the other.

(i) MICs. Examples of the synergistic effects obtained with ticarcillin and tobramycin or carbenicillin and gentamicin are illustrated in Table 1, showing typical MIC values obtained with the antibiotics alone and in combination. It can be seen that the higher activities of the new compounds, ticarcillin and tobramycin, against *P. aeruginosa*, compared with carbenicillin and gentamicin, respectively, were reflected also in their combined effects. Synergy

was demonstrated not only against strains of *P. aeruginosa* that were susceptible to the penicillins but also against strains that were moderately resistant, but β-lactamase-producing strains with a high level of resistance to carbenicillin and ticarcillin, e.g., Dal and FR11, were always indifferent to the combinations. The pseudomonas strains with a moderate level of resistance to gentamicin usually showed increased susceptibility to ticarcillin + tobramycin and sometimes to carbenicillin + gentamicin, but the former combination was notably more active.

The penicillin/aminoglycoside combinations also showed enhanced activity against gram-negative bacilli other than *P. aeruginosa*, and both combinations were equally active against these organisms, reflecting the activities of the individual antibiotics (Table 1). In general, synergy was most pronounced against bacteria that were susceptible or only moderately resistant to the penicillins and aminoglycosides. Enhancement of activity was never seen with β-lactamase-producing strains of bacteria with a high level of resistance to the penicillins. Synergy was demonstrated against two tobramycin/gentamicin-resistant strains of *Providencia stuartii* tested, but the concentrations of aminoglycosides in the synergistic combinations make the clinical relevance of such synergism doubtful.

The numbers of strains against which synergistic effects were demonstrated by the penicillin/aminoglycoside combinations are shown in Table 2. Ticarcillin + tobramycin showed enhanced activity against 27 of 34 strains of *P. aeruginosa* and against 17 of 20 enterobacteria. Only β-lactamase-producing strains highly resistant to ticarcillin were indifferent to the combination. Essentially similar results were obtained with the combination of carbenicillin + gentamicin, synergistic effects being observed against 24 of 34 strains of pseudomonas and 16 of 20 other gram-negative bacteria. There was no evidence of antagonism in any of these tests.

(ii) MBCs. Table 2 also shows that both ticarcillin + tobramycin and carbenicillin + gentamicin produced enhanced bactericidal activity against most of the strains of *P. aeruginosa* tested. As was the case in the MIC tests, the combination of ticarcillin + tobramycin was two- to fourfold more effective in the MBC tests than was the carbenicillin + gentamicin combination.

(iii) Viable count tests. The bactericidal synergism shown by the ticarcillin + tobramycin combination in MBC tests was reproduced also in viable count experiments against *P. aeruginosa*. For example, Fig. 1a shows that a combination of 100 μg of ticarcillin per ml + 0.1 μg of tobramycin per ml was rapidly bactericidal against *P. aeruginosa* PU4 and caused a 99.99% kill by 6 h, whereas the antibiotics alone were ineffective or only moderately effective. Increasing the tobramycin concentration to 1.0 μg/ml resulted in increased bactericidal activity, and the compound alone caused a 99% kill of the culture, but when combined with ticarcillin (100 μg/ml) the culture was sterilized within

TABLE 1. *Examples of responses of P. aeruginosa and other gram-negative bacilli to combinations of ticarcillin + tobramycin and carbenicillin + gentamicin*

Organism	MIC (μg/ml)					
	Ticarcillin	Tobramycin	Ticarcillin + tobramycin	Carbenicillin	Gentamicin	Carbenicillin + gentamicin
P. aeruginosa R2	31	0.12	8.0 + 0.03	62	0.5	16 + 0.12
R4	62	0.25	8.0 + 0.06	125	1.0	16 + 0.25
R139	16	0.25	8.0 + 0.06	31	1.0	16 + 0.25
Dal	>500	0.25	>500 + 0.25	>500	1.0	>500 + 1.0
PMG-1	31	0.25	8.0 + 0.12	62	12.5	31 + 6.2
WM627	31	6.2	4 + 1.6	125	25	16 + 6.2
PA100	31	>100	8.0 + 25	62	>100	31 + >100
FR11	>500	31	>500 + 31	>500	500	>500 + 500
E. coli JT1	8.0	0.5	2.0 + 0.12	8.0	0.5	2.0 + 0.12
Proteus mirabilis 977	4.0	1.0	1.0 + 0.25	8.0	1.0	2.0 + 0.5
Proteus rettgeri I	4.0	0.5	1.0 + 0.12	4.0	0.5	1.0 + 0.12
Klebsiella aerogenes A	125	0.5	31 + 0.12	250	0.25	62 + 0.06
E. coli JT4	>500	0.5	>500 + 0.5	>500	1.0	>500 + 1.0
P. stuartii	500	50	125 + 25	500	250	250 + 50

6 h. The bactericidal effects produced by the combination were measured also in tests carried out in human serum, and Fig. 1b compares the relative bactericidal activities of ticarcillin and tobramycin against *P. aeruginosa* NCTC 10662 in nutrient broth and in serum. In this test neither antibiotic was particularly effective in broth, whereas the combination demon-

TABLE 2. *Synergy of ticarcillin + tobramycin and carbenicillin + gentamicin combinations against P. aeruginosa and other gram-negative bacilli*[a]

Test	Organism	Combination	No. of strains		
			Synergy	Partial synergy	Indifference
Bacteriostatic (MIC)	*P. aeruginosa* (34 strains)	Ticarcillin + tobramycin	12	15	7
		Carbenicillin + gentamicin	9	15	10
	Other gram-negative bacilli (20 strains)	Ticarcillin + tobramycin	9	8	3
		Carbenicillin + gentamicin	10	6	4
Bactericidal (MBC)	*P. aeruginosa* (18 strains)	Ticarcillin + tobramycin	7	10	1
		Carbenicillin + gentamicin	8	7	3

[a] See text for definition of synergy.

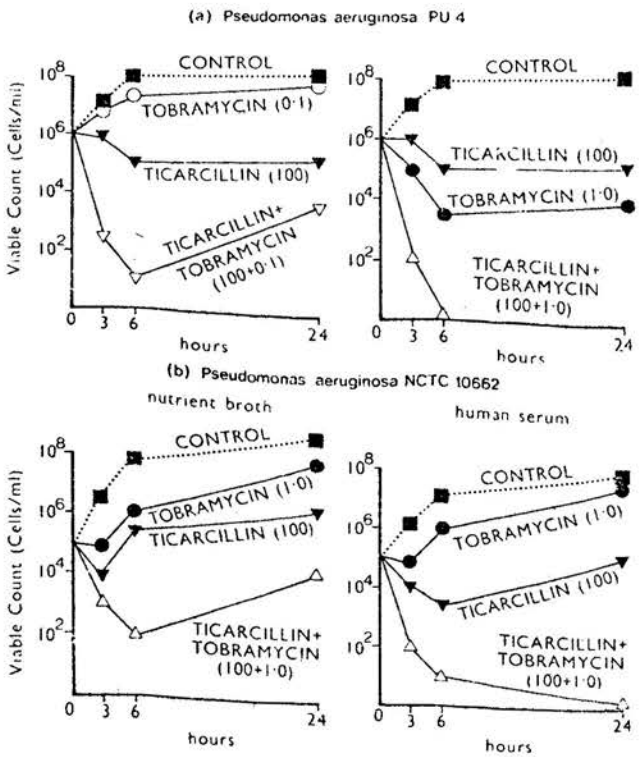

FIG. 1. *Bactericidal activities of ticarcillin and tobramycin, alone and in combination, against two strains of P. aeruginosa (a) in nutrient broth and (b) in nutrient broth and human serum. Figures in parentheses show antibiotic concentrations (micrograms per millilitre).*

strated pronounced bactericidal activity in the early part of the test, causing a 99.9% reduction in the viable count by 6 h, although regrowth of the culture occurred between 6 and 24 h. Ticarcillin showed greater activity in human serum than in broth and caused a 99.9% kill by 6 h but failed to prevent subsequent regrowth. Tobramycin was relatively ineffective, as was the case in broth, but the combination of ticarcillin + tobramycin was rapidly bactericidal, producing a 99.9% kill at 6 h and reducing the 24-h count to a very low level.

Increased bactericidal activity was demonstrated also by ticarcillin + tobramycin against a strain of *P. aeruginosa* resistant to gentamicin (MIC, 15.6 μg/ml). In this experiment, carbenicillin at 100 μg/ml was bactericidal and caused a 99.9% kill in 6 h, gentamicin (10 μg/ml) had little effect, and a combination of the two antibiotics (100 + 10 μg/ml) was no more effective than was carbenicillin itself (Fig. 2). In contrast, ticarcillin (100 μg/ml) showed bactericidal activity slightly greater than that of carbenicillin, and tobramycin (1.0 μg/ml) was markedly bactericidal, reducing the viable count 99.9% by 6 h. The combination of ticarcillin plus tobramycin (100 + 1.0 μg/ml) showed an enhanced rate of bactericidal activity during the first 6 h of the test, although there was some regrowth of the culture during overnight incubation.

Activity against experimental infections. (i) Intraperitoneal infections. The results of parenteral treatment of mouse intraperitoneal infections are shown in Tables 3, 4, and 5. In all these tests the doses of the individual antibiotics were chosen so as to produce only small numbers of survivors (usually less than 20%). Table 3 shows that combinations of ticarcillin + tobramycin produced significantly greater protective effects against the three test strains of *P. aeruginosa* than were produced by the individual antibiotics. Thus, doses of the compounds that were ineffective on their own produced significant increases in the numbers of survivors when the compounds were administered in combination. Similarly, ticarcillin + tobramycin was notably more active against an infection due to *E. cloacae* than when either ticarcillin or tobramycin was administered alone (Table 4).

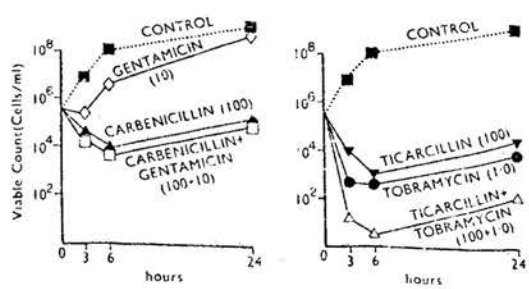

FIG. 2. *Bactericidal activities of ticarcillin and tobramycin and carbenicillin and gentamicin against a gentamicin-resistant strain of P. aeruginosa, PMG1. Figures in parentheses show antibiotic concentrations (micrograms per milliliter).*

TABLE 3. *Synergy between ticarcillin and tobramycin against three intraperitoneal pseudomonas infections in mice*

Treatment	Total dose[a] (mg/kg)	% Survivors[b]		
		P. aerugi-nosa 4	*P. aerugi-nosa* 11	*P. aerugi-nosa* 31
Ticarcillin	78	15	5	20
	39	5	5	0
Tobramycin	6.3	10	5	5
	3.1	0	0	0
Ticarcillin + tobramycin	78 + 6.3	80[c]	85[c]	100[c]
	39 + 6.3	55[d]	45[d]	100[c]
	78 + 3.1	75[c]	55[c]	95[c]
	39 + 3.1	30[e]	5[f]	55[c]

[a] Dosed at 1, 3, 5, 7, and 9 h after infection.
[b] Mean of two tests, 20 mice per group.
[c] $P < 0.001$.
[d] $P < 0.01$.
[e] $P < 0.05$.
[f] P is not significant.

The activity of ticarcillin + tobramycin was also compared with that of carbenicillin + gentamicin against the pseudomonas infections (Table 5). Ticarcillin and tobramycin were relatively ineffective when administered alone, and carbenicillin and gentamicin were, if anything, less effective than the newer compounds against these infections. As before, the combinations of ticarcillin + tobramycin usually resulted in a marked increase in protection of the infected mice, whereas carbenicillin + gentamicin was relatively ineffective, and it was evident that combined therapy with ticarcillin + tobramycin was significantly more effective.

(ii) Bactericidal activity in vivo. The effects of subcutaneously administered ticarcillin, tobramycin, and a combination of ticarcillin + tobramycin on the proliferation of bacteria in the peritoneal cavity of mice after intraperitoneal injection with *P. aeruginosa* 4 are illustrated in Fig. 3. In untreated mice the bacterial peritoneal count fell from the initial inoculum of 7×10^5 cells to 4.9×10^1 cells over the first 3 h and then increased to a count in excess of 10^8 cells at 14 h. Administration of tobramycin had

little effect, and the bacterial counts were not significantly different from those of the untreated mice. Treatment with ticarcillin resulted in a reduction of the viable count to 5×10^4 cells for up to 14 h, after which time the count increased to a level of 7.8×10^7 cells. A combination of ticarcillin (78 mg/kg) + tobramycin (6.3 mg/kg) resulted in a notably greater reduction in the viable counts in the peritoneal cavity than either antibiotic alone. Thus, more than 99% of the bacterial cells were killed by 9 h; there was little increase in the bacterial count from then up to 25 h after infection, and at 25 h it was evident that the combination was notably more effective than the individual antibiotics.

TABLE 4. *Synergy between ticarcillin and tobramycin against an intraperitoneal mouse infection caused by E. cloacae T753*

Treatment	Total dose[a] (mg/kg)	% Survivors[b]	
		Test 1	Test 2
Ticarcillin	312	10	10
	156	0	0
Tobramycin	1.6	0	0
	0.8	0	0
Ticarcillin + tobramycin	312 + 1.6	90[c]	90[c]
	156 + 1.6	70[c]	100[c]
	312 + 0.8	30[d]	20[d]
	156 + 0.8	0[d]	0[d]

[a] Dosed at 1, 3, 5, 7, and 9 h after infection.
[b] Ten mice per group.
[c] $P < 0.001$.
[d] P is not significant.

TABLE 5. *Comparative activities of ticarcillin and tobramycin and carbenicillin and gentamicin against intraperitoneal pseudomonas infections in mice*

Treatment	Total dose[a] (mg/kg)	% Survivors[b]		
		P. aerugi-nosa 4	*P. aerugi-nosa* 11	*P. aerugi-nosa* 31
Ticarcillin	78	30	10	30
	39	10	10	0
Tobramycin	6.3	20	10	0
	3.1	0	0	0
Ticarcillin + tobramycin	78 + 6.3	100	100	100
	39 + 6.3	80	70	100
	78 + 3.1	100	90	90
	39 + 3.1	50	0	60
Carbenicillin	78	0	10	0
	39	0	0	0
Gentamicin	6.3	0	0	0
	3.1	0	0	0
Carbenicillin + gentamicin	78 + 6.3	10	10	20
	39 + 6.3	0	0	0
	78 + 3.1	10	0	20
	39 + 3.1	30	0	0

[a] Dosed at 1, 3, 5, 7, and 9 h after infection.
[b] Ten mice per group.

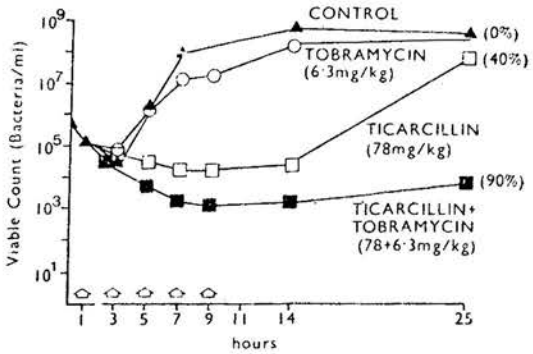

FIG. 3. *Bactericidal effects of a combination of ticarcillin and tobramycin on the growth of P. aeruginosa 4 in the peritoneal cavity of mice. The animals were dosed subcutaneously 1, 3, 5, 7, and 9 h after infection, and the percentages in parentheses show survivors from control groups.*

The efficacy of treatment of this infection in reducing the mortality of the infected mice in this experiment is also shown in Fig. 3, and it can be seen that the combination of ticarcillin + tobramycin increased the amount of protection afforded. In untreated mice and mice treated with tobramycin, there were no survivors; ticarcillin produced 40% survivors, but half of these were moribund up to 48 h after infection. However, treatment with the combination provided 90% protection of infected animals.

(iii) Experimental pyelonephritis. The effects of therapy with ticarcillin, tobramycin, and ticarcillin + tobramycin in reducing bacterial kidney counts and kidney abscess formation in mice surviving from 7 to 15 days after intravenous infections with *P. aeruginosa* or *E. coli* are illustrated in Fig. 4 and 5. In both infections, some 80% of the untreated control mice had significant kidney bacterial counts, and there was a high incidence of visible kidney lesions.

Interpretation of the results of therapy of the pseudomonas infection was complicated by the fact that a number of mice had to be killed during the experiment because of central nervous system toxicity. This was particularly evident in groups treated with tobramycin, where 33 to 58% of the animals were affected, and was also seen, albeit to a lesser extent, in untreated animals. Also, several deaths occurred within

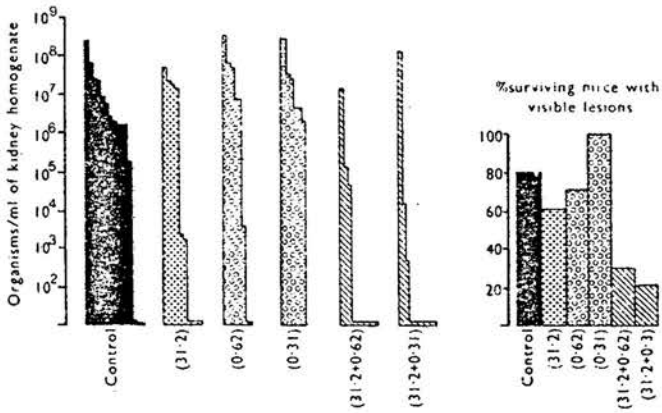

FIG. 4. *Efficacy of ticarcillin and tobramycin, alone and in combination, against an experimental pyelonephritis in mice infected with P. aeruginosa 11. Each bar in the histograms representing the bacterial kidney counts shows the counts measured in an individual animal. Symbols: (dots) ticarcillin; (circles) tobramycin; (stripes) ticarcillin + tobramycin.*

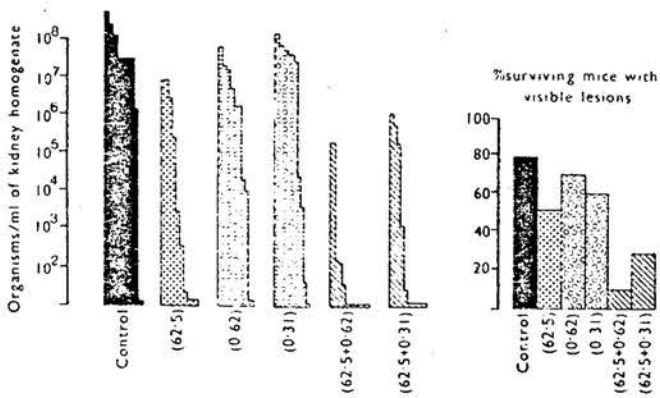

FIG. 5. *Efficacy of ticarcillin and tobramycin, alone and in combination, against an experimental pyelonephritis in mice infected with E. coli JT415. Each bar in the histograms representing the bacterial kidney counts shows the counts measured in an individual animal. Symbols: (dots) ticarcillin; (circles) tobramycin; (stripes) ticarcillin + tobramycin.*

48 h in these groups. Interestingly, central nervous system toxicity was not observed in the groups receiving ticarcillin or combined therapy, although the dose of tobramycin administered to the latter animals was the same, and none of the mice died within 48 h.

In both infections, treatment with the individual antibiotics was relatively ineffective in reducing kidney bacterial counts or in preventing visible abscess formation, whereas ticarcillin + tobramycin produced a much greater degree of protection of the infected animals. Thus, in both experiments the combination of antibiotics was notably more effective than either ticarcillin or tobramycin alone in the treatment of this experimental pyelonephritis, and these results would appear to provide further evidence for synergy between ticarcillin and tobramycin in vivo.

DISCUSSION

The extent of synergy produced by ticarcillin + tobramycin was generally of the same order as that demonstrated by carbenicillin + gentamicin, and consequently the ticarcillin + tobramycin combination was usually four times more effective than the carbenicillin + gentamicin combination against *P. aeruginosa* in the MIC and MBC tests. The greatest differences in activity between the combinations were seen against the gentamicin-resistant, tobramycin-susceptible strains, and these were notably more susceptible to ticarcillin – tobramycin.

Against gram-negative bacilli other than *P. aeruginosa* there was little difference between the combinations in any of the tests. Synergy was observed most often in tests with bacteria susceptible to both the penicillin and the aminoglycoside constituents of the combinations but was also seen on occasion against strains with a moderate level of resistance to the antibiotics. In general, synergistic effects were not seen with bacteria with a high level of resistance to either or both of the antibiotics in the test combinations.

In the in vivo studies described here, the combination of ticarcillin + tobramycin demonstrated a significant enhanced antibacterial activity not only against *P. aeruginosa* but also against *E. coli* and *E. cloacae*, the two organisms tested as examples of nonpseudomonads. Thus, in the intraperitoneal infection model the combination was significantly more effective against all three pseudomonas infections and against the enterobacter infection than either ticarcillin or tobramycin alone. The viable count study of the effect of the antibiotics on the growth of *P. aeruginosa* in the peritoneal cavity of infected animals showed that the combination of ticarcillin + tobramycin produced greater bactericidal effects than either antibiotic alone and that the bactericidal activity correlated with the degree of protection produced. Moreover, ticarcillin + tobramycin was more efficacious than carbenicillin + gentamicin against all three pseudomonas infections, reflecting the superior activities of the newer compounds in this infection model.

Enhancement of activity was also observed against the experimental mouse pyelonephritis when ticarcillin was combined with tobramycin at doses that, alone, showed only little effect in reducing the counts of viable bacteria in the kidneys of infected animals or in preventing visible kidney abscess formation. It was not possible to make a direct comparison of the effect of ticarcillin + tobramycin with that of carbenicillin + gentamicin in this model because of the size of the study involved, but comparison with earlier (unpublished) studies with carbenicillin + gentamicin suggest that the ticarcillin + tobramycin combination would be the more effective of the two combinations. The results of treatment of the experimental pyelonephritis described here might be more significant than those obtained with the intraperitoneal infection, as in this infection model dosing is delayed for 20 h after infection, by which time there is substantial lodgment of the bacteria in the kidney (4). Thus, therapy is not started until the infection is established, as is normally the case in clinical practice.

The observations of McLaughlin and Reeves (13) of the inactivation of gentamicin by carbenicillin caused concern, but it is now generally agreed that the interaction is likely to be of clinical importance only under special circumstances, notably, in patients with renal failure (17). Recent studies by Davies et al. (5) and Holt et al. (6) have shown that there is no significant difference between ticarcillin and carbenicillin with respect to inactivation of aminoglycosides, in agreement with results (unpublished) obtained in this laboratory. The results of the present study, showing evident synergy in vitro and in vivo with ticarcillin + tobramycin, support the view that inactivation of tobramycin by ticarcillin is only rarely likely to be of clinical significance.

In clinical studies, Klastersky et al. (8) have reported better responses in cancer patients treated with combinations of antibiotics, including ticarcillin + tobramycin, that were synergistic than with nonsynergistic combinations. More recently, Parry and Neu (16) found that ticarcillin + tobramycin compared favorably with ticarcillin alone, or with ticarcillin + gentamicin, in eradicating *P. aeruginosa* from the sputum of patients with cystic fibrosis. The results of the in vitro and in vivo studies described in this report support these preliminary clinical observations that combined therapy with ticarcillin and tobramycin may be advantageous in the treatment of infections due to *P. aeruginosa* and other gram-negative bacilli.

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SYNERGY BETWEEN TICARCILLIN AND AMINOGLYCOSIDES AGAINST GRAM-NEGATIVE BACILLI IN VITRO AND IN VIVO

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Serious infections caused by Pseudomonas aeruginosa or other Gram-negative bacilli are often treated with a combination of carbenicillin and an aminoglycoside antibiotic, not only because of the broad-spectrum of activity displayed by this antibiotic combination but also because of the synergy that can be demonstrated in vitro (3, 4, 5) or against experimental infections (1). The effectiveness of combined carbenicillin/aminoglycoside therapy in the treatment of such infections appears to be well established. Ticarcillin, an analogue of carbenicillin, is a broad-spectrum penicillin which is more active than the latter against Ps. aeruginosa and, in a number of studies, synergy has been demonstrated between ticarcillin and gentamicin or tobramycin (2, 5). In the study reported here the activities of combinations of ticarcillin and aminoglycosides, namely, gentamicin, tobramycin, sisomicin, amikacin and dibekacin against Ps. aeruginosa and Enterobacteriaceae were measured in vitro and against experimental mouse infections.

Antibacterial activity in vitro

The Minimum Inhibitory Concentrations (MIC) of the antibiotics alone, and in combination, were measured by two-fold serial dilutions in nutrient broth in microtitre plates (2).

Ticarcillin inhibited 18/22 strains of Ps. aeruginosa at a concentration of 62.5 µg/ml, and was twice as active as carbenicillin against the test organisms but was very much less active than the aminoglycosides. Tobramycin was the most active of the aminoglycosides against strains of Ps. aeruginosa and was about two-fold more active than gentamicin and dibekacin. Amikacin was rather less active than the latter two compounds and inhibited all strains at a concentration of 2 µg amikacin/ml. Aminoglycoside-resistant strains of Ps. aeruginosa were as sensitive to ticarcillin as typical pseudomonas strains but these strains had various distinctive patterns of sensitivity to the aminoglycosides. In general, strains that were resistant to gentamicin were resistant also to sisomicin but could be sensitive to the other aminoglycosides. Similarly, tobramycin-resistant strains of Ps. aeruginosa showed complete cross-resistance to dibekacin but not to the other compounds. A number of pseudomonas were resistant to gentamicin, sisomicin, tobramycin and dibekacin but were sensitive to amikacin and some strains are insensitive to all five aminoglycosides. Against Enterobacteriaceae, ticarcillin was no more active than carbenicillin, and gentamicin, tobramycin and sisomicin generally showed similar activities, being rather more active than amikacin and dibekacin. The anaerobic Bacteroides fragilis were uniformly insensitive to the aminoglycosides and moderately sensitive to the penicillins (MIC 16-32 µg/ml).

Synergy in vitro

The inhibitory activities of ticarcillin in combination with the aminoglycosides were measured by the chessboard technique

using the microtitre serial-dilution method described above. The bactericidal effects were measured by subculture of 0.01 ml volumes from wells showing no growth in the chessboard MIC tests into antibiotic-free broth. Minimum bactericidal concentrations (MBC) were measured after 18 h incubation at 37°C as the lowest concentrations showing no visible growth. Combinations of ticarcillin and aminoglycoside were recorded as being synergistic when one-quarter the MIC or MBC of each constituent of the combination inhibited or killed the test organism. The effects were considered to be partially synergistic when the effective combination contained one-quarter the MIC or MBC of one antibiotic combined with one half the MIC or MBC of the other constituent. The combination was reported as being indifferent in effect when the concentration of both drugs was no more effective or was only twice as effective in combination as each was alone.

The frequency of synergistic effects demonstrated by ticarcillin/aminoglycoside combinations in the MIC tests was similar for all five combinations tested (Table 1). In general, synergy was observed against approximately half of the pseudomonas strains and enhanced activity (partial synergy) was produced against most of the remainder. Likewise, the ticarcillin/aminoglycoside combinations produced a marked increase in activity against most of the strains of Enterobacteriaceae tested, provided that the organism was not highly resistant to ticarcillin or to the aminoglycoside under test. Strains of B. fragilis were no more sensitive to the combinations than to ticarcillin alone. The patterns of

synergy in the MBC tests to measure bactericidal activity were similar to those observed in the MIC tests.

Table 1

Synergy between ticarcillin and aminoglycosides against Ps. aeruginosa and other Gram-negative bacilli

Organism	Combination		% strains		
			Synergy	Partial synergy	Indifference
Ps. aeruginosa	ticarcillin +	gentamicin (22)[a]	52	43	4
		tobramycin (22)	36	64	0
		sisomicin (16)	50	50	0
		amikacin (16)	50	50	0
		dibekacin (16)	56	38	6
Other Gram-negative bacteria[b]	ticarcillin +	gentamicin (16)	64	18	18
		tobramycin (16)	38	44	18
		sisomicin (20)	55	25	20
		amikacin (20)	45	35	20
		dibekacin (20)	45	35	20

[a] Number of strains

[b] E. coli, K. aerogenes, Enterobacter sp., S. marcescens, Proteus sp., B. fragilis.

Synergy against experimental infections

The efficacy of ticarcillin and the aminoglycosides, alone and in combination, were measured against mouse peritoneal infections due to Ps. aeruginosa and to Ent. cloacae. Mice were infected by the intraperitoneal route with suspensions of the test organism in 3% hog gastric mucin standardised to produce an infective inoculum of approximately 100 MLD.

The antibiotics were administered by the subcutaneous route 1, 3, 5, 7 and 9 hours after infection, the numbers of animals surviving 4 days after infection were recorded and the percentage of survivors per treatment group was calculated. The results of treatment of infections due to Ps. aeruginosa 4 are shown in Table 2.

Table 2

Synergy between ticarcillin and aminoglycosides against intraperitoneal mouse infections due to Ps. aeruginosa 4

Treatment	Total dose[a] (mg/kg)	% Protection[b]
ticarcillin	78	0
gentamicin	25	0
ticarcillin + gentamicin	78 + 25	85
ticarcillin	78	15
tobramycin	3.1	0
ticarcillin + tobramycin	78 + 3.1	75
ticarcillin	78	0
sisomicin	12.5	5
ticarcillin + sisomicin	78 + 12.5	100
ticarcillin	78	0
amikacin	25	15
ticarcillin + amikacin	78 + 25	100
ticarcillin	78	0
dibekacin	12.5	5
ticarcillin + dibekacin	78 + 12.5	75

[a] Animals dosed subcutaneously 1, 3, 5, 7 and 9 hr after infection.

[b] 20 mice per group.

In all tests the individual antibiotics had little effect in protecting the mice from the lethal effects of the infection and there were only small numbers of survivors (0-15%) in groups treated with ticarcillin or the aminoglycosides alone. In contrast, the combinations of ticarcillin and the aminoglycosides at the same doses were significantly more effective ($p > 0.05$) and protected 75 to 100% of the infected animals. Similar results were also obtained against an infection due to a different test strain of Ps. aeruginosa, II. Likewise, therapy of an intraperitoneal mouse infection due to Ent. cloacae T 753 with combinations of ticarcillin and aminoglycosides was significantly more effective ($p < 0.05$) than treatment with the antibiotics alone. Results in Table 3 show that combined therapy resulted in 75 to 95% protection of the infected animals compared with 5 to 15% survival in the groups treated with ticarcillin or with the individual aminoglycosides.

Table 3

Synergy between ticarcillin and aminoglycosides against intraperitoneal infections due to Enterobacter cloacae T 753

Treatment	Total dose[a] (mg/kg)	% Protection[b]
ticarcillin	106	5
gentamicin	1.8	10
ticarcillin + gentamicin	100 + 1.8	85
ticarcillin	106	0
tobramycin	1.6	0
ticarcillin + tobramycin	106 + 1.6	85
ticarcillin	106	10
sisomicin	0.4	10
ticarcillin + sisomicin	106 + 0.4	80
ticarcillin	106	5
amikacin	3.0	15
ticarcillin + amikacin	106 + 3.0	95
ticarcillin	106	5
dibekacin	2.5	15
ticarcillin + dibekacin	106 + 2.5	75

[a] Animals dosed subcutaneously 1, 3, 5, 7 and 9 hr after infection

[b] 20 mice per group.

Conclusions

The results of the studies described here show that synergistic effects were produced in growth inhibition tests and in bactericidal tests by ticarcillin in combination with the five aminoglycosides tested. The extent and frequency of synergy against the strains of Ps. aeruginosa and other Gram-negative bacilli was generally similar for all the combinations tested. Synergistic effects were not usually observed with aminoglycoside-resistant bacteria or in tests with bacteria with a high level of resistance to ticarcillin. The synergy observed in vitro was reproduced against experimental mouse infections and ticarcillin/aminoglycoside combinations produced significantly greater protective effects than the individual antibiotics. The results reported here indicate that combined therapy with ticarcillin and an aminoglycoside antibiotic would be justified in the treatment of serious infection caused by Gram-negative bacilli.

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Synergistic Activity of Penicillins against Penicillinase-producing Gram-negative Bacilli

THE semi-synthetic penicillins, methicillin and cloxacillin, which are highly stable to staphylococcal penicillinase are also stable to the penicillinases of many Gram-negative bacteria[1,2]. However, these penicillins have little activity against Gram-negative bacteria because of intrinsic insensitivity of the organisms, and consequently penicillinase-producing and non-penicillinase-producing Gram-negative bacteria are equally insensitive to methicillin and cloxacillin[3]. With ampicillin and benzylpenicillin, however, this is not the case, and with certain Gram-negative bacteria penicillinase production may be important in determining the activity of ampicillin or benzylpenicillin[3].

In the case of certain penicillinase-producing Gram-negative bacteria, penicillinase activity can be inhibited with methicillin[4] or cloxacillin, with the result that in the presence of one of these penicillinase-stable penicillins the rate of inactivation of ampicillin or benzylpenicillin is diminished. The results in Table 1 show the rate of inactivation of ampicillin in the presence of either methicillin or cloxacillin as a percentage of the rate of inactivation of ampicillin alone; similar results are also shown for benzylpenicillin in the presence of these two penicillinase-stable penicillins. In the experiments shown in Table 1 ampicillin and benzylpenicillin were at a concentration of 10 μg/ml., and methicillin and cloxacillin were at 1 μg/ml. Penicillinase activity was estimated by following the formation of penicilloic acid using the microiodometric method[5]. It will be seen that the rate of inactivation by penicillinase could be diminished in the presence of methicillin and cloxacillin. With certain bacteria, the degree of inhibition of penicillinase activity was considerable while with other strains the effect was small or non-existent. When methicillin and cloxacillin were tested in equal concentration to benzylpenicillin and ampicillin (that is, 10 μg/ml.) inhibition was much more frequently encountered, and almost complete inhibition was often observed.

In antibacterial tests *in vitro*, mixtures of methicillin or cloxacillin with ampicillin or benzylpenicillin show synergistic effects against certain strains of Gram-negative bacteria. These organisms are intrinsically sensitive to ampicillin or benzylpenicillin but by virtue of their ability to produce penicillinase are normally able to destroy these labile penicillins[3]. In the presence of the penicillinase-stable penicillins, methicillin and cloxacillin, the penicillinase activities of these bacteria are diminished and the organisms are more sensitive to ampicillin and benzylpenicillin. Examples of synergism of this type are shown in Table 2. In these experiments, serial dilutions

Table 1. Rate of Inactivation of Ampicillin or Benzylpenicillin in the Presence of Methicillin or Cloxacillin, as a Percentage of the Rate in the Absence of Methicillin or Cloxacillin

Organism	Ampicillin 10 μg/ml. + methicillin 1 μg/ml.	Ampicillin 10 μg/ml. + cloxacillin 1 μg/ml.	Benzylpenicillin 10 μg/ml. + methicillin 1 μg/ml.	Benzylpenicillin 10 μg/ml. + cloxacillin 1 μg/ml.
Klebsiella pneumoniae C	<1	45	15	50
Escherichia coli 83	14	86	19	100
Escherichia coli 70	42	100	7	70
*Escherichia coli R*136	62	38	86	100
Aerobacter aerogenes A	64	90	45	100
Aerobacter aerogenes G	67	100	27	77
Pseudomonas pyocyanea	—*	—*	24	<1
Proteus vulgaris A	11	45	28	100

* Ampicillin was highly stable to this penicillinase.

of ampicillin or benzylpenicillin were made in nutrient broth, and also in nutrient broth containing 10 μg/ml. of methicillin or cloxacillin, and the minimal inhibitory concentrations were determined after overnight incubation at 37° C. Methicillin and cloxacillin showed no activity against any of the bacteria at concentrations of up to 50 μg/ml., and the concentration of 10 μg/ml. used in the tests was chosen as being one which can be readily obtained in the serum in human beings with normal dosage.

It will be seen from Table 2 that a striking degree of synergism could be demonstrated with certain strains of bacteria. For example, against *Escherichia coli R*136 ampicillin was inhibitory only at a concentration of 25 μg/ml., and cloxacillin alone at a concentration of 10 μg/ml. was without effect. In combination with 10 μg/ml. of cloxacillin, however, ampicillin was inhibitory at 2·5 μg/ml. Similar synergistic effects were seen with various strains of *E. coli*, *Aerobacter aerogenes* and *Proteus* sp.

In general, synergism was observed more frequently with benzylpenicillin than with ampicillin, and this may be associated with the fact that ampicillin is more stable than benzylpenicillin to the penicillinases produced by certain Gram-negative bacteria[1-3,6]. As a result of this, certain organisms are sensitive to ampicillin but are relatively insensitive to benzylpenicillin and it was with this group of bacteria that synergism with benzylpenicillin was most marked. However, although the minimum inhibitory concentrations of benzylpenicillin were in certain cases diminished considerably in the presence of a penicillinase-stable penicillin, they were in general no lower than those obtained with ampicillin alone. Moreover, no one combination of penicillins was consistently more synergistic than any other, and there was a distinct variation from strain to strain, so that with certain bacteria synergism was more marked with methicillin than with cloxacillin, while with other strains the converse was the case.

It was observed that in certain cases inhibition of penicillinase was not reflected in the results of the minimum inhibitory concentration tests because of intrinsic

Table 2. Enhancement of the Antibacterial Activities of Ampicillin or Benzylpenicillin against Gram-negative Bacteria in the Presence of Methicillin or Cloxacillin

	Minimal inhibitory concentration (μg/ml.) (Serial-dilution in broth; inoculum, one drop of an overnight broth culture)					
	Ampicillin	Ampicillin with methicillin*	Ampicillin with cloxacillin*	Benzylpenicillin	Benzylpenicillin with methicillin*	Benzylpenicillin with cloxacillin*
Escherichia coli R136	25	25	2·5	> 250	125	25
Escherichia coli T340	2·5	2·5	2·5	50	50	5·0
Escherichia coli R9	5·0	5·0	5·0	50	50	5·0
Aerobacter aerogenes A	>100	5·0	50	50	50	50
Aerobacter aerogenes R92	10	10	< 5·0	—	—	—
Proteus vulgaris A	50	10	2·5	25	2·5	10
Proteus mirabilis R26	5·0	5·0	5·0	25	5·0	5·0
Proteus mirabilis P34	1·25	1·25	1·25	50	50	5·0
Paracolon A	10	2·5	2·5	50	50	25

* Methicillin or cloxacillin were present at a concentration of 10 μg/ml. These penicillins were inactive against the test organisms at concentrations up to 50 μg/ml.

insensitivity of the organisms, penicillinase playing little part in the resistance of these bacteria. In other cases where synergism was apparent in the serial-dilution tests, the amounts of penicillinase produced were too low to permit inhibition of the enzyme to be studied by the methods used. Moreover, quantitative differences would also arise because fixed concentrations of 1 μg/ml. of methicillin or cloxacillin and 10 μg/ml. of ampicillin and benzylpenicillin were used in the enzyme investigations, whereas in the serial-dilution tests concentrations of ampicillin, and benzylpenicillin from 1 μg/ml. to 500 μg/ml. were combined with a concentration of 10 μg/ml. of methicillin or cloxacillin.

In order to assess the possible clinical value of such synergism, a survey was carried out with 350 freshly isolated clinical strains of Gram-negative bacilli. In these experiments, strains of bacteria which had been found to require more than 5 μg/ml. of ampicillin or benzylpenicillin for inhibition were tested for sensitivity to these penicillins alone and in combination with 10 μg/ml. of methicillin or cloxacillin. Of the total of 350 isolates, 62 per cent (217 strains) were inhibited by ampicillin alone, at a concentration of 5 μg/ml. or less. With the strains which required a concentration greater than 5 μg/ml. for inhibition, 9 per cent (12 strains) showed a significant degree of synergism, but only in 4 per cent (5 strains) of these isolates was this effect adequate to reduce the minimal inhibitory concentrations to values of 5 μg/ml. or less. With benzylpenicillin the situation was similar. As would be expected, sensitivity to benzylpenicillin alone was not so frequent as was the case with ampicillin and only 18 per cent of the isolates (63 strains) were sensitive to concentrations of 5 μg/ml. of this penicillin or less. Of the strains requiring greater than 5 μg/ml. of benzylpenicillin for inhibition (287 strains) a significant degree of synergism was demonstrated with 17 per cent (49 strains), but in only 6 per cent (17 strains) was the minimum inhibitory concentration reduced to 5 μg/ml. or less. Therefore, although synergism is clearly demonstrable with certain strains of Gram-negative bacteria, these constitute only a small proportion of the bacteria encountered routinely, and the degree of synergism would seem to be inadequate for this to have useful clinical application.

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Antibacterial Activities of Combinations of Ampicillin and Cloxacillin

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Infections in premature infants or newborn babies may require empirical treatment with a broad-spectrum antibiotic before bacteriological data are available. However, several of these agents, including sulfonamides, chloramphenicol, and tetracycline, are potentially toxic to this age group, whereas ampicillin, although of low toxicity, is not active against penicillinase-producing staphylococci. On the other hand, a combination of ampicillin with cloxacillin is relatively nontoxic and would be expected to be active against a wide range of bacteria. Accordingly, experiments were designed to evaluate the antibacterial activities of various mixtures of these penicillins in vitro and in experimental infections in mice. There was no evidence of antagonism between the penicillins, and, in general, the mixtures demonstrated the activities of the individual substituents, except that synergistic effects were observed in vitro and in vivo against certain penicillinase-producing gram-negative bacteria. The synergism between the penicillins was shown to be due to inhibition by cloxacillin of the penicillinases produced by these bacteria and subsequent reduction of the extent of inactivation of ampicillin. It was concluded that a mixture of ampicillin with cloxacillin should provide a broad-spectrum antibiotic combination effective against penicillinase-producing staphylococci and most strains of gram-negative bacteria.

The treatment of suspected, or confirmed, bacterial infections in premature infants or neonates may necessitate the use of broad-spectrum antibiotics or of combinations of antibacterial agents to provide adequate antibiotic cover. This age group is, however, particularly susceptible to the hazards of treatment with these substances, and several compounds are known to be potentially dangerous, especially sulfonamides, chloramphenicol, and tetracyclines (Lancet 2:562-563, 1963). An alternative broad-spectrum antibiotic, ampicillin, is not toxic to neonates (Lancet 2:562-563, 1963) and is active against a wide range of bacteria (5), but this penicillin is ineffective against penicillinase-resistant staphylococci which are frequently implicated in neonatal infections. The penicillinase-stable penicillin, cloxacillin, like ampicillin, is nontoxic, but unlike the latter is active against penicillin-resistant staphylococci. Accordingly, the use of a combination of these two penicillins, namely, the penicillinase-stable penicillin, cloxacillin, together with the broad-spectrum penicillin, ampicillin, has been recommended for use in the treatment of neonatal infections (Lancet 2:562-563, 1963; Lancet 1:863, 1966). The experiments described here were designed to evaluate the antibacterial activity in vitro and in vivo of combinations of these two penicillins.

Materials and Methods

The minimal inhibitory concentrations (MIC) of ampicillin and cloxacillin, alone and in combination, required to prevent growth of

the test organism for 18 hr at 37 C were determined by serial dilution in nutrient broth. The cultures of bacteria used in these studies were mainly obtained from clinical sources. When mixed cultures of *Staphylococcus aureus* and *Escherichia coli* were tested for susceptibility to the penicillins, samples were removed from the tubes after incubation overnight and plated on selective media containing no antibiotic. Staphylococci were identified by their growth on mannitol-salt agar, and coliform organisms, by their appearance on MacConkey agar. In certain of the serial-dilution experiments, the extent of destruction of ampicillin, whether tested alone or in combination with cloxacillin, was measured by microbiological assay for ampicillin. Samples were taken from these tests after overnight incubation and were assayed by an agar diffusion method (7) with a strain of *E. coli* which was susceptible to ampicillin and resistant to cloxacillin. The bactericidal activities of the penicillins were determined by measurement of reduction in viable count of bacterial cultures after exposure to the drugs by use of a standard drop-plate technique.

The activities of the penicillins against experimental staphylococcal infections in mice were determined by infecting the animals in a hind leg via the intramuscular route and measuring the effect of therapy on the thigh lesion produced. In some experiments, the animals were infected with a penicillin-susceptible and a penicillin-resistant staphylococcus, each culture being injected into a separate hind limb (2). Groups of 10 mice were treated at 0, 24, and 48 hr with the individual penicillins and with the combination of ampicillin and cloxacillin. The results of treatment were measured in the same way as for the single thigh lesion.

Mouse protection experiments were also carried out to measure the curative doses of the penicillins in mice infected with ampicillin-resistant strains of *E. coli*. The cultures were suspended in 5% hog mucin and injected via the intraperitoneal route. The penicillins were administered orally or subcutaneously to groups of 10 mice immediately after infection, and the animals were observed for a period of 4 days.

Results

The results in Table 1 show the activities of ampicillin, cloxacillin, and of a combination of equal parts of ampicillin and cloxacillin against two strains of *S. aureus* and *E. coli*. Ampicillin was active against the nonpenicillinase-producing cultures *S. aureus* (Oxford) and *E. coli* 372, but was destroyed by the penicillinase-producing organisms *S. aureus* (Russell) and *E. coli* 80. The staphylococci were susceptible to cloxacillin, whereas both strains of *E. coli* were resistant to this penicillin; however, this was not due to destruction of

Table 1. *Relative activities in vitro of ampicillin and cloxacillin alone and in combination against various bacteria*[a]

Culture	MIC (μg/ml)		
	Ampicillin	Cloxacillin	Ampicillin + cloxacillin (50/50)
Staphylococcus aureus (Oxford)[b]	0.1	0.1	0.1
S. aureus (Russell)[c]	250	0.5	1.25
Escherichia coli 372[b]	1.25	500	2.5
E. coli 80[c]	500	500	50
S. aureus (Oxford)	0.25	0.25	0.5
E. coli 372	1.25	125	2.5
S. aureus (Russell)	250	0.5	1.25
E. coli 372	250	250	50
S. aureus (Russell)	>500	1.25	1.25
E. coli 80	>500	250	125

[a]Nutrient broth containing graded amounts of the penicillins was inoculated with the test organism. After overnight incubation at 37 C, subcultures were made onto penicillin-free MacConkey agar and penicillin-free mannitol-salt agar. Minimal inhibitory concentrations were determined after overnight incubation at 37 C.

[b]Nonpenicillinase-producing culture.

[c]Penicillinase-producing culture.

cloxacillin. The combination of ampicillin and cloxacillin was active against both strains of staphylococci whether cultured alone or in the presence of *E. coli*. Likewise, the growth of a mixed culture of the ampicillin-susceptible strain of *E. coli* and the penicillin-susceptible staphylococcus was inhibited by the two penicillins. However, the combination of penicillins failed to inhibit the growth of the ampicillin-susceptible coliform (*E. coli* 372) in the presence of *S. aureus* (Russell) owing to destruction of the ampicillin moiety by the penicillin-resistant staphylococcus. On the other hand, the combination of ampicillin and cloxacillin was more active against the penicillinase-producing strain of *E. coli* than was ampicillin alone.

Table 2 shows the results of experiments designed to test the combination of ampicillin and cloxacillin against the double infection in mice resulting from both penicillin-susceptible and penicillin-resistant bacteria. In these tests, the double thigh lesion technique was used: the mice were infected in one hind limb with a penicillin-susceptible staphylococcus and in the other with a penicillinase-producing strain of *S. aureus*. Ampicillin exerted significant activity against the infection produced by the penicillin-susceptible staphylococcus (strain 2187) despite the fact that the mice were also infected with a penicillin-resistant culture. The activity of ampicillin against *S. aureus* 2187 in the double-thigh lesion test was about the same as that obtained against that particular organism in a single-thigh lesion test when the mice were infected with the susceptible organism alone. The combination of ampicillin and cloxacillin was as active against the experimental infections as would be predicted from the activities of the individual penicillins.

The results of tests with ampicillin and cloxacillin, alone and in combination, against penicillinase-producing strains of gram-negative bacteria are shown in Table 3. On occasion, the combination of ampicillin and cloxacillin demonstrated activity considerably greater than that of ampicillin alone. Cloxacillin was virtually inactive against these organisms. Microbiological assay of the ampicillin content of the cultures after overnight incubation in the serial-dilution tests showed that these penicillinase-producing bacteria destroyed ampicillin during the course of the tests, whereas the addition of cloxacillin to ampicillin brought about a substantial reduction in the extent of destruction of ampicillin. This is illustrated in Fig. 1, which shows the results of a viable-count experiment with an ampicillin-resistant strain of *Proteus morganii*. Ampicillin at a concentration of 500 μg/ml produced a significant fall in the viable count of the organism after

Table 2. *Relative activities in vivo of ampicillin and cloxacillin, alone and in combination*[a]

Penicillin	Dose	Protection[b] (%)			
		Staphylococcus aureus Russell		*S. aureus* 2187	
		Oral	Subcutaneous	Oral	Subcutaneous
	mg/kg				
Ampicillin	50	27.1	26.7	72.6	91.3
Cloxacillin	50	42.8	87.4	45.2	66.6
Ampicillin + cloxacillin	50 + 50	40.0	90.6	84.1	89.1

[a]Groups of 10 mice were infected in one hind limb with *S. aureus* Russell (penicillinase-producing strain) and in the other hind limb with penicillin-susceptible *S. aureus* 2187.

[b]Per cent protection = [(mean daily thigh enlargement of infected controls − mean daily thigh enlargement of penicillin group) / (mean daily thigh enlargement of infected controls)] × 100.

Table 3. *Synergism between ampicillin and cloxacillin against gram-negative bacteria in vitro*

Organism	MIC (μg/ml)		
	Cloxacillin	Ampicillin	Ampicillin + cloxacillin (50/50)
Escherichia coli B6	1,000	>1,000	1,000
E. coli E80	500	>1,000	100
E. coli 83	1,000	250	50
Aerobacter aerogenes 1390	1,000	1,000	50
A. aerogenes N1	>1,000	1,000	100
A. aerogenes N2	>1,000	>1,000	>1,000
Proteus mirabilis 8	>1,000	1,000	250
P. morganii A	1,000	250	25
P. morganii H	1,000	100	25
P. rettgeri A	1,000	50	50
P. vulgaris A	250	50	25
P. vulgaris E	>1,000	>1,000	250

7 and 24 hr, and there was no destruction of the penicillin. At a concentration of 100 μg/ml, ampicillin reduced the viable count at 7 hr, at which time there was no appreciable loss in activity of the antibiotic, but after 24 hr there was no ampicillin level and the culture showed considerable growth. With the mixtures of ampicillin and cloxacillin, it was obvious that the cloxacillin had

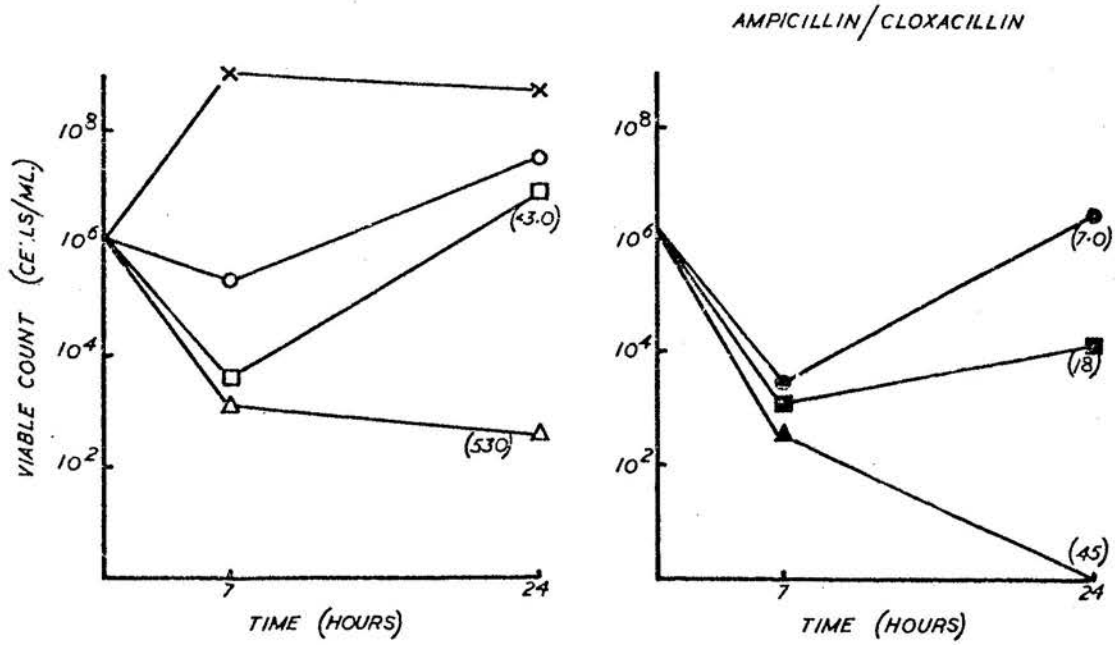

Fig. 1. *Bactericidal activities of ampicillin and cloxacillin, alone and in combination, against a penicillinase-producing strain of Proteus morganii. Symbols: ×, antibiotic-free control; ○, cloxacillin, 500 μg/ml; □, ampicillin, 100 μg/ml; △, ampicillin, 500 μg/ml; ●, ampicillin (10 μg/ml) + cloxacillin (10 μg/ml); ■, ampicillin (25 μg/ml) + cloxacillin (25 μg/ml); ▲, ampicillin (50 μg/ml) + cloxacillin (50 μg/ml). Figures in brackets show ampicillin concentrations after 24 hr.*

reduced the penicillinase activity of the organism, allowing the residual ampicillin content to inhibit growth of the organism.

The results of the tests with the combination of ampicillin and cloxacillin in experimental infections in mice with penicillinase-producing gram-negative bacteria were less uniform and less impressive than were the results of in vitro tests with these organisms, but enhanced activity was demonstrated with the combination of penicillins against certain bacteria. The results of experimental infections with two strains of *E. coli* are shown in Table 4, and these are expressed as the dose required to cure 50% of the infected animals (CD_{50}, mg/kg).

Discussion

The results of the experiments reported here demonstrate that, in general, when ampicillin is combined with cloxacillin the penicillins function independently, producing additive effects. Consequently, the combination is active against a wide range of gram-positive and gram-negative bacteria, including penicillin-resistant staphylococci. Although penicillin-resistant staphylococci are capable of destroying the ampicillin moiety of the combination of penicillins in vitro, this may not necessarily occur in vivo. The results of treatment with a combination of ampicillin and cloxacillin of experimental infections due to mixed cultures of bacteria show that, in certain circumstances at least, ampicillin retains its activity in vivo in the presence of a penicillinase-producing staphylococcus. A combination of ampicillin and cloxacillin might, therefore, be effective against mixed infections of ampicillin-susceptible gram-negative bacteria and penicillin-resistant staphylococci. The results of clinical trials in the United Kingdom with a combination of ampicillin and cloxacillin (Ampiclox) in the treatment of infections of premature and newborn infants have been encouraging (1, 9) and are in accord with the data presented here. (Ampiclox is the registered trade mark of Beecham Research Laboratories for a combination of ampicillin and cloxacillin containing two parts of the former and one part of the latter.)

Synergism may be demonstrated in the laboratory with ampicillin and cloxacillin against penicillinase-producing strains of gram-negative bacteria as a result of inhibition of penicillinase activity by cloxacillin (4, 8). In the previous report from our laboratory (8), we questioned whether such synergism was likely to have much clinical significance, but this observation was based on the results

Table 4. *Synergism between ampicillin and cloxacillin against penicillinase-producing strains of Escherichia coli in vivo*[a]

Culture	Penicillin	MIC	CD_{50} (mg/kg)			
			Oral		Subcutaneous	
			Expt 1	Expt 2	Expt 1	Expt 2
		μg/ml				
Escherichia coli R101	Ampicillin	100	>400	>400	200	310
	Cloxacillin	>50	>400	>400	370	>400
	Ampicillin + cloxacillin, 50/50	10	>400	>400	50	76
E. coli R136	Ampicillin	25	27	50	76	50
	Cloxacillin	>50	>400	>400	>400	>400
	Ampicillin + cloxacillin, 50/50	2.5	76	32	23	31

[a]Groups of 10 mice were infected by the intraperitoneal route and treated immediately after infection.

of experiments with combinations of ampicillin and cloxacillin containing a low concentration of cloxacillin, i.e., 10 μg of cloxacillin per ml. (The concentration of 10 μg of cloxacillin per ml was chosen as being one which can be readily obtained in the serum of human beings with normal dosage.) With higher concentrations of cloxacillin, examples of synergism with ampicillin become more numerous in vitro. It is conceivable, therefore, that synergism between ampicillin and cloxacillin might on occasion be achieved under circumstances during which higher serum or tissue concentrations of both penicillins are achieved during therapy. A clinical example of synergism between penicillins as a result of inhibition of penicillinase activity is that reported by Shirley and Moore (6), who successfully treated a refractory urinary infection due to *Pseudomonas aeruginosa* with high doses of benzylpenicillin and methicillin after in vitro tests had shown synergism between the two penicillins. In the newborn, serum concentrations obtained with therapeutic doses of ampicillin or cloxacillin may be much higher than those obtained in older subjects (3; Knudsen and Sutherland, *in preparation*). Zaidi (9) has reported satisfactory response in neonates treated with a combination of ampicillin and cloxacillin, although the coliforms which were isolated from these infants were resistant to ampicillin in vitro. This may be a reflection of high ampicillin levels or, alternatively, may indicate synergism between the penicillins.

The laboratory results reported here substantiate clinical reports that a combination of ampicillin and cloxacillin provides a nontoxic, bactericidal, broad-spectrum antibiotic combination especially suitable for the treatment of infections in the newborn.

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Synergism Between Penicillins

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The resistance of ampicillin-resistant strains of Gram-negative bacteria is frequently associated with penicillinase production and the ability of these organisms to destroy the penicillin. Synergism can be demonstrated between ampicillin and penicillinase-stable penicillins against certain ampicillin-resistant bacteria and it is generally assumed that the synergism observed is the result of competitive inhibition of the penicillinase of the test organism by the penicillinase-stable penicillin. However, the resistance of many Gram-negative bacteria to ampicillin may be due to both penicillinase-production and to an intrinsic resistance which apparently does not involve drug destruction. Data are presented to show that the effect of combining cloxacillin or flucloxacillin with ampicillin may result not only in protection of ampicillin from destruction by penicillinase but also in a pronounced increase in the activity of ampicillin against intrinsically resistant cells. Further evidence to suggest a synergistic action between ampicillin and cloxacillin or flucloxacillin which is not associated with inhibition of penicillinase activity is shown by the enhanced activity of combinations of ampicillin with cloxacillin or flucloxacillin against certain ampicillin-sensitive bacteria which do not produce penicillinase.

The resistance of Enterobacteriacae to ampicillin is often associated with β-lactamase production and the ability of these organisms to destroy the penicillin, but with some bacteria β-lactamase activity may be accompanied by an intrinsic insensitivity not involving drug destruction (3). Synergism can be demonstrated with combinations of ampicillin and penicillinase-stable penicillins against some ampicillin-resistant bacteria and it is generally assumed that this is due to competitive inhibition of β-lactamase activity by the penicillinase-stable penicillin (1, 2, 4). In the present study, data are presented to suggest that the synergistic activities of combinations of ampicillin and penicillinase-stable penicillins may be due not only to inhibition of β-lactamase but also to potentiation of the intrinsic activity of ampicillin.

The results in Table 1 illustrate the relative activities of ampicillin and a combination of equal parts of ampicillin and cloxacillin against 300 strains of bacteria. The combination showed distinct synergistic activity against ampicillin-resistant strains of Shigella sonnei and certain Escherichia coli but not against E. coli carrying ampicillin R-factors. Enhancement of activity, of a lower order, was also observed against the majority of strains of Klebsiella/Enterobacter and with resistant indole positive Proteus species. Examination of a number of these ampicillin-resist-

ant bacteria showed that destruction of ampicillin took place during the course of the sensitivity tests and that this was reduced in the presence of cloxacillin, indicating that synergism was associated with inhibition of β-lactamase activity.

TABLE 1. Relative activities of ampicillin and a combination of equal parts of ampicillin and cloxacillin against 300 strains of bacteria

Organism	Ampicillin sensitivity	No. of strains	Fold-increase in activity of ampicillin-cloxacillin combination*					
			-2	0	2	4	8	16
Escherichia coli	resistant	29	-	15	-	2	11	1
	sensitive	43	3	16	22	2	-	-
Shigella sonnei	resistant	18	-	-	-	6	6	6
	sensitive	24	-	12	12	-	-	-
Salmonella species	sensitive	20	1	18	1	-	-	-
Proteus species	sensitive	10	-	8	2	-	-	-
	resistant	30	-	3	11	8	4	4
Klebsiella/ Enterobacter	resistant	81	1	11	45	19	5	-
Staphylococcus aureus	resistant	19	-	18	1	-	-	-
	sensitive	6	-	4	2	-	-	-
Streptococcus faecalis	sensitive	20	-	2	18	-	-	-
		300	5	107	114	37	26	11

* $\frac{\text{M.I.C. of ampicillin against test organism}}{\text{Concentration of ampicillin in M.I.C. of combination}}$

However, the data in Table 1 also show that the ampicillin/cloxacillin combination appeared to be as active as ampicillin alone against a number of ampicillin-sensitive strains of E. coli and Sh. sonnei, although cloxacillin was inactive against these bacteria (MIC 250 µg/ml or greater). This was not the case with strains of Salmonella species or sensitive strains of Proteus mirabilis where the combination showed only the activity to be expected from its ampicillin content. The ampicillin/cloxacillin combination also showed enhanced activity against enterococci, but showed no synergistic effects against sensitive or resistant strains of Staphylococcus aureus. The ampicillin-sensitive bacteria did not destroy ampicillin in the sensitivity tests and so inhibition of β-lactamase was unlikely to be a reason for the enhanced activity of the combination of ampicillin and cloxacillin.

The activities of ampicillin, alone, and in combination with cloxacillin were investigated in greater detail in the viable count experiment with an ampicillin-resistant strain of E. coli (425) illustrated in Fig. 1. Cloxacillin (10 µg/ml and 100 µg/ml) had little effect on the growth of the or-

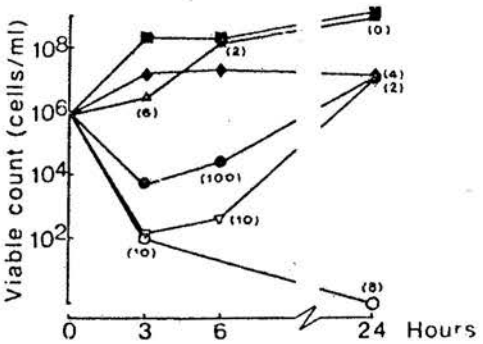

Fig. 1. Bactericidal activities of ampicillin and ampicillin/cloxacillin combinations against E. coli 425
■ control; ▲ ampicillin 10 µg/ml; ● ampicillin 100 µg/ml; cloxacillin 100 µg/ml; Δ ampicillin/cloxacillin 10 + 10 µg/ml; o ampicillin/cloxacillin 10 + 10 µg/ml.
Figures in brackets show ampicillin concentrations.

ganism, and ampicillin at a concentration of 10 µg/ml was inhibitory for only a short period of time. A higher concentration of ampicillin (100 µg/ml) produced a marked bactericidal effect over the first three hours of the test, but three hours later there was an increase in the viable count although there was no obvious destruction of ampicillin during this period of time. The ampicillin/cloxacillin combinations (10 + 10 µg/ml, 10 + 100 µg/ml) produced even greater bactericidal effects than 100 µg/ml ampicillin, although the ampicillin content of each combination was only one tenth that of the culture containing 100 µg ampicillin/ml, and although there was no evidence that the resistance of the culture growing in the presence of 100 µg ampicillin/ml was due to β-lactamase activity. These results sug-

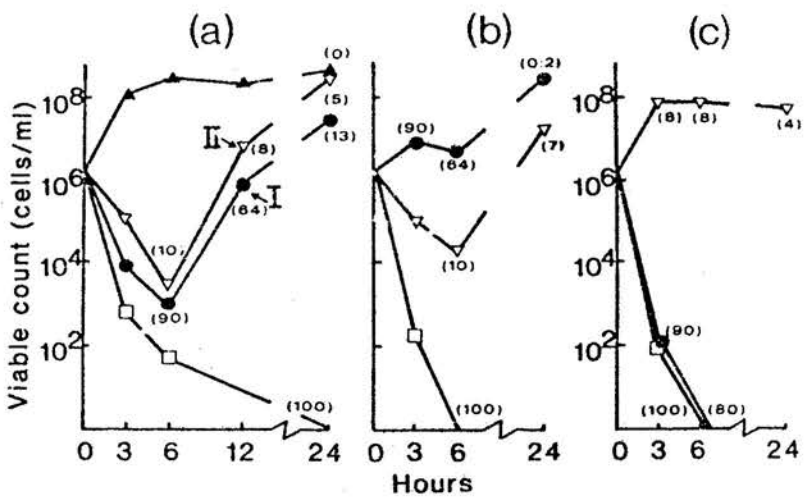

Fig. 2. Bactericidal activities of ampicillin and ampicillin/cloxacillin combinations against (a) E. coli 425; (b) Culture I, derived from E. coli 425 growing in the presence of 100 µg ampicillin/ml; (c) Culture II, derived from E. coli 425 growing in the presence of ampicillin/cloxacillin (10 + 10 µg/ml).
▲ control; ● ampicillin 100 µg/ml; Δ ampicillin/cloxacillin 10+10 µg/ml; □ ampicillin/cloxacillin 100 + 100 µg/ml.
Figures in brackets show ampicillin concentrations.

gest that growth was occurring in the presence of a high concentration of ampicillin due to the presence of cells which had an intrinsic resistance to ampicillin and that these cells were more sensitive to the combinations of ampicillin and cloxacillin than to ampicillin alone.

A further experiment was performed in which E. coli 425 was grown in the presence of ampicillin and ampicillin/cloxacillin combinations (Fig. 2). As in the previous experiment ampicillin (100 µg/ml) and the ampicillin/cloxacillin combination (10 + 10 µg/ml) exerted an initial marked bactericidal effect, but at 12 hours there was substantial regrowth, although significant amounts of ampicillin remained in the cultures (Fig. 2a). Aliquots were taken from these two 12 hr cultures (I, ampicillin 100 µg/ml and II, ampicillin/cloxacillin 10 + 10 µg/ml) and subcultured into antibiotic-free broth. After incubation for 12 hr at 37°C, cultures I and II were tested for sensitivity to ampicillin and ampicillin/cloxacillin combinations. Culture I, selected from the cells growing in the presence of 100 µg/ml ampicillin, grew well in the presence of 100 µg/ml ampicillin, but was as sensitive to ampicillin/cloxacillin combinations as was the parent culture, E. coli 425 (Fig. 2b). In a similar fashion, culture II, selected from cells growing in the presence of ampicillin/cloxacillin (10 + 10 µg/ml) was totally resistant to ampicillin/cloxacillin (10 + 10 µg/ml) but was sensitive to ampicillin and to the higher concentration of the ampicillin/cloxacillin combination (Fig. 2c). These results indicate that populations of cells with differing sensitivities to ampicillin or to the combination of ampicillin and cloxacillin had been selected after initial exposure to the drugs and that these cells had an intrinsic resistance to the penicillins in addition to possessing β-lactamase.

Data similar to those illustrated above have been obtained with other strains of E. coli and with other bacteria e.g. Sh. sonnei, P. morganii and were demonstrated with different combinations of penicillins e.g. ampicillin and flucloxacillin or amoxycillin and flucloxacillin. The results suggest that in certain cases enhancement of activity of ampicillin against ampicillin-resistant bacteria by penicillinase-stable penicillins may be due to effects other than inhibition of β-lactamase or in some cases to both an intrinsic activity and inhibition of β-lactamase. Further evidence to support a synergistic effect between penicillins which is not inhibition of β-lactamase appears to be provided by the enhanced activity of ampicillin combination against some ampicillin-sensitive bacteria.

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ANTIBACTERIAL ACTIVITY AND SYNERGY, IN VITRO AND IN VIVO, OF A COMBINATION OF AMOXYCILLIN AND FLUCLOXACILLIN

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Abstract

The antibacterial activity of a combination of equal parts of amoxycillin and flucloxacillin was compared *in vitro* and *in vivo* with that of amoxycillin and flucloxacillin against a range of Gram-positive and Gram-negative bacteria. The combination generally showed additive effects against bacteria sensitive to the individual penicillins and there was no evidence of antagonism, but synergistic effects were observed between amoxycillin and flucloxacillin against certain amoxycillin-resistant Gram-negative bacilli. The extent of synergism varied according to the particular bacterial species under test and synergy was observed only against bacteria with chromosomally-mediated β-lactamases and not against bacteria with R-factor mediated β-lactamases. In general, amoxycillin + flucloxacillin demonstrated activity against experimental mouse infections in good agreement with its in vitro activity, and synergy was produced against a range of Gram-negative bacilli *in vivo*. The data suggest that clinical trial with amoxycillin + flucloxacillin in the treatment of selected infections including those due to some amoxycillin-resistant bacteria may well be justified.

INTRODUCTION

Amoxycillin is a broad-spectrum penicillin which inhibits the growth of a wide range of Gram-positive and Gram-negative bacteria at concentrations readily attainable in the body at normal dosage (16). However, amoxycillin is usually not stable to the β-lactamases produced by a variety of bacteria and is readily inactivated by these organisms. As a consequence, amoxycillin is not active against β-lactamase-producing strains of staphylococci or of many enterobacteria, although β-lactamase production is not always the sole mechanism of resistance of the latter group of organisms. In contrast, flucloxacillin is stable to most bacterial β-lactamases and is rarely inactivated by β-lactamase-producing bacteria. Nevertheless, flucloxacillin is relatively inactive against Gram-negative bacteria and shows activity of interest solely against Gram-positive cocci including penicillin-resistant staphylococci (15). Combining flucloxacillin with amoxycillin might therefore be expected to extend the broad antibacterial spectrum of amoxycillin to include β-lactamase-producing strains of Staphylococcus aureus.

In addition, it has been shown that certain combinations of β-lactamase-stable and β-lactamase-labile penicillins demonstrate synergistic effects against various Gram-negative bacilli (1, 3, 4, 5, 14). The synergy exhibited arises as a result of the inhibition of the β-lactamase of the organism by the β-lactamase-stable member of the combination enabling the labile penicillin to exert its antibacterial activity against the organism. As the combination of ampicillin and cloxacillin has been shown to demonstrate synergistic

activity as a result of this mechanism (2, 6, 7, 8, 11) it would be expected that amoxycillin + flucloxacillin would exhibit similar effects.

The study presented here was designed to measure the antibacterial activity of a combination of equal parts of amoxycillin and flucloxacillin *in vitro* and against a variety of experimental infections, and to determine the extent and frequency of synergy produced by combining amoxycillin and flucloxacillin.

MATERIALS AND METHODS

Amoxycillin was used in *in vitro* tests and in *in vivo* oral absorption studies as amoxycillin trihydrate (860 µg/mg). In *in vivo* parenteral studies amoxycillin sodium salt was administered (93% pure). Flucloxacillin sodium salt was tested in all *in vitro* and *in vivo* experiments (915 µg/mg). All preparations were obtained from Beecham Pharmaceuticals, Worthing, England.

Antibacterial activity in vitro

Minimum Inhibitory Concentrations (MIC) of amoxycillin, flucloxacillin and of the combination of equal parts of amoxycillin + flucloxacillin required to inhibit growth of the test organism for 18 hr at 37°C were measured by two-fold serial dilution of the substances in 20 ml volumes of nutrient agar (Blood Agar Base, Oxoid). When set, the agar plates were inoculated with 0.001 ml of undiluted overnight broth culture delivered by a multiple inoculating device, an inoculum of about 5×10^5 cells.

The bactericidal activity of the substances against a β-lactamase-producing strain of Escherichia coli (425) was measured by inoculating 20 ml volumes of nutrient broth containing selected concentrations of the compounds with 0.05 ml of an overnight broth culture of the organism. Samples taken at 0, 3, 6 or 24 hr after incubation at 37°C were suitably diluted and 0.02 ml volumes were dropped with a standard dropping pipette onto blood agar containing penicillinase. Colonies were counted after 18 hr incubation at 37°C and the viable counts were calculated. The antibiotic concentrations present in the samples were measured by large plate agar diffusion assay, using a β-lactamase-producing strain of S. aureus (1555) to measure flucloxacillin and an amoxycillin-sensitive (flucloxacillin-resistant) strain of E. coli (NCTC 86) for the assay of amoxycillin.

Activity against experimental infections

Albino mice (Olac strain, 18-22 g) were injected by the intraperitoneal route with 0.5 ml of a suspension in hog gastric mucin (1701-W, Wilson Laboratories) of a dilution of an overnight broth culture (Veal Infusion, Difco) of the test organism standardised to give an infective inoculum of 10 to 100 Median Lethal Dose (MLD). The antibiotics were administered immediately after infection as a single dose, 0.2 ml/20 g, as acacia suspensions in phosphate-buffered saline for oral administration, or as solutions in phosphate-buffered saline for injection. Groups of ten mice were treated at each dose level. The numbers of animals surviving 4 days after infection were recorded and the dose of penicillin required to produce 50% protection of infected animals was calculated.

The activities of the combination of the penicillins were also determined against an experimental mouse pyelonephritis produced by intravenous injection of E. coli. Mice (Olac strain 18-22 g) were injected intravenously with 0.2 ml of a suitable dilution in veal infusion broth (Difco) of an overnight culture of E. coli JT 415, an inoculum of approximately 10^8 cells/mouse. The renal virulence of the bacteria was enhanced by intramuscular administration of iron sorbitol citrate (Jectofer, Astra Chemicals Ltd.), 30 mg/kg, at 18 hr, 42 hr, 66hr and 90 hr after infection. Subcutaneous treatment with the penicillins started 20 h after infection and continued 4 times a day at two-hourly intervals. Groups of 10 mice were killed at each dose level, 10 to 15 days after infection. The kidneys were removed aseptically and examined for macroscopic abscesses, and bacterial counts were made from homogenates prepared from the kidneys.

RESULTS

Antibacterial activity in vitro

The antibacterial activities of amoxycillin and of flucloxacillin against a representative range of Gram-negative and Gram-positive bacteria are illustrated in Table 1. It can be seen that amoxycillin was active at low concentrations against Gram-positive cocci, except penicillin-resistant staphylococci which were resistant as a result of β-lactamase activity. Gram-negative bacteria sensitive to amoxycillin included gonococci, Haemophilus influenzae, E. coli, Proteus mirabilis and Salmonella and Shigella species.

Table 1

Antibacterial spectrum of amoxycillin and flucloxacillin

Organism	MIC (μg/ml)	
	amoxycillin	flucloxacillin
Escherichia coli	5.0	500
Salmonella typhi	1.25	500
Shigella sonnei	5.0	250
Klebsiella aerogenes	250	>500
Enterobacter aerogenes	250	>500
Pseudomonas aeruginosa	>500	>500
Proteus mirabilis	2.5	250
Proteus morganii	250	250
Haemophilus influenzae	0.25	12.5
Neisseria gonorrhoeae	0.01	0.1
Bacteroides fragilis	25	250
Staphylococcus aureus	0.1	0.1
Staphylococcus aureus*	250	0.5
Streptococcus pyogenes	0.01	0.1
Streptococcus pneumoniae	0.02	0.25
Streptococcus faecalis	1.25	25

* β-lactamase-producing strain.

Strains of Pseudomonas aeruginosa, Klebsiella aerogenes and Enterobacter species, indole-positive Proteus species Serratia marcescens and Bacteroides fragilis were relatively insensitive to the broad-spectrum penicillin. Flucloxacillin was active against pyogenic streptococci, pneumococci and staphylococci, including β-lactamase-producing strains of S. aureus, but was generally less active than amoxycillin against penicillin-sensitive cocci. In general, Gram-negative bacteria were relatively insensitive to

flucloxacillin with the exception of gonococci which were quite sensitive to the compound.

The combination of equal parts of amoxycillin + flucloxacillin usually produced additive effects against amoxycillin-sensitive bacteria and against β-lactamase-producing strains of S. aureus so that the activity observed was that to be expected for the activities of the individual penicillins. In a number of cases, however, the combination appeared to be active as amoxycillin against amoxycillin-sensitive strains of E. coli and Sh. sonnei and against Str. faecalis although flucloxacillin was relatively inactive against these organisms.

In contrast, against certain β-lactamase-producing strains of Gram-negative bacilli, distinct synergistic effects were produced by amoxycillin + flucloxacillin and results in Table 2 illustrate examples. It can be seen that a wide range of Gram-negative bacteria are represented in the table. Flucloxacillin was virtually inactive against these organisms (MIC 500 µg flucloxacillin/ml or greater) and amoxycillin MIC values varied from 25 to 500 µg amoxycillin/ml, i.e. these bacteria were markedly less sensitive to amoxycillin than are sensitive strains of enterobacteria (MIC values 1.0-5.0 µg ampicillin/ml). The combination showed a distinct degree of synergism against the test organisms so that in most cases one-tenth or less of the inhibitory concentration of amoxycillin combined with one-tenth or less of the inhibitory concentration of flucloxacillin produced inhibition of these particular penicillinase-producing bacteria.

Table 2

Examples of synergy between amoxycillin and flucloxacillin against amoxycillin-resistant strains of Gram-negative bacilli

Organism	Strain	MIC (µg/ml)		
		amoxycillin	flucloxacillin	amoxycillin + flucloxacillin
Escherichia coli	JT 410	250	500	12.5 + 12.5
	JT 460	250	>500	25 + 25
	JT 497	125	500	12.5 + 12.5
Shigella sonnei	4052	500	>500	50 + 50
	4036	500	>500	50 + 50
	4342	250	500	25 + 25
Proteus morganii	718	500	>500	50 + 50
	709	250	500	25 + 25
Enterobacter aerogenes	R 140	250	>500	12.5 + 12.5
	T 627	250	>500	25 + 25
Citrobacter freundii	W 98	50	500	6.3 + 6.3
	T 745	25	>500	6.3 + 6.3
Klebsiella aerogenes	T 3	500	>500	125 + 125
	T 4	500	>500	125 + 125
	66	500	>500	50 + 50

The frequency and extent of synergism demonstrated by amoxycillin + flucloxacillin against 138 strains of amoxycillin-resistant bacteria with MIC values of 125->500 µg amoxycillin/ml are illustrated in Table 3. The most pronounced synergistic effects were observed with certain amoxycillin-resistant strains of E. coli, Sh. sonnei, P. morganii and Enterobacter species and with a significant proportion of these organisms the combination showed a five to ten-fold

increase in activity compared with amoxycillin. Against K. aerogenes the extent of synergism was evident with only a limited number of strains. Similarly, synergism was not demonstrated with strains of R-factor enterobacteria with transferable amoxycillin resistance and the combination was inactive against these bacteria. Examples of this in Table 3 are 10 strains of E. coli with amoxycillin R-factors which were no more sensitive to amoxycillin + flucloxacillin than to amoxycillin alone. In this study synergism was demonstrated only against those amoxycillin-resistant enterobacteria possessing non-transferable chromosomally-mediated β-lactamases.

Table 3

Distribution of synergistic effects prodyced by amoxycillin + flucloxacillin in MIC tests with 138 amoxycillin-resistant strains of Gram-negative bacilli

Organism	No. of strains	Increase in activity of combination*					
		¼	½	1	2	5	10
Escherichia coli	20			10	3	6	1
Shigella sonnei	9			2	-	5	2
Proteus mirabilis	8			4	4		
Proteus morganii	6			2	-	4	
Proteus rettgeri	5		1	3	1		
Proteus vulgaris	7			5	1	1	
Enterobacter species	16			6	4	2	4
Citrobacter freundii	10		2	3	3	2	
Klebsiella aerogenes	34	1	6	21	5	1	
Pseudomonas aeruginosa	4			4			
Bacteroides fragilis	19		5	12	2		
Total	138	1	14	72	23	21	7

* Increase in activity = $1 \Big/ \frac{\text{MIC of combination}}{\text{MIC of amoxycillin alone}}$

Synergism between amoxycillin and flucloxacillin against amoxycillin-resistant bacteria was also demonstrated in disc sensitivity tests using amoxycillin and flucloxacillin filter paper discs. Thus, the results in Figure 1 illustrate a disc sensitivity test with a strain of E. coli, and it can be seen that the organism was resistant to amoxycillin (25 µg) and flucloxacillin (25 µg) discs but appeared sensitive to a sensitivity disc containing 25 µg amoxycillin and 25 µg flucloxacillin. Similar results were obtained with other amoxycillin-resistant strains of E. coli, Sh. sonnei and P. morganii.

Examination of a number of amoxycillin-resistant bacteria showed that amoxycillin was destroyed during the course of the sensitivity tests and that the extent of inactivation was reduced in the presence of flucloxacillin, confirming that the synergism observed with these bacteria was associated with inhibition of β-lactamase activity by flucloxacillin. An example of the protection of amoxycillin by flucloxacillin is given in Figure 2 which shows the bactericidal activity against a β-lactamase-producing strain of E. coli 425. Amoxycillin (10 µg/ml) was inactive and the viable count was similar to that of the control culture. At a higher concentration (100 µg/ml) amoxycillin was active during the early part of the experiment but at 24 hr the concentration had fallen to 4 µg/ml and the viable count had increased to greater than 10^7 cells/ml. In the presence of flucloxacillin, however, the bactericidal effects were markedly increased and a combination of 10 µg amoxycillin/ml + 10 µg flucloxacillin/ml was significantly more active than 10 µg amoxycillin/ml during the first six hours of the test.

A higher concentration of flucloxacillin (100 µg/ml) brought about almost total protection of amoxycillin (100 µg/ml) and caused a total bactericidal effect at 24 hours.

Antibacterial activity in vivo

The results in Table 4 compare the activities of amoxycillin, flucloxacillin and the combination of equal parts of amoxycillin + flucloxacillin against intraperitoneal mouse infections due to sensitive strains of staphylococci, streptococci and coliform bacilli. In these tests flucloxacillin was much less active than amoxycillin against the Gram-positive cocci and was inactive against the Gram-negative bacilli. Amoxycillin was effective against all the infections and amoxycillin + flucloxacillin showed the activity to be expected from the amoxycillin content of the combination.

Similarly, additive effects were also obtained against an intraperitoneal infection due to a β-lactamase-producing strain of S. aureus where amoxycillin was inactive and flucloxacillin showed good activity, as would be expected from the in vitro sensitivity of the organism to the compounds. In this case, amoxycillin + flucloxacillin showed activity corresponding to the flucloxacillin moiety of the combination(Table 5).

The production of synergistic antibacterial effects in vivo was investigated in a number of mouse intraperitoneal infections caused by β-lactamase-producing strains of Gram-negative bacilli which had been shown to be more sensitive in vitro to amoxycillin + flucloxacillin than to amoxycillin alone.

Table 4

Activity of amoxycillin + flucloxacillin against intraperitoneal mouse infections caused by amoxycillin-sensitive bacteria

Organism	Route of dosing	PD_{50} (mg/kg)*		
		amoxycillin	flucloxacillin	amoxycillin + flucloxacillin
Staphylococcus aureus Smith	oral	0.15	26.2	0.14 + 0.14
	subcutaneous	0.14	11.3	0.13 + 0.13
Streptococcus pyogenes CN10	oral	0.16	21.5	0.30 + 0.30
	subcutaneous	1.5	13.2	1.25 + 1.25
Escherichia coli 8	oral	23	>500	23 + 23
	subcutaneous	72	>500	64 + 64
Escherichia coli 9	oral	21	>500	19 + 19
	subcutaneous	29	>500	25 + 25
Salmonella typhimurium 10	oral	6.0	>500	4.4 + 4.4
	subcutaneous	5.6	>500	6 + 6
Proteus mirabilis 13	oral	50	>500	35 + 35
	subcutaneous	50	>500	71 + 71

* Median protective dose.

Table 5

Activity of amoxycillin/flucloxacillin against an intraperitoneal mouse infection caused by a penicillinase-producing strain of S. aureus 1555

Route of dosing	PD_{50} (mg/kg)		
	flucloxacillin	amoxycillin	amoxycillin + flucloxacillin
oral	56 (41 - 77)	>400	81 + 81 (72 - 364)*
subcutaneous	36 (21 - 60)	790 (220 - 2850)	21 + 21 (22 - 81)

* 95% confidence limits

The results in Table 6 show the relative activities of the compounds and of the combination administered by the oral route and by subcutaneous injection against two strains of each of E. coli, Sh. sonnei, P. morganii, Ent. aerogenes and K. aerogenes and one strain of Ent. cloacae. Flucloxacillin was ineffective by both routes against all the infections, as was amoxycillin by the oral route against the E. coli, Sh. sonnei and P. morganii infections. It can be seen that the combination of amoxycillin + flucloxacillin was notably more effective by the oral route against all infections except for the two Ent. aerogenes infections and the infection due to K. aerogenes I112. Amoxycillin showed greater efficacy against these infections when administered by subcutaneous injection but amoxycillin + flucloxacillin was also significantly more active ($p < 0.05$) by this route against all the infections caused by these amoxycillin-resistant Gram-negative bacteria. In contrast to these findings, the combination was ineffective against infections caused by two strains of E. coli and two strains of K. aerogenes, all of which were insensitive to the combination of amoxycillin + flucloxacillin in vitro. (Results not shown in table).

Table 6

Synergistic activity of amoxycillin/flucloxacillin against intraperitoneal mouse infections caused by amoxycillin-resistant bacteria

Organism	Route of dosing	PD_{50} (mg/kg)		
		amoxycillin	flucloxacillin	amoxycillin + flucloxacillin
Escherichia coli JT 415	oral	>1000	>1000	410 + 410
	subcutaneous	580	>1000	70 + 70
Escherichia coli JT 417	oral	>1000	>1000	315 + 315
	subcutaneous	360	>1000	115 + 115
Shigella sonnei CAM3463	oral	>1000	>1000	335 + 335
	subcutaneous	422	>1000	75 + 75
Shigella sonnei CAM3606	oral	>1000	>1000	500 + 500
	subcutaneous	500	>1000	125 + 125
Proteus morganii Du10	oral	>1000	>1000	158 + 158
	subcutaneous	820	>1000	90 + 90
Proteus morganii Du718	oral	>1000	>1000	330 + 330
	subcutaneous	760	>1000	105 + 105
Enterobacter aerogenes US6	oral	232	>1000	150 + 150
	subcutaneous	1000	>1000	310 + 310
Enterobacter aerogenes US11	oral	153	>1000	190 + 190
	subcutaneous	250	>1000	129 + 129
Enterobacter cloacae T754	oral	>1000	>1000	600 + 600
	subcutaneous	1500	>1000	360 + 360
Klebsiella aerogenes R66	oral	580	>1000	65 + 65
	subcutaneous	300	>1000	63 + 63
Klebsiella aerogenes I112	oral	500	>1000	750 + 750
	subcutaneous	500	>1000	210 + 210

The combination of penicillins also demonstrated synergy against an amoxycillin-resistant strain of E. coli in a different infection model, namely an experimental mouse pyelonephritis. Data in Figure 3 show that in 70% of the mice treated with the individual penicillins the kidney bacterial counts were of the order of 10^4 to 10^8 cells per ml of kidney homogenate, whereas in mice treated with the combination of amoxycillin + flucloxacillin less than 100 cells/ml were isolated from the renal tissues. The differences in efficacy were also apparent from the macroscopic appearance of the kidneys. Thus, 90% of the untreated animals and 70% of the mice treated with amoxycillin or flucloxacillin had visible abscesses in the cortical and medullary regions. In contrast abscess formation was markedly reduced after treatment with amoxycillin + flucloxacillin and abscesses were observed in only 0 to 10% of these animals.

DISCUSSION

Various reasons can be advanced to show why the use of a combination of antibiotics may be preferred to single drug therapy, and among the more important are the need to extend the antibacterial spectrum for the treatment of serious undiagnosed infections or of mixed infections, and the production of synergistic effects. From the results reported here it is evident that a combination of amoxycillin and flucloxacillin meets both these criteria to a greater or lesser extent. Thus amoxycillin + flucloxacillin is active against a broad spectrum of Gram-positive and Gram-negative bacteria,

including penicillinase-producing strains of staphylococci that are resistant to amoxycillin on its own. There is no evidence of antagonism between the two penicillins and the combination displays at the very least additive effects and, in a number of cases, synergistic effects. Amoxycillin + flucloxacillin therefore extends the spectrum of amoxycillin to incorporate penicillin-resistant staphylococci, but what is less obvious is the significance of the synergism shown experimentally by the combination.

Gram-negative bacilli produce a wide variety of β-lactamases (9) and it is not unexpected that flucloxacillin should be inhibitory against only a limited range of β-lactamases. The evidence presented here indicates that flucloxacillin has its greatest effect against certain chromosomally-mediated β-lactamases which confer a medium level of resistance to amoxycillin. Thus, amoxycillin + flucloxacillin produces synergistic effects most frequently against strains of E. coli, Sh. sonnei, P. morganii and Enterobacter species with MIC values of 125-500 μg amoxycillin/ml and the combination may be from five to ten times more active than amoxycillin against these organisms. This group of organisms is generally resistant to cephalosporins and is relatively sensitive to carbenicillin as well as to amoxycillin + flucloxacillin. On the other hand, strains of Enterobacteriaceae with β-lactamases mediated by R-factors show a high level of resistance to amoxycillin (MIC >1000 μg/ml) and the combination is rarely active at concentrations less than 500 μg/ml or greater. R-factor strains now constitute a high proportion of amoxycillin-resistant strains of Enterobacteriaceae and amoxycillin + flucloxacillin would not be expected to be effective against these bacteria.

The good correlation between the *in vitro* sensitivity of amoxycillin-resistant bacteria to amoxycillin + flucloxacillin and the results of treatment of experimental mouse infections is of considerable interest as it appears to show that bacteria with an increased sensitivity to amoxycillin + flucloxacillin *in vitro* also display an increased response to the combination *in vivo*. Conversely, bacteria that are resistant to the combination *in vitro* may be expected to be resistant *in vivo*. The good response of experimental mouse infections to amoxycillin + flucloxacillin may encourage belief that the combination could produce satisfactory results in the treatment of suitable clinical infections. . Clinical studies on the treatment of infections due to resistant bacteria with combinations of penicillins have produced mixed results. In some cases, combined treatment was considered to have produced a synergistic effect and to be advantageous but in others no enhancement of activity was observed (6, 8, 10, 12, 13). However, it is not always clear that the ampicillin-resistant bacteria causing the infections were in fact sensitive to the penicillin combination under trial, a necessary requirement for successful outcome of treatment.

Overall, it is evident that amoxycillin + flucloxacillin demonstrates synergistic effects against a broad spectrum of amoxycillin-resistant bacteria but that with only a limited number of bacteria could the degree of synergism be expected to be sufficient to produce satisfactory therapeutic results. On the other hand, the evidence of these *in vitro* and *in vivo* studies suggests that infections due to amoxycillin-resistant bacteria that are sensitive

to amoxycillin + flucloxacillin *in vitro* may well respond satisfactorily to appropriate therapy with the combination. The data indicate that the clinical trial of amoxycillin + flucloxacillin in the treatment of suitable infections would be justified, including urinary tract infections due to appropriate β-lactamase-producing Gram-negative bacilli, where high concentrations of the agents can be achieved in the urine and where synergy would be most likely to occur.

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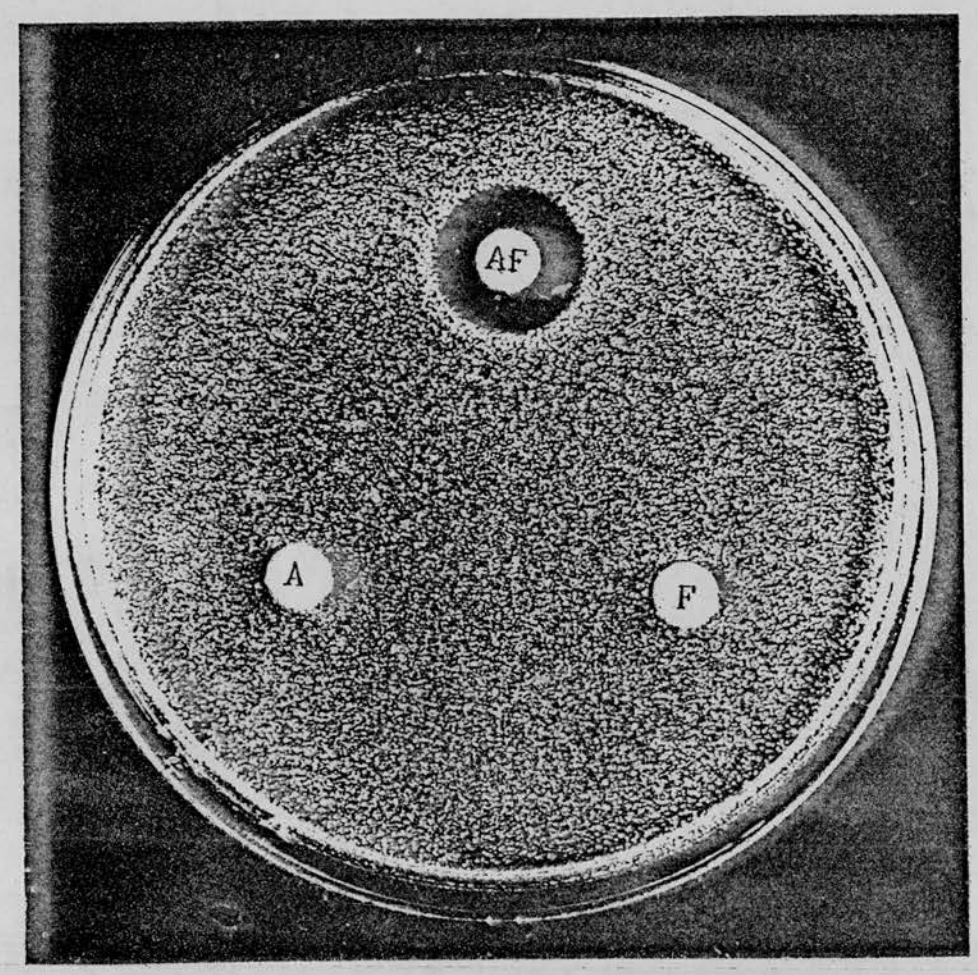

Figure 1. Synergy between amoxycillin and flucloxacillin in an antibiotic-sensitivity disc test against an amoxycillin-resistant strain of Escherichia coli. A, amoxycillin 25 µg; F, flucloxacillin 25 ;g; AF, amoxycillin 25 µg + flucloxacillin 25 µg.

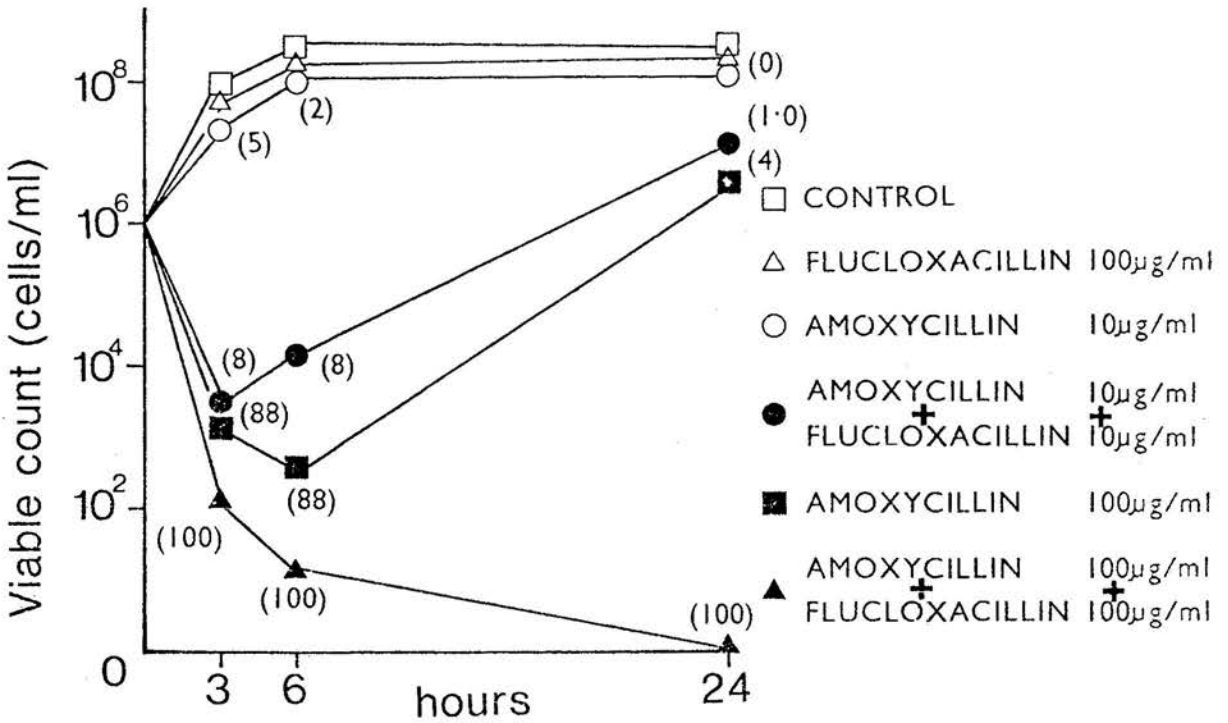

Figure 2. Synergistic bactericidal effects produced by amoxycillin + flucloxacillin against an amoxycillin-resistant strain of Escherichia coli. Figures in brackets show the amoxycillin concentrations (µg amoxycillin/ml).

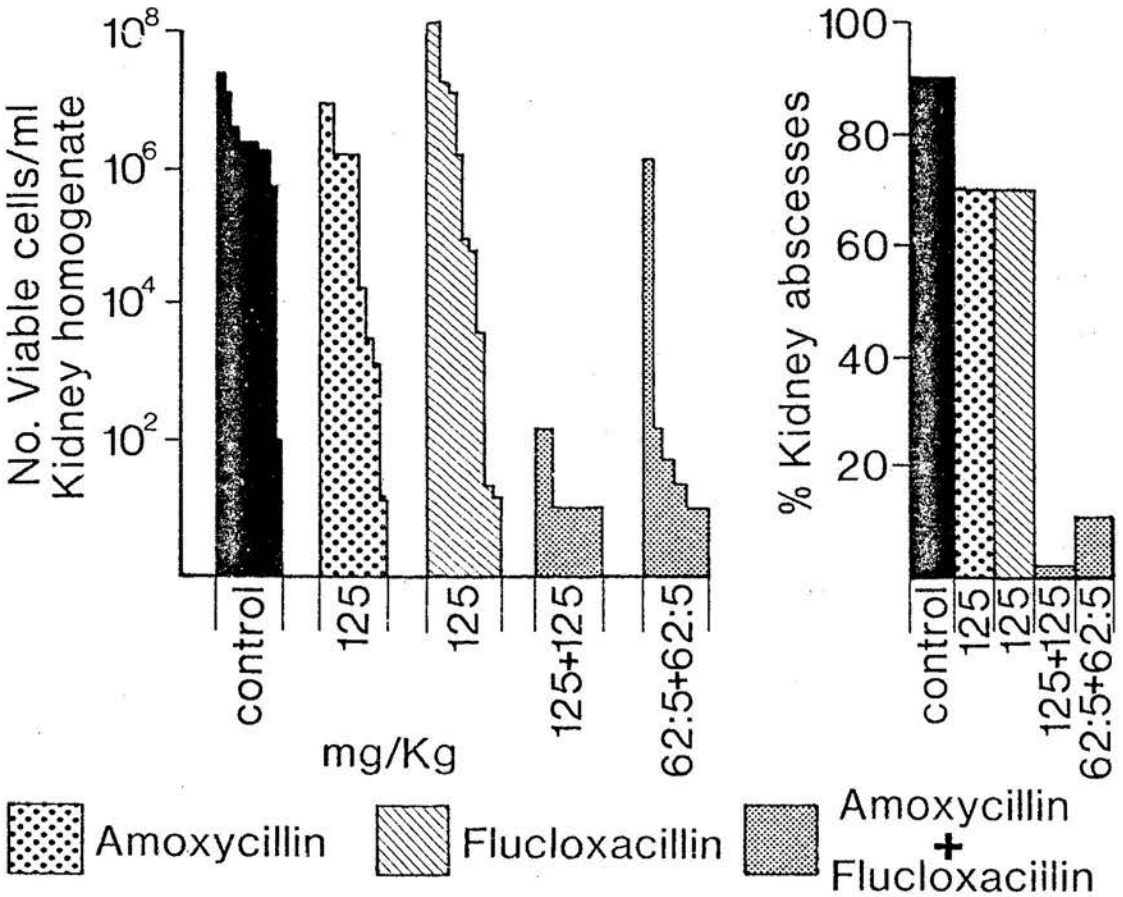

Figure 3. Synergistic activity of amoxycillin + flucloxacillin against an experimental mouse pyelonephritis due to an amoxycillin-resistant strain of Escherichia coli.

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SPIRAMYCIN: A REAPPRAISAL OF ITS ANTIBACTERIAL ACTIVITY

BY

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Spiramycin was less active than erythromycin *in vitro* against sensitive strains of *Staphylococcus aureus*, but was as effective against staphylococcal infections in mice when the drugs were administered immediately after infection: spiramycin was relatively more effective in prophylactic experiments when the antibiotics were administered at 4 or at 6 hr before infection. Strains of staphylococci which had been habituated to either spiramycin or erythromycin *in vitro* showed complete cross-resistance to both antibiotics *in vitro*, but the majority of a number of erythromycin-resistant strains isolated from patients were sensitive to spiramycin *in vitro*. Spiramycin was effective against a number of such strains *in vivo*, but erythromycin had little effect against these infections. Investigation into the distribution of the antibiotics in the serum and tissues of mice showed that spiramycin was maintained in tissues (lung, liver, kidney, spleen and heart) at higher concentrations and for a longer period than erythromycin, although erythromycin was initially present in serum in higher concentrations. It is suggested that the efficacy of spiramycin against experimental infections is due to its persistence in the animal body.

Soon after the isolation of spiramycin (Rovamycin) in 1954 (Pinnert-Sindico, Ninet, Preud'Homme & Cosar), two years after the discovery of erythromycin (McGuire, Bunch, Anderson, Boaz, Flynn, Powell & Smith, 1952), it became apparent that, although the physical, chemical and biological properties of these two antibiotics were closely related, erythromycin was more active than spiramycin *in vitro*.

Chabbert (1955) demonstrated that the antibacterial spectrum of spiramycin corresponded closely with that of erythromycin, but that the latter was more effective *in vitro* against susceptible organisms. These findings were confirmed by Garrod & Waterworth (1956) and by Garrod (1957), who made extensive studies *in vitro* of the relative antibacterial activities of the erythromycin group of antibiotics. Jones & Finland (1957) found that spiramycin was less effective than erythromycin in tests in which the antibacterial activity of sera was measured *in vitro* after administration of these antibiotics to healthy human volunteers. As it had also been established that some degree of cross-resistance existed between the two substances, it is not surprising that it was concluded that spiramycin was far less effective than erythromycin (Garrod, 1957; Editorial, 1957).

On the other hand, Cosar (1956) reported that spiramycin was as effective as erythromycin against a number of experimental infections. Chabbert, Boyer, Saviard,

Boulingre & Hervé (1957) concluded that spiramycin was more effective in the treatment of experimental staphylococcal infections and, more recently, Benazet & Dubost (1959) reported that spiramycin was more active than erythromycin against streptococci and staphylococci in mice.

The dissimilarity of the two antibiotics was further emphasized by the diversity of results of various studies concerning the nature and extent of the cross-resistance existing between the two antibiotics. Although habituation *in vitro* to either of the antibiotics produced strains of bacteria completely resistant to the other antibiotic, the degree of cross-resistance reported from clinical studies was very variable. For example, while Garrod (1957) reported complete cross-resistance in one-third of the erythromycin-resistant strains examined in his study, Finland (1956) found very little cross-resistance between the two drugs, and Lowbury & Hurst (1959) noted only a slight reduction in sensitivity to spiramycin in about one-quarter of the erythromycin-resistant staphylococci used by them.

The results reported in this paper were derived from experiments designed to compare the relative antibacterial activities of spiramycin and erythromycin *in vitro* and *in vivo* and, if possible, to provide some explanation for the diverse results reported by various workers.

METHODS

In vitro

Bacteriostatic activity. Spiramycin and erythromycin were dissolved in aqueous methanol and serially diluted by 2-fold steps in ox-heart digest broth. Each tube was inoculated with 0.1 ml. of a 6-hr-old broth culture of *Staphylococcus aureus* and incubated at 37° C for 24 hr, when the minimal inhibitory concentrations (M.I.C.) were determined as the lowest concentrations of antibiotic which prevented growth visible to the naked eye.

The effect of serum on the antibacterial activity of the antibiotics was determined by adding horse serum to a final concentration of 10% or 20% to digest broth and by reading the M.I.C. as before. Similarly the effect of inoculum size was determined by inoculating series of antibiotic-broth solutions with varying dilutions of the infecting organism.

Bactericidal activity. Bactericidal tests were carried out by subculturing from tubes showing no growth in the bacteriostatic tests on to nutrient agar containing no antibiotic. After overnight incubation, the absence or presence of growth was noted.

The effect of the antibiotics on the viable counts of cultures was measured as follows. Three concentrations of spiramycin and erythromycin (equivalent to M.I.C.×4; M.I.C.×1; and M.I.C.×0.25) were prepared in 45 ml. digest broth. For spiramycin these concentrations were 12.5, 3.125, and 0.8 μg/ml., and for erythromycin 1.25, 0.32, and 0.08 μg/ml., respectively. Each bottle of broth, and one containing no antibiotic, were inoculated with 5 ml. of a 24-hr-old broth culture of *Staphylococcus aureus* (Oxford) standardized to contain 10^8 organisms/ml. At intervals of 1, 2, 4, 7 and 24 hr after inoculation 1 ml. aliquots were diluted in 1/4 strength Ringer solution prepared from tablets supplied by Oxoid giving final salt concentrations in g/l. of NaCl 2.25, KCl 0.105, $CaCl_2$ 0.12, $NaHCO_3$ 0.05. Viable counts were made by a drop-plate method, modified from Miles, Misra & Irwin (1938) and readings were made after incubation at 37° C for 48 hr.

Habituation. Serial dilutions of spiramycin and erythromycin were made in digest broth, and the tubes were inoculated with 0.1 ml. of 18-hr broth cultures of *Staph. aureus*. After incubation at 37° C for 24 hr, subcultures were made, from the tubes containing the highest concentration of antibiotic in which there was obvious growth, into a fresh series of antibiotic-broth solutions. Transfers were made daily until resistance developed to the antibiotics.

Coagulase tests. Tubes containing 1 ml. volumes of fresh rabbit plasma, diluted 1/10 with saline, were inoculated with 0.1 ml. of 18-hr broth cultures of *Staph. aureus*, and incubated at 37° C in a water bath. Results were read after 0.5, 1, 3, 6 and 24 hr.

In vivo

Activity in mice

Therapeutic activity. Albino mice were infected intravenously with 0.2 ml. of 18-hr broth cultures of *Staph. aureus*, and dosed orally with 0.5 ml. of suspensions of the antibiotics, ½, 6, 24, 48 and 72 hr after infection. Deaths were noted daily for 10 days, when surviving mice were killed and examined macroscopically for the presence of abscesses on kidneys; all kidneys were cultured for staphylococci.

Prophylactic activity. Groups of mice were given orally a single dose of 0.5 mg/g of the antibiotics at 6, 4 or 2 hr or immediately before intravenous infection with, in the first experiment, 0.1 ml. of an 18-hr broth culture, and in the second experiment 0.5 ml. of a 10^{-1} dilution of an 18-hr broth culture of *Staph. aureus* (CN 491). Deaths were recorded daily for 7 days.

Antibiotic levels in tissues of mice

The methods adopted for the extraction and assay of the tissue antibiotic concentrations were similar to those used by Benazet & Dubost (1959) in their studies on the erythromycin group of antibiotics, except that the French workers used aqueous methanol in their extraction procedures, and preferred a filter-paper technique for the assay of antibiotic concentrations.

Mice were given a single oral dose of 0.5 mg/g of spiramycin or erythromycin, and groups of 10 were killed 2, 6, 24 and 48 hr after injection. Heart blood was collected from the groups of mice killed at 2 hr and 6 hr, and the pooled sera were assayed for antibiotic concentrations. The heart, liver, lungs, kidney and spleen were removed aseptically from the animals killed 6, 24 and 48 hr after dosing, and pools of each of these organs were weighed. Each of the organ pools was triturated with sterile sand (1 g/2 g tissue) and phosphate buffer, *p*H 8.0 (25 ml. for the pooled organs of each group, but 100 ml. for pooled livers), centrifuged, and the supernatant fluids assayed for antibiotic content.

The sera and extracts were assayed for spiramycin and erythromycin as described for erythromycin by Grove & Randall (1955), *Sarcina lutea* (N.C.T.C. 8340) being used as the assay organism. The extracts were added to antibiotic cylinders on Pen-assay agar (Difco), *p*H 8.0, pre-seeded with *Sarcina lutea*. Zone diameters were measured after incubation at 30° C for 24 hr, when the tissue antibiotic concentrations were calculated from standard curves.

RESULTS

Activity in vitro

The inhibitory concentrations of spiramycin and erythromycin to 45 strains of antibiotic-sensitive staphylococci are shown in Table 1. Erythromycin was more active than spiramycin, the growth of all 45 strains being inhibited by 1.0 μg/ml. or less of erythromycin, whereas only 7 of these strains were affected by similar concentrations of spiramycin. The average inhibitory concentrations of spiramycin

TABLE 1

BACTERIOSTATIC ACTIVITY OF SPIRAMYCIN AND ERYTHROMYCIN AGAINST 45 SENSITIVE STRAINS OF *STAPH. AUREUS*

Antibiotic	No. of strains tested	No. of strains inhibited. Minimal inhibitory concentrations (μg/ml.) 0·03	0·06	0·13	0·25	0·5	1·0	2·0	4·0	8·0	16·0
Spiramycin	45	0	0	0	0	2	5	17	16	4	1
Erythromycin	45	4	9	14	11	5	2	0	0	0	0

in this series were 2.0 to 4.0 μg/ml. of antibiotic, about 20-fold greater than those of erythromycin (average 0.1 to 0.2 μg/ml.).

In a series of experiments with antibiotic-resistant staphylococci (naturally resistant and derived from patients), the inhibitory concentrations of spiramycin against 18 out of 20 coagulase-positive, erythromycin-resistant strains were very similar to those found necessary to inhibit sensitive organisms (Table 2). The remaining two strains were inhibited by 25 μg/ml. and 32 μg/ml. of spiramycin.

TABLE 2

BACTERIOSTATIC ACTIVITY OF SPIRAMYCIN AND ERYTHROMYCIN AGAINST 20 ANTIBIOTIC-RESISTANT STRAINS OF *STAPH. AUREUS* DERIVED FROM PATIENTS

The sensitivity of the staphylococci to antibiotics other than spiramycin or erythromycin was determined by a filter paper disc–agar diffusion technique (R=resistant: S=sensitive)

	M.I.C. (μg/ml.)		Antibiotic sensitivity			
Strain no.	Spiramycin	Erythromycin	Penicillin	Streptomycin	Tetracyclines	Chloramphenicol
1	4·0	250	R	R	R	S
2	8·0	250	R	R	R	S
3	32·0	125	R	R	R	S
4	8·0	125	R	R	R	S
5	4·0	125	R	R	R	S
6	8·0	63	R	R	R	S
7	4·0	63	R	R	R	S
8	4·0	8·0	R	R	R	S
9	4·0	63	R	R	R	S
10	2·0	125	R	R	R	S
11	2·0	63	R	R	R	S
12	<1·0	4·0	R	R	R	S
13	3·2	3·2	R	R	R	S
14	12·5	12·5	R	S	R	S
15	12·5	6·3	R	S	S	S
16	25·0	25·0	R	S	R	S
17	2·0	16·0	—	—	—	—
18	8·0	125	R	R	R	S
19	<1·0	1·0	R	R	R	mod. S
20	2·0	125	—	—	—	—
21	2·0	0·1	S	S	S	S

Horse serum added to digest broth at concentrations of 10% and 20% had little or no effect on the activity of spiramycin and erythromycin, there being a 2- or 4-fold change in the M.I.C. at the most. Likewise, altering the inoculum size had little effect on the inhibitory concentration of either antibiotic, there being only a 4-fold difference between series of tubes inoculated with an 18-hr-old culture and those inoculated with a 10^{-6} dilution of the same culture.

The bactericidal effects of both antibiotics were also closely related (Fig. 1), so that equivalent multiples of the M.I.C. of each antibiotic produced similar changes in the viable counts of the Oxford strain of *Staph. aureus*. For example, 3.2 μg/ml. of spiramycin, equivalent to the M.I.C., prevented multiplication of the original inoculum of 10^{-5} organisms/ml. over a period of 24 hr, while 0.32 μg/ml. of erythromycin, also equivalent to the M.I.C., produced a similar effect. Likewise concentrations of M.I.C. × 4 of the antibiotics produced comparable falls in the numbers of organisms (Fig. 1).

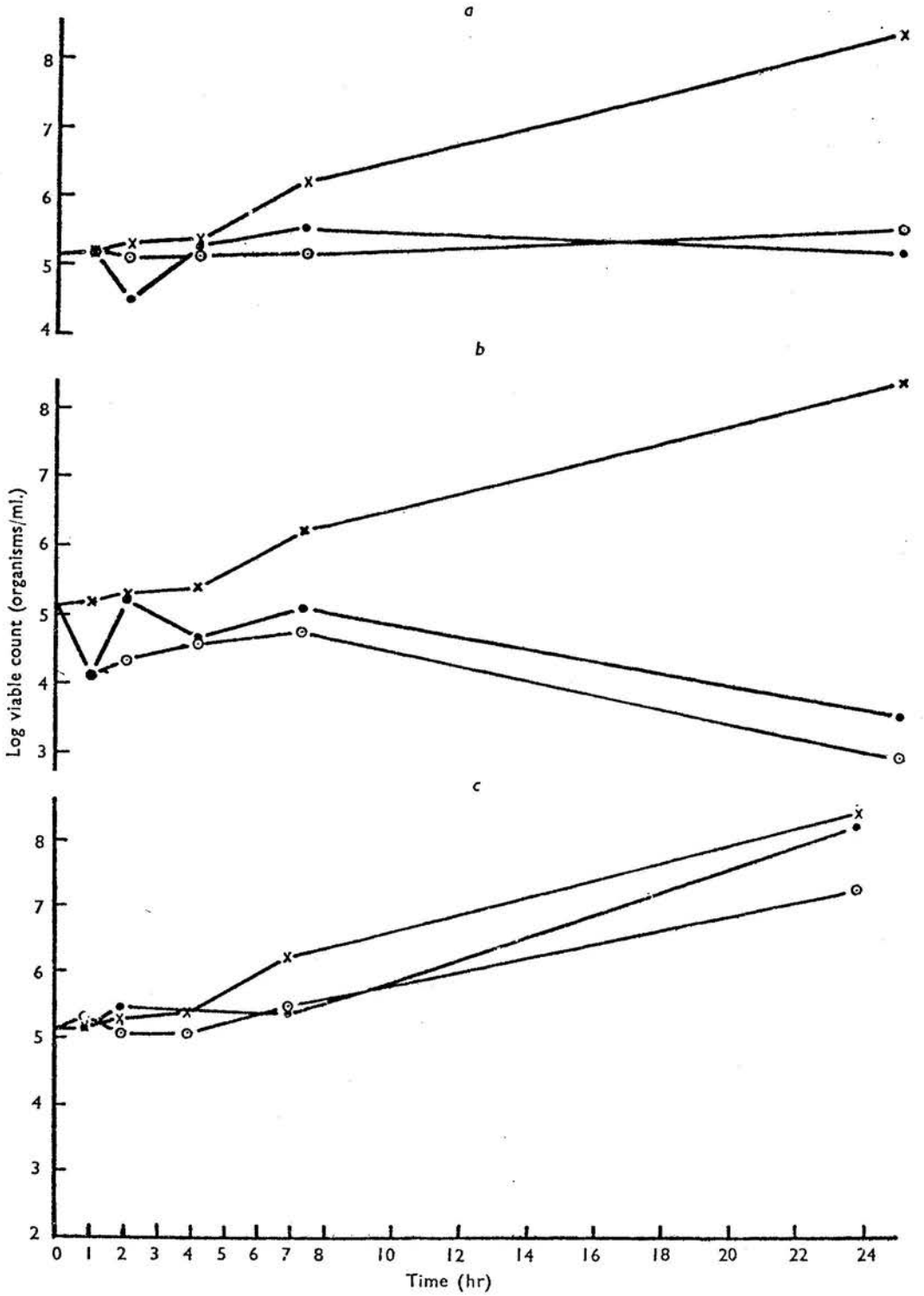

Fig. 1. The bactericidal effects of equivalent inhibitory concentrations of spiramycin (○——○) and erythromycin (●——●) against *Staph. aureus* (Oxford) *in vitro*. The line ×——× denotes the growth over 24 hr of the control culture of *Staph. aureus*. (*a*) Minimal inhibitory concentrations (M.I.C.): spiramycin 3.2 μg/ml.; erythromycin 0.32 μg/ml. (*b*) M.I.C.×4: spiramycin 12.5 μg/ml.; erythromycin 1.25 μg/ml. (*c*) M.I.C.×0.25: spiramycin 0.8 μg/ml.; erythromycin 0.08 μg/ml. Ordinates: Log viable count (organisms/ml.). Abscissae: time in hr.

Similarly, subcultures taken at 24 hr from the bacteriostatic tests showed both antibiotics to be bactericidal at concentrations within one or two tubes of the bacteriostatic inhibitory concentrations.

Two sensitive strains of *Staph. aureus*, cultured in subinhibitory concentrations of either spiramycin or erythromycin, soon developed resistance to the antibiotics in which they were cultured, and highly resistant strains were obtained after 15 transfers in increasing concentrations of the antibiotics. These strains (made resistant in the laboratory) showed complete cross-resistance to both antibiotics, and it was noted that some of the characteristics of the resistant variants differed from those of the parent (sensitive) strains. In general (Table 3) these strains did not ferment mannitol, the coagulase reaction was less marked, and their pathogenicity to mice was reduced.

TABLE 3

CHARACTERISTICS OF STRAINS OF *STAPH. AUREUS* HABITUATED *IN VITRO* TO EITHER SPIRAMYCIN OR ERYTHROMYCIN

Mannitol fermentation was determined on mannitol salt agar (Oxoid) and pathogenicity to mice was estimated by a comparison of the effect of graded dilutions of cultures of the respective strains injected intravenously

Strain	Habituated to	M.I.C. (μg/ml.) Spiramycin	M.I.C. (μg/ml.) Erythromycin	Coagulase	Mannitol fermentation	Pathogenicity to mice
CN 491	—	2·0	0·1	+++	+	+++
CN 491 SR	Spiramycin	>50	>50	+	—	+
CN 491 ER	Erythromycin	>50	>50	+	—	—
133	—	2·0	0·1	++	+	+++
133 SR	Spiramycin	>50	>50	+	—	—
133 ER	Erythromycin	50	>50	+	—	+
Oxford	Spiramycin	>100	>100	Not tested		

Activity in mice

In these experiments, the intravenous infection of untreated mice normally caused the death of most of the animals within 2 to 5 days; after 2 days, abscesses visible to the naked eye could be observed in the kidneys of most of the infected mice, and staphylococci could be cultured from the kidneys of surviving mice in nearly all cases.

Therapeutic activity. Spiramycin was as active as erythromycin in *in vivo* experiments against a mouse-virulent laboratory strain, *Staph. aureus* (133), although erythromycin was considerably more active *in vitro* against this organism (Table 4). Post-mortem examination of surviving mice, 10 days after infection, showed that there was a lower incidence of kidney infection in the groups of mice treated with spiramycin, as judged by the presence of macroscopic abscesses and by the numbers of healthy-looking kidneys from which staphylococci were cultured.

Spiramycin was also highly effective against mouse infections caused by recently isolated strains of erythromycin-resistant staphylococci from patients (Table 5). In contrast, erythromycin was inactive, or relatively so, against these experimental infections.

TABLE 4

COMPARATIVE ACTIVITIES OF SPIRAMYCIN AND ERYTHROMYCIN AGAINST AN INTRAVENOUS STAPHYLOCOCCAL INFECTION IN MICE

Organism: *Staph. aureus* 133. The drugs were administered orally 0·5, 6, 24, 48 and 72 hr after infection. Numbers in parentheses are fiducial limits of error. $P=0·05$

Antibiotic	M.I.C. (μg/ml.)	Single oral dose (mg/g)	No. surviving after 10 days	ED50 mg/g
Spiramycin	2·0	0·5	10/10	
		0·25	10/10	
		0·13	8/10	0·075
		0·06	4/10	(0·05–0·11)
Erythromycin	0·1	0·5	10/10	
		0·25	8/10	0·11
		0·13	6/10	(0·07–0·17)
		0·06	3/10	
Untreated	—	—	0/10	—

TABLE 5

COMPARATIVE ACTIVITIES OF SPIRAMYCIN AND ERYTHROMYCIN AGAINST EXPERIMENTAL INFECTIONS CAUSED BY RECENTLY ISOLATED ERYTHROMYCIN-RESISTANT STRAINS OF *STAPH. AUREUS*

The drugs were administered orally 0·5, 6, 24, 48 and 72 hr after infection

Strain	M.I.C. (μg/ml.) Spiramycin	M.I.C. (μg/ml.) Erythromycin	Treatment	Single oral dose (mg/g)	No. surviving after 10 days
MB 1	4·0	250	Untreated	—	1/10
			Spiramycin	0·5	10/10
			Erythromycin	0·5	2/10
MB 2	8·0	250	Untreated	—	0/10
			Spiramycin	0·5	10/10
			Erythromycin	0·5	1/10
MB 7	4·0	63·0	Untreated	—	1/10
			Spiramycin	0·5	8/10
			Erythromycin	0·5	4/10
MB 9	4·0	63·0	Untreated	—	0/10
			Spiramycin	0·5	9/10
			Erythromycin	0·5	2/10
MB 11	2·0	63·0	Untreated	—	1/10
			Spiramycin	0·5	10/10
			Erythromycin	0·5	4/10
MB 12	<1·0	4·0	Untreated	—	1/10
			Spiramycin	0·5	10/10
			Erythromycin	0·5	4/10

On the other hand, neither antibiotic was active *in vivo* against a strain of *Staph. aureus* (133 ER), which had been habituated to erythromycin *in vitro*, although both were effective *in vivo* against a strain (CN 491 SR) which had been habituated to spiramycin (Table 6). These strains were completely resistant *in vitro* to both antibiotics.

Prophylactic activity. Spiramycin was more effective than erythromycin when the antibiotics were administered to mice at intervals before infection with *Staph. aureus* (CN 491). The results of two experiments are given in Table 7. Thus, in the first

TABLE 6

EFFECT OF SPIRAMYCIN AND ERYTHROMYCIN *IN VIVO* AGAINST STRAINS OF *STAPH. AUREUS* HABITUATED *IN VITRO* TO THESE ANTIBIOTICS

The compounds were administered orally 0·5, 6, 24, 48 and 72 hr after infection

Strain	Habituated to	Antibiotic	M.I.C. (μg/ml.)	Dose (mg/g)	No. surviving after 10 days
133 ER	Erythromycin	Spiramycin	50	0·5	3/8
				0·25	2/8
				0·13	1/8
		Erythromycin	>50	0·5	3/8
				0·25	2/8
				0·13	0/8
		—	—	Untreated	0/15
CN 491 SR	Spiramycin	Spiramycin	>50	0·5	8/8
				0·25	6/8
				0·13	2/8
		Erythromycin	>50	0·5	8/8
				0·25	7/8
				0·13	4/8
		—	—	Untreated	0/6

TABLE 7

PROPHYLACTIC EFFECT OF ONE ORAL DOSE OF SPIRAMYCIN AND ERYTHROMYCIN ADMINISTERED TO MICE 0, 2, 4 OR 6 HR BEFORE INTRAVENOUS INFECTION WITH *STAPH. AUREUS* CN 491

Experiment 1: infecting dose, 0·1 ml. of 18-hr culture. Experiment 2: infecting dose, 0·5 ml. of 1/10 dilution of 18-hr culture

Treatment	Dose (mg/g)	Time of administration before infection (hr)	No. surviving after 7 days		Mean survival time (days)	
			Expt. 1	Expt. 2	Expt. 1	Expt.
Untreated	—	—	1/5	0/10	2·8	2·5
Spiramycin	0·5	0	4/5	0/10	6·8	7·0
		2	4/5	3/10	6·8	6·0
		4	4/5	8/10	6·6	6·8
		6	3/5	6/10	6·0	6·1
Erythromycin	0·5	0	3/5	3/10	5·8	5·5
		2	3/5	2/10	5·8	5·4
		4	0/5	2/10	3·0	4·9
		6	0/5	0/10	2·0	2·9

experiment, erythromycin afforded some protection to animals treated 2 hr before, and immediately before, infection, but none of the mice dosed with this drug 4 hr and 6 hr beforehand survived the duration of the experiment. Spiramycin, on the other hand, was effective at all four intervals of time.

In the second experiment the protection afforded by spiramycin was repeated except for the group of animals dosed 2 hr before infection, where 5 mice died suddenly on day 6. The pattern of survival in the case of erythromycin-dosed animals was similar to that of the first experiment, except for some slight extension of protection to the group treated 4 hr before infection.

Antibiotic concentrations in the organs of uninfected mice

The results of these experiments are summarized in Table 8.

Both antibiotics were present in high concentrations in the serum within 2 hr after administration (15.0 μg/ml. of erythromycin, 9.4 μg/ml. of spiramycin), but the antibiotic concentrations in the serum fell to fairly low values (3.5 μg/ml. of erythromycin, 1.8 μg/ml. of spiramycin) 6 hr after administration. The concentrations of erythromycin in the serum were almost twice those of spiramycin at both periods.

TABLE 8

TISSUE CONCENTRATIONS OF SPIRAMYCIN AND ERYTHROMYCIN AFTER ONE ORAL DOSE OF 0.5 MG/G TO MICE

Expt.	Antibiotic	Time after administration (hr)	Serum concn. (μg/ml.)	Tissue concentration (μg/g)				
				Heart	Kidney	Liver	Lung	Spleen
1	Spiramycin	2	9·4	—	—	—	—	—
		6	1·8	34	95	110	82	111
		24	—	9·0	16·0	7·0	18·0	18·0
		48	—	0 (<5·0)	8·0	2·5	Trace (<3·0)	Trace (<2·0)
	Erythromycin	2	15·0	—	—	—	—	—
		6	3·5	16	59	120	46	51
		24	—	<1·5	6·0	<0·5	1·0	5·0
		48	—	—	—	—	—	—
2	Spiramycin	24	—	10	16	7·0	17	18
	Erythromycin	24	—	<2·0	<1·0	<1·0	<2·0	<2·0

Six hours after administration of the antibiotics, high concentrations of spiramycin and erythromycin were present in the organs. In general, erythromycin produced lower concentrations than spiramycin except in the liver. After 24 hr, however, while substantial amounts of spiramycin (about 1/5 to 1/10 of the 6-hr values) were still present in all the tissues, low concentrations only of erythromycin could be detected in some organs. Spiramycin could still be detected in the organs, except the heart, 48 hr after administration. In a further experiment to confirm these results, spiramycin and erythromycin were administered at the same time and the 24-hr tissue extracts were assayed. The concentrations of spiramycin found in the tissues in this experiment were very similar to those found previously, but erythromycin could not be detected in any of the extracts.

DISCUSSION

Spiramycin is 1/10 to 1/20 as active *in vitro* as erythromycin against antibiotic-sensitive strains of staphylococci. This fact has already been demonstrated in Great Britain (Garrod & Waterworth, 1956), in France (Chabbert, 1955) and in the United States (Jones & Finland, 1957). Likewise, although the results of the bactericidal experiments indicate that the mode of action on one antibiotic is very

similar to that of the other, the equivalent bactericidal concentrations of each differ to about the same extent as do the bacteriostatic concentrations.

Spiramycin, however, is undoubtedly much more active against experimental infections than might be expected from the activity of the compound *in vitro*. Garrod (1957) suggested that the ratio of the *in vitro* activity of spiramycin to that of erythromycin would be reflected in the body, but this is not so. In this series of experiments spiramycin was as effective *in vivo* as erythromycin against the laboratory strain of *Staph. aureus* (133), in spite of the fact that the latter antibiotic was 20 times as active *in vitro* against this strain. Spiramycin, too, was active against naturally occurring antibiotic-resistant staphylococci *in vivo*, whereas erythromycin had little or no effect against these infections. Finally, the extent and duration of prophylactic activity of spiramycin was greater than that of erythromycin. Our experiments have been limited to a study of staphylococcal infections, but others, Benazet & Dubost (1959), Cosar (1956) and Freeman (personal communication), have demonstrated similar anomalous results against other micro-organisms, such as streptococci or pneumococci.

An explanation for this phenomenon would be apparent if spiramycin were metabolized *in vivo* to a highly active form, but extensive studies in France have produced no evidence of the existence of such a metabolite (Benazet, personal communication). An alternative hypothesis put forward by Chabbert *et al.* (1957) is that spiramycin is active *in vivo* because it is retained in the body in high concentrations for a considerable period. To confirm this, these workers carried out an elegant series of experiments, which demonstrated that the activity of spiramycin in mouse infections was related to the tissue concentrations of the antibiotic. More recently, Benazet & Dubost (1959) reported similar findings in an independent study.

The experiments reported here confirm that spiramycin is retained in the body of the mouse longer, and in higher concentrations, than is the case with erythromycin, and it seems reasonable to suggest that the pronounced *in vivo* activity of spiramycin against mouse infections may be due to this factor. The same explanation would account for the superiority of spiramycin over erythromycin in prophylactic experiments. Recently, too, Maniar, Eidus & Greenberg (1960) have reported that, after subcutaneous administration of a single dose of antibiotic to mice, spiramycin was present in various organs at higher concentrations than erythromycin over a period of 24 hr; after prolonged treatment spiramycin accumulated in the tissues whereas erythromycin did not. These workers also demonstrated that spiramycin was more effective against a pneumococcal infection in mice, although erythromycin was 4 times more active *in vitro*, and concluded, accordingly, that the *in vivo* activity of spiramycin was related to its affinity for animal tissues (Greenberg, Maniar & Eidus, 1960).

It is important to note that in these experiments the serum concentrations of erythromycin were almost twice as great as those of spiramycin after administration of equivalent doses of the antibiotics. If, then, therapeutic activity were related directly to antibiotic serum concentrations, as is the rationale of the *in vivo–in vitro* type of experiment used by Jones & Finland (1957) in the evaluation of these two antibiotics, and which is favoured by many workers for the comparison of related

antibiotics, erythromycin might be expected to be more active *in vivo* than spiramycin—but this is not so. This work therefore appears to support the doubts which have been expressed recently (Editorials, 1961a, 1961b) as to the validity of the assessment of antibiotics by measurement of the antibacterial activity of sera.

The nature of the cross-resistance which exists between these two antibiotics is more obscure, and it is possible that at least two mechanisms are involved. Garrod (1957) and Jones, Nichols & Finland (1956) have demonstrated that strains habituated *in vitro* show complete cross-resistance to each other, but that cross-resistance between strains isolated from clinical infections is variable. For example, Lowbury & Hurst (1959) have shown that, of 305 strains of staphylococci fully resistant to erythromycin, none was resistant to spiramycin *in vitro*.

Our experiments with a limited number (20) of typical antibiotic-resistant strains of staphylococci derived from clinical sources show that not only is spiramycin active against most naturally occurring erythromycin-resistant strains *in vitro*, but also that it is very effective *in vivo* against these organisms. The therapeutic activity of spiramycin against these staphylococci is of considerable practical importance in view of the increasing numbers of infections caused by antibiotic-resistant staphylococci.

A possible explanation for the divergent results reported from various laboratories might be that strains habituated in the laboratory to spiramycin and erythromycin bear as little relationship to recently isolated resistant staphylococci as penicillin-resistant strains produced in the laboratory bear to resistant penicillinase-producing staphylococci. This conclusion might appear to be borne out not only by the fact that the biochemical characteristics of the habituated strains differed from those of the parent strain but also from the unexpected results derived from experimental infections caused by these organisms.

It may be concluded that spiramycin is an active antistaphylococcal antibiotic, effective against many erythromycin-resistant strains of staphylococci. For the purpose of antibiotic-sensitivity tests, and for use in clinical practice, spiramycin, because of its effectiveness against antibiotic-resistant organisms, and by virtue of its high and prolonged tissue concentrations, should be considered as an antibiotic in its own right, rather than as a member of the erythromycin group of antibiotics. It is also suggested that the evaluation of the therapeutic activity of antibiotics from data based largely on *in vitro* studies—the method in common use to-day—may be misleading. Discrepancies between *in vivo* and *in vitro* results in the evaluation of antibiotics should be explored. Light may be shed on these by estimates of tissue concentrations of an antibiotic as well as serum concentrations. Moreover, the assessment of cross-resistance solely by the use of organisms habituated to antibiotics *in vitro* may also be misleading; results should be checked by the use of naturally occurring resistant strains, if any are available.

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Comparative Effects of Amoxycillin and Ampicillin in the Treatment of Experimental Mouse Infections

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Amoxycillin was significantly more active than ampicillin in the treatment of intraperitoneal mouse infections when administered by oral and parenteral routes, although the causal bacteria were equally susceptible in vitro to the two penicillins. Amoxycillin produced higher antibiotic blood concentrations in mice than ampicillin after oral administration, and this was a possible explanation for the superior oral activity of amoxycillin. In contrast, antibiotic blood concentrations were the same for both compounds after subcutaneous injection, but it was demonstrated that amoxycillin was more effective than ampicillin by this route in reducing bacterial counts in the peritoneal cavity and in the blood of mice infected with *Escherichia coli*. Amoxycillin was also significantly more active than ampicillin in the treatment of infection by intraperitoneal dosing as a result of greater bactericidal activity in infected mice together with the production of higher antibiotic blood levels. The results of these studies on the effects of parental treatment of experimental infections with the two penicillins show that the superior chemotherapeutic activity of amoxycillin was associated with the greater bactericidal activity of amoxycillin in vivo and with differences in the distribution of the two penicillins in the infected animal.

Although amoxycillin is similar to ampicillin with respect to antibacterial spectrum and general level of antibacterial activity in vitro (4, 5), initial chemotherapeutic studies showed that the compound was more active than ampicillin against a variety of mouse infections by oral and parenteral routes (1). The superior activity of amoxycillin by the oral route might be explained largely by the better oral absorption characteristics of amoxycillin compared with ampicillin, resulting in higher serum concentrations of amoxycillin, but this would not be the explanation for the results obtained by subcutaneous injection where the blood levels produced by amoxycillin were no different from those of ampicillin (1). Further investigations of the activity of amoxycillin after parenteral treatment of an experimental mouse thigh lesion infection showed that amoxycillin produced greater bactericidal effects than ampicillin in vivo and suggested that this was the explanation for the superior chemotherapeutic activity of amoxycillin (2).

The data reported here were derived from studies designed to investigate differences between amoxycillin and ampicillin in the treatment of experimental intraperitoneal mouse infections and involved measurement of antibiotic levels and bacterial counts in the blood and peritoneal cavity of infected mice and correlation of these parameters with effectiveness of treatment.

MATERIALS AND METHODS

Antibiotics. The penicillins were used as commercial preparations of amoxycillin trihydrate (850 μg/mg; Amoxil, Bencard) and ampicillin trihydrate (855 μg/mg; Penbritin, Beecham Research Laboratories) except in parenteral treatment studies, where the sodium salts were used.

Mice. Male or female albino mice, 18 to 22 g, CS1 strain (Charles River, U.K. Ltd.) or OLAC strain (Oxford Laboratory Animal Colony), were used throughout.

MICs. Twofold serial dilutions of amoxycillin and ampicillin were prepared in 5-ml volumes of nutrient broth (Oxoid no. 2). Each tube was inoculated with a drop (0.03 ml) of an overnight broth culture of the test organism, and the minimal inhibitory concentrations (MICs) were determined after 18 h of incubation at 37 C.

Bactericidal activity in vitro. The bactericidal activities of amoxycillin and ampicillin in vitro were compared in (i) veal infusion broth (Difco), (ii) mouse peritoneal washings prepared by intraperitoneal injection of 5.0 ml of saline (see below), and (iii) mouse peritoneal washings prepared by intraperitoneal injec-

tion of 0.5 ml of 3% hog gastric mucin (1701-W, Wilson Laboratories, Chicago), followed after a short interval with 4.5 ml of saline.

The antibiotics were incorporated in 20-ml volumes of the test media at a final concentration of 20 μg/ml, and each flask was inoculated with 2.0 ml of a 1:1000 dilution in 0.2% yeast extract (Difco) of an overnight broth culture of *Escherichia coli* 8. The flasks were incubated at 37 C, 1.0-ml aliquots were removed at intervals, and 0.1-ml volumes of suitable dilutions in 0.2% yeast extract were plated in duplicate on nutrient agar (Oxoid) plates. Colonies were counted after 18 h of incubation at 37 C and the number of viable bacteria in the samples was estimated.

Mouse protection tests. Mice were injected by the intraperitoneal route with 0.5 ml of a suspension in hog gastric mucin of a dilution of an overnight broth culture of the test organism standardized to give an infective inoculum of 100 to 1,000 median lethal doses. Strains of *E. coli* 8, *Salmonella typhimurium* 10, *Staphylococcus aureus* Smith., and *Streptococcus pyogenes* CN10 were suspended in 3% mucin, *Klebsiella aerogenes* 18 in 1% mucin, and *Proteus mirabilis* 13 in 5% mucin. The antibiotics were administered immediately after infection as a single dose, 0.2 ml/20 g, as acacia suspensions in phosphate-buffered saline for oral administration or as solutions in phosphate-buffered saline for subcutaneous or intraperitoneal injection. In general, group of 5 to 10 mice were treated at each dose level, but in a number of tests groups of 20 or 25 animals were used. The numbers of animals surviving 4 days after infection were recorded, and the dose of penicillin required to produce 50% survival of infected animals was calculated by using log probability paper (3).

Bactericidal activity in vivo. Mice were infected intraperitoneally with *E. coli* 8 and dosed with amoxycillin or ampicillin immediately after infection. Groups of five mice were killed by dislocation of the neck at intervals during the 8-h period after infection. Samples of blood were collected from the axillary region and 0.3-ml volumes were added to 0.3 ml of heparin (1,000 U/ml; Weddal Laboratories Ltd.). For peritoneal counts, the abdomen was swabbed with 70% alcohol and the skin was reflected, care being taken not to open the peritoneal cavity. The peritoneum was grasped in a fine pair of forceps and tented, and 4.5 ml of sterile phosphate-buffered saline was injected forcefully. The abdomen was carefully massaged 20 times between the thumb and first two fingers to ensure adequate mixing. A small incision was made into the peritoneum, and a sample of washings was collected with a sterile Pasteur pipette and diluted in 0.2% yeast extract. Volumes (0.1 ml) of yeast extract dilutions of blood and peritoneal washings were plated in dulpicate on nutrient agar plates, and viable colonies were counted after overnight incubation at 37 C.

Antibiotic concentrations in blood and peritoneal washings. Specimens of blood and peritoneal washings from mice infected intraperitoneally with *E. coli* 8 were collected as described above and assayed by large-plate agar diffusion assay with *Sarcina lutea* NCTC 8340 as assay organism. Specimens of heparinized mouse blood were assayed against standard solutions of amoxycillin or ampicillin in whole horse blood, a diluent shown not to differ appreciably from mouse blood in the assay procedures. Specimens of peritoneal washings were suitably diluted in phosphate-buffered saline and assayed against standard antibiotic solutions prepared in the same diluent. The assay plates were incubated overnight at 30 C, inhibition zone diameters were measured, and antibiotic concentrations were derived from standard lines prepared from the standard solutions.

RESULTS

In vitro sensitivities of infecting organisms. MICs of amoxycillin and ampicillin against the bacteria selected for the production of experimental intraperitoneal mouse infections are shown in Table 1. In those tests the organisms were equally susceptible to both penicillins, although there were wide differences in the MIC values found against the different bacteria. For example, the staphylococci and streptococci were much more susceptible to amoxycillin and ampicillin than were the gram-negative bacilli.

In the studies reported here, the bactericidal activities of amoxycillin and ampicillin against *E. coli* 8 were compared both in vitro and in vivo, and results in Fig. 1 illustrate the in vitro bactericidal effects of the compounds in veal infusion broth and in mouse peritoneal washings (with and without mucin). These latter two media were tested to find the influence of the milieu to be found at the site of infection in the mouse on the bactericidal activities of the penicillins. The compounds were tested at a concentration of 20 μg/ml, one that is readily attained in the peritoneal cavity of the mouse. In all three media, amoxycillin produced slightly more rapid and slightly greater bactericidal effects against *E. coli* 8, but at no time was the difference between the compounds very pronounced.

Activity against various intraperitoneal infections. Data in Table 2 compare the relative activities of amoxycillin and ampicillin against various intraperitoneal mouse infections and show the mean median protective doses of both compounds by oral and subcutaneous routes of administration. It can be seen that in the large number of tests reported, amoxycillin was significantly more active than ampicillin by the oral route ($P < 0.01$) against all six strains of test bacteria (*E. coli* 8, *Salmonella typhimurium* 10, *K. aerogenes* 18, *P. mirabilis* 13, *Staphylococcus aureus* Smith, and *Streptococcus pyogenes* CN10). After subcutaneous administration, amoxycillin was more effective than ampicillin ($P > 0.01$) in the

treatment of the infections due to the gram-negative bacilli, but there was no significant difference between the compounds ($P < 0.1$) against the staphylococcal or streptococcal infections.

Antibiotic blood levels after oral administration. The antibiotic concentrations measured in the blood of mice infected with *E. coli* 8 after single oral doses of amoxycillin or ampicillin are shown in Fig. 2. Peak antibiotic concentrations occurred from 30 to 60 min after dosing and fell to relatively low levels at 120 min. At both dose levels tested, 12.5 and 25 mg/kg, the concentrations of amoxycillin were generally two to three times higher than the corresponding ampicillin concentrations.

TABLE 1. *Minimal inhibitory concentrations of amoxycillin and ampicillin against infecting organisms*

Organism	Minimal inhibitory concn (μg/ml)	
	Amoxycillin	Ampicillin
Escherichia coli 8	5.0	5.0
Salmonella typhimurium 10	0.5	0.5
Klebsiella aerogenes 18	2.5	2.5
Proteus mirabilis 13	1.25	1.25
Staphylococcus aureus Smith	0.05	0.05
Streptococcus pyogenes CN10	0.01	0.01

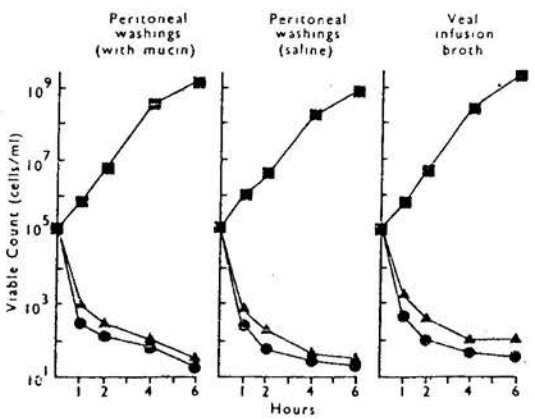

FIG. 1. *Bactericidal effects of amoxycillin and ampicillin against E. coli 8 in vitro. Symbols: ■, Control; ●, amoxycillin (20 μg/ml); ▲, ampicillin (20 μg/ml).*

Effects against an intraperitoneal infection due to E. coli 8. (i) Development of infection. The development of infection in mice after intraperitoneal injection of *E. coli* 8 is illustrated in Fig. 3. When the organism was administered as a suspension in saline, the bacterial count fell within 10 min from the initial inoculum of 10^5 cells per ml of peritoneal washings to a count of approximately 10^3 cells/ml and remained at that value for the next 7 h. Bacteria appeared rapidly in the blood after intraperitoneal injection, reaching a count of about 50 cells/ml within 10 min, but thereafter there was only a slight increase in the count to just above 100 cells per ml of blood. In contrast, the virulence of *E. coli* 8 was significantly enhanced when the organism was injected as a suspension in 3% hog gastric mucin. Thus, in the peritoneal cavity the viable count increased from 10^5 cells per ml of peritoneal fluid after injection to a

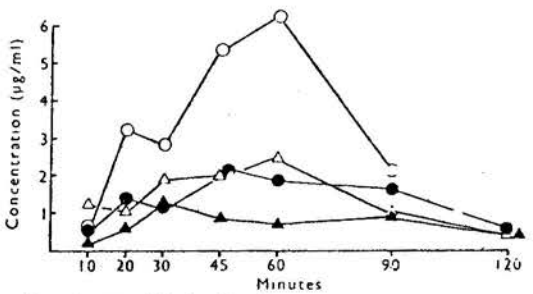

FIG. 2. *Antibiotic blood concentrations after oral administration of amoxycillin and ampicillin to mice infected with E. coli 8. Symbols: ●, Amoxycillin (12.5 mg/kg); ○, amoxycillin (25 mg/kg); ▲, ampicillin (12.5 mg/kg); △, ampicillin (25 mg/kg). Each point represents the mean of five mice.*

TABLE 2. *Relative activities of amoxycillin and ampicillin against intraperitoneal infections in mice*

Organism	Mean PD_{50} (mg/kg) oral[a]				Mean PD_{50} (mg/kg) subcutaneous			
	No. of expts	Ampicillin	Amoxycillin	P[b]	No. of expts	Ampicillin	Amoxycillin	P
Escherichia coli 8	31	77.9	30.5	<0.01	40	63.7	19.7	<0.01
Salmonella typhimurium 10	41	20.8	7.3	<0.01	33	24.0	11.0	<0.01
Klebsiella aerogenes 18	31	17.3	5.1	<0.01	31	27.0	9.5	<0.01
Proteus mirabilis 13	9	18.2	6.7	<0.01	12	38.2	22.2	<0.01
Staphylococcus aureus Smith	42	0.35	0.18	<0.01	39	0.33	0.27	>0.1
Streptococcus pyogenes CN10	8	0.55	0.15	<0.01	7	0.19	0.12	>0.1

[a] PD_{50}, Median protective dose.
[b] *P*, Wilcoxon signed ranks test (6).

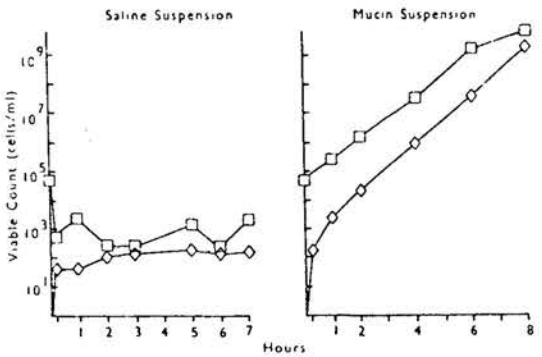

FIG. 3. *Effect of mucin on the proliferation of E. coli 8 in mice after intraperitoneal injection. Symbols:* □, *Peritoneal washings;* ◇, *blood.*

count in excess of 10^9 cells/ml by the end of 8 h. The bacterial count in the blood of these mice rose to 5×10^3 cells per ml of blood after 1 h and 7×10^8 cells/ml at 8 h. In the presence of mucin, therefore, the culture proliferated rapidly in the peritoneal cavity after intraperitoneal injection, resulting in the rapid appearance of bacteria in blood and the production of progressive bacteremia.

Accordingly, in all tests reported below *E. coli* 8 was injected as a suspension in 3% mucin.

(ii) Effects after subcutaneous administration of the penicillins. The effect of treatment of mouse intraperitoneal infections due to *E. coli* 8 with a single subcutaneous dose of amoxycillin or ampicillin is illustrated in Table 3. In this test groups of 25 mice were treated at each dose level, and it can be seen that amoxycillin was more effective than ampicillin at all doses. The median protective dose of amoxycillin was significantly lower than that of ampicillin ($P < 0.05$), in agreement with results reported in Table 2.

The average antibiotic concentrations measured in the blood and peritoneal cavities after a single subcutaneous dose of amoxycillin or ampicillin to mice infected with *E. coli* 8 are shown in Fig. 4. The antibiotic blood concentrations were the same for both compounds after a single subcutaneous dose of 12.5 mg/kg, falling rapidly from a peak level of about 8.5 μg/ml at 10 min to 0.5 μg/ml at 2 h. The antibiotic concentrations in peritoneal washings reached peak values within 20 min and fell to relatively low concentrations at 2 h. The levels obtained with amoxycillin were consistently higher than those of ampicillin at both doses shown, 12.5 and 25 mg/kg, although the differences were less than twofold.

The inhibitory effects of a subcutaneous dose of amoxycillin on the proliferation of bacteria in the peritoneal cavity and in the blood of mice after intraperitoneal injection of *E. coli* 8 are illustrated in Fig. 5. In untreated mice the bacterial peritoneal count increased rapidly from the initial inoculum of 5×10^4 cells to a count in excess of 10^9 cells at 8 h. Administration of a single subcutaneous dose of amoxycillin resulted in an initial fall in the viable count, and the highest dose (200 mg/kg) resulted in 99.9% reduction of the inoculum by 4 h. However, the bacterial count rose between 4 and 8 h so that by 8 h the peritoneal counts were of the same order as the original infecting inoculum.

After intraperitoneal injection of *E. coli* 8, the bacterial count in the blood of untreated mice increased rapidly, reaching 5×10^3 cells/ml at 1 h and 7×10^8 cells/ml at 8 h. The rate of increase in the blood bacterial count was markedly lower in the amoxycillin-treated mice, and at the higher doses the blood count barely exceeded 10 cells/ml over the 8-h period.

The effect of treatment with a subcutaneous dose of amoxycillin in reducing the mortality of

TABLE 3. *Relative activities of amoxycillin and ampicillin by the subcutaneous route against an intraperitoneal infection due to E. coli 8*

Dose (mg/kg)	Amoxycillin		Ampicillin	
	No. of survivors[a]	PD_{50}[b] (mg/kg)	No. of survivors	PD_{50} (mg/kg)
200	24		22	
100	23		19	
50	20	21.0	14	48.0
25	14		6	
12.5	10	(14.3-30.9)[c]	2	(32.6-70.6)[c]
6.25	3		4	
3.12	7		1	
0	0		0	

[a] 25 mice per group.
[b] PD_{50}, Median protective dose.
[c] 95% confidence limits (3).

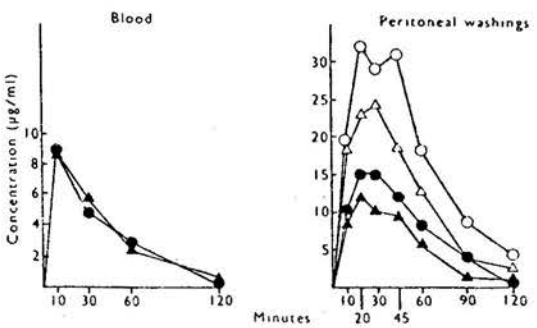

FIG. 4. *Concentrations of amoxycillin and ampicillin in blood and peritoneal washings after subcutaneous injection in mice. Symbols:* ●, *Amoxycillin (12.5 mg/kg);* ○, *amoxycillin (25 mg/kg);* ▲, *ampicillin (12.5 mg/kg);* △, *ampicillin (25 mg/kg).*

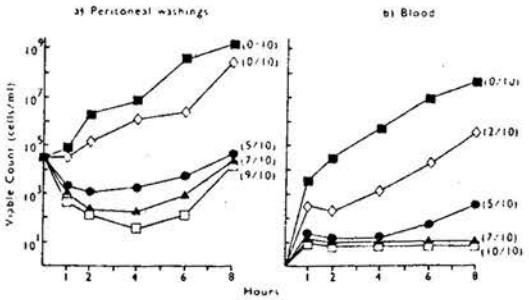

FIG. 5. *Bactericidal activity of amoxycillin after subcutaneous administration to mice infected with E. coli 8. Symbols: ■, Control; □, 200 mg/kg; ▲, 50 mg/kg; ●, 12.5 mg/kg; ◇, 3.1 mg/kg. In parentheses, no. of survivors/no. infected.*

the infected mice in this test is also shown in Fig. 5; increasing the dose of amoxycillin resulted in an increase in the amount of protection afforded. Thus, in untreated mice, there were no survivors in the untreated control groups, whereas the top dose of amoxycillin (200 mg/kg) produced 100% protection of infected mice and the intermediate doses resulted in corresponding responses.

Results in Fig. 6 compare the effects of a single subcutaneous dose of amoxycillin or ampicillin on the bacterial counts in blood and peritoneal washings of mice infected intraperitoneally with *E. coli* 8. The values shown represent the mean values obtained in five tests and are typical of values obtained in each individual study. As was found in the previous study with amoxycillin (Fig. 5), the blood and peritoneal counts increased rapidly after infection in untreated mice, but treatment with the penicillins resulted in pronounced inhibitory effects depending upon the dose and the compound. At both dose levels tested (12.5 and 3.1 mg/kg), amoxycillin caused a greater reduction in the viable counts in the peritoneal cavity and in the blood than did the equivalent doses of ampicillin, and amoxycillin was more effective than ampicillin in protecting the mice.

Intraperitoneal administration. (i) Activity. The curative effects of treating mice by intraperitoneal administration of the compounds immediately after intraperitoneal injection of *E. coli* 8 are shown in Table 4; amoxycillin was significantly more effective than ampicillin in the tests shown. The differences between the compounds were statistically significant ($P < 0.05$) and were of the same order as those observed in the test reported in Table 3, where the compounds were administered by subcutaneous injection.

(ii) Antibiotic levels in blood and peritoneal washings. After intraperitoneal injection of a 12.5 mg/kg dose to mice infected with *E. coli* 8, the concentrations of both compounds in peritoneal washings fell rapidly from greater than 400 μg/ml immediately after injection to between 15 and 30 μg/ml after 30 min and to barely detectable levels at 2 h (Fig. 7). In general, there was little difference between amoxycillin and ampicillin, and the concentrations in the peritoneal washings of infected mice were similar for both compounds. In contrast, in blood the amoxycillin concentrations in these mice were distinctly higher than those obtained with ampicillin, being twice as high for amoxycillin at all time intervals up to 90 min after dosing (Fig. 7).

(iii) Comparative bactericidal effects of amoxycillin and ampicillin. The effects of a single intraperitoneal dose of amoxycillin or ampicillin on the bacterial counts in blood and peritoneal washings of mice infected intraperitoneally with *E. coli* 8 are shown in Fig. 8. At the higher dose level tested (12.5 mg/kg), amoxycillin caused a 99% reduction in the viable

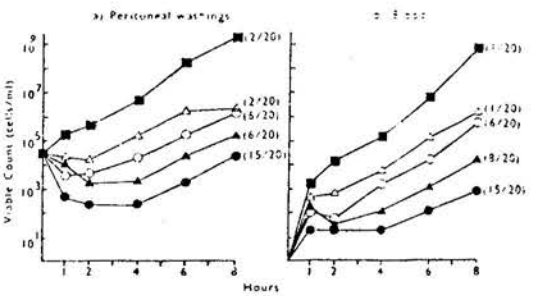

FIG. 6. *Bactericidal activities of amoxycillin and ampicillin after subcutaneous administration to mice infected with E. coli 8 (mean of five tests). Symbols: ■, Control; ●, amoxycillin (12.5 mg/kg); ○, amoxycillin (3.1 mg/kg); ▲, ampicillin (12.5 mg/kg); △, ampicillin (3.1 mg/kg). In parentheses, no. of survivors/no. infected.*

TABLE 4. *Relative activities of amoxycillin and ampicillin by the intraperitoneal route against an intraperitoneal infection due to E. coli 8*

Dose (mg/kg)	Amoxycillin		Ampicillin	
	No. of survivors[a]	PD_{50}[b] (mg/kg)	No. of survivors	PD_{50} (mg/kg)
200	19		19	
100	19		17	
50	18	12.5	13	24.5
25	11		6	
12.5	13	(8.0-19.3)[c]	6	(15.3-39.2)[c]
6.25	9		3	
3.12	4		6	
0	0		0	

[a] 20 mice per group.
[b] PD_{50}, Median protective dose.
[c] 95% confidence limits (3).

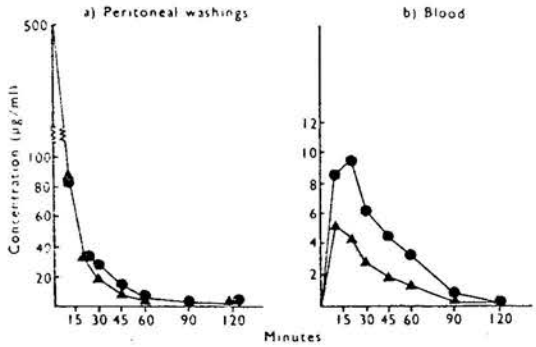

FIG. 7. *Concentrations of amoxycillin and ampicillin in blood and peritoneal washings after intraperitoneal administration to mice (mean of three tests). Symbols: ●, Amoxycillin (12.5 mg/kg); ▲, ampicillin (12.5 mg/kg).*

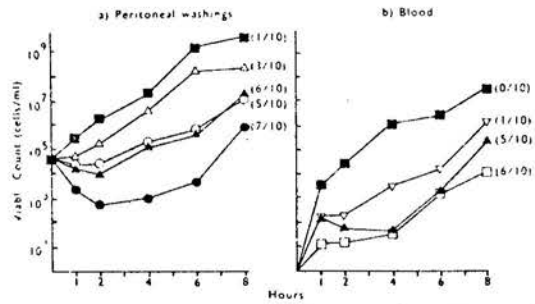

FIG. 8. *Bactericidal activities of amoxycillin and ampicillin after intreaperitoneal administration to mice infected with E. coli 8. Symbols: a) ■, Control; ●, amoxycillin (12.5 mg/kg); ○, amoxycillin (3.1 mg/kg); ▲, ampicillin (12.5 mg/kg); △ ampicillin (3.1 mg/kg). b) ■, Control; □, amoxycillin (6.25 mg/kg); ▲, ampicillin (12.5 mg/kg); ▽, ampicillin (6.25 mg/kg). In parentheses, no. of survivors/no. infected.*

count in the peritoneal cavity within 2 h, whereas the corresponding dose of ampicillin was markedly less effective and was not much more active than the lower dose of amoxycillin (3.1 mg/kg). Similarly, amoxycillin was distinctly more active than ampicillin in reducing the blood bacterial counts, and the bactericidal activity resulting from the dose of 6.25 mg of amoxycillin per kg was as great or greater than that of 12.5 mg of ampicillin per kg.

DISCUSSION

Effective antimicrobial therapy requires the administration of an appropriate agent so as to produce inhibitory levels at the site of infection. Factors that are important to achieve this effect and that can be measured readily in the laboratory include the in vitro susceptibility of the organism to the antibiotic, its extent of binding to serum protein, and the antibacterial serum concentrations obtained after dosing; knowledge of these parameters is necessary for prediction of the outcome of therapy. However, production of adequate antibiotic concentrations at the site of infection in the body is dependent not only upon the factors already mentioned but upon others that are not so easily determined, e.g., diffusion through membranes, tissue distribution, etc. Previous studies with amoxycillin have shown that the compound demonstrates therapeutic activity against experimental infections that cannot be explained entirely on the basis of in vitro activity, protein binding, or antibiotic blood concentrations (1, 2). The studies reported here were designed to investigate factors other than these that might also be contributing to the activity of amoxycillin against experimental infections.

The results reported confirm the findings of Acred et al. (1) that amoxycillin was more active than ampicillin against a variety of bacterial infections in mice. The superiority of amoxycillin was most marked when the compounds were administered by the oral route, and amoxycillin was significantly more active than ampicillin against the four test strains of gram-negative bacilli and the two strains of gram-positive cocci. By subcutaneous injection, amoxycillin was the more active of the compounds against the enterobacteria but was no more effective than ampicillin in the treatment of the streptococcal and staphylococcal infections. The infecting organisms used in these tests appeared to be equally sensitive in vitro to both amoxycillin and ampicillin by conventional serial dilution tests, so the greater oral activity of amoxycillin might be reasonably attributed to the good absorption characteristics of the compound that result in higher concentrations of amoxycillin in the blood of infected mice (1, 2). This is not the explanation, however, for the differences in activities resulting from subcutaneous injection of the penicillins, since the antibiotic blood concentrations were the same for both amoxycillin and ampicillin by this route as has been reported by others (1, 2).

After subcutaneous injection, amoxycillin was more effective than ampicillin in reducing the bacterial count in the peritoneal cavity of mice infected intraperitoneally with *E. coli* 8. In these tests, the penicillins exerted an initial bactericidal effect on the bacterial count that persisted for up to 4 h after injection. At this time, the antibiotic concentrations in the peritoneal fluid had fallen well below inhibitory concentrations and at all dose levels there was resumption of growth from 4 h onwards. How-

ever, the increase in the peritoneal bacterial count between 4 and 8 h did not necessarily produce a corresponding increase in the blood bacterial count since the mucin necessary to ensure the proliferation of *E. coli* 8 had disappeared from the peritoneal cavity by this time. As a result, there was a significant increase in the blood count only if the peritoneal count at 4 to 6 h was of the same order as that immediately after injection. The inhibitory effects of the penicillins on blood bacterial counts depended therefore not only upon the antibiotic concentrations in the blood but also upon the bactericidal effects of the compounds in reducing the numbers of organisms in the peritoneal cavity in the early stages of the infection.

The superior bactericidal activity of amoxycillin compared with ampicillin in the peritoneal cavity of infected mice after subcutaneous injection can be explained in part by the better peritoneal concentrations of amoxycillin, but other factors must also be responsible since the differences in the peritoneal concentrations of the two compounds were less than twofold, whereas the bactericidal activity of amoxycillin in the peritoneal cavity appeared to be two to four times greater than that of ampicillin. In vitro experiments suggested that the bactericidal activity of amoxycillin against *E. coli* 8 might be slightly greater than that of ampicillin, but the differences between the compounds were never very pronounced. In these studies, therefore, amoxycillin was producing greater bactericidal effects in vivo after subcutaneous injection than would be predicted on the basis of in vitro activity or antibiotic concentrations, in agreement with findings reported by Hunter et al. (2) in a different experimental model.

The results obtained after intraperitoneal administration of the penicillins confirmed the superior activity of amoxycillin compared with ampicillin in reducing the bacterial peritoneal counts in mice infected with *E. coli* 8, although the peritoneal concentrations of amoxycillin were no higher than those of ampicillin. However, the concentratiosn of amoxycillin in the blood of the infected mice were found to be twice as high as those of ampicillin after intraperitoneal dosing; consequently the superior therapeutic activity of amoxycillin by this route can be attributed not only to its superior bactericidal activity in the peritoneal cavity but also to the higher concentrations found in the blood.

The results reported here confirm that amoxycillin is more effective than ampicillin in the treatment of experimental mouse infections, and the evidence suggests that the greater efficacy of amoxycillin by injection compared with ampicillin is due to its superior in vivo bactericidal activity combined with better distribution characteristics in the infected animal.

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Comparative Effects of Amoxycillin and Ampicillin on the Morphology of *Escherichia coli* In Vivo and Correlation with Activity

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An experimental mouse intraperitoneal infection due to *Escherichia coli* was treated with subcutaneous amoxycillin or ampicillin. Specimens of blood and peritoneal washings from the infected animals were assayed for antibiotic concentrations and examined microscopically for observation of the effects produced by the two penicillins on the morphology of bacteria growing in vivo. Amoxycillin was significantly more effective than ampicillin in protecting the animals from the lethal effects of the infection, although the antibiotic concentrations in the body fluids were very similar for both compounds. However, microscopic examination showed marked differences in the morphological effects produced at equivalent dose levels by the two compounds against the bacteria present in blood and peritoneal fluid. Treatment with amoxycillin at dose levels that produced peak antibiotic concentrations in the body fluids ranging from one-quarter to three times the minimum inhibitory concentration resulted in the formation of spheroplast forms, which lysed rapidly. In contrast, at the same concentrations, ampicillin produced relatively stable filaments or long cell forms, which lysed much more slowly, although at higher dose levels the effects produced were generally similar to those seen with amoxycillin. It is concluded that the superior therapeutic activity of amoxycillin compared with ampicillin is due to its greater bacteriolytic activity in vivo.

Amoxycillin [D-(−)-α-amino-*p*-hydroxybenzylpenicillin] and ampicillin [D-(−)-α-aminobenzylpenicillin] show similar activities in conventional growth inhibition tests, but amoxycillin has been reported to exert more rapid bactericidal effects in vitro than ampicillin (10, 14). In more recent studies by Rolinson and his colleagues (15; G. N. Rolinson, A. C. Macdonald, and D. A. Wilson, Abstr. 9th Int. Congr. Chemother, London, abstr. 302, 1975), microscopic examination of cultures of *Escherichia coli* exposed to the two penicillins revealed that at concentrations just above minimum inhibitory concentration (MIC) values amoxycillin caused the formation of spheroplasts, which lysed readily, whereas exposure to ampicillin resulted in the formation of filamentous cells, which were relatively stable. The more rapid rate of lysis demonstrated by amoxycillin as a result of its production of spheroplasts accounts for its greater bactericidal activity compared with ampicillin.

It is reasonably well established that amoxycillin is significantly more active than ampicillin against experimental infections, even when blood and tissue concentrations of the two compounds are similar (1, 3, 10). Also, there is evidence to show that in these infections amoxycillin produces greater bactericidal effects in the infected animal than does ampicillin, and it is suggested that this may largely explain the superior chemotherapeutic activity of amoxycillin compared with ampicillin (3, 10).

It seemed desirable to investigate the effects produced by amoxycillin and ampicillin on the morphology of bacteria during the course of experimental infections to determine if there was any correlation between activity and morphological effects in vivo, as has been demonstrated in vitro. The experiments reported describe results observed during the treatment of an intraperitoneal mouse infection due to a strain of *E. coli* with ampicillin and amoxycillin.

MATERIALS AND METHODS

Antibiotics. The penicillins tested were preparations of sodium amoxycillin and sodium ampicillin (Beecham Pharmaceuticals, Worthing, Sussex).

Mice. Male or female albino mice (18 to 22 g),

MFI strain (Olac 1976 Ltd., Bicester, Oxon), were used throughout.

Mouse protection tests. Mice were injected by the intraperitoneal route with 0.5 ml of a suspension in 3% hog gastric mucin (1701W, Wilson Laboratories, Chicago, Ill.) of a dilution of an overnight broth culture of *E. coli* 8 standardized to give an infective inoculum of 100 median lethal doses. The antibiotics were administered by the subcutaneous route immediately after infection, 0.2 ml/20-g mouse in phosphate-buffered saline. In a second series, mice were infected with 10,000 median lethal doses, the inoculum used for the microscopical studies, and were treated at the time of infection and 4 h later. Groups of 20 mice were treated at each dose level. The numbers of animals surviving 4 days after infection were recorded, and the dose of penicillin required to produce 50% survival of infected animals was calculated (11).

Antibiotic concentrations in mouse blood and peritoneal washings. Mice were infected intraperitoneally with *E. coli* 8 and dosed subcutaneously with amoxycillin or ampicillin immediately after infection. Groups of five mice were killed by dislocation of the neck at intervals during the 2-h period after infection. Samples of blood were collected from the axillary region, and 0.3-ml volumes were added to 0.3-ml volumes of heparin (100 U/ml; Weddal Pharmaceuticals Ltd., Wrexham, Clwyd). For determination of antibiotic concentrations in the peritoneum, the abdomen was swabbed with 75% alcohol and the skin was reflected, care being taken not to open the peritoneal cavity, and 2 ml of saline was injected forcefully. The abdomen was carefully massaged to ensure adequate mixing. A small incision was made in the peritoneum, and a sample of washings was collected with a sterile Pasteur pipette. The specimens of blood and peritoneal washings were assayed by large-plate agar diffusion assay with *Sarcina lutea* NCTC 8340 as the assay organism. Specimens of heparinized mouse blood were assayed against standard solutions of amoxycillin or ampicillin in whole horse blood, a diluent shown not to differ significantly from mouse blood in the assay procedures. Specimens of peritoneal washings were suitably diluted in phosphate-buffered saline and assayed against standard antibiotic solutions prepared in the same diluent. The assay plates were incubated overnight at 30°C, inhibition zone diameters were measured, and antibiotic concentrations were derived from standard lines prepared from standard solutions.

Microscopy and photography. Mice were infected intraperitoneally with approximately 10^7 cells of a suspension in 3% mucin of an overnight broth culture of *E. coli* 8 and were treated with a single subcutaneous dose of amoxycillin or ampicillin. The animals were killed at intervals after administration of the penicillins, and specimens of blood and peritoneal fluid were collected for microscopic examination. For the observations made on the peritoneal washings, the mice were dosed by the subcutaneous route immediately after infection, but for the studies on the blood the mice were not treated with penicillins until 6 h after intraperitoneal infection to ensure there was a sufficient bacteremia at the time of therapy. Specimens of peritoneal washings were suspended in an equal volume of 2% glutaraldehyde fixative in 0.05 M sodium cacodylate (osmolality, 305 mOsmol/kg); blood samples were suspended in 2% glutaraldehyde fixative containing 20 U of heparin (osmalality, 320 mOsmol/kg). The osmolalities of the fixatives were prepared to be the same as previously determined osmolalities of mouse peritoneal washings and blood. The glutaraldehyde fixative was shown in preliminary experiments to preserve the morphology of the bacteria and to prevent any further antibacterial activity of the penicillin. Specimens for microscopy were mounted on a thin film of agar prepared by spreading 0.6 ml of molten 0.8% brain heart infusion agar over the surface of a no. 1 cover slip (22 by 56 mm), which was trimmed to a 10-mm square after the agar had solidified. The sample was placed in the center of the agar and covered with a second cover slip for high-resolution microscopy, using a Ziess WL microscope fitted with differential interference contrast optics. Photography of the samples was carried out with a 120-format roll film camera and a 100-W tungsten-base lamp.

RESULTS

Activity against *E. coli* intraperitoneal infection. The relative activities of amoxycillin and ampicillin in protecting mice from the lethal effects of intraperitoneal infection with *E. coli* 8 are shown in Table 1. In the first study, in which the infective inoculum was approximately 10^5 cells (100 median lethal doses), the animals were treated with a single subcutaneous dose immediately after infection, as was the case in earlier studies to compare the in vivo activities of amoxycillin and ampicillin (1, 3). At all dose levels, amoxycillin was more effective than ampicillin, and the median protective dose of amoxycillin was significantly lower than that of ampicillin ($P < 0.05$). In the second study, the inoculum was much larger (10^7 cells) and was chosen for the microscopy studies. Against this larger inoculum, representing about 10,000 median lethal doses, treatment with a single dose of either compound was relatively ineffective, except at doses of 200 mg/kg or greater. However, administration of a second dose 4 h after infection resulted in effective therapy (Table 1), and amoxycillin was again signficantly more effective than ampicillin ($P < 0.05$).

Antibiotic levels in blood and peritoneal fluid. Data in Fig. 1 show the mean values obtained in three experiments to measure the antibiotic concentrations obtained in the blood and peritoneal cavities of mice infected with *E. coli* 8 after a single subcutaneous dose of amoxycillin or ampicillin. The concentrations of amoxycillin or ampicillin were the same in the

TABLE 1. *Relative activities of amoxycillin and ampicillin by the subcutaneous route against an intraperitoneal mouse infection due to E. coli 8*

Inoculum (no. of cells)	Dose (mg/kg)	Amoxycillin		Ampicillin	
		No. of survivors (20 mice/group)	PD_{50}[a] (mg/kg)	No. of survivors (20 mice/group)	PD_{50} (mg/kg)
10^{5}[b] (100 MLD[c])	200	19		19	
	100	19		17	
	50	17		12	
	25	16	4.0	11	23.5
	12.5	13	(2.4-8.6)[d]	8	(15.5-35.5)
	6.25	12		1	
	3.12	9		2	
	0	0		0	
10^{7}[e] (10,000 MLD)	200	20		20	
	50	19	7.2	19	22 0
	12.5	16	(4.6-11.2)	3	(16.3-29.7)
	3.12	3		0	
	0	0		0	

[a] PD_{50}, Median protective dose.
[b] Dosed immediately after infection.
[c] MLD, Median lethal dose.
[d] 95% confidence limits.
[e] Dosed immediately and 4 h after infection.

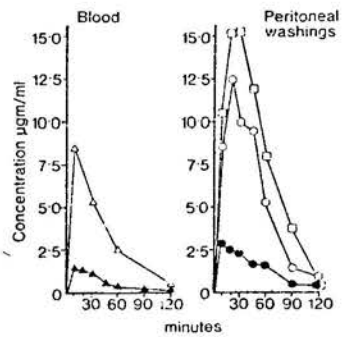

FIG. 1. *Concentrations of amoxycillin and ampicillin in blood and peritoneal washings after a single subcutaneous dose to mice infected with E. coli 8. Symbols: Blood—(△) 12.5 mg of amoxycillin and ampicillin per kg; (▲) 3.1 mg of amoxycillin and ampicillin per kg. Peritoneal washings—(□) 12.5 mg of amoxycillin per kg; (○) 12.5 mg of ampicillin per kg; (●) 3.1 mg of amoxycillin and ampicillin per kg*

blood at both dose levels, 12.5 and 3.1 mg/kg, and in the peritoneal fluid at the 3.1-mg/kg dose level. In the blood, the dose of 12.5 mg/kg produced a peak level of 8.5 μg/ml at 10 min, which fell rapidly to undetectable levels (<0.01 μg/ml) by 120 min; at 3.1 mg/kg, the concentrations also fell rapidly from the peak value of 1.5 μg/ml measured at 10 min. The antibiotic concentrations measured in the peritoneal cavity were rather higher than those obtained in the blood. At 12.5 mg/kg, the peak level of ampicillin was 12 μg/ml at 20 min compared with 15 μg/ml for amoxycillin, and the levels of both penicillins fell rapidly to low values 2 h after treatment. In general, at this dose level the amoxycillin levels were about 20% higher than those of ampicillin. At the 3.1-mg/kg dose, peak concentrations of about 3.0 μg of amoxycillin and ampicillin per ml were measured in the peritoneal washings after 10 min.

Morphological effects in vivo. (i) Peritoneal fluid. The effects produced by a single subcutaneous dose of ampicillin (12.5 mg/kg) on the morphology of *E. coli* 8 in the peritoneal cavity of infected mice are illustrated in Fig 2. Normal rod-shaped cells were observed up to 30 min after dosing, after which there was a progressive increase in cell length. Thus, at 1 h most bacteria observed were two to three cells in length, and these were replaced by cells of increasing length: at 2 h, with filaments 5 to 10 cells long, and from 3 h onwards with filaments 10 to 20 cells in length (Fig. 2a). Most of the filaments had normal motility, and many of them showed small bulges at one or more points along the cell. By 4 h many of the filaments had lysed, but some showed evidence of fragmentation, with regeneration of cells of normal morphology. Septation generally occurred at the tip of the filaments, though some cells showed multiple fragments (insert, Fig. 2a). At 5 h large numbers of filaments were still present, and increased macrophage activity was also evident so that many of the resid-

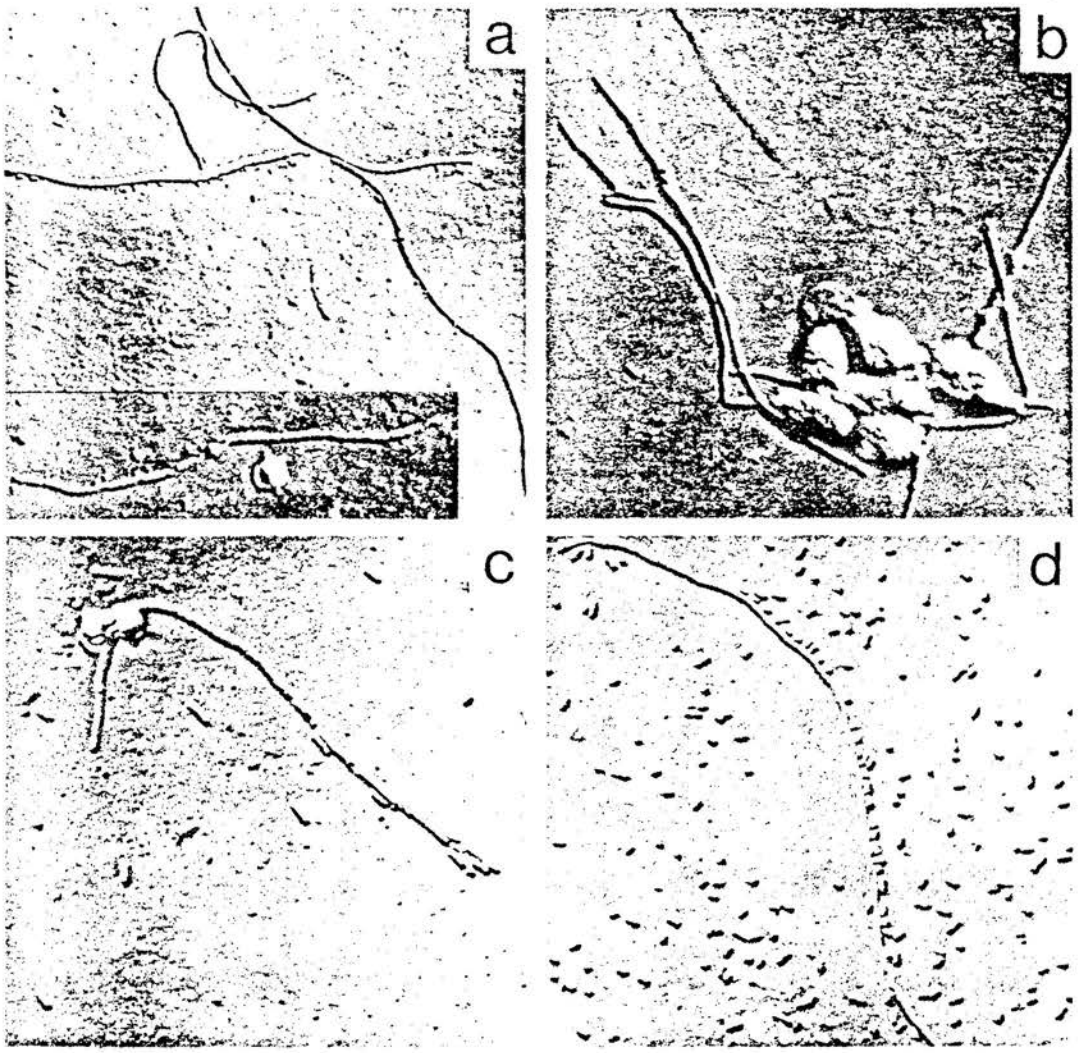

FIG. 2. *Effects of a single subcutaneous dose of ampicillin (12.5 mg/kg) on the morphology of E. coli 8 in the mouse peritoneum at (a) 3 h, (b) 5 h, (c) 9 h, and (d) 14 h. Differential interference phase contrast (×1,100).*

ual long forms and normal bacteria were in the process of being engulfed by the macrophages (Fig. 2b). At 9 h, large numbers of normal cells were being regenerated from the filaments, and by cell division (Fig. 2c), so that by 14 h very large numbers of normal cells were seen in each microscope field, together with the occasional greatly elongated cell (Fig. 2d).

At a higher dose level (50 mg/kg), treatment with ampicillin initially caused spheroplast formation and a rapid reduction in the numbers of bacteria, after which there was a progressive increase in cell length so that from 90 min to 2 h small numbers of elongated cells four to eight cells in length were seen in each microscope field. Between 3 and 9 h, when the ampicillin concentration in the peritoneal fluid had fallen to subinhibitory levels, filaments 10 to 20 cells long were present. Many of these gradually lysed over the next few hours, so that 6 h after dosing ghost forms of filaments were seen and surviving filaments were showing signs of fragmenting, and at 9 h normal cells were more numerous. There was no evidence of filament formation after treatment with a dose of 200 mg of ampicillin per kg, and only small numbers of normal cells were seen during the 14-h period after dosing.

In contrast to the findings with ampicillin, a

dose of 12.5 mg of amoxycillin per kg caused the rapid formation of spheroplasts, which lysed readily, and there was no evidence of filament formation. Instead, slightly elongated cells, about two cells in length, with spheroplasts, were seen 40 to 60 min after dosing (Fig. 3a), 10 to 30 min after the peak antibiotic concentrations (15 μg/ml) were measured in the peritoneal fluid. Soon thereafter, the majority of spheroplasts had lysed, and only small numbers of normal cells and an occasional spheroplast were seen 2 h after dosing. Similar results were obtained with higher doses of amoxycillin.

Treatment with a lower dose of amoxycillin (3.1 mg/kg) also resulted initially in the rapid formation of spheroplasts 60 to 80 min after dosing (Fig. 3b), i.e., 50 to 70 min after peak antibiotic levels (3.2 μg/ml) were measured in the peritoneum, and only small numbers of slightly elongated cells were seen up to 90 min after dosing. However, from 2 h onwards, by which time the antibiotic concentrations in the fluid had fallen to very low levels, the surviving cells elongated and filament formation became evident. In contrast to ampicillin-induced filaments, many of these long cells had bulbous swellings (Fig. 3c), and by 3 h there was a great variety of cell forms, including long forms with multiple bulbous swellings and bulges

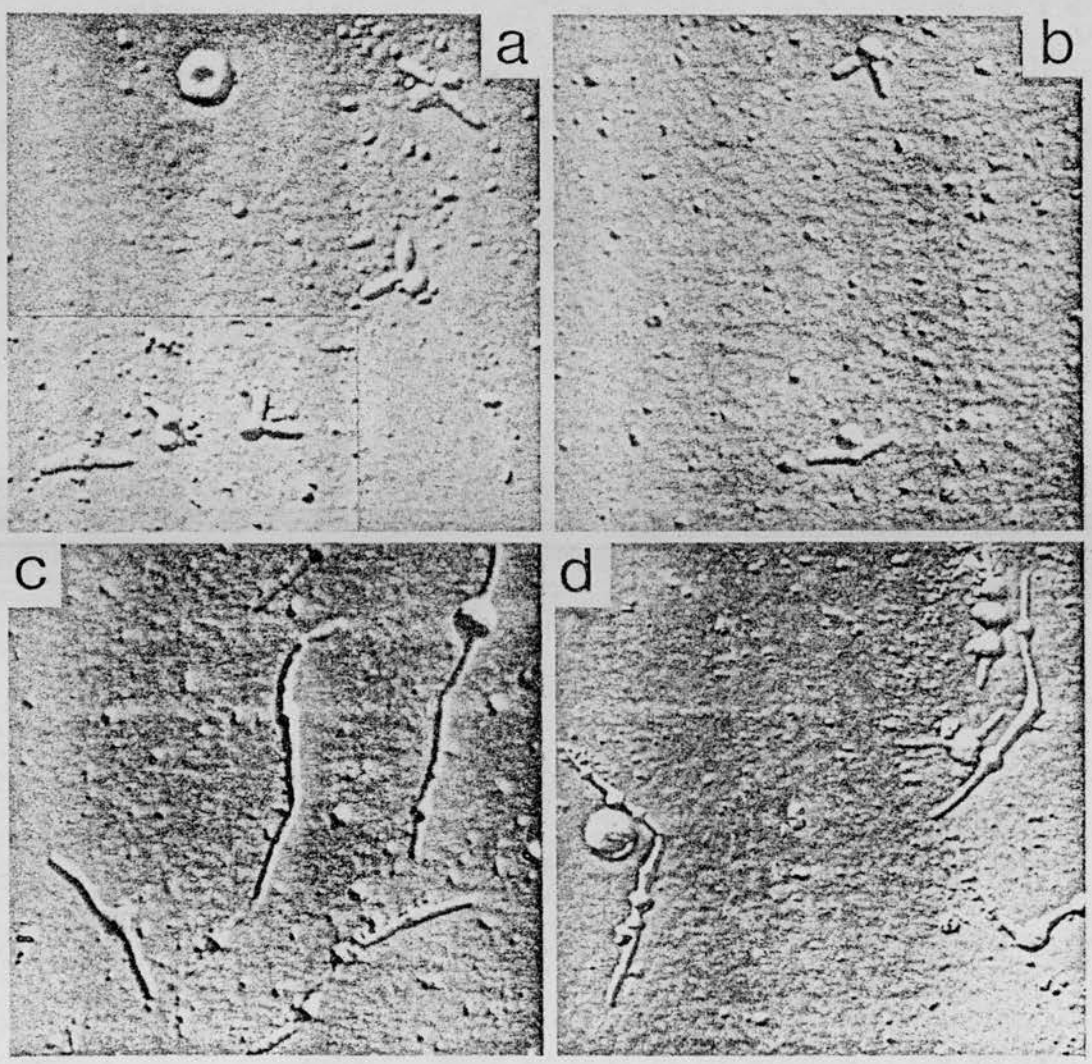

FIG. 3. *Effects of a single subcutaneous dose of amoxycillin on the morphology of E. coli 8 in the mouse peritoneum: (a) 12.5 mg/kg, 40 min; (b) 3.1 mg/kg, 80 min; (c) 3.1 mg/kg, 2 h; (d) 3.1 mg/kg, 3 h. Differential interference phase contrast (×1,500).*

along the filaments (Fig. 3d). At 4 h many of the long cells were still present, some showing evidence of lysis and others of fragmentation. By 9 h macrophage activity had increased, large numbers of normal cells were seen, and a number of filaments were still present.

(ii) **Blood.** The results of treatment with a single subcutaneous dose of 12.5 mg of ampicillin per kg on the morphology of *E. coli* 8 in the blood of infected mice are illustrated in Fig. 4. As was the case in the peritoneum, this dose of ampicillin caused characteristic cell elongation and filament formation. Relatively large numbers of normal cells were observed up to 30 min after dosing, but these were replaced after 60 to 90 min, when the antibiotic concentrations had fallen to subinhibitory levels, by filaments four to six cells in length, many with central bulges or collars (Fig. 4a). By 2 h, large numbers of elongated cell forms were seen, and filaments six to eight cells long were present (Fig. 4b). Fragmentation of filaments and the reappearance of normal cells were observed 3 to 5 h after dosing (Fig. 4c), and, by 9 h, normal cells were seen in large numbers of the blood of surviving animals. At a higher dose, 50 mg/kg, there was some evidence of cell elongation and cells of two to four cells in length were observed 1 to 2 h after treatment, but there was no sign of filament formation during this period, although it is likely this would have occurred later after the fall in ampicillin concentrations in the body.

The administration of amoxycillin at 12.5 and 50 mg/kg resulted in the formation of spheroplasts and rapid lysis of bacteria in the blood, as in the peritoneal cavity, and there was no evidence of filament formation. Typically, large numbers of spheroplasts were observed in the blood after 40 to 50 min (Fig. 4d), and by 60 min there was a significant reduction in the number of bacteria present, so that only the occasional spheroplast or normal cell was seen.

Again, rather different effects were observed at the lower dose of amoxycillin, namely, 3.1 mg/kg. Relatively large numbers of normal cells were observed after 30 min, but these evolved between 50 to 60 min to slightly elongated forms, about two cells in length, many with large swellings or spheroplasts (Fig. 4e). In addition, numerous ghost cells were seen. Between 1 and 2 h there was a marked decrease in the number of spheroplasts and a few normal cells were observed, together with slightly elongated cells with central collars. At 3 h many of the surviving bacteria had elongated, and filamentous cells were observed, many with large, single or multiple swellings (Fig. 4f). Between 3 and 5 h after dosing, a number of the bizarre forms lysed while fragmentation occurred in some of the filamentous forms, so that at 5 h a variety of cell forms was seen, including elongated cells with swellings as well as increased numbers of normal cells.

DISCUSSION

In the studies reported here amoxycillin was significantly more effective than ampicillin in protecting mice from the lethal effects of intraperitoneal infection with a strain of *E. coli*. Both compounds produced similar antibiotic concentrations in blood and peritoneal washings of the infected animals, in agreement with the results of earlier studies (3), so that the difference in the activities of the compounds cannot be explained in this fashion. In the earlier studies mentioned above, amoxycillin was shown to produce greater bactericidal effects than ampicillin in the infected animals, but this aspect was not investigated here.

In this study, microscopic examination of specimens of blood and peritoneal fluid from infected animals revealed striking differences in the effects produced by equivalent doses of the two penicillins on the morphology of the bacteria growing in vivo. In essence, amoxycillin typically produced spheroplast forms, which lysed rapidly, whereas ampicillin only did so at relatively high doses compared with amoxycillin. Characteristically, ampicillin produced filamentous or long cell forms at dose levels at which amoxycillin was producing spheroplasts, and these variants lysed relatively slowly. However, at high doses ampicillin caused spheroplast formation in the same way as amoxycillin, whereas at low dose levels treatment with amoxycillin resulted in the formation of filamentous cells after initial spheroplast formation, so that the differences between the compounds are essentially concentration dependent rather than reflecting differences in modes of action.

To a great extent, the morphological effects produced by the two penicillins during the course of treatment of this experimental infection resembled the effects described by Rolinson and his colleagues in their in vitro studies (15; Rolinson et al., Abstr. 9th Int. Congr. Chemother., abstr. 302, 1975). The similarities in the effects produced in vitro and in vivo are somewhat unexpected in view of the differences between the two experimental systems. For instance, in the infected animal the bacteria are exposed to the body fluids and to the host immune mechanism, factors that are not present in cultures incubated in vitro. In addition, in in vitro studies the bacteria are exposed to a

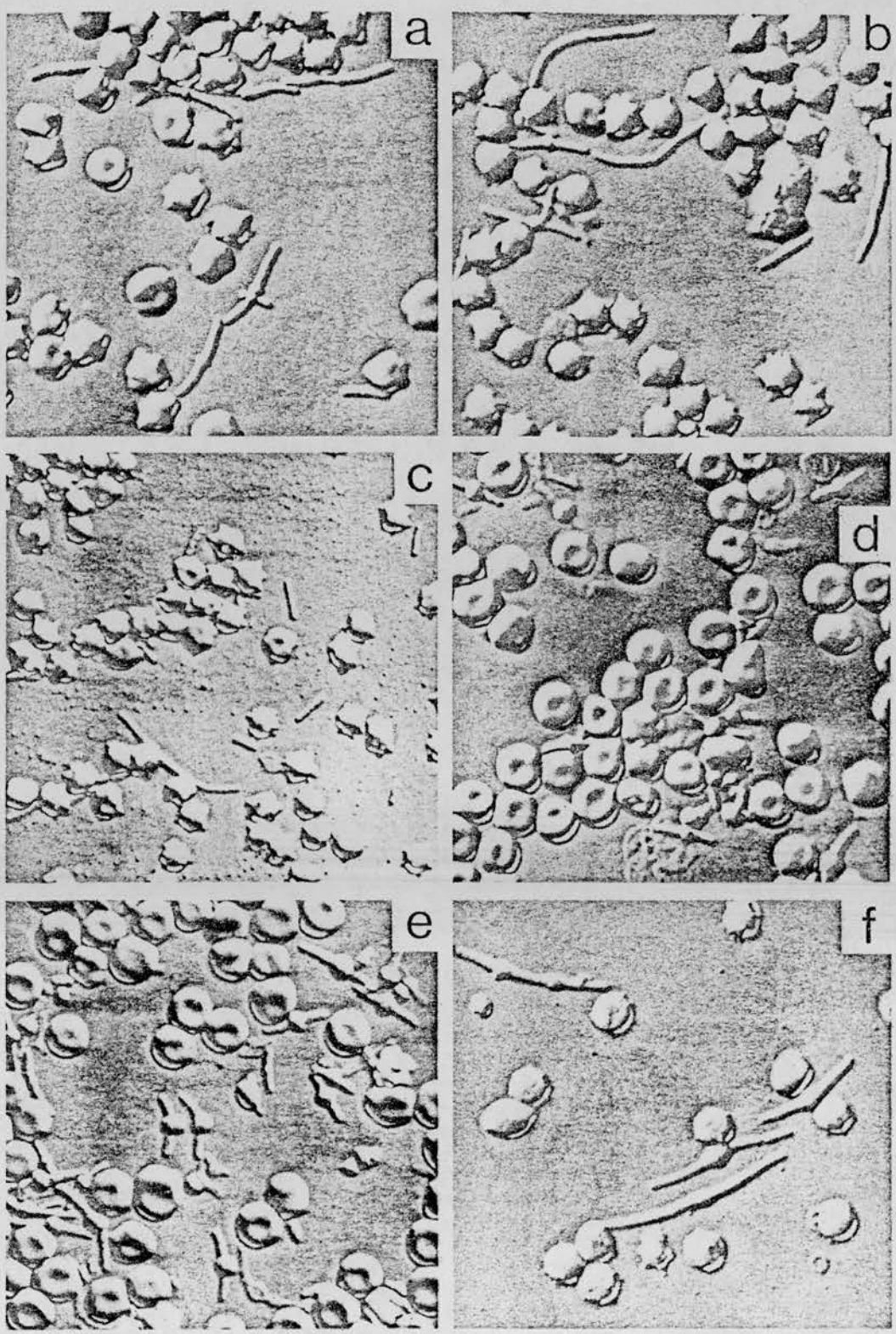

FIG. 4. *Effects of a single subcutaneous dose of ampicillin or amoxycillin on the morphology of E. coli 8 in the blood of infected mice. Ampicillin (12.5 mg/kg) at: (a) 60 min; (b) 2 h; (c) 4 h. Amoxycillin: (d) 12.5 mg/kg, 40 min; (e) 3.1 mg/kg, 1 h; (f) 3.1 mg/kg, 3 h. Differential interference phase contrast (×1,100).*

constant concentration of antibiotic for a relatively long period of time, whereas in the animal studies described here, peak antibiotic concentrations were attained rapidly (within 15 min) and the levels declined rapidly as the antibiotics were excreted from the host. The dynamic nature of the in vivo system is illustrated by the changing morphology of the bacteria in the infected animals with the passage of time. For example, at a low dose of amoxycillin (3.1 mg/kg), spheroplasts were produced soon after peak antibiotic levels were attained in blood and peritoneal fluid, but later, as the compound was being excreted from the animal, the low concentrations remaining evoked the production of filamentous forms. Similarly, with a dose of 50 mg of ampicillin per kg, spheroplast formation, seen soon after peak antibiotic levels were attained, was followed by cell elongation as the ampicillin concentrations in the body fell to subinhibitory levels.

Although filament formation was evident with low doses of amoxycillin as well as at higher doses of ampicillin, there were obvious differences in the appearances of the long cell forms. For instance, with amoxycillin, the filaments had characteristic multiple swellings, like inflated balloons, along the length of the cells, whereas these abnormal forms were not seen with ampicillin. These swellings became evident some length of time after the initial rapid formation of spheroplasts by amoxycillin had ceased and at a time when amoxycillin was no longer detectable in the body fluids.

Perhaps the most striking feature of this study is the very rapid bacteriolytic effect produced in vivo during treatment with amoxycillin at dose levels that resulted in the bacteria being exposed to subinhibitory concentrations of the compound for only short periods of time. For example, at a dose level of 3.1 mg/kg, amoxycillin produced peak blood antibiotic concentrations of about 1.5 μg/ml at 10 min, and these fell to about 0.25 μg/ml at 60 min and were undetectable at 120 min. The MIC of amoxycillin (and ampicillin) against the test organism, *E. coli* 8, was found in repeated tests to be 5.0 μg/ml, so that at best the bacteria in the blood of the infected animal were exposed to a concentration of one-fourth the MIC for a very short period of time. Nevertheless, treatment with this dose of amoxycillin resulted in the rapid formation of spheroplasts in the blood followed by rapid lysis, i.e., within 60 min after administration of the compound. Consequently, amoxycillin appeared to be notably more bacteriolytic in vivo than was observed in the in vitro studies of Rolinson and his colleagues (15), where filament formation occurred at subinhibitory concentrations and lysis of the filaments only took place after a period of hours. In contrast, the effects produced in vivo by ampicillin were more in keeping with those observed in vitro by Rolinson et al. (15) and Greenwood and O'Grady (6), in that spheroplast formation was observed in vitro and in vivo only when the bacteria were exposed to ampicillin concentrations well in excess of MIC levels. At concentrations not greatly in excess of MIC values (up to two to three times the MIC), and at subinhibitory concentrations, treatment with ampicillin resulted typically in the formation of filaments in vitro and in vivo.

The morphological effects produced by benzylpenicillin on susceptible bacteria were observed soon after the antibiotic was isolated (5), and the presence of abnormal varieties, in animals or patients, after penicillin therapy has been described (2, 4, 8, 9, 12). More recently, there has been a resurgence of interest in the various morphological effects produced by β-lactam antibiotics as a result of awareness of the variety of effects caused by compounds of differing structures (4, 6, 14, 17), and differences in effects on morphology have been correlated with differences in affinity for binding sites (7, 13, 16). However, to the best of our knowledge, the study reported here represents the first attempt to seek a correlation in vivo between antibiotic effect on morphology and the results of therapy. The apparent similarity between in vitro and in vivo observations is of obvious interest.

It is evident from the results of this study that the differences in the therapeutic activities of amoxycillin and ampicillin against experimental infections are associated with the differences produced on the morphology of the bacteria in vivo by the relatively low transient concentrations of the compounds present in the blood and tissues of the infected animals during therapy. The rapid production of spheroplasts and the rapid lysis observed with amoxycillin in vivo contrasts markedly with the filament formation and slower rate of lysis seen with ampicillin. The significantly greater bacteriolytic activity of amoxycillin in infected animals compared with ampicillin provides the explanation for its greater bactericidal activity in vivo and is the reason for the superior therapeutic activity of amoxycillin.

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Development *in vitro* of Bacterial Cross-resistance involving Penicillins, Chloramphenicol and Tetracycline

THE selection of antibiotic-resistant variants of bacteria can readily be demonstrated *in vitro* by repeated transfer of the culture in the presence of the particular antibiotic in question. Resistant variants isolated in this way show cross-resistance to other antibiotics of similar chemical structure to the antibiotic in which the original organism was cultured. Cross-resistance among unrelated antibiotics has been reported on occasion[1–5] with strains of bacteria selected from *in vitro* experiments, but the phenomenon has not become well established and it is usually accepted that cross-resistance is not seen among chemically dissimilar antibiotics. We now wish to report the development of cross-resistance between certain penicillins, chloramphenicol and tetracycline, with bacteria cultured in the presence of any of these antibiotics.

The results of experiments with a strain of *Salmonella paratyphi B* (B.R.L. 1824) are shown in Table 1. This culture was sensitive to ampicillin, benzylpenicillin, chloramphenicol, streptomycin and tetracycline before repeated transfer in sub-inhibitory concentrations of these antibiotics. After resistance had developed to the particular antibiotic in which the organism was transferred, the resistant culture was tested for sensitivity to the other antibiotics. It was found that there was cross-resistance between the penicillins, chloramphenicol and tetracycline, such that the increase in resistance which developed as a result of culture in the presence of one of these antibiotics was accompanied by a lesser, but nevertheless significant, increase in resistance to the other unrelated, antibiotics. The development of this multiple resistance occurred in a gradual stepwise manner, and

Table 1. SELECTION *in vitro* OF ANTIBIOTIC-RESISTANT VARIANTS OF *Salmonella paratyphi* B (B.R.L. 1824) SHOWING CROSS-RESISTANCE INVOLVING PENICILLINS, CHLORAMPHENICOL, AND TETRACYCLINE

Antibiotic in which culture was transferred	No. of transfers	Minimum inhibitory concentration (μg/ml.) Ampicillin	Benzylpenicillin	Chloramphenicol	Tetracycline	Streptomycin
None (parent culture)	0	1·25	12·5	5·0	1·25	12·5
Ampicillin	12	250	> 500	50	25	25
Benzylpenicillin	12	125	> 500	50	25	12·5
Chloramphenicol	10	25	125	125	50	5·0
Tetracycline	10	25	125	25	125	25
Streptomycin	4	1·25	5·0	5·0	1·25	> 500

there was no evidence that polyresistant variants were present in the original antibiotic-sensitive cultures.

In contrast to these findings, the multiple-resistant variants described here were, in general, as sensitive to streptomycin as was the parent strain, and streptomycin-resistant variants of the original culture of *Salm. paratyphi B* (B.R.L. 1824) remained sensitive to the penicillins, chloramphenicol and tetracycline. Likewise, a culture of this organism repeatedly transferred in the presence of ampicillin showed no change in sensitivity to cycloserine, kanamycin, neomycin or polymyxin.

The development of cross-resistance involving penicillins, chloramphenicol and tetracycline was also demonstrated with a strain of *Escherichia coli*, a strain of *Klebsiella pneumoniae* and several *Salmonella* species. Cross-resistance was not seen with two strains of *Proteus mirabilis*, and one strain each of *Shigella flexneri* and *Shigella sonnei*.

It would seem, therefore, on the basis of the findings reported here, that the emergence of antibiotic-resistance *in vitro* should not be regarded as a phenomenon which is highly specific for the antibiotic in which the bacterial culture is transferred. On the contrary, it is evident that cross-resistance among dissimilar antibiotics can readily be demonstrated *in vitro* with certain bacteria, particularly species of *Salmonella*, although the clinical significance of this is as yet not known.

These results will be reported in detail elsewhere.

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CHARACTERISTICS OF METHICILLIN-RESISTANT STAPHYLOCOCCI

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ABSTRACT

SUTHERLAND, R. (Beecham Research Laboratories Ltd., Betchworth, Surrey, England), AND G. N. ROLINSON. Characteristics of methicillin-resistant staphylococci. J. Bacteriol. 87:887-899. 1964.—Cultures of naturally occurring methicillin-resistant staphylococci obtained from a number of hospitals were examined for the nature and degree of resistance to methicillin and other antibiotics. All the cultures tested were similar in that they consisted of mixed populations in which the majority of cells were of normal sensitivity to methicillin with a minority showing methicillin resistance. The resistant members also differed from the rest of the population in that they grew more slowly even in the absence of methicillin. Pure cultures of the resistant minority were obtained readily but, on repeated transfer in the absence of methicillin, resistance was lost and the cultures reverted to mixed populations similar to the original naturally occurring strains. When methicillin-sensitive staphylococci were repeatedly subcultured in the presence of methicillin, a mixed population was obtained in which only a minority of cells were resistant to the antibiotic; in this respect, the cultures of methicillin-resistant staphylococci selected in vitro resembled the naturally occurring strains. The original cultures of methicillin-resistant staphylococci comprised populations of cells with uniform sensitivity or insensitivity to other antibiotics. The resistance of these staphylococci to methicillin was not due to increased ability to inactivate the drug but to intrinsic insensitivity to methicillin.

At the time methicillin was introduced into clinical practice, naturally occurring strains of *Staphylococcus aureus* resistant to the drug had not been encountered in the course of extensive tests. Soon after, however, Jevons (1961) described three strains, of a total of 5,440 isolates tested, which showed increased resistance to methicillin. These strains showed no increased ability to destroy methicillin, but did differ from normal staphylococci in that the cultures comprised mixed populations of cells with differing sensitivities to methicillin; the majority were sensitive to methicillin and a minority were highly resistant (Knox, 1961; Rolinson, 1961).

Since the first report of methicillin-resistant staphylococci, further strains have also been encountered elsewhere (Barber, 1961; Barber and Waterworth, 1962; Chabbert and Baudens, 1962; Cetin and Ang, 1962; Stewart and Holt, 1963; Eriksen and Erichsen, 1963; Jevons, Coe, and Parker, 1963; Beck and Evans, 1963; Harding, 1963), and, like the original strains isolated at Colindale, some of these strains have been reported to comprise cultures with a minority population of highly resistant cells (Barber, 1961; Barber and Waterworth, 1962; Chabbert and Baudens, 1962; Harding, 1963).

In contrast, other strains of naturally occurring methicillin-resistant staphylococci have been reported to differ from this pattern. For example, Stewart and Holt (1963) described methicillin-resistant strains comprising cultures uniform in appearance, growth, and insensitivity to methicillin. Moreover, these strains were reported to be able to inactivate the penicillinase-stable penicillin, cloxacillin, to a greater extent than did normal strains of penicillinase-producing staphylococci. Eriksen and Erichsen (1963) also described strains of staphylococci capable of destroying methicillin.

The incidence of methicillin-resistant staphylococci would appear to be very low (Jevons et al., 1963), but there is little doubt that such strains can, on occasion, cause serious infection which may not be controlled with methicillin (Stewart and Holt, 1963; Harding, 1963). It is important, therefore, that as much should be known about these strains as possible, and, particularly, that reliable methods of detecting these strains during routine clinical testing should be determined. In the present paper, we describe experiments with subcultures of many of the methicillin-resistant strains described in the literature from 1961 to date, and include tests for population composition, degree and stability of resistance to

methicillin, ability to inactivate methicillin and cloxacillin, and resistance to other antibiotics. Consideration is also given to reliable methods of detecting methicillin-resistant strains during the course of routine antibiotic-sensitivity testing.

MATERIALS AND METHODS

Cultures. Strain 13137 was received from P. M. Jevons, and was one of the original isolates of methicillin-resistant staphylococci (Jevons, 1961). R. W. Riddell of the Brompton Hospital supplied strain 7059. Strain P89 was one of several cultures sent to our laboratory from Poland by J. Parnas. G. T. Stewart supplied strains QMH I, QMH II, 6467, and 8054, which had been isolated at Queen Mary's Hospital, Carshalton. Strains QMH I and QMH II had been isolated before and after the death of a child who failed to respond to methicillin treatment (Stewart and Holt, 1963). J. W. Harding of the Central Middlesex Hospital supplied strains 1244/62 and 1247/62, which were the cause of a serious infection (Harding, 1963); K. R. Eriksen of Copenhagen sent strains 5974 and 5982, which were described in the literature earlier (Eriksen and Erichsen, 1963). Strains BRL 1776 and BRL 1800 had been isolated at The Children's Hospital, Birmingham, and were sent to us by K. B. Rogers. Mary Barber of the Hammersmith Hospital supplied strains M11-M25, and A. Beck of Paddington General Hospital provided strains 19223/62–1529/63. Strains 8/5, 97/2, and 71/5, isolated at St. Thomas' Hospital, were sent to us by M. Ridley.

Minimal inhibitory concentrations (MIC). MIC levels of antibiotics were determined by serial dilution of graded concentrations of the drugs in 5-ml volumes of broth or 18-ml volumes of agar. Inocula comprised 1 drop (approximately 0.03 ml) of an overnight broth culture of the test organism diluted as specified, and the cultures were incubated at 37 C for 24 and 48 hr.

Determination of population composition. The population composition of the methicillin-resistant cultures was determined by spreading 0.1-ml amounts of dilutions of broth cultures on agar containing graded concentrations of methicillin. The numbers of colonies visible after 24 and 48 hr of incubation were counted, and the proportions of the total population resistant to different concentrations of methicillin were calculated.

Selection of resistant cells. Resistant cells were selected from methicillin-resistant cultures by spreading these strains on agar containing 100 to 500 μg/ml of methicillin and, after 48 hr of incubation, subculturing into antibiotic-free broth.

Strains of normal staphylococci were habituated in vitro to methicillin by daily transfer in broth containing increasing concentrations of the penicillin. As inoculum, a drop from the culture growing in the highest concentration of methicillin in the previous test was used.

Bactericidal effects. Bactericidal effects were measured by inoculating Erlenmeyer flasks containing 100-ml volumes of nutrient broth and the required concentrations of methicillin with 1 ml of an overnight broth culture of the test organism, giving an inoculum of approximately 10^8 cells. Samples (1 ml) were taken from the flasks at intervals after inoculation, and viable counts were made by pipetting 0.02-ml volumes of suitable dilutions of the cultures onto blood-agar plates.

Sensitivity disc tests. Sensitivity disc tests were carried out in the usual fashion by flooding agar plates with suitable dilutions of overnight broth cultures of the test organisms and placing freshly prepared filter-paper discs (diameter, 6 mm) containing 5 μg of cloxacillin or 10 μg of methicillin on the surface of the plates. The diameters of zones of inhibition were measured after overnight incubation at 37 C.

RESULTS AND DISCUSSION

Determination of MIC levels of methicillin. The MIC levels of methicillin in broth against methicillin-resistant staphylococci are shown in Table 1. In these experiments, a large inoculum (approximately 10,000,000 organisms) and a small inoculum (approximately 10 organisms) were employed, and inhibition of growth was measured after incubation at 37 C for 24 and 48 hr.

With all strains, the growth of the light inoculum was inhibited after 24 hr by a concentration of 5 μg/ml of methicillin, and most strains were inhibited by concentrations below this level. In the same test, the MIC of methicillin against a normal penicillinase-producing strain of staphylococcus (BRL 1089) was 1.25 μg/ml. so that under these conditions methicillin-resistant strains were only slightly more resistant to methicillin than were other staphylococci. After 48 hr with the light inoculum, the MIC values of these cultures were slightly higher, but all were

TABLE 1 *Effect of inoculum size and length of incubation time on sensitivity of staphylococci to methicillin*

Source	Strain	Phage type	MIC (μg/ml)			
			Light inoculum[a]		Heavy inoculum[b]	
			24 hr	48 hr	24 hr	48 hr
Colindale	13137	7/47/53/54/75/77	2.5	5.0	5.0	125
Brompton Hospital	7059	—	1.25	2.5	5.0	125
Poland	P89	Group III	2.5	5.0	12.5	500
Queen Mary's Hospital Carshalton	QMHI	53/75/77	2.5	5.0	12.5	500
	QMHII	53/75/77	2.5	5.0	12.5	500
	6467	—	5.0	5.0	5.0	125
	8054	—	5.0	12.5	12.5	500
Birmingham	BRL 1776	—	2.5	5.0	12.5	>500
	BRL 1800	47/53/77	2.5	12.5	12.5	>500
Central Middlesex Hospital	1247/62	7/42E/53/54/75/77	2.5	5.0	12.5	500
	1244/62	7/47/53/54/75/77	2.5	12.5	25	>500
Hammersmith Hospital	M11	47/53/54/77	2.5	5.0	12.5	125
	M15	53/75/77	1.25	1.25	5.0	250
	M25	54/75/77	2.5	5.0	25	250
Copenhagen	5974	Group III	1.25	5.0	12.5	250
	5982	Group III	2.5	5.0	5.0	125
Paddington Hospital	19223/62	47/53/77	5.0	12.5	25	250
	25191/62	47/53/75/77	2.5	5.0	12.5	250
	24414/62	47/53/75/77	2.5	5.0	12.5	250
	1702/63	47/53/75/77	2.5	5.0	12.5	250
	1529/63	47/75/77	2.5	12.5	25	250
St. Thomas' Hospital	8/5	75/77	2.5	5.0	12.5	125
	97/2	53/75/77	0.5	2.5	12.5	250
	71/5	53/75/77	2.5	2.5	12.5	500
	BRL 1727[c]		0.5	2.5	2.5	>500
	BRL 1089[d]		1.25	2.5	2.5	5.0
	Staphylococcus aureus Oxford		0.25	1.25	0.5	2.5

[a] One drop of a 10^{-6} dilution of an overnight culture.
[b] One drop of an overnight culture.
[c] Strain of *S. aureus*, coagulase-negative.
[d] Typical methicillin-sensitive, penicillinase-producing strain of *S. aureus*.

TABLE 2. *Population analysis of a methicillin-resistant strain of Staphylococcus aureus 8054**

Dilution of inoculum†	No. of cells	Incubation period (hr)	Concn of methicillin (μg ml)										
			500	250	125	50	25	12.5	5.0	2.5	1.25	0.5	Control
None	30×10^6	24	−	−	−	±	±	±	+	+	+	+	+
		48	106	250	±	±	±	±	+	+	+	+	+
10^{-2}	30×10^4	24	−	−	−	−	−	−	+	+	+	+	+
		48	−	−	2	18	40	100	+	+	+	+	+
10^{-4}	30×10^2	24	−	−	−	−	−	−	−	±	±	±	±
		48	−	−	−	−	−	−	±	±	±	±	±
10^{-6}	30	24	−	−	−	−	−	−	−	50	30	20	30
		48	−	−	−	−	−	−	30	50	33	25	30

* Symbols: + = confluent growth; ± = discrete colonies (too numerous to count); numbers = number of colonies visible; − = no growth.

† Inoculum, 0.1 ml of overnight broth culture dilated as specified and spread over surface of blood-agar plate.

inhibited by 5.0 to 12.5 μg/ml of methicillin. In contrast, with the heavy inoculum the MIC values were generally as high as 12.5 μg/ml even after 24 hr of incubation, and after 48 hr most of the strains were inhibited only by concentrations as high as 250 μg/ml or greater. Under the same conditions, the MIC values for a methicillin-sensitive staphylococcus were 2.5 μg/ml after 24 hr and 5.0 μg/ml at 48 hr.

With all the strains of methicillin-resistant staphylococci, the MIC values therefore ranged from as low as 1.25 to 2.5 μg/ml to as high as 250 μg/ml and over, depending entirely on the size of the inoculum and the length of incubation. It is well known that there is an analogous inoculum effect with benzylpenicillin and penicillinase-producing staphylococci, giving rise to results similar to those shown in Table 1. With benzylpenicillin, the inoculum effect results from the destruction of the drug, which becomes greater as the inoculum size and incubation times are increased. With methicillin and the resistant strains used in this work, however, preliminary experiments showed that destruction of the drug was negligible. Since drug inactivation is not a factor in these experiments, the results shown in Table 1 suggest that these strains are mixed populations with respect to methicillin sensitivity, the majority of the population being of normal sensitivity but with a minority highly resistant. This interpretation is consistent with the effect of inoculum size and incubation period on the MIC. If the strains are mixed populations as suggested, a very small inoculum would contain primarily, or even entirely, sensitive organisms, and the MIC for methicillin would be low. Moreover, the MIC would not vary substantially as a result of further incubation. A very large inoculum, on the other hand, would be expected to include at least a certain number of cells of the resistant proportion.

Population analysis. The population composition of methicillin-resistant strains was determined as described in Materials and Methods, and typical results for one of the strains are shown in Table 2. With an inoculum of 0.1 ml of a 10^{-6} dilution of an overnight broth culture, 30 colonies grew on the control plate after 24 hr; and approximately the same number also grew on agar containing methicillin at concentrations up to 5 μg/ml, but there was no growth at 5 μg/ml and over. Similar results were obtained with an inoculum of a 10^{-4} dilution of broth culture. In this case, however, the colonies were too numerous to count but growth was uniform on the plates containing less than 5 μg/ml of methicillin, whereas at 5 μg/ml and over there was no growth. As the inoculum and therefore the number of organisms plated increased, resistant members became evident so that, with 0.1 ml of undiluted overnight culture, visible growth was present even after 24 hr at concentrations of methicillin as high as 50 μg/ml. However, growth on the plates containing from 12.5 to 50 μg/ml of methicillin was not identical with growth on the control plates or on those containing low concentrations of methicillin. At the higher concentrations of methicillin, the colonies were much less numerous and were very much smaller and less pigmented than normal. These resistant

TABLE 3. *Determination of proportion of methicillin-resistant cells in seven strains of methicillin-resistant staphylococci**

Strain	Methicillin concn (μg ml)									
	500	250	125	50	25	12.5	5.0	2.5	1.25	Control
13137	0	0.1	0.8	0.6	0.6	2	5×10^5	10^6	10^6	10^6
QMH I	0	0.75	1.3	4	35	25	7×10^5	10^6	10^6	10^6
8054	2.5	6	6	45	100	250	10^6	10^6	10^6	10^6
5974	1	10	4	5	6	35	5×10^4	10^6	10^6	10^6
BRL 1776	0.3	0.5	0.8	1.0	1.2	6	30	10^6	10^6	10^6
BRL 1800	0	0.1	0.3	2	2	5	—	10^6	10^6	10^6
1244/62	1	3	3	6	40	50	10^6	10^6	10^6	10^6
BRL 1727†	0	0	0.3	3	6	8	10	10^6	10^6	10^6
BRL 1089‡	0	0	0	0	0	0	0.5	10^6	10^6	10^6

* Results are expressed as the number of colonies per million plated growing at indicated concentration of methicillin. Colonies were counted after 48 hr at 37 C.

† Strain of *Staphylococcus aureus*, coagulase-negative.

‡ Typical methicillin-sensitive, penicillinase-producing strain of *S. aureus*.

members of the population also grew more slowly in the presence of methicillin than did the rest of the population, and, on incubation of the plates for a further 24 hr, colonies were then visible at concentrations as high as 500 μg/ml of methicillin. These highly resistant members grew as a mixture of small pigmented colonies and minute nonpigmented colonies, this difference in colony appearance being due possibly to different rates of growth. In contrast, the methicillin-sensitive members obtained by plating a small inoculum grew quickly and normally in the presence of low concentrations of methicillin, and the number and appearance of the colonies did not alter as a result of further incubation from 24 to 48 hr.

Although certain members of the culture were highly resistant to methicillin (Table 2), they were present only as a minority of the total population. These figures are expressed in Table 3 as the numbers of colonies growing at different concentrations of methicillin per million colonies growing on the control drug-free plate. With this strain (culture 8054), only 250 per million of the population were able to grow at a concentration of 12.5 μg/ml and only 2.5 cells per million were capable of growing at 500 μg/ml of methicillin. This type of analysis was also carried out for six other strains of methicillin-resistant staphylococci, and for a typical penicillin-resistant, methicillin-sensitive culture (BRL 1089). The figures obtained from these experiments to determine the proportions of the populations with different degrees of resistance are shown in Table 3. All these cultures displayed the same pattern of growth and population composition as illustrated in Table 2 for strain 8054. That is to say, the highly resistant cells were visible only after 48 hr of incubation, and the proportion capable of growing at a concentration of 12.5 μg/ml of methicillin or over was small and in no case exceeded 250 cells per million plated.

Although the highly resistant members of the strains shown in Table 3 are clearly a minority of the total culture, the basic population of these cultures does show a slight but distinct degree of resistance compared with typical penicillinase-producing staphylococci. Results for the latter type of culture (BRL 1089) are shown in Table 3; the population was uniformly able to grow in a concentration of 2.5 μg/ml of methicillin, but only 0.5 colonies per million were evident at concentrations of 5 μg/ml. With the methicillin-resistant strains, however, all except one culture contained at least 5×10^4 cells per million of the population capable of growth at 5 μg ml. With these methicillin-resistant strains, therefore, the basic population itself would seem to be slightly more resistant to methicillin than are typical staphylococci, but this degree of resistance is only in the order of a single twofold dilution.

Characteristics of the highly methicillin-resistant members of the populations. Results are shown in Table 4 for the highly resistant members selected

TABLE 4. *Effect of methicillin on the growth of resistant fractions isolated from methicillin-resistant strains of Staphylococcus aureus**

Strain†	Dilution of inoculum	Incubation (hr)	Methicillin concn (μg/ml)											
			500	250	125	50	25	12.5	5.0	2.5	1.25	0.5	0.25	0.1	Control
13137R	None	24	±	+	+	+	+	+	+	+	+	+	+	+	+
		48	+	+	+	+	+	+	+	+	+	+	+	+	+
	10^{-6}	24	−	−	M	M	M	M	M	M	M	M	M	M	M
		48	25	27	23	35	27	25	28	29	20	23	27	25	31
5974R	None	24	±	±	+	+	+	+	+	+	+	+	+	+	+
		48	±	+	+	+	+	+	+	+	+	+	+	+	+
	10^{-6}	24	−	−	−	−	−	M	M	M	M	M	28	31	35
		48	25		17	30	22	37	21	19	24	29	30	36	35
8054R	None	24	±	+	+	+	+	+	+	+	+	+	+	+	+
		48	±	+	+	+	+	+	+	+	+	+	+	+	+
	10^{-6}	24	−	−	M	M	M	M	M	M	M	M	20	28	30
		48	40	33	27	31	21	28	30	29	35	27	28	31	32

* Symbols: + = confluent growth; ± = discrete colonies; numbers = number of colonies; − = no growth; M = minute colonies.

† Strains 13137R, 5974R, and 8054R were the highly resistant members isolated from cultures 13137, 5974, and 8054, respectively.

from three methicillin-resistant cultures. Selection of these resistant members was carried out by picking off colonies growing after 48 hr on agar containing 500 μg/ml of methicillin, and culturing these colonies overnight in antibiotic-free broth. Volumes (0.1 ml) of suitable dilutions of these broth cultures were then used to inoculate agar plates containing graded concentrations of methicillin, in the manner of the experiment shown in Table 2. The methicillin-resistant fraction isolated in this way gave rise to a uniformly resistant population, since a small inoculum resulted in approximately the same number of colonies on a plate containing 500 μg/ml as on the control drug-free plate. These resistant cells also grew at a slower rate, as was seen in the experiments with the original cultures. Similar observations were also reported by Barber (1961). Growth was slow even on drug-free agar, resulting in rather small white colonies after 24 hr of incubation. In the case of the resistant fraction isolated from culture 13137, almost no growth at all could be detected after 24 hr. The presence of methicillin exerted an additional inhibitory effect so that colonies growing in high concentrations were nonpigmented and were smaller than those colonies growing at lower concentrations of methicillin. After 48 hr of incubation, growth was considerably heavier and colonies could be counted clearly, although again colony size became smaller as the concentration of methicillin increased. It is apparent, therefore, that this resistant minority is a clearly distinguishable fraction of the total population and that the extremely limited number of colonies growing on high concentrations of methicillin represents not the total population growing poorly but the distinct resistant members of a mixed population.

Stability of the methicillin-resistant fractions. The three cultures shown in Table 4, representing the resistant fraction present in cultures 13137, 5974, and 8054, were subcultured in drug-free nutrient broth for six transfers, and the population analysis was again carried out by plating 0.1-ml amounts of undiluted broth cultures and 10^{-6} dilutions of broth cultures on agar containing graded concentrations of methicillin (Table 5). After six transfers in the absence of methicillin, all three cultures had reverted to a mixed population similar to the original cultures from which the resistant fractions had been obtained, which is in agreement with findings reported by Barber (1962). The proportion of resistant cells was still somewhat higher than that seen in the original cultures, but the majority of the population was sensitive as indicated by the results with the small inoculum, which showed colonies only at concentrations of 5 μg/ml and below. The resistant fraction still showed slower growth, whereas the sensitive members grew normally after only 24 hr

TABLE 5. *Stability of resistant fractions after subculture (six transfers) in the absence of methicillin**

Strain†	Dilution of inoculum	Incubation (hr)	Methicillin concn (μg/ml)											
			500	250	125	50	25	12.5	5.0	2.5	1.25	0.5	0.25	0.1	Control
13137R	None	24	−	−	−	20	50	±	+	+	+	+	+	+	+
		48	5	50	80	±	±	±	+	+	+	+	+	+	+
	10^{-6}	24	−	−	−	−	−	−	25	26	30	26	41	37	27
		48	−	−	−	−	−	−	25	26	30	26	41	37	27
5974R	None	24	−	M	M	M	M	±	+	+	+	+	+	+	+
		48	±	±	±	±	±	±	+	+	+	+	+	+	+
	10^{-6}	24	−	−	−	−	−	−	−	27	27	14	35	20	
		48	−	−	−	−	−	−	25	27	27	14	35	20	
8054R	None	24	M	M	±	±	±	±	+	+	+	+	+	+	
		48	±	±	±	±	±	+	+	+	+	−	+	+	
	10^{-6}	24	−	−	−	−	−	−	−	32	18	24	23	19	23
		48	−	−	−	−	−	−	12	32	18	24	23	19	23

* Symbols: + = confluent growth; ± = discrete colonies; numbers = number of colonies; − = no growth; M = minute colonies.

† Strains 13137R, 5974R, and 8054R were the highly resistant members isolated from cultures 13137, 5974, and 8054, respectively.

of incubation. Resistance to methicillin would, therefore, appear to be an unstable factor, and loss of resistance also appears to be accompanied by resumption of normal rate of growth and colony appearance.

Effect of methicillin on the viable count of broth cultures of methicillin-resistant staphylococci. Since the methicillin-resistant staphylococci used in this work appear to comprise cultures with mixed populations, of which the majority are sensitive to methicillin, a substantial fall in the viable count of such cultures might be expected in the presence of methicillin during the first few hours before the resistant fraction has been able to proliferate. Bactericidal activity of methicillin against different strains of staphylococci is shown in Fig. 1 to 4. The strain shown in Fig. 1 was a typical penicillin-resistant, methicillin-sensitive strain, almost the entire population of which was sensitive to a concentration of 5 μg/ml of methicillin after 48 hr of incubation. In this culture, therefore, resistant members were not detectable. In Fig. 2 and 3, both cultures were naturally occurring methicillin-resistant strains. One of these (culture 5974) contained resistant members but only as a very small proportion, and even at 5 μg/ml the growth of a significant proportion of the population was inhibited. In the other culture (strain 8054), the level of resistance of the basal population itself was slightly higher, and the majority of the

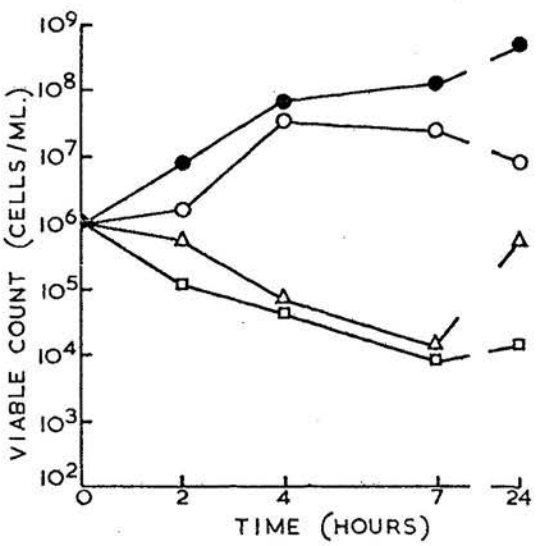

FIG. 1. *Effect of methicillin on the growth of Staphylococcus aureus BRL 1089. Methicillin concentrations: ●, antibiotic-free control; ○, 1.25 μg/ml; △, 2.5 μg/ml; □, 5.0 μg/ml.*

population was capable of growth at a concentration of 5 μg/ml, although again only a small minority was capable of growth at 12.5 μg/ml and above. As shown in Fig. 1, 2, and 3, the results of the viable counts are in accord with the population analysis for these cultures shown in Table 3. With strain BRL 1089, a concentration of 1.25 μg/ml of methicillin permitted growth

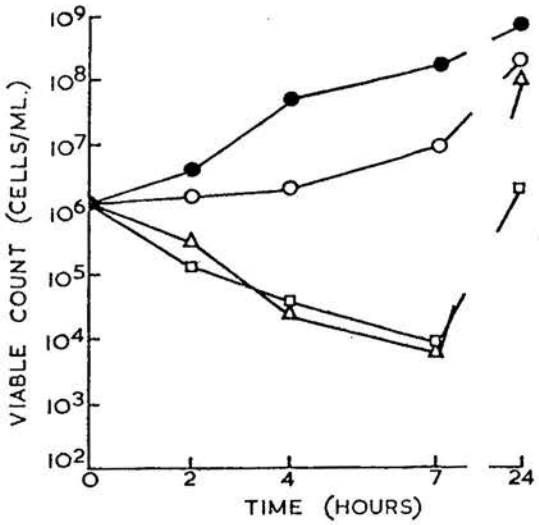

FIG. 2. *Effect of methicillin on the growth of Staphylococcus aureus 5974. Methicillin concentrations: ●, antibiotic-free control; ○, 2.5 μg/ml; △, 5.0 μg/ml; □, 20 μg/ml.*

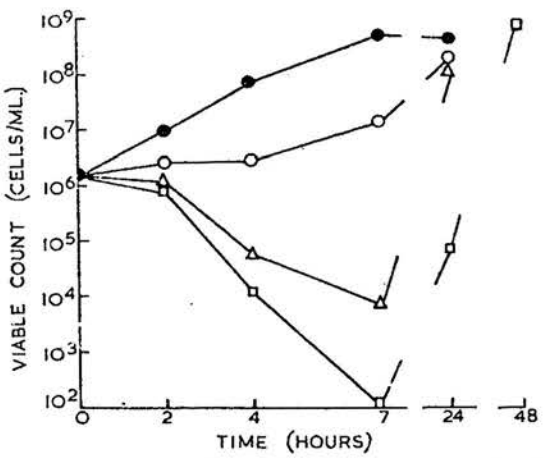

FIG. 3. *Effect of methicillin on the growth of Staphylococcus aureus 8054. Methicillin concentrations: ●, antibiotic-free control; ○, 5.0 μg/ml; △, 10 μg/ml; □, 20 μg/ml.*

over the first 7 hr, but a concentration of 2.5 μg/ml or greater resulted in a kill of over 99% of the original population during this period. With further incubation up to 24 hr, little change took place and, although some growth did occur at 2.5 μg/ml, the viable count, even at 24 hr, was still below the initial figure. With the methicillin-resistant culture 5974 (Fig. 2), considerable bactericidal activity again took place during the first 7 hr with relatively low levels of methicillin, but thereafter the highly resistant members grew; even at a concentration of 20 μg/ml, the viable count increased over 100-fold between 7 and 24 hr. A similar picture was also obtained with the other methicillin-resistant culture (8054), but in this case the basal population itself showed some degree of resistance; with 5 μg/ml only a diminished rate of increase in viable count was obtained. With 10 μg/ml, however, a kill of over 99% was again obtained in the first 7 hr, but thereafter the resistant variants proliferated; at 24 hr the viable count with 10 μg/ml of methicillin was almost as high as in the control culture. As would be expected, a pure culture of a resistant fraction isolated from strain 8054 was not readily affected by methicillin, and no bactericidal activity was seen with concentrations of up to 40 μg/ml of the drug (Fig. 4).

Sensitivity of methicillin-resistant staphylococci to cloxacillin. MIC values of cloxacillin against certain methicillin-resistant cultures are shown in Table 6. Even with a heavy inoculum, most of the cultures were inhibited by a concentration of 2.5 μg/ml of cloxacillin after 24 hr of incubation. The corresponding figures for methicillin were 12.5 to 25 μg/ml. This difference in activities is similar to that obtained with typical methicil-

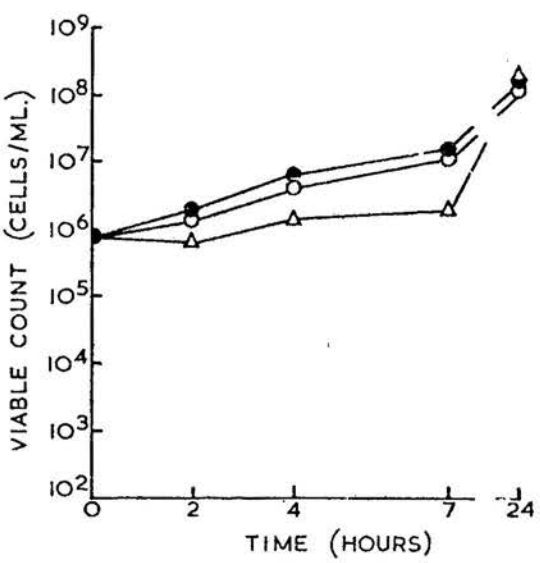

FIG. 4. *Effect of methicillin on the growth of Staphylococcus aureus 8054R, a resistant culture isolated from strain 8054. Methicillin concentrations: ●, antibiotic-free control; ○, 10 μg/ml; △, 40 μg/ml.*

TABLE 6. *Sensitivity of methicillin-resistant staphylococci to cloxacillin*

Source	Strain	MIC (μg/ml)			
		Light inoculum[a]		Heavy inoculum[b]	
		24 hr	48 hr	24 hr	48 hr
Colindale	13137	0.25	1.25	0.5	125
Brompton Hospital	7059	0.25	0.5 0.5	1.25	125
Poland	P89	0.5		1.25	>500
Queen Mary's Hospital Carshalton	QMH I	0.25	2.5	1.25	250
	QMH II	0.25	1.25	2.5	250
	6467	0.25	0.5	5.0	>500
	8054	0.5	0.5	1.25	250
Birmingham	BRL 1776	0.5	1.25	2.5	250
	BRL 1890	0.05	0.1	1.25	125
Central Middlesex Hospital	1247/62	0.25	0.5	0.5	50
	1244/62	0.05	0.5	0.5	50
Hammersmith Hospital	M11	0.25	1.25	1.25	50
	M15	0.25	0.25	0.5	12.5
	M25	0.5	0.5	2.5	250
Copenhagen	5974	0.1	0.5	1.25	25
	5982	0.1	0.5	1.25	50
Paddington Hospital	19223/62	0.25	0.5	1.25	12.5
	24414/62	0.05	0.5	0.5	50
	1529/63	0.25	0.5	2.5	12.5
St. Thomas' Hospital	8/5	0.5	0.5	1.25	>500
	97/2	0.25	0.5	2.5	>500
	71/5	0.25	0.5	1.25	25
	BRL 1727[c]	0.01	0.25	5.0	>500
	BRL 1089[d]	0.25	2.5	0.5	1.25
	Staphylococcus aureus Oxford	0.025	0.1	0.1	0.25

[a] One drop of a 10^{-6} dilution of an overnight culture.
[b] One drop of an undiluted overnight broth culture.
[c] Strain of *Staphylococcus aureus*, coagulase-negative.
[d] Typical methicillin-sensitive, penicillinase-producing strain of *S. aureus*.

lin-sensitive staphylococci where cloxacillin is approximately five times more active than methicillin. The minority population, highly resistant to methicillin, is also resistant to cloxacillin, however, and with a heavy inoculum, after 48 hr of incubation, the MIC values of cloxacillin for some strains were as high as 250 μg/ml.

Sensitivity of methicillin-resistant staphylococci to other antibiotics. The MIC values of tetracycline, streptomycin, chloramphenicol, and erythromycin for certain methicillin-resistant cultures of staphylococci are shown in Table 7. Graded concentrations of the antibiotics were prepared in agar in petri dishes, and the surface

TABLE 7. *Sensitivity of seven strains of methicillin-resistant staphylococci to streptomycin, tetracycline, chloramphenicol, and erythromycin*

Strain	Inoculum*	MIC (μg/ml)							
		Streptomycin		Tetracycline		Chloramphenicol		Erythromycin	
		24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
13137	Neat	>500	>500	250	500	5.0	12.5	2.5	2.5
	Dilute	>500	>500	125	125	5.0	5.0	0.5	0.5
8054	Neat	>500	>500	125	250	5.0	12.5	1.25	2.5
	Dilute	500	500	50	250	1.25	5.0	0.1	0.1
BRL 1800	Neat	>500	>500	250	500	5.0	12.5	2.5	2.5
	Dilute	>500	>500	125	125	5.0	5.0	0.5	0.5
1247/62	Neat	>500	>500	250	500	5.0	12.5	2.5	2.5
	Dilute	>500	>500	50	125	5.0	5.0	0.5	0.5
M11	Neat	>500	>500	250	500	5.0	12.5	2.5	2.5
	Dilute	>500	>500	125	125	5.0	5.0	0.5	0.5
5974	Neat	>500	>500	250	500	5.0	12.5	2.5	2.5
	Dilute	>500	>500	125	125	5.0	5.0	0.5	1.25
24414/62	Neat	>500	>500	125	250	50	125	1.25	2.5
	Dilute	>500	>500	125	125	25	50	0.5	1.25
Oxford	Neat	5.0	5.0	0.5	2.5	5.0	12.5	2.5	2.5
	Dilute	1.25	2.5	<0.1	0.5	5.0	5.0	0.25	0.5

* Neat inoculum, one drop of an undiluted overnight broth culture; dilute inoculum, one drop of a 10^{-5} dilution of overnight culture.

of the agar was inoculated. Again, two levels of inoculum were used, a large inoculum comprising one drop of an overnight broth culture, and a small inoculum consisting of one drop of a 10^{-5} dilution of the same broth culture. Growth was recorded after 24 and 48 hr of incubation. All the cultures appeared to consist of fairly uniform populations with respect to antibiotic sensitivity. This is indicated by the fact that the MIC values for the heavy inoculum were very similar to those obtained with the small inoculum. The strains also showed a rather similar spectrum of resistance. All the cultures were highly resistant to tetracycline and streptomycin, with MIC values generally of 250 μg/ml or over. All the strains also showed the same level of sensitivity to erythromycin, with MIC values of 2.5 μg/ml for a large inoculum and 0.5 μg/ml with a small inoculum, the Oxford staphylococcus being inhibited in these tests at the same concentrations. With chloramphenicol, all strains except one were relatively sensitive, with MIC values of 5 μg/ml for a heavy inoculum after 24 hr of incubation. One of the methicillin-resistant staphylococci, however, was highly resistant to chloramphenicol, and required 50 to 125 μg/ml for inhibition.

In addition to the sensitivity tests with the original cultures of methicillin-resistant staphylococci, tests were also carried out to determine the antibiotic sensitivity of pure cultures of the methicillin-resistant fractions present in these strains. The MIC values were determined with graded concentrations of antibiotic in agar, and the plates were inoculated with 10^{-3} dilutions of overnight broth cultures of the original strains and of the isolated resistant fractions. In the case of the original cultures, this dilution ensured that the inoculum consisted essentially of sensitive members compared with the pure cultures of resistant variants. No significant difference in sensitivity was observed with respect to tetracycline, streptomycin, chloramphenicol, and erythromycin. Consequently, these resistant fractions appeared to differ from their parent cultures only with respect to sensitivity to methicillin and not to any of the other antibiotics tested.

Methicillin-resistant staphylococci selected in vitro. In view of the results with naturally occurring methicillin-resistant strains indicating them to be characteristically mixed populations of differing sensitivities, experiments were carried out on the population composition of methicillin-

resistant staphylococci habituated in vitro. The Oxford staphylococcus and a typical penicillin-resistant, methicillin-sensitive staphylococcus (strain 452) were transferred repeatedly in broth containing methicillin. By means of this selection, a culture was obtained from the Oxford strain which grew in 50 μg/ml of methicillin; from strain 452, a culture was obtained which grew in 125 μg/ml of methicillin. These cultures were examined for population composition, as above, on agar containing graded concentrations of methicillin, with broth cultures containing 50 and 125 μg/ml of methicillin, respectively, as inocula. The cultures were not transferred in the absence of methicillin prior to the population analysis. The proportions of the total population capable of growth at different levels of methicillin were calculated as previously (Table 3); the results are shown in Table 8. With the resistant variant from the Oxford staphylococcus, although the inoculum was a culture growing in broth containing 50 μg/ml of methicillin, no growth occurred on agar at this concentration, and at 12.5 to 25 μg/ml less than 0.1% of the population was capable of growth. At 1.25 to 5.0 μg/ml of methicillin, 90% of the population was sensitive to the drug. Similar results were also obtained with the resistant variants from culture 452. The culture analyzed had been taken from a broth culture containing 125 μg/ml of methicillin. At this concentration, less than 0.02% of the population was capable of growth on agar, and 90% of the population was inhibited by 5 μg/ml. Both cultures, therefore, showed a mixed population similar to that of naturally occurring strains. Although these cultures had been selected by transfer in the presence of the drug and were capable of showing some growth in high concentrations of methicillin, the populations were still largely sensitive with only a very small proportion actually resistant. A further reduction in the numbers of the resistant proportion of the population also occurred when the cultures were taken through six transfers in methicillin-free broth. This instability was seen with the variants selected from the penicillinase-producing strain as well as with that obtained from the Oxford staphylococcus.

Inactivation of methicillin and cloxacillin by methicillin-resistant staphylococci. Serial dilution tests in broth culture were carried out with methicillin and cloxacillin by use of large inocula of two strains of methicillin-resistant staphylococci, cultures 8054 and 5974. Culture 8054 was one of the Carshalton strains which were reported to inactivate cloxacillin (Stewart and Holt, 1963), and culture 5974 was reported by Eriksen and Erichsen (1963) to inactivate methicillin. After 48 hr of incubation, the penicillin concentration in each tube was estimated by assay with *Sarcina lutea*; a set of uninoculated serial dilutions incubated for the same period were used as standards. Some assay error is evident in this type of experiment, but the results (Table 9) indicate that the extent of inactivation of both methicillin and cloxacillin was very slight. Cloxacillin is known to be less stable to staphylococcal penicillinase (Nayler et al., 1962) than methicillin, and in this experiment inactivation of cloxacillin was more readily detectable than was inactivation of methicillin. Inactivation of low concentrations of cloxacillin (2 to 5 μg/ml) by methicillin-resistant staphylococci is undoubtedly greater than is the case with typical staphylococci, but this is only because the latter type of staphylococci do not grow at such con-

TABLE 8. *Determination of numbers of methicillin-resistant cells present in two strains of Staphylococcus aureus habituated to methicillin in vitro**

Strain†	Methicillin concn (μg/ml)								
	125	50	25	12.5	5.0	2.5	1.25	0.5	Control
Oxford R	0	0	$<10^3$	$<10^3$	$<10^5$	$<10^5$	$<10^5$	10^6	10^6
452R	$<2.5 \times 10^2$	2.5×10^4	5×10^4	5×10^4	10^5	10^6	10^6	10^6	10^6

* Results are expressed as number of colonies per million plated growing in the indicated concentrations of methicillin after 48 hr at 37 C.

† Strain Oxford R was a resistant culture obtained from *S. aureus* (Oxford) by repeated transfer in the presence of methicillin. Strain 452R was a resistant culture obtained from a penicillinase-producing, methicillin-sensitive staphylococcus.

centrations; destruction of the antibiotic would therefore not be expected. Methicillin-resistant strains show intrinsic resistance to methicillin and cloxacillin; this is clearly indicated by the results in Table 9 which show growth in broth containing residual concentrations of methicillin and cloxacillin as high as 200 μg/ml after the period of incubation. Where drug inactivation occurs, this is the consequence, and not the cause, of the bacterial growth.

Routine testing for resistance to methicillin and cloxacillin. All the cultures of methicillin-resistant staphylococci described in this report were mixed populations with respect to sensitivity to methicillin. This feature presents certain difficulties with regard to routine sensitivity testing. Thus, in serial dilution tests, if a large inoculum was employed these cultures appeared to be resistant, but with a small inoculum they generally appeared to be sensitive. Results of experiments with methicillin and cloxacillin sensitivity discs with several strains of methicillin-resistant staphylococci and three strains of normal staphylococci are shown in Table 10 where the same inoculum effect is apparent. Undiluted overnight broth culture was used as a large inoculum, resulting in dense confluent growth; a dilution of 10^{-4} was used to provide a small inoculum, giving numerous but separate colonies on the plates. With the Oxford strain of staphylococcus, the level of inoculum had little effect on zone diameter. On the other hand, with the typical penicillin-resistant strains (BRL 1089 and Russell), the inhibition zones with a large inoculum were considerably smaller than those obtained with small inocula. This was particularly marked with cloxacillin and would seem to be a reflection of the penicillinase activities of the cultures. With the methicillin-resistant strains, all the cultures showed zones of inhibition with methi-

TABLE 9. *Inactivation of methicillin and cloxacillin by two methicillin-resistant strains of Staphylococcus aureus*

Strain	Initial concn	Methicillin		Cloxacillin	
		Residual concn*	Growth*	Residual concn*	Growth*
	μg/ml	μg/ml		μg/ml	
8054	250	220	±	250	±
	125	120	+	140	±
	50	60	+	45	+
	25	22	+	23.5	+
	12.5	11.0	+	11.5	+
	5.0	3.3	+	<2.5	+
	2.5	1.7	+	<2.5	+
	1.25	0.5	+		+
	0.5	0	+		+
5974	250	220	+	230	±
	125	130	+	130	±
	50	47	+	50	+
	25		+	26	+
	12.5	15.0	+	12.5	+
	5.0	3.4	+	<2.5	+
	2.5	1.5	+	<2.5	+
	1.25	0.25	+		+
	0.5	0	+		+

* Growth and residual penicillin concentration measured at 48 hr. Symbols: + = full growth; ± = light growth.

TABLE 10. *Sensitivity testing of methicillin-resistant strains with antibiotic-sensitivity discs*

Strain	Inoculum*	Zone diameter† (mm)	
		Methicillin (10 μg)	Cloxacillin (5 μg)
13137	Neat	3.0‡	0
	Dilute	12	15
QMH I	Neat	0	7
	Dilute	17	23
QMH II	Neat	3	5
	Dilute	9	14
8054	Neat	0	0
	Dilute	16	21
5974	Neat	8‡	10
	Dilute	12‡	14
5982	Neat	8	9
	Dilute	9	14
BRL 1800	Neat	7‡	0
	Dilute	12	15
1247/62	Neat	7‡	10
	Dilute	13	15
Oxford	Neat	23	22
	Dilute	24	24
BRL 1089 (methicillin-sensitive)	Neat	16	12
	Dilute	20	16
Russell (methicillin-sensitive)	Neat	15	8
	Dilute	20	24

* Neat = overnight culture; dilute = diluted to give discrete colonies (10^{-4}).

† Zone diameter = diameter of zone of inhibition minus diameter of disc (6 mm).

‡ Few isolated colonies growing within zone.

cillin and cloxacillin when the small inoculum was used; on the basis of this type of test, these strains might be judged to be sensitive. With the heavy inoculum, the zones of inhibition were very much smaller, yet some of the strains gave zones of inhibition notwithstanding the fact that the cultures were known to contain methicillin-resistant cells.

These results were quite consistent with the characteristics of these strains, as determined in the experiments on population composition. A complete absence of an inhibition zone with these cultures would only be expected with an inoculum sufficiently heavy to provide a uniform growth of the resistant fraction, and in many of the cultures the resistant proportion was too small to allow this. Not infrequently, the resistant members could be seen growing as minute isolated colonies within an otherwise clear zone of inhibition, but even these were not always apparent until after 48 hr of incubation. The disc method is, therefore, not entirely satisfactory for routine testing for resistance to methicillin. A more reliable procedure would be a serial dilution test, with a heavy inoculum and incubation for 48 hr. Under these conditions, typical methicillin-sensitive staphylococci showed MIC values of 5.0 μg/ml or less, whereas all the strains of methicillin-resistant staphylococci examined in our laboratory required 125 μg/ml or more of methicillin for inhibition. In this way, it should be possible to identify clearly the presence of methicillin-resistant staphylococci.

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DISCUSSION

Chairman: A. MANTEN (*Utrecht*); Participants: P. C. FLEMING (*Toronto*); M. T. PARKER (*London*); K. R. ERIKSEN (*Copenhagen*); MARY BARBER (*London*); G. AYLIFFE (*Birmingham*); R. SUTHERLAND (*Brockham*).

Clinical significance of Methicillin-resistant staphylococci

FLEMING: In Toronto we have for three years been looking for methicillin-resistant strains. We have examined 8,000 routine isolates in our own hospital, and not found them. The Provincial Health Laboratory have authorised us to say that they have examined 45,000 strains in the 18 months, and not found them. I have approached each of the major teaching hospitals in Toronto, with the same result.

This is in line with the lack of reports of these strains coming from the United States. During the last three years we have used 95 kilograms of penicillinase-resistant penicillins—about two cwt.—and about 75% of this has been used by a relatively small group of patients in the fibrocystic clinic. About 40 of these fibrocystic patients are on continual inhalation therapy, of 3 grammes of methicillin a day; some of them are also, in addition, on oral cloxacillin. I examine their flora every month in the vast majority of instances, very carefully, because I had expected that in this situation we might find tolerant strains, and so far we have not seen them.

We have heard a lot to-day about the resistant strains, and this resistance is so far only laboratory resistance: there has been no clinical experience of cases treated with methicillin who have failed to respond, and who have been described in large enough numbers to make any observations of clinical importance on the clinical side. Therefore I suggest that we go on for the moment referring to them as methicillin-tolerant strains.

What are the clinical implications? The results from normal plates with no sodium chloride added show that the vast majority of cells are killed very rapidly by relatively small quantities of methicillin. Therefore you might assume that in a patient who was infected, the staphylococcal load would so be reduced by therapy that his natural defence mechanisms might reasonably produce a cure. If one considers the situation, where there is a breakthrough of the tolerant strains, Mary Barber and many others have pointed out that these strains are different from their parent strains in that they grow more slowly and have a different colonial appearance. It might then be reasonable to think that, if you compare their virulence with their parent strain, the virulence might be less.

However, because an organism has less virulence does not mean that it cannot be pathogenic. For example, *Staphylococcus albus* can be pathogenic on a heart valve, it can be pathogenic in patients with leukæmia or other impairment of the host defence mechanisms. I think it is against this background that we must consider the scanty clinical information which we have.

Assuming that these strains do break through, do you stop the methicillin? I suggest this is the wrong thing to do as the strains should revert back to the more normal and therefore more virulent form. It would probably be more correct to add another antibiotic.

The only clinical information we have to go on are the two cases described in the literature by Dr. Stewart, and another one by Dr. Harding. They are clearly just not enough, because we do not know fully about the host responses, and in some of these cases we do not know the full clinical details. We have only reached the situation where we cannot talk about methicillin-resistant strains in a clinical sense, but only about methicillin-tolerant strains in a laboratory sense.

As Mary Barber has pointed out, all these strains will grow apparently normally on the plate if you add some sodium chloride. Perhaps we should not think of pencillin as being primarily bactericidal, but rather primarily as interfering with the cell-wall formation, and that it is the environment which is bactericidal —in the case of the laboratory, it is the tonicity of the broth, and in the case of the human we do not really know what is the bactericidal factor. It may well be that L-forms and protoplasts are formed, and the body has a special mechanism for getting rid of this type of micro-organism; this would fit in with cases where one might have expected a response but do not necessarily find one.

It is quite clear that these strains were in existence long before methicillin was produced.

I would like to ask Dr. Parker if any of these strains are neomycin-resistant. I am merely wondering what the selective factor is—if the habit of using Naseptin cream could be causing selection of neomycin-resistant strains, which perhaps also happen to be examples of these naturally-occurring methicillin-resistant strains?

PARKER: Neomycin resistance is, in our experience, almost entirely confined to the "new" untypable members of phage-group III. Some of these organisms are indeed methicillin resistant, but so are many strains in which neomycin-resistance has not yet become common, so there is no suggestion of a significant link-up here.

ERIKSEN: All our methicillin-resistant strains have been neomycin-sensitive, and the reason they were selected in our research department was because they were tetracycline-resistant.

BARBER: In one particular case Dr. Fleming did, I think, misquote, when he suggested I had said these methicillin-tolerant organisms were less virulent than are methicillin-sensitive cultures. That is not the case. One of the interesting things with methicillin is that the methicillin-tolerant culture in the absence of methicillin appears to be a fully virulent staphylococcus.

ELEK: I believe Professor Barber was responsible for introducing the terminology of resistant and tolerant staphylococci. A point she made was that these tolerant organisms are as virulent as the parent. She left out the words "to mice". There is no agreed method of establishing the virulence of organisms at the present moment. Some people accept mouse virulence; others accept virulence to man, and yet others have an even more composite concept of what virulence means. It seems to me that Dr. Parker's evidence is very valuable in that respect.

If we assume that these tolerant organisms are in fact virulent, we could reasonably assume that in the course of four years they would increase in numbers, as far as clinical infections are concerned; and that does not seem to be the case.

The other point Professor Barber has made is fascinating. The fact that she has biochemical mutants which have a partial susceptibility to methicillin very strongly suggests that there are at least two mechanisms in cell-wall formation. What she did not say again was that she must have worked with first-stage mutants, because the first-stage mutant may be very different from the original wild strain. What appears to be the case is that these organisms are capable of manufacturing some cell-wall, but not enough cell-wall under normal circumstances. I think as a fundamental observation this must be very interesting indeed, and I hope Professor Barber will follow it up.

AYLIFFE: In view of the lack of clinical evidence, or very little, on the treatment of methicillin-resistant infections with methicillin, I might mention some mouse experiments that were done on this subject.

Mice were infected by intraperitoneal injection with a methicillin-resistant staphylococcus, and another group with a sensitive strain. These mice were then treated with methicillin or cloxacillin, in doses of 100 mg. per kg. daily given in two doses for the first three days. The results were that the mortality in the mice infected with the methicillin-resistant strain was the same, both in the treated and in the untreated mice, both for methicillin and cloxacillin; whereas with the sensitive strain mortality was much lower in the treated mice.

Assessment of resistance to methicillin

SUTHERLAND: I would like to stress the very great variation seen in sensitivity tests with naturally occurring strains of methicillin-resistant staphylococci. The results shown in Table I are representative of those seen in tests in our laboratories. I would suggest that Dr. Parker, in using a concentration of 12.5 μg./ml. with an incubation period of 18 hours, may have missed a certain number of these resistant strains. We would consider it essential to incubate for a period of 48 hours.

This variation in sensitivity with inoculum size and incubation period is analogous to that seen with penicillin G and penicillinase-producing staphylococci but, with methicillin-resistant strains, resistance to methicillin is not due to destruction of the antibiotic. It is our experience that these strains produce very little destruction of methicillin and we have been unable to confirm destruction of methicillin as extensive as that reported.

Our interpretation of the results shown in the table is that these cultures consist of mixed populations in which the majority of cells are of normal sensitivity to methicillin, with a minority showing resistance. This is demonstrated in Figure 1. The figure on the left hand

TABLE I

Effect of Incubation Period and Inoculum Size on the Sensitivity to Methicillin of Naturally Occurring Methicillin-Resistant Strains of *Staphylococcus aureus*.

	Minimal inhibitory concentration (μg./ml.)	
Inoculum*	24 hours	48 hours
undiluted	5.0-25	500
1/100,000	2.5-5.0	5.0-12.5

*Inoculum, one drop of an overnight broth culture diluted as specified.

side shows the effect of varying concentrations of methicillin on a naturally occurring methicillin-resistant strain of staphylococcus. It can be seen that a concentration of 5 μg./ml. of methicillin had little effect on growth of the organism, while at a concentration of 10 μg./ml. there was a marked bactericidal effect for seven hours, but thereafter the culture grew so that at 24 hours growth was equal to that in the drug-free medium. With the concentration of 20 μg./ml. there was again a marked bactericidal effect over seven hours after which there was an increase in growth. After 24 hours the growth was less than that in the drug-free medium, but at 48 hours there was complete growth. If a sample is taken from the 48 hour growth in the tube containing 20 μg./ml. methicillin and inoculated into a fresh series of tubes containing methicillin, the growth curves obtained are as shown in the second figure. It can be seen that even at the upper concentration growth was almost as rapid as in the drug-free tube, although growth in the drug-free medium was not as rapid as that seen with the parent naturally occurring methicillin-resistant strain. Thus, after one transfer in methicillin a resistant variant has been selected from the original culture which comprised a mixed population of cells.

This is further illustrated in Figure 2. There are two parameters, firstly, drug concentration and, secondly, medium. The plates contain graded concentrations of methicillin, those on the left containing 100 μg./ml. methicillin, the next 50 μg./ml., the next 25 μg./ml., and the plates on the right hand side 12.5 μg./ml. methicillin. Plates in the upper row contain nutrient agar, and in the lower row, nutrient agar containing 5% sodium chloride. The upper half of each plate was inoculated with a series of dilutions of a naturally occurring methicillin-resistant strain of staphylococcus.

The drop on the left hand side was undiluted overnight broth culture and would comprise an inoculum of approximately one million cells. The drop in the middle was a 1/1,000 dilution of this culture, and that on the right hand side a 1/100,000 dilution. The plates were incubated for 48 hours. After this period, growth on plates containing 12.5 μg./ml. of methicillin was the same as that seen on the drug-free control plate, which is not shown here. At a concentration of 25 μg./ml. methicillin, however, the inocula diluted 1/1,000 and 1/100,000 failed to grow and only the undiluted inoculum shows growth on this plate. Likewise, at a concentration of 50 μg./ml. methicillin only the undiluted inoculum grew, this time with a certain reduction in growth and at 100 μg./ml. methicillin only a few isolated, and in some cases, small colonies, grew. This illustrates the nature of the mixed population of the naturally occurring methicillin-resistant strain. If now one transfers cells growing on the plate containing 100 μg./ml. methicillin into drug-free broth, and after overnight incubation uses this as an inoculum in a similar test, the results obtained are shown on the lower half of each plate. It can be seen there was uniform growth on all plates with all three inocula, illustrating the uniform resistance of this methicillin-resistant fraction.

The results of some experiments with electrolytes are also illustrated in Figure 2 where the plates in the lower row contain nutrient agar plus 5% sodium chloride. With a concentration of 12.5 μg./ml. methicillin, there was good growth of all inocula, the growth was, in fact, rather better than that seen on the corresponding plate containing nutrient agar. Moreover, at a concentration of 25 μg./ml. methicillin, all three inocula grew, unlike the growth on nutrient agar where only the undiluted inoculum grew. However, with a concentration of 50 μg./ml. methicillin only the undiluted inoculum grew and at 100 μg./ml. methicillin only a small number of isolated colonies could be seen. Thus, in our experiments, the addition of 5% sodium chloride increased the growth of methicillin-resistant staphylococci in the presence of methicillin only over a limited range of concentrations. The effect of electrolytes on the growth of methicillin-resistant staphylococci is a very interesting phenomenon but its significance is still to be wholly determined.

It is possible, however, that the phenomenon may have an application in sensitivity testing. As Dr. Parker has illustrated, routine sensitivity testing of these strains with antibiotic sensitivity discs is very misleading. Some of our results with sensitivity discs are shown in Figure 3

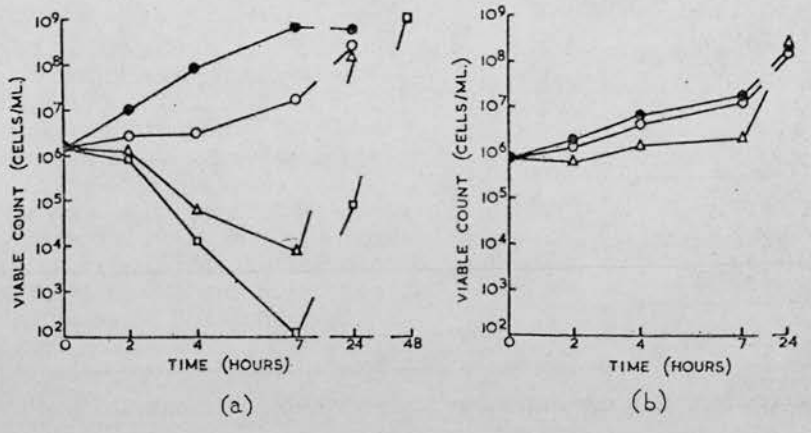

FIG. 1.—The effect of methicillin on the viable count of methicillin-resistant staphylococci.

(a) naturally occurring methicillin-resistant strain ●, drug-free medium; O, 5 μg./ml.; △, 10 μg./ml.; □, 20

(b) methicillin-resistant fraction isolated from (a) ●, drug-free medium; O, 10 μg./ml.; △ 40 μg./ml. methicillin.

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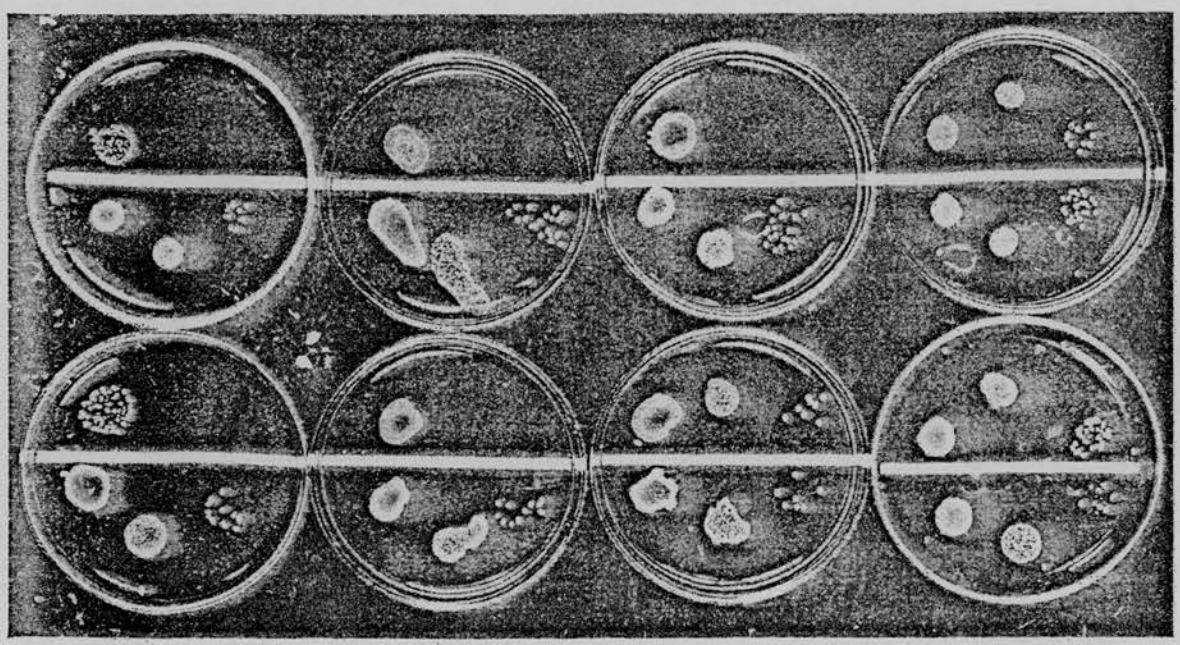

FIG. 2.—The effects of electrolytes on the action of methicillin against methicillin-resistant staphylococci.

From left to right the plates contain 100 μg./ml., 50 μg./ml., 25 μg./ml. and 12.5 μg./ml. methicillin, respectively.

Upper row, nutrient agar. Lower row, nutrient agar + 5% sodium chloride.

The upper half of each plate has been inoculated with an undiluted, a 1/1,000 dilution and a 1/100,000 dilution of an overnight broth culture of a naturally occurring methicillin-resistant staphylococcus.

The lower half of each plate has been inoculated with similar dilutions of a methicillin-fraction isolated from the strain shown in the upper half.

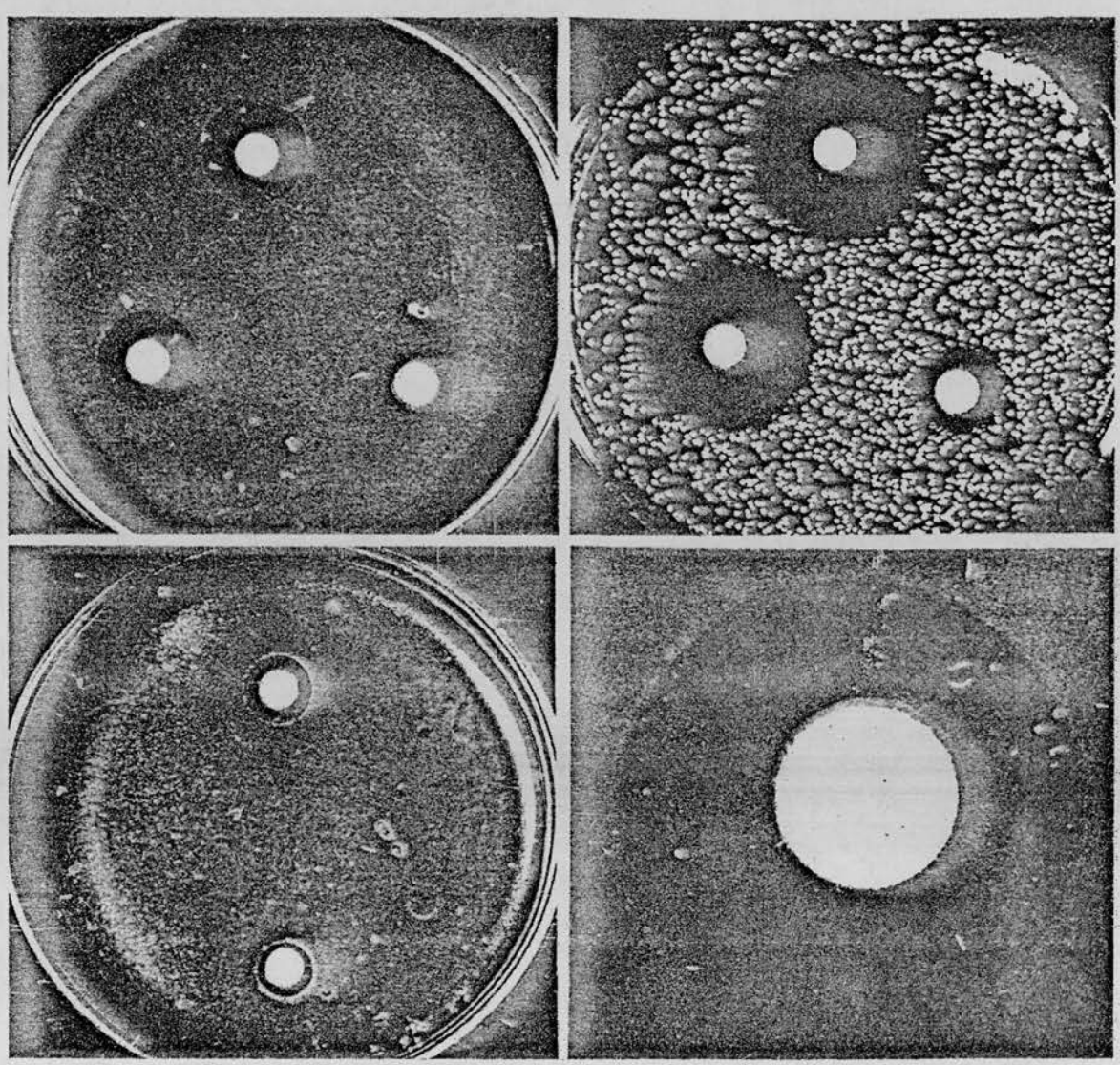

FIG. 3.—Antibiotic-sensitivity disc tests with naturally occurring methicillin-resistant staphylococci.

Discs contain 10 μg. methicillin or 5 μg. cloxacillin, (in upper two plates, disc on right-hand side of plate contains 5 μg. benzylpenicillin).

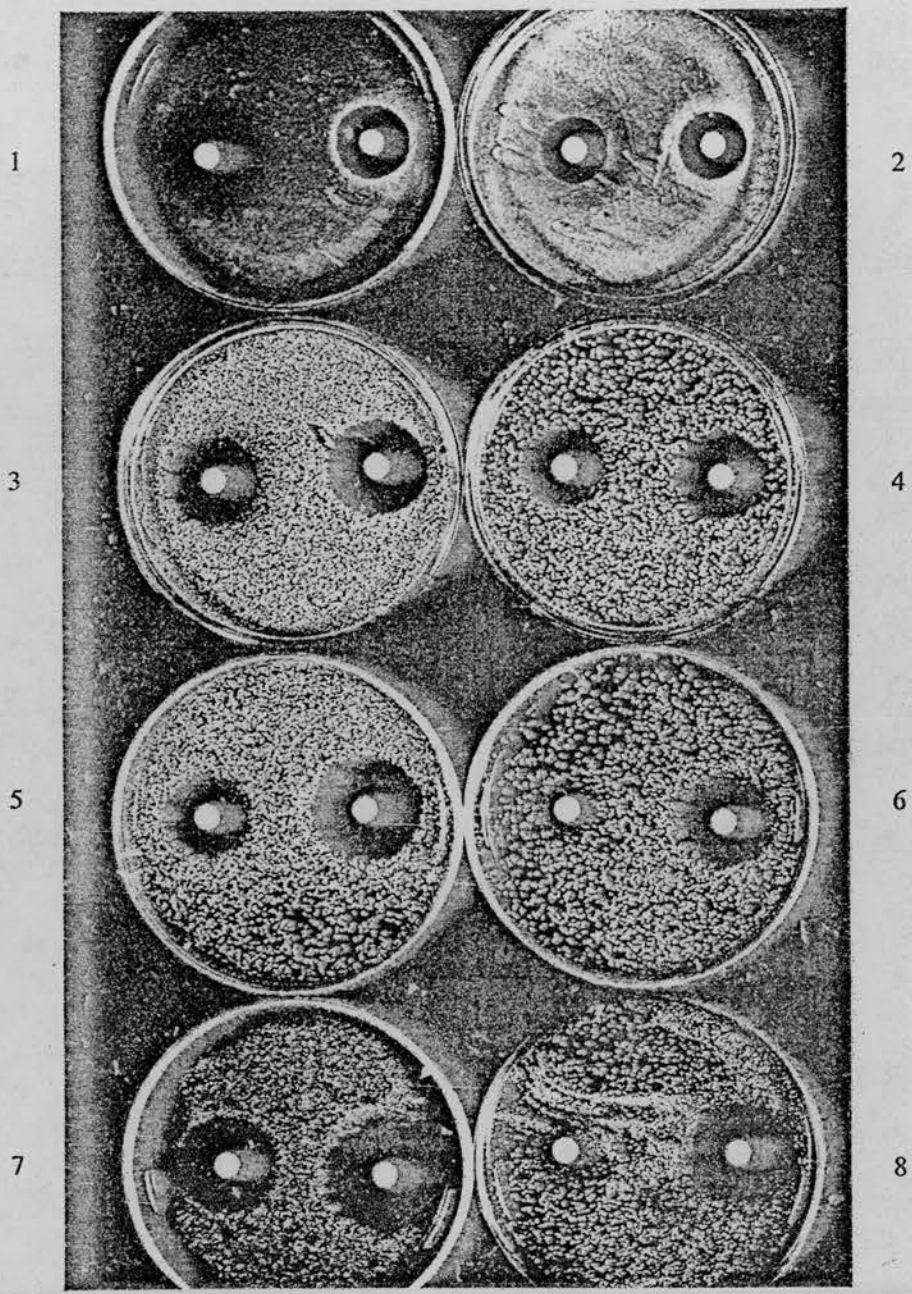

FIG. 4.—Effects of electrolytes on the results of antibiotic-sensitivity disc tests with methicillin-resistant staphylococci.

Dishes on the left-hand side have been inoculated with a penicillinase-producing, methicillin-sensitive strain of *Staph. aureus*, and those on the right-hand side with a naturally occurring methicillin-resistant staphylococcus.

Discs on the left-hand side of each plate contain 10 μg. methicillin and those on the right-hand side, 5 μg. cloxacillin.

Dishes 1, 2, 3, and 4 contain agar. Dishes 5 and 6 contain nutrient agar + 5% sodium chloride. Dishes 7 and 8 contain mannitol-salt agar.

where it can be seen that the variation demonstrated in M.I.C. tests occurs also in sensitivity disc tests. Thus, with a light inoculum of cells, large zones of sensitivity can be seen with the methicillin and cloxacillin discs, and there is no doubt that any worker would report these strains as being sensitive to these penicillins. Even with the undiluted inocula fairly large zones of sensitivity can be seen with some staphylococci, although with other strains the zones are indeed very small and these latter strains would be classified as being resistant. With some strains, resistant colonies may be seen within a zone of inhibition after 24 hours, but other strains require 48 hours incubation. Therefore, we would recommend a test in which broth or agar containing a concentration of 12.5 or 20 μg./ml. of methicillin was inoculated with the test organism and incubated for a period of 48 hours. In our experience, all methicillin-sensitive staphylococci are inhibited by these concentrations over this period of time, but all naturally occurring strains of methicillin-resistant staphylococci will grow.

However, as in clinical practice the results of laboratory tests are required as rapidly as possible the following test might be more satisfactory from a clinical point of view. Figure 4 shows the results of disc sensitivity tests on agar containing electrolytes. The plates on the left hand side of the slide have been inoculated with a penicillinase-producing staphylococcus sensitive to methicillin, and those on the right with a naturally occurring methicillin-resistant strain of staphylococcus. The upper four plates contain nutrient agar and illustrate the effect of inoculum size. The plates in the third row from the top contain nutrient agar plus 5% sodium chloride, and it can be seen that in this case the resistant colonies in the methicillin-resistant culture have grown up to the edge of the disc containing 10 μg. of methicillin. The effect with the cloxacillin discs (5 μg.) is not so obvious, but there are usually quite a large number of resistant colonies growing up within the inhibition zone after 24 hours. The plates at the bottom contain mannitol salt agar (Chapman agar), a selective medium often used for isolating staphylococci, and again it can be seen that the naturally occurring methicillin-resistant staphylococci appear to be resistant to the disc containing 10 μg. of methicillin. It is possible that this test, or an adaptation of it, may be suitable for the routine testing of strains of staphylococci for sensitivity to methicillin.

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The Nature of the Insensitivity of Gram-Negative Bacteria towards Penicillins

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SUMMARY

The insensitivity of Gram-negative bacteria towards different penicillins has been correlated with the inactivation of these compounds by the penicillinases produced by these bacteria. Penicillins such as phenethicillin, propicillin, methicillin, and cloxacillin were relatively inactive against Gram-negative bacteria, but resistance was not accompanied by inactivation of these compounds. With ampicillin and benzylpenicillin, however, bacterial resistance was associated with destruction of the penicillins. Ampicillin was more stable to the penicillinases produced by certain Gram-negative bacteria than was benzylpenicillin and was correspondingly more active against these organisms. Gram-negative bacteria which produced little or no penicillinase were two to four times more sensitive to ampicillin than to benzylpenicillin, but ampicillin was at least ten times more active than benzylpenicillin against several penicillinase-producing coliform organisms. All strains of bacteria which were resistant to both ampicillin and benzylpenicillin were capable of inactivating both compounds, but the insensitivity of these bacteria was not necessarily due solely to penicillinase production. Strains of *Pseudomonas aeruginosa* and certain strains of *Escherichia coli* and *Proteus mirabilis* were able to grow in high concentrations of these antibiotics, whereas with the strains of *Aerobacter aerogenes*, *Proteus morganii* and *Proteus vulgaris* examined, the resistance appeared to be due solely to inactivation of the penicillins.

INTRODUCTION

The destruction of benzylpenicillin by certain Gram-negative bacteria was reported first by Abraham & Chain (1940) and, later, by Bondi & Dietz (1944), but both groups of workers also observed that the resistance of bacteria to penicillins was not invariably associated with penicillinase formation. Similar findings with ampicillin were reported by Rolinson & Stevens (1961) who showed that certain Gram-negative bacteria were capable of destroying the antibiotic, but that others were capable of growing in high concentrations of it; these results have been confirmed by other workers (Stewart, Coles, Nixon & Holt, 1961; Trafford *et al.* 1962; Ayliffe, 1963). Thus, with ampicillin, as with benzylpenicillin, penicillinase appeared to occupy an indeterminate role in the resistance of Gram-negative bacteria towards penicillins. More recently, the characteristics of the penicillinases elaborated by certain Gram-negative bacteria have been investigated by various workers who have reported striking differences in the activities of these enzymes against various penicillins. For example, Auhagen *et al.* (1962) and Smith (1963) described strains of *Escherichia coli* which destroyed benzylpenicillin but which

had little or no effect on ampicillin, propicillin, methicillin or oxacillin. Ayliffe (1963) showed that ampicillin was more stable than benzylpenicillin to the penicillinase produced by a strain of *E. coli*, but, in addition, reported that ampicillin was inactivated as rapidly, or more rapidly, by penicillinases produced by strains of Proteus and Klebsiella. In these laboratories, Mr F. R. Batchelor (personal communication) has measured marked variation in the reaction of several penicillins to the penicillinases produced by a number of Gram-negative bacteria. It seemed possible that in some cases the differences in the sensitivities of some strains of Gram-negative bacteria towards various penicillins might be correlated with the relative stabilities of these compounds to the bacterial penicillinases. The results reported here were derived from experiments designed to investigate the relationship between the penicillinase activity of Gram-negative bacteria and the sensitivity of these organisms to penicillins, with a view to assessing the significance of these enzymes as factors in the resistance of bacteria to these antibiotics. The experiments comprised tests for antibacterial activity, measurement of the destruction of penicillins by bacteria, and observation of the growth of bacteria in the presence of penicillins. In addition, certain tests were also carried out to distinguish between penicillin inactivation by β-lactamases (penicillinases), which yield penicilloic acids, and deacylases (penicillin amidases) which yield 6-amino-penicillanic acid (6-APA).

METHODS

Measurement of the antibacterial activities of penicillins. Minimal inhibitory concentrations of various penicillins against bacteria were determined by twofold serial dilution in nutrient broth (Oxoid No. 2). The antibiotics were incorporated in graded concentrations in 5 ml. volumes of nutrient broth which were inoculated with 1 drop (0·03 ml.) of an overnight culture of the test organism. Inhibitory concentrations were measured after overnight incubation at 37°. In other experiments, the effect on the antibacterial activity of the penicillins of altering the inoculum size was measured.

Measurement of penicillin destruction. The destruction of penicillins incubated overnight in the antibacterial tests described above was estimated by microbiological assay of residual penicillin activity. Samples were taken from the cultures and were plated in punch-holes in large rectangular plates containing nutrient agar seeded with *Bacillus subtilis* (ATCC 6633). In these assays, standard lines were prepared from standard solutions of penicillins which had been incubated overnight at 37° in nutrient broth to compensate for thermal inactivation of penicillin. The plates containing test and standard samples were incubated overnight at 29° when the diameters of zones of inhibition were measured in the usual way. Tests were also carried out to estimate the extent of penicillin inactivation due to the experimental procedure adopted, and which represented destruction additional to that occurring in the antibacterial tests. Likely factors appeared to be change in pH value of cultures, and the penicillinase activities of test cultures during the microbiological assay procedures.

Measurement of the effect of penicillins on the growth of bacteria. Optically matched tubes containing selected concentrations of penicillins in 10 ml. volumes of nutrient broth were incubated with 0·5 ml. or 1·0 ml. of an overnight culture of test organism.

The solutions were incubated at 37° in a water bath, and growth was measured turbidimetrically over a period of 24 hr by means of an EEL nephelometer. In addition, a replicate set of cultures was prepared in an identical fashion and samples were taken from these at regular intervals and assayed for penicillin content as described above.

Quantitative estimation of penicillins by chromatography. Samples were taken from broth cultures of *Pseudomonas aeruginosa* which had been incubated overnight at 37° with known concentrations of ampicillin or benzylpenicillin and these were assayed as follows. One drop (about 6 μl.) was placed on the origin of filter-paper tapes (1 cm. wide, Whatman No. 1) and developed by descending chromatography in a butanol+pyridine+water (1+1+1, by vol.) system. Tapes spotted with nutrient broths containing from 2·5 to 100 μg. ampicillin or benzylpenicillin/ml. were treated in a like manner. After 16 hr, the tapes were placed on the surface of large rectangular assay plates containing nutrient agar seeded with *Bacillus subtilis* (ATCC 6633) and incubated for 24 hr at 29°. The penicillin concentrations of the test samples were estimated from standard lines derived from standard samples.

Chromatographic estimation of 6-aminopenicillanic acid. Samples were taken from the antibacterial tests and treated as above for quantitative estimation of penicillin by chromatography. The tapes were sprayed with 5% sodium bicarbonate solution and 5% phenylacetyl chloride in acetone before being plated for microbiological assay (Batchelor, Doyle, Nayler & Rolinson, 1959).

Chromatographic examination for penicilloic acid. Samples from antibacterial tests with ampicillin and benzylpenicillin were spotted on large sheets (Whatman paper No. 1) and developed in the butanol+pyridine+water system as described above. The chromatograms were sprayed with the starch-iodine reagent of Thomas (1961), and the zones which developed compared with those produced by the penicilloic acids which had been prepared by treatment of standard solutions of ampicillin and benzylpenicillin with penicillinase.

RESULTS

Preliminary

Destruction of penicillin due to change in pH values of bacterial cultures was considered to be negligible as a result of experiments which showed that there was little inactivation of standard solutions of benzylpenicillin incubated overnight in nutrient broth buffered at pH 5·0. This was the lowest pH value recorded for a number of overnight cultures of Enterobacteriaceae.

The amount of destruction of ampicillin or benzylpenicillin by bacterial penicillinase during the microbiological assay procedures was estimated by finding the effect of adding, at measured intervals, a culture of a penicillinase-producing strain of *Staphylococcus aureus* to standard solutions of these penicillins contained in punch-holes in an assay plate. There was significant inactivation only when the enzyme system was added within 30 min. of placing the penicillin solution on the assay plate, owing to the very rapid diffusion of the penicillins into the surrounding medium. It seemed improbable therefore that there would be any marked destruction of penicillins during the microbiological assay as a result of the penicillinase produced by the Gram-negative bacteria being examined.

Examination for production of 6-aminopenicillanic acid (6-APA)

Chromatographic examination of broth cultures in which there was marked destruction of penicillin as judged by microbiological assay, showed the presence of large amounts of the cognate penicilloic acid, and little or no 6-APA. The organisms tested included strains of *Escherichia coli*, Proteus, *Pseudomonas aeruginosa* and *Aerobacter aerogenes*. With one strain of *Proteus rettgeri*, however, an appreciable quantity of 6-APA was detected; the penicilloic acids of benzylpenicillin and 6-APA were also observed in cultures of this strain.

The activities of seven penicillins against Gram-negative bacteria

The relative activities of ampicillin, benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin, methicillin, and cloxacillin, against a variety of Gram-negative bacteria are shown in Table 1. From this it can be seen that of these compounds, ampicillin and benzylpenicillin alone showed appreciable activity. In general, ampicillin was two to ten times more active than benzylpenicillin, but certain strains were insensitive to both penicillins.

Table 1. *The relative activities of seven penicillins against certain Gram-negative bacteria*

	Antibiotic						
Organism	Ampicillin	Benzyl-penicillin	Phenoxy-methyl-penicillin	Phene-thicillin	Propi-cillin	Methi-cillin	Cloxa-cillin
	Minimal inhibitory concentration (μg./ml.)						
Escherichia coli 2	2·5	12·5	125	> 500	500	> 500	> 500
E. coli 6	5·0	25	250	500	250	> 500	500
E. coli 37	25	500	> 500	500	250	> 500	500
E. coli 83	500	> 500	> 500	> 500	500	> 500	> 500
Aerobacter aerogenes A	250	125	500	> 500	500	500	500
Klebsiella pneumoniae E	1·25	5·0	125	500	250	.	.
Proteus mirabilis 977	2·5	2·5	50	250	125	125	500
P. mirabilis 889	> 500	> 500	.	.	.	.	.
P. morganii A	125	> 500	> 500	500	500	.	.
Pseudomonas aeruginosa A	> 500	> 500	> 500	> 500	> 500	> 500	> 500
Salmonella typhi	1·25	2·5	50	250	125	> 500	500
S. paratyphi A	1·25	5·0	250	500	250	> 500	> 500
Shigella sonnei	5·0	25	125	500	250	> 500	> 500
S. flexneri	2·5	12·5	125	250	125	500	500

The relative stabilities of seven penicillins to the penicillinases of Gram-negative bacteria

The results of experiments designed to correlate the antibacterial activities of seven penicillins against two strains of *Escherichia coli* and the stabilities of these compounds to the penicillinases produced by the test bacteria are shown in Table 2. In these tests the minimal inhibitory concentrations of the antibiotics were measured in the usual fashion in nutrient broth using an ampicillin-sensitive strain and an

ampicillin-resistant strain of *E. coli*; in addition, the amount of destruction of penicillin which had occurred after overnight incubation was measured by microbiological assay. It will be seen that phenethicillin, propicillin, methicillin and cloxacillin were almost completely unaffected by the penicillinases produced by

Table 2. *The relative stabilities of ampicillin, benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin, methicillin, and cloxacillin to the penicillinases produced by two strains of Escherichia coli*

Minimal inhibitory concentrations of the penicillins against two strains of *Escherichia coli* were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Penicillin	Organism	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.								
Bacteria-free control*		500	250	125	50	25	12·5	5·0	2·5	1·25
Benzylpenicillin	*E. coli* 2	−	−	−	−	−	+	+	+	+
		470	245	96	45	17·2	2·2	2·2	0·9	0·4
	E. coli 83	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Ampicillin	*E. coli* 2	−	−	−	−	−	−	−	+	+
		560	245	120	53	24·0	13·4	5·3	2·4	1·3
	E. coli 83	±	+	+	+	+	+	+	+	+
		400	82	24	1·3	0·7	0	0	0	0
Phenoxymethylpenicillin	*E. coli* 2	±	±	+	+	+	+	+	+	+
		520	240	115	34	13·0	5·8	3·0	1·3	0·6
	E. coli 83	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Phenethicillin	*E. coli* 2	+	+	+	+	+	+	+	+	+
		530	250	132	53	27·0	13·0	4·7	2·6	1·25
	E. coli 83	+	+	+	+	+	+	+	+	+
		550	195	93	41	16·5	8·4	2·6	0·6	0
Propicillin	*E. coli* 2	±	+	+	+	+	+	+	+	+
		490	250	120	45	26·0	12·0	4·4	2·1	0·9
	E. coli 83	±	+	+	+	+	+	+	+	+
		510	250	127	49	23·0	11·0	3·0	1·3	0·4
Methicillin	*E. coli* 2	.	+	+	+	+	+	+	+	+
		.	250	128	54	25·0	13·0	5·1	2·6	1·2
	E. coli 83	.	+	+	+	+	+	+	+	+
		.	250	130	49	25·0	12·5	4·6	1·8	0·9
Cloxacillin	*E. coli* 2	.	+	+	+	+	+	+	+	+
		.	235	128	51	24·0	13·0	4·5	3·1	.
	E. coli 83	.	+	+	+	+	+	+	+	+
		.	245	130	51	25·0	13·0	4·0	0	.

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
\+ = Growth.
− = No growth.
± = Partial growth.

either strain of *E. coli*, but exerted little or no inhibitory effect on these cultures. In contrast, there was complete destruction of benzylpenicillin and marked destruction of ampicillin by the resistant strain of *E. coli* strain 83, and there was some

obvious destruction of benzylpenicillin by the sensitive strain of *E. coli* 2 at concentrations below the inhibitory value. Ampicillin was, however, unaffected by this strain. Phenoxymethylpenicillin appeared to occupy a mid-way position between these two groups of penicillins, inasmuch as this compound was almost inactive against one strain and was completely inactive against the other; but, nevertheless, there was obvious destruction of this penicillin by the ampicillin-sensitive organism and complete destruction by the second strain.

Table 3. *The activities of ampicillin and benzylpenicillin against Gram-negative bacteria causing minimal destruction of penicillins*

Minimal inhibitory concentrations of the penicillins against five strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.								
Bacteria-free control*		50	25	12·5	5·0	2·5	1·25	0·5	0·25	0·1
Salmonella typhi	Benzyl-penicillin	.	.	−	−	±	+	+	+	+
		.	.	.	5·0	2·3	1·4	0·7	0·28	0·16
	Ampicillin	.	.	−	−	−	−	±	+	+
		.	.	.	5·0	2·7	1·4	0·53	0·28	0·1
S. paratyphi A	Benzyl-penicillin	.	.	.	−	−	±	+	+	+
		.	.	.	5·0	1·9	1·4	0·7	0·28	0·16
	Ampicillin	.	.	.	.	−	−	±	+	+
		.	.	.	.	2·5	1·8	0·44	0·23	0·12
Proteus mirabilis 4	Benzyl-penicillin	.	.	−	−	−	+	+	+	+
		.	.	12·5	5·0	2·0	1·0	0·52	0·29	0·16
	Ampicillin	.	.	−	−	−	−	−	+	+
		.	.	.	5·0	2·6	1·4	0·53	0·22	0·16
P. mirabilis c977	Benzyl-penicillin	.	.	−	−	+	+	+	+	+
		.	.	10·0	4·2	1·9	1·4	0·44	0·29	0·16
	Ampicillin	.	.	.	−	−	−	+	+	+
		.	.	.	5·0	2·6	1·4	0·53	0·21	0·1
Klebsiella pneumoniae E	Benzyl-penicillin	.	.	−	−	−	±	+	+	+
		.	.	10·5	5·0	2·4	1·4	0·45	0·28	< 0·1
	Ampicillin	.	.	.	−	−	−	−	+	+
		.	.	.	5·6	2·9	1·3	0·6	0·23	< 0·1

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
+ = Growth.
− = No growth.
± = Partial growth.

The antibacterial activities and penicillinase stabilities of ampicillin and benzylpenicillin towards Gram-negative bacteria

As a result of numerous experiments to compare the relative antibacterial activities of ampicillin and benzylpenicillin, the Gram-negative bacteria were grouped as follows:

Group 1. *Gram-negative bacteria sensitive to both ampicillin and benzylpenicillin.* The results in Table 3 show the relative activities of ampicillin and benzylpenicillin against two Salmonella species, two strains of *Proteus mirabilis* and one strain of

Klebsiella pneumoniae. These organisms were inhibited by concentrations of 0·5–5·0 μg. penicillins/ml. but, in general, ampicillin was two to four times more active than was benzylpenicillin. Microbiological assay of these samples showed that there had been little or no destruction of either penicillin after incubation with the test organisms at 37° for 16–20 hr.

Group 2. Gram-negative bacteria sensitive to ampicillin but relatively resistant to benzylpenicillin. From Table 4 it can be seen that ampicillin inhibited the growth of three strains of *Escherichia coli* and one strain of each of *Shigella flexneri* and

Table 4. *Penicillinase activities of certain Gram-negative bacteria sensitive to ampicillin but moderately insensitive to benzylpenicillin*

Minimal inhibitory concentrations of the penicillins against five strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.								
Bacteria-free control*		50	25	12·5	5·0	2·5	1·25	0·5	0·25	0·1
Shigella flexneri	Benzyl penicillin	.	−	±	+	+	+	+	+	+
		.	13·5	13·0	0·44	0·2	0·16	0·12	< 0·1	< 0·1
	Ampicillin	.	.	.	−	−	−	+	+	+
		.	.	.	5·0	2·6	1·7	0·44	0·2	< 0·1
S. sonnei	Benzyl-penicillin	−	±	±	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
	Ampicillin	.	.	.	−	−	−	+	+	+
		.	.	.	5·0	2·1	1·05	0·12	< 0·1	< 0·1
Escherichia coli 2	Benzyl-penicillin	.	−	+	+	+	+	+	+	+
		.	17·0	1·9	0·64	0·46	1·8	0·12	< 0·1	< 0·1
	Ampicillin	.	.	.	−	−	±	+	+	+
		.	.	.	5·2	2·6	1·4	0·53	0·14	< 0·1
E. coli 61	Benzyl-penicillin	.	−	−	+	+	+	+	+	+
		.	20·0	6·8	0·34	0	0	0·12	0	0
	Ampicillin	.	.	.	−	−	−	+	+	+
		.	.	.	5·0	2·6	1·4	0·41	0·22	< 0·1
E. coli T 341	Benzyl-penicillin	−	−	+	+	+	+	+	+	+
		.	27·0	1·5	0	0	0	0	0	0
	Ampicillin	.	.	−	−	±	+	+	+	+
		.	.	15·0	4·4	2·65	0·63	0·24	0	0

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
\+ = Growth.
− = No growth.
± = Partial growth.

S. sonnei at concentrations of 1·25–2·5 μg./ml., whereas benzylpenicillin was inhibitory only at 12·5–25 μg./ml., thus showing a tenfold difference in the activities of these penicillins. Moreover, all five strains of bacteria destroyed most or all of the benzylpenicillin at concentrations below the minimal inhibitory concentrations, but there was little or no destruction of ampicillin by these organisms. These results showed the greater stability of ampicillin to the penicillinases produced by these bacteria, and suggested the possibility that the sensitivity of each strain to these

penicillins was related to the stabilities of the compounds to the penicillinase elaborated by each particular strain. This is supported by the results of an experiment shown in Fig. 1, illustrating the growth of one of these strains of bacteria, *E. coli* T341, in the presence of different concentrations of benzylpenicillin, and in Table 5 which shows the rate of destruction of the penicillin by this strain. It can be seen that at the lowest benzylpenicillin concentration (5 μg./ml.) growth was as rapid as in the control tube containing no antibiotic, while the concentration of antibiotic diminished steadily until none could be detected after 7 hr. At the next higher concentration (12·5 μg./ml.), there was little increase in growth for the first

Table 5. *Destruction of benzylpenicillin by cultures of Escherichia coli T341*

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Escherichia coli* T341 and incubated at 37° for 24 hr.; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration (μg./ml.)		
0	25	12·5	5·0
1	24	11·5	5·4
2	20	9·2	5·0
3	16	6·3	4·5
4	15	3·0	2·5
5	12	1·3	1·3
6	11	1·3	0·9
7	10	0	0
24	0	0	0

3–4 hr., while the penicillin concentration fell gradually to 3·0 μg./ml.; during the next 3 hr the turbidity of the culture increased steadily and the penicillin concentration fell to zero. At the highest concentration (25 μg./ml.), there was no increase in turbidity over a period of 7½ hr, during which time the penicillin concentration decreased from 25 to 10 μg./ml. After incubation overnight at 37° the turbidities of all these cultures were approximately equivalent to that of the control culture containing no penicillin, and no penicillin was detected for any concentration.

Group 3. *Gram-negative bacteria resistant to both ampicillin and benzylpenicillin.* Examples of bacteria resistant to ampicillin and to benzylpenicillin are shown in Table 6; it can be seen that all strains brought about marked destruction of both penicillins. In some cases, no penicillin could be detected in any of the culture tubes in which there was growth, e.g. *Escherichia coli* 70, *Proteus mirabilis* 889, *Klebsiella pneumoniae* C, *Aerobacter aerogenes* A and G. In other cases there was complete destruction of benzylpenicillin, but only partial destruction of ampicillin, for example, with strains of *P. rettgeri* A and *Ps. aeruginosa* A and C9. Yet again, with some strains there was only partial destruction of either penicillin, e.g. *E. coli* 37 and Paracolon WM29.

Quantitative estimation of penicillinase activity by *Pseudomonas aeruginosa* was made by bioautographic techniques, as it was noted that antibiotic-free cultures of this species displayed marked antibacterial activity against the assay organism, *Bacillus subtilis* ATCC 6633, with the result that microbiological assays showed little

Table 6. *Penicillinase activities of Gram-negative bacteria resistant to ampicillin and benzylpenicillin*

Minimal inhibitory concentrations of the penicillins against ten strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.								
Bacteria-free control*		500	250	125	50	25	12·5	5·0	2·5	1·25
Escherichia coli 70	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Proteus mirabilis 889	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae C	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Aerobacter aerogenes A	Benzyl-penicillin	−	−	+	+	+	+	+	+	+
		170	8	0	0	0	0	0	0	0
	Ampicillin	−	+	+	+	+	+	+	+	+
		90	0	0	0	0	0	0	0	0
A. aerogenes G	Benzyl-penicillin	−	+	+	+	+	+	+	+	+
		55	0	0	0	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa A	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	.	.	.	.	.	.	.
	Ampicillin	+	+	+	+	+	+	+	+	+
		530	135	.	.	.	.	.	.	.
Ps. aeruginosa C9	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	.	.	.	.	.	.	.
	Ampicillin	+	+	+	+	+	+	+	+	+
		375	150	.	.	.	.	.	.	.
Proteus rettgeri A	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	−	−	−	±	+	+	+	+	+
		520	260	120	40	9·2	0·88	0·2	0	0
E. coli 37	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		8·5	18·5	12·5	3·2	0·3	< 0·1	0·5	0·4	< 0·1
	Ampicillin	−	−	+	+	+	+	+	+	+
		505	220	105	31	13·0	6·5	1·7	0·6	0·2
Paracolon WM 29	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		160	72	15	15	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		330	135	82	29	9·3	7·1	1·5	0·35	0·1

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
+ = Growth. − = No growth. ± = Partial growth.

or no destruction of either penicillin by this species. In fact, most strains of *P. aeruginosa* were capable of causing some destruction of both penicillins, ampicillin being more resistant than benzylpenicillin.

It is of interest that of some 30 strains of Gram-negative bacteria tested which were resistant to ampicillin and benzylpenicillin, all were capable of causing some degree of destruction of penicillin. In this series, no organisms which did not produce penicillinase and were inherently penicillin insensitive were isolated. In general, ampicillin was more stable than benzylpenicillin to the penicillinases produced by these Gram-negative bacteria, save that strains of *Aerobacter aerogenes* appeared to destroy ampicillin to a rather greater extent than benzylpenicillin.

Further experiments were designed to attempt to determine to what extent the resistance of these bacteria was due to penicillinase production. A simple type of experiment appeared to be estimation of the effect of decrease in inoculum size on

Table 7. *The effect of alteration of inoculum size on the sensitivity of Gram-negative bacteria to ampicillin and benzylpenicillin*

Organism	Inoculum*	Ampicillin	Benzylpenicillin
		Minimal inhibitory concentrations (μg./ml.)	
Escherichia coli 70	Neat	> 500	500
	10^{-3}	12·5	25
	10^{-6}	12·5	25
Proteus vulgaris D	Neat	> 500	> 500
	10^{-3}	5·0	12·5
	10^{-6}	1·25	2·5
P. morganii A	Neat	125	> 500
	10^{-3}	25	125
	10^{-6}	2·5	12·5
Aerobacter aerogenes A	Neat	500	125
	10^{-3}	12·5	12·5
	10^{-6}	2·5	5·0
A. aerogenes G	Neat	500	125
	10^{-3}	12·5	12·5
	10^{-6}	2·5	5·0
Pseudomonas aeruginosa A	Neat	> 500	> 500
	10^{-3}	500	> 500
	10^{-6}	500	> 500
E. coli 83	Neat	> 500	> 500
	10^{-3}	> 500	> 500
	10^{-6}	500	> 500
P. mirabilis 889	Neat	500	500
	10^{-3}	500	250
	10^{-6}	250	250

* 1 drop of an overnight culture diluted as specified.

the sensitivity of the organism to penicillin, the rationale being that intrinsically sensitive, penicillinase-producing bacteria would show a marked increase in sensitivity to penicillins with smaller inocula, whereas very small inocula of inherently insensitive organisms would still be capable of proliferating in high

concentrations of penicillin. It can be seen from Table 7 that *Escherichia coli* 70, *Proteus vulgaris* D and *P. morganii* A, and *Aerobacter aerogenes* A and G were very much more sensitive to ampicillin and benzylpenicillin when small inocula were employed, whereas with *Pseudomonas aeruginosa* A, *E. coli* 83, and *P. mirabilis* 889 there was little or no change in sensitivity to ampicillin or benzylpenicillin, even when the inoculum was diminished by a factor of one in a million. From these results it would seem that the former five strains might be examples of intrinsically sensitive, penicillinase-producing bacteria, and that the latter three strains might serve as examples of intrinsically insensitive organisms. These results prompted further experiments designed to confirm these findings by correlating growth with concomitant penicillin destruction.

Table 8. *Destruction of benzylpenicillin by cultures of Aerobacter aerogenes* A

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Aerobacter aerogenes* A and incubated at 37° for 24 hr; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration (μg./ml.)		
0	100	25	5·0
1	100	25	4·3
2¼	90	20	2·6
4	37	2·8	1·6
5½	4·7	2·2	0·7
24	0	0	0

An example of an intrinsically sensitive, but penicillinase-producing organism is illustrated in Fig. 2, which shows the growth of *Aerobacter aerogenes* A in the presence of different concentrations of benzylpenicillin; the rate of destruction of the penicillin by this culture is shown in Table 8. The minimal inhibitory concentration of this culture after overnight incubation was 500 μg. benzylpenicillin/ml., but during the first 6 hr a concentration of 100 μg./ml. completely inhibited growth; during this period the penicillin concentration fell from 100 to 5 μg./ml. Thereafter growth took place so that after 24 hr the turbidity of this culture equalled that of a penicillin-free control culture. These results appear to confirm that the growth of this culture in the presence of benzylpenicillin was related to the rate of destruction of the antibiotic by the organism.

In contrast, an intrinsically insensitive strain of *Escherichia coli* 83 multiplied in the presence of concentrations of up to 500 μg. benzylpenicillin/ml. as rapidly as did an antibiotic-free culture (Fig. 3), although the rate of destruction of penicillin was relatively slow (Table 9), showing that the organism was able to proliferate readily in the presence of high concentrations of penicillin.

Thus, the inferences drawn from the inoculum-dilution experiments regarding the nature of the insensitivity of various Gram-negative bacteria to penicillins appeared to be supported by the growth experiments illustrated above, and would seem to confirm that whereas some penicillinase-producing strains of Gram-negative bacteria are inherently insensitive to penicillins, with other strains resistance is due mainly to destruction of the antibiotic.

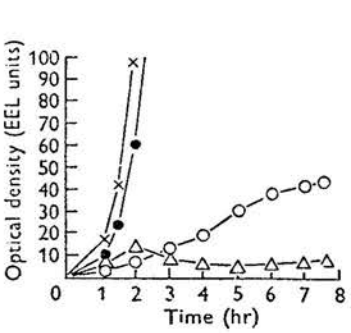

Fig. 1

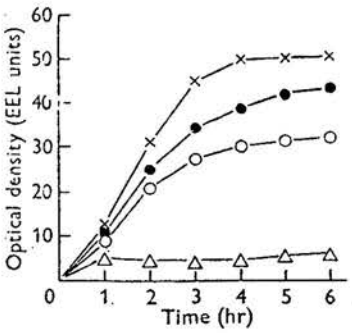

Fig. 2

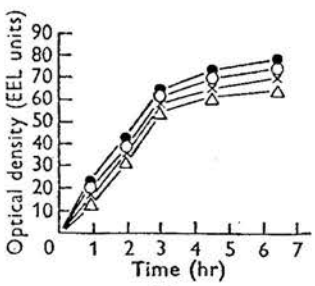

Fig. 3

Fig. 1. Growth of *Escherichia coli* (T341) in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 5 μg./ml.; ○, culture containing penicillin, 12·5 μg./ml.; △, culture containing penicillin, 25 μg./ml.

Fig. 2. Growth of *Aerobacter aerogenes* A in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 5 μg./ml.; ○, culture containing penicillin, 25 μg./ml.; △, culture containing penicillin, 100 μg./ml.

Fig. 3. Growth of *Escherichia coli* (83) in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 25 μg./ml.; ○, culture containing penicillin, 125 μg./ml.; △, culture containing penicillin, 500 μg./ml.

Table 9. *Destruction of benzylpenicillin by cultures of Escherichia coli 83*

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Escherichia coli* 83 and incubated at 37° for 24 hr; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration (μg./ml.)		
0	500	125	25
1	490	124	20
3½	465	115	16
5½	425	90	8
24	0	0	0

DISCUSSION

On the basis of the results reported here, the penicillins which display a relatively low order of activity against any of the Gram-negative bacteria examined may be readily distinguished from those penicillins which show a significant effect against certain strains. Compounds in the former group include phenethicillin, propicillin, methicillin and cloxacillin, and it is plain that the insensitivity of the Gram-negative bacteria to these penicillins is in no way associated with penicillinase. In contrast, the sensitivities of Gram-negative bacteria to ampicillin and benzylpenicillin were very varied, and in many cases the reaction of the organism to these drugs appeared to depend upon the ability of the organism to produce a penicillinase capable of destroying the specific penicillin. From the experiments described above, the bacteria tested could be divided into three groups, according to the effect of the specific penicillinase upon ampicillin or benzylpenicillin, e.g.:

(*a*) Strains which produced little or no penicillinase and were sensitive to

ampicillin and to benzylpenicillin, e.g. one Salmonella species, and some strains of *Proteus mirabilis* and Klebsiella.

(*b*) Strains which caused little or no destruction of ampicillin but more marked destruction of benzylpenicillin, and which were, accordingly, sensitive to ampicillin but less sensitive to benzylpenicillin, e.g. strains of *Escherichia coli*, Shigella species.

(*c*) Strains which were capable of destroying both compounds. This group, comprising penicillin-resistant organisms, could be further divided into: (i) intrinsically sensitive, penicillinase-producing, bacteria, e.g. strains of *Aerobacter aerogenes*, *P. vulgaris*, *P. morganii;* (ii) intrinsically insensitive, penicillinase-producing, bacteria, e.g. *Pseudomonas aeruginosa*, strains of *E. coli* and *P. mirabilis*.

It follows from these findings that the main difference in the activities of ampicillin and benzylpenicillin is associated with the increased stability of ampicillin towards the penicillinases produced by certain Gram-negative bacteria. Consequently, while ampicillin is from two to four times more active than benzylpenicillin against Gram-negative bacteria which produce little or no penicillinase, it is at least ten times more active against many strains which do produce penicillinase. The latter would apparently include the large number of strains of *Escherichia coli* which are sensitive to relatively low concentrations of ampicillin, but which require considerably higher concentrations of benzylpenicillin for inhibition. It is worth noting, however, that with these organisms growth is dependent upon destruction of the penicillin in the culture medium, so that benzylpenicillin might seem to be considerably more effective if antibacterial activity were measured over a shorter period of time than is conventional. Strains resistant to both penicillins are usually capable of destroying both compounds, although, again, ampicillin is generally more stable to the action of the penicillinases produced by these organisms. An exception to this finding is that benzylpenicillin appears to be more stable to *Aerobacter aerogenes* than is ampicillin but there is usually extensive destruction of both compounds. These findings are in good agreement with those of various other workers who have reported that ampicillin displays increased stability to the penicillinases produced by Gram-negative bacteria (Auhagen *et al.* 1962; Ayliffe, 1963; Smith, 1963). The last two workers also noted the superior stability of benzylpenicillin to the penicillinases produced by Aerobacter species.

In this series of experiments, all the resistant strains of bacteria produced penicillinase, whereas Trafford *et al.* (1962) reported that a relatively high proportion of bacteria which did not produce penicillinase were resistant to ampicillin. On the other hand, Barber (1962) reported that the majority of coliform bacteria are penicillinase-producing organisms capable of destroying penicillins. It is possible that in some cases at least, divergences in findings may be due to differences in techniques; this is being investigated. Likewise, although Knox & Smith (1962) have described strains of Gram-negative bacteria which are sensitive to ampicillin but with inherent resistance to benzylpenicillin, such strains have not been isolated in the experiments described above.

It thus seems that benzylpenicillin is active against strains of Gram-negative bacteria which produce little or no penicillinase, but is ineffective against penicillinase-producing bacteria. In contrast, ampicillin is relatively stable to the penicillinases produced by certain of the Gram-negative bacteria, and is, as a consequence, substantially more active than benzylpenicillin against many of these

bacteria. With resistant strains of bacteria, however, the situation is much more complex; here insensitivity may be due to penicillinase activity alone, or may be due to inherent resistance, although in the results reported here this inherent resistance was always accompanied by some degree of penicillin destruction.

It is a pleasure to thank Dr M. O. Moss for his assistance in carrying out the chromatographic procedures and Dr G. N. Rolinson for much helpful advice and criticism.

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The Role of Penicillin Acylase in the Resistance of Gram-Negative Bacteria to Penicillins

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SUMMARY

The resistance of 148 clinical isolates of Gram-negative bacteria to ampicillin and benzylpenicillin was determined in serial-dilution sensitivity tests, together with the extent of inactivation of the penicillins, and the production of 6-aminopenicillanic acid (6-APA). Many of the cultures were resistant to the penicillins and inactivated the compounds, but only one culture, a strain of *Escherichia coli*, showed penicillin acylase activity, as indicated by the production of 6-APA. However, the cultural conditions prevailing in serial-dilution tests, namely, stationary culture at 37°, although favourable for the functioning of penicillin acylases, were shown to be highly unfavourable for production of the enzyme by coliform bacteria.

INTRODUCTION

The resistance of certain Gram-negative bacteria to ampicillin and benzylpenicillin has been shown to be associated with the destruction of the penicillins by the β-lactamase (penicillinase, Fig. 1) activity of these organisms (Ayliffe, 1963; Percival, Brumfitt & de Louvois, 1963; Sutherland, 1964). However, the occurrence of penicillin acylase activity, leading to the hydrolysis of penicillins with the production of the relatively inactive 6-aminopenicillanic acid (6-APA) (Fig. 1) has been reported in many species of Gram-negative bacteria (Kaufmann & Bauer, 1960; Rolinson *et al.* 1960; Claridge, Gourevitch & Lein, 1960; Huang, Seto & Shull, 1963), and resistance to penicillins as a result of penicillin acylase activity has been described by English, McBride & Huang (1960) and by Holt & Stewart (1964*a*). The latter authors reported that 40% of 310 clinical strains of *Escherichia coli* examined produced penicillin amidase (penicillin acylase), but the properties of the enzyme extracted from one of these cultures, *E. coli* NCIB 9465, (Holt & Stewart, 1964*b*) differed very markedly from those of the penicillin acylases produced by other strains of *E. coli* (Rolinson *et al.* 1960; Kaufmann, Bauer & Offe, 1960; Kaufmann & Bauer, 1964).

The experiments described here were designed to investigate the occurrence of penicillin acylase activity among Gram-negative bacteria of clinical origin and to ascertain the role of this enzyme in the resistance of bacteria to penicillins. Because optimum cultural conditions for the formation of penicillin acylase by *Escherichia coli* (Kaufmann & Bauer, 1964) are different from those prevailing in antibiotic sensitivity tests, a lack of activity in these tests might be due either to intrinsic inability of the organism to form the enzyme or to the cultural conditions being unfavourable for production of penicillin acylase. Accordingly, the Gram-negative

bacteria used in these experiments were examined for penicillin acylase activity, not only in the serial-dilution tests employed routinely for the determination of bacterial sensitivity to antibiotics, but also in experiments using cultural conditions found to be especially favourable for the formation of penicillin acylase by Entero-

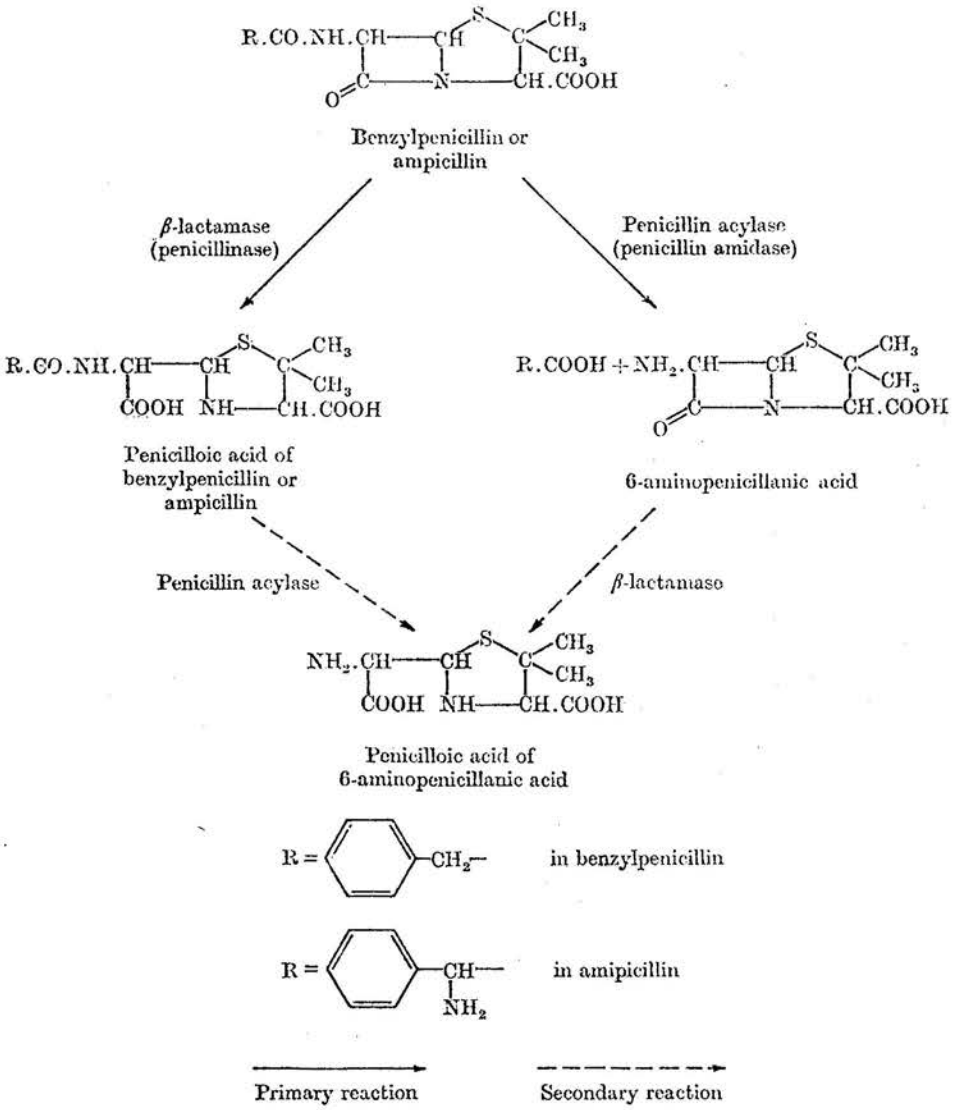

Fig. 1. Degradation of penicillins by Gram-negative bacteria.

bacteriaceae. It was hoped by this means to obtain a true measure of the occurrence of penicillin acylase among Gram-negative bacteria. Three strains of *E. coli* reported by Holt & Stewart (1964*a*, *b*) to be penicillin acylase-producing cultures were also included in the studies reported here.

METHODS

Bacterial cultures. A total of 148 strains of Gram-negative bacteria which were predominantly resistant to ampicillin and benzylpenicillin were obtained from several hospitals. In addition, ten strains of bacteria of non-clinical origin known to produce penicillin acylase were included in these experiments.

Effects of cultural conditions on penicillin acylase formation in Escherichia coli. strain c 975. Conical flasks (500 ml.) containing 50 ml. of medium were inoculated with 0·5 ml. of an overnight nutrient broth culture of a clinical strain of *E. coli*. c 975. The cultures were incubated stationary or on a rotary shaker, at 26° or 37° (see Table 1 for details). After 18 hr sodium benzylpenicillin was added to all flasks to a final concentration of 1 mg. penicillin/ml. The penicillin was added in M/4 phosphate buffer (pH 8·5) as penicillin acylase is known to be most active under alkaline conditions (Rolinson *et al.* 1960). All flasks were shaken for 4 hr at 30° after which samples were assayed for 6-APA as described below. The following media were employed:

(*a*) Yeatex medium consisting of 2·5% Yeatex paste (Standard grade, English Grains Ltd., Shobnall Road, Burton-on-Trent, Notts.) in water, adjusted to pH 7·0.

(*b*) Yeatex medium + 0·15% phenylacetic acid. This acid was dissolved in NaOH to give a 5% solution at pH 7·0 which was added to the Yeatex medium to the desired concentration, and the whole was adjusted to pH 7·0 before sterilization at 15 lb./sq. in. for 15 min.

(*c*) Nutrient broth (Oxoid no. 2) adjusted to pH 7·0.

(*d*) Nutrient broth + 0·15% phenylacetic acid as in (*b*).

Measurement of antibacterial activity. Minimal inhibitory concentrations of ampicillin and benzylpenicillin required to inhibit growth of the test bacteria were measured by two-fold serial dilution in nutrient broth (Oxoid no. 2). The penicillins were incorporated in graded concentrations in 5 ml. volumes of nutrient broth which were inoculated with one drop (0·03 ml.) of an overnight culture of the test organism. Inhibitory concentrations were determined after overnight incubation at 37°.

Measurement of penicillin destruction in penicillin sensitivity tests. The destruction of penicillins in the antibacterial tests described above was estimated by microbiological assay of residual penicillin with an agar diffusion method (Sutherland, 1964). Certain samples were also examined for β-lactamase activity by chromatographic examination for penicilloic acids.

Penicillin acylase activity in penicillin sensitivity tests. Samples were taken from cultures containing the highest concentration of penicillin allowing growth after overnight incubation and these were examined for 6-APA.

Penicillin acylase in shaken flask cultures. Two 500 ml. conical flasks containing 50 ml. of a solution consisting of 2·5% Yeatex paste and sodium phenylacetate at a final concentration equivalent to 0·1% phenylacetic acid were inoculated with 0·5 ml. of an overnight nutrient broth culture of the test organism. The flasks were shaken on a rotary shaker (1 in. throw) at 24° for 24 hr Ten ml. of a 6 mg./ml. solution of sodium benzylpenicillin in M/4 phosphate buffer (pH 8·5) were added to one of the flasks to give a concentration of 1 mg./ml. penicillin. A similar quantity of ampicillin was added to the second flask. The flasks were transferred to a rotary shaker and shaken at 30°. After 4 hr 0·5 ml. of *n*-butyl acetate was added to each

flask and the shaking was continued for a further hour by which time the cultures were non-viable. The flasks were assayed for 6-APA content by paper chromatography as described below. Solutions of benzylpenicillin and ampicillin were also chromatographed to provide standards for the estimation of the amount of residual penicillin in the reaction mixtures.

Analytical methods. Detection and estimation of 6-APA. Filter paper strips, Whatman no. 1, 1 cm. wide, were spotted in duplicate with 6 μl. of the samples under test. The benzylpenicillin reaction mixtures were chromatographed with a butanol + ethanol solvent system (*n*-butanol + ethanol + water 4 + 1 + 5 v/v top phase). Ampicillin reaction mixtures were chromatographed in a butanol + acetic acid solvent system (*n*-butanol + acetic acid + water, 12 + 3 + 5 v/v). In both cases chromatography was carried out overnight at 5°. The chromatograms were dried at 40° and one of each pair was sprayed with 5% aqueous sodium bicarbonate, 5% phenylacetyl chloride in acetone and 5% aqueous sodium bicarbonate, consecutively, to convert 6-APA into benzylpenicillin (Batchelor, Doyle, Naylor & Rolinson, 1959). The zones of ampicillin, benzylpenicillin and phenylacetylated 6-APA were located by placing dried chromatograms on nutrient agar seeded with a spore suspension of *B. subtilis* ATCC 6633 and incubated at 37°. The appearance on only the phenylacetylated chromatogram of an antibacterial zone at the R_F given below was taken to indicate the presence of 6-APA. Solutions of 6-APA in M/20, pH 7, phosphate buffer were also chromatographed alongside samples of reaction mixtures so as to provide standards for the estimation of 6-APA. On the phenylacetylated chromatograms a solution of 6-APA 5 μg./ml. yielded an inhibition zone 14–15 mm. wide at an R_F of about 0·44 in butanol + acetic acid and 16–18 mm. wide at an R_F of about 0·12 in butanol + ethanol. Under these conditions 6-APA 1 μg./ml. could be detected. Phenylacetylated chromatograms of the benzylpenicillin and ampicillin solutions showed no zones of 6-APA. Benzylpenicillin had an R_F of 0·89 in butanol + ethanol and ampicillin an R_F of 0·74 in butanol + acetic acid.

Detection of penicilloic acids. Test samples (6–30 μl.), containing about 3 μg. penicilloic acid were applied to sheets of Whatman No. 1 chromatography paper and 3 μg. of the penicilloic acids of ampicillin, benzylpenicillin and 6-APA were also treated in the same way, as standards. The penicilloic acids were prepared in two ways. In the first method 5 mg. of penicillin or 6-APA in 5 ml. of water was treated with 0·75 ml. of 5 N-NaOH for 30 min. at 37°, after which the solution was neutralized with 0·75 ml. of 5 N-HCl and the volume made up to 10 ml. with water. In the second method 5 mg. of penicillin or 6-APA in 5 ml. M/20 phosphate buffer (pH 7) was treated for 30 min. at 37° with 1 ml. of the supernatant fluid of a penicillinase (β-lactamase)-producing culture of *Bacillus cereus*, after which the volume was adjusted to 10 ml. The chromatograms were developed with a butanol + pyridine solvent (*n*-butanol + pyridine + water, 1 + 1 + 1 v/v) overnight at 5° after which they were dried at 40° and sprayed with the starch + iodine + acetic acid spray described by Thomas (1961), to reveal white penicilloic acid zones on a dark background. The approximate R_F values of the various compounds in the above solvent system were:

Benzylpenicillin	0·75	Ampicillin penicilloic acid	0·37
Ampicillin	0·62	6-APA penicilloic acid	0·28
Benzylpenicilloic acid	0·51		

RESULTS

Factors affecting penicillin acylase formation by Escherichia coli c 975

The results given in Table 1 illustrate the effect of cultural conditions on the formation of penicillin acylase in *Escherichia coli* c 975, one of the clinical strains of bacteria examined. Under the most favourable conditions, namely, growth at 26° in a shaken flask of Yeatex medium containing phenylacetic acid, all the available benzylpenicillin was converted to 6-APA. It can be seen that the cultural conditions had a significant effect on the amount of growth, but enzyme formation was not necessarily proportional to bacterial growth. For example, the shaken cultures grown in nutrient broth at 26° and at 37° produced similar quantities of bacteria, but the penicillin acylase activity of the culture grown at 26° was at least a hundred-fold greater than that of the culture grown at 37°. Likewise, the growth-promoting effect of phenylacetic acid did not account entirely for the observed increase in enzyme activity.

Table 1. *Effect of cultural conditions on the production of penicillin acylase by a clinical isolate of Escherichia coli c 975*

E. coli c 975 was grown for 18 hr under the conditions shown below, after which benzylpenicillin to a final concentration of 1 mg./ml. was added to each flask. The cultures were incubated at 30° for 4 hr, when the concentration of 6-APA was measured.

Basal medium	Cultural conditions: Temperature	Cultural conditions: Aeration	0·15 % phenylacetic acid	Growth: dry wt. bacteria (mg./ml.)	Enzyme activity: 6-APA (μg./ml.)
2·5 % nutrient broth pH 7·0	37°	Static	Absent	0·38	Trace
	37°	Shaken	Absent	1·13	Trace
	26°	Static	Absent	0·33	18
	26°	Shaken	Absent	1·39	100
	26°	Shaken	Present	2·14	515
2·5 % Yeatex pH 7·0	26°	Shaken	Present	3·6	635

Distribution of minimal inhibitory concentrations

The organisms tested for sensitivity to ampicillin and benzylpenicillin, together with the respective minimal inhibitory concentrations, are shown in Table 2. Because the organisms used in this work were predominantly resistant to the penicillins the proportion sensitive to the penicillins in these tests was smaller than is typical of strains encountered routinely in clinical practice (Sutherland & Rolinson, 1964; Sutherland & Batchelor, 1964).

Penicillin acylase activity in penicillin sensitivity tests

In the sensitivity tests with 148 clinical cultures of bacteria, 133 strains (90 %) destroyed benzylpenicillin and 91 strains (61 %) caused some destruction of ampicillin. However, only one culture, *Escherichia coli* 1187, showed any penicillin acylase in these tests. With this culture a trace of 6-APA (< 1 μg./ml.) was detected in the test with benzylpenicillin but none was found with ampicillin. The conditions of pH (alkaline) and temperature (37°) prevailing in these sensitivity tests were favourable for penicillin acylase activity (Rolinson *et al.* 1960), hence lack of enzyme activity in these tests was due to absence of the enzyme.

Table 2. *Distribution of minimal inhibitory concentrations of ampicillin and benzylpenicillin against 148 strains of Gram-negative bacteria*

Organism	No. of strains	Penicillin	Minimal inhibitory concentrations (μg./ml.) and number of strains inhibited									
			> 500	500	250	125	50	25	12·5	5·0	2·5	1·25
Escherichia coli	51	Ampicillin	4	2	3	1	4	3	2	14	15	3
		Benzylpenicillin	10	1	2	3	22	9	2	2	.	.
Aerobacter aerogenes	17	Ampicillin	5	3	2	.	3	.	2	.	1	1
		Benzylpenicillin	8	2	2	3	.	1	1	.	.	.
E. coli (intermediate) and paracolon	18	Ampicillin	8	2	.	1	.	.	5	.	1	1
		Benzylpenicillin	10	1	.	.	3	3	.	1	.	.
Proteus mirabilis	16	Ampicillin	8	.	.	.	.	.	.	1	.	7
		Benzylpenicillin	8	.	.	.	.	.	.	1	6	1
P. morganii	7	Ampicillin	.	.	2	5	.	.	.	.	.	.
		Benzylpenicillin	6	.	.	1	.	.	.	.	.	.
P. rettgeri	8	Ampicillin	2	1	2	1	1	.	1	.	.	.
		Benzylpenicillin	8	.	.	.	.	.	.	.	.	.
P. vulgaris	11	Ampicillin	4	4	.	.	2	.	1	.	.	.
		Benzylpenicillin	7	1	.	3	.	.	.	.	.	.
Pseudomonas pyocyanea	13	Ampicillin	13	.	.	.	.	.	.	.	.	.
		Benzylpenicillin	13	.	.	.	.	.	.	.	.	.
Salmonella sp.	5	Ampicillin	.	.	.	.	.	.	.	3	2	.
		Benzylpenicillin	.	.	.	.	.	.	5	.	.	.
Shigella sp.	2	Ampicillin	.	.	.	.	.	.	.	2	.	.
		Benzylpenicillin	.	.	.	.	2	.	.	.	.	.
Total	148	Ampicillin	44	12	9	8	10	3	11	20	19	12
		Benzylpenicillin	70	5	4	10	27	13	8	4	6	1

Table 3. *Effect of cultural conditions on penicillin acylase activity of Gram-negative bacteria*

The minimal inhibitory concentrations of ampicillin and benzylpenicillin were determined after incubation overnight at 37°. Penicillin destruction was measured by microbiological assay of residual penicillin content and penicillin acylase activity was determined by measurement of 6-APA. In the shaken culture test for production of penicillin acylase activity under favourable conditions the cultures were shaken overnight at 24° in Yeatex medium containing 0·1 % phenylacetic acid. Penicillin (1 mg./ml.) was added and the cultures were shaken at 30° for 4 hr after which the 6-APA content was measured.

	Benzylpenicillin					Ampicillin				
	Shaken culture test for penicillin acylase		Serial-dilution test			Shaken culture test for penicillin acylase		Serial-dilution test		
Strain	6-APA (μg./ml.)	Residual penicillin (μg./ml.)	Minimal inhibitory concentration (μg./ml.)	Penicillin destruction	Penicillin acylase activity 6-APA (μg./ml.)	6-APA (μg./ml.)	Residual penicillin (μg./ml.)	Minimal inhibitory concentration (μg./ml.)	Penicillin destruction	Penicillin acylase activity 6-APA (μg./ml.)
E. coli I 187	105	0	50	±	Trace*	> 200	> 200	5	±	0
E. coli C 975	90	0	50	+	0	> 200	> 200	5	−	0
E. coli T 432	> 200	0	12·5	−	0	> 200	190	1·25	−	0
E. coli I 199	> 200	> 200	25	+	0	180	> 200	5	±	0
P. rettgeri A	> 200	68	> 500	+	0	32	> 200	50	±	0
P. rettgeri C	> 200	> 200	> 500	+	0	24	> 200	> 500	±	0
P. rettgeri D	> 200	0	> 500	+	0	32	> 200	250	±	0
P. rettgeri F	Trace	> 200	> 500	+	0	3	> 200	12·5	±	0
P. rettgeri G	> 200	68	> 500	+	0	37	> 200	500	±	0
P. rettgeri I	> 200	0	> 500	+	0	100	> 200	> 500	±	0
E. coli NCIB 8742†	> 200	0	50	±	0	> 200	170	5	±	0
E. coli NCIB 8744†	80	> 200	25	±	0	25	> 200	5	−	0
E. coli BRL 1009†	> 200	1	12·5	±	0	> 200	42	5	−	0
E. coli NCIB 8741†	> 200	0	50	±	0	> 200	70	5	±	0
A. faecalis BRL 1238†	> 200	> 200	12·5	±	Trace	160	> 200	12·5	±	0
A. faecalis BRL 1237†	> 200	130	25	±	1	200	> 200	25	−	0
A. faecalis NCTC 415†	28	200	50	±	2	32	> 200	25	±	0
A. faecalis NCIB 8156†	3	0	125	±	2	5	0	250	±	Trace
Achr. arsenoxydans NCIB 8687†	12	190	12·5	−	0	0	150	12·5	−	0
Ps. pyocyanea BRL 1058†	Trace	> 200	> 500	+	0	3	> 200	> 500	±	Trace

+ = complete destruction; ± = partial destruction; − = no destruction.

† Known penicillin-acylase producing bacteria of non-clinical origin.

* In a repeat test no 6-APA was detected and no residual benzylpenicillin was found.

Formation of penicillin acylase by clinical strains of Gram-negative bacteria in shaken flask cultures

Of the 148 clinical strains of Gram-negative bacteria tested in the shaken flask test with Yeatex medium containing phenylacetic acid, ten strains were found to show penicillin acylase activity (see Table 3). Most of these strains formed large amounts of 6-APA from benzylpenicillin and ampicillin but the ratio of penicillin acylase activities against the two substrates varied from culture to culture. One of the reasons for this is the simultaneous production of penicillin acylase and β-lactamase in some cultures. For example, with benzylpenicillin as substrate, far less than the theoretical maximum yield of 6-APA (viz. 608 μg./ml.) was obtained from cultures of *Escherichia coli* I 187 and C 975, although the amount of residual benzylpenicillin in the reaction mixture had fallen to zero, suggesting that some other penicillin-destroying factor, such as β-lactamase, was operating. However, when ampicillin was used as substrate for these two cultures, the yields of 6-APA were greater and residual ampicillin was present presumably because it was not destroyed by β-lactamase. These results are consistent with the greater stability of ampicillin to penicillinase (β-lactamase) activities of certain Gram-negative bacteria (Ayliffe, 1963; Percival, Brumfitt & de Louvois, 1963; Smith, 1963; Sutherland, 1964). Where the penicillin acylase activity of cultures was not complicated by the presence of β-lactamase, as evidenced by the presence of substantial quantities of residual benzylpenicillin, viz. *Escherichia coli* I 199 and *Proteus rettgeri* C, benzylpenicillin was a better substrate for penicillin acylase than ampicillin. Cultures of *E. coli* T 432 and *P. rettgeri* A, D, G and I, incubated with benzylpenicillin contained more than 200 μg./ml. 6-APA, and low levels of residual penicillin, suggesting that penicillin acylase was the predominant enzyme, although the presence of some β-lactamase activity cannot be ruled out. Of these cultures, *E. coli* T 432 would appear to be the most active penicillin acylase producer judging by the high yield of 6-APA obtained from ampicillin.

The results in Table 3 also include data obtained with these cultures in the serial-dilution sensitivity tests. Although all ten strains produced penicillin acylase in the shaken culture test, this enzyme was absent in serial-dilution tests except in the case of *Escherichia coli* I 187 for which a trace of 6-APA was detected. These cultures were also examined in the serial-dilution tests for the presence of penicilloic acids. In all cultures where there was significant destruction of the penicillin, the cognate penicilloic acid was detected signifying β-lactamase activity. In no case was the penicilloic acid of 6-APA observed.

Comparative tests with known penicillin acylase-producing bacteria

The yields of 6-APA produced by ten strains of saprophytic bacteria in penicillin acylase-producing shaken culture tests are shown in Table 3. Comparison of the yields of 6-APA with the amount of residual benzyl penicillin or ampicillin showed that two strains of *Alcaligenes faecalis* (BRL 1237 and BRL 1238) produced high penicillin acylase activity and in view of the high residual penicillin values, little or no β-lactamase activity. Three strains of *Escherichia coli* (NCIB 8742, NCIB 8741 and BRL 1009) also produced high amounts of penicillin acylase but as the residual benzyl-

penicillin values were of a low order it is possible that β-lactamase was also present. The remaining bacteria demonstrated lower penicillin acylase activity and with *A. faecalis* NCIB 8156, which produced only 6-APA 3–5 μg./ml., substantial β-lactamase activity was present.

In serial-dilution tests with these cultures, penicillin acylase activity was not observed with any of the strains of *Escherichia coli*. Small amounts of 6-APA were detected with *Alcaligenes faecalis* in the tests with benzylpenicillin and only one of these strains produced 6-APA in the test with ampicillin. A trace of 6-APA was found in the culture of *Pseudomonas pyocyanea* BRL 1058 incubated with ampicillin. In no instance did the 6-APA measured account for all of the penicillin destruction observed in the sensitivity tests indicating that β-lactamase activity was also present.

Destruction of penicillins by Escherichia coli NCIB *9464, NCIB 9465 and* NCIB *9466*

Cultures of *Escherichia coli* NCIB 9464 NCIB 9465 and NCIB 9466, reported by Holt & Stewart (1964*a*, *b*) to produce 6-APA from penicillins, were obtained from the National Collection of Industrial Bacteria. All three cultures were resistant to

Table 4. *Destruction of penicillins by Escherichia coli* NCIB *9464, NCIB 9465 and* NCIB *9466 in sensitivity tests*

Minimal inhibitory concentrations of benzylpenicillin and 6-APA required to inhibit the growth of *E. coli* NCIB 9464, NCIB 9465, and NCIB 9466 for 18 hr at 37° were determined by serial dilution in nutrient broth. After overnight incubation, residual antibiotic activity was measured by microbiological assay, and 6-APA and penicilloic acid production in the benzylpenicillin sensitivity tests was determined by chromatography

Strain	Penicillin	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.						
		500*	250	125	50	25	12·5	5·0
Escherichia coli NCIB 9464	Benzylpenicillin	+	+	+	+	+	+	+
		0†	0	0	.	.	.	.
	6-APA	−	−	−	+	+	+	+
		.	.	.	36	16	10	4·2
E. coli NCIB 9465	Benzylpenicillin	+	+	+	+	+	+	+
		0†	0	0	.	.	.	.
	6-APA	−	−	−	+	+	+	+
		.	.	.	33	18	7·6	3·8
E. coli NCIB 9466	Benzylpenicillin	−	+	+	+	+	+	+
		360	0†	0	.	.	.	.
	6-APA	−	−	−	+	+	+	+
		.	.	.	38	19	6·1	3·8

+ = growth; − = no growth.

* The concentrations represent the initial concentrations (μg./ml.) of penicillins prior to incubation with the cultures.

† Chromatographic examination of these cultures showed large zones of benzylpenicilloic acid and no trace of 6-APA.

benzylpenicillin in serial-dilution tests and brought about complete destruction of the penicillin in these tests (Table 4). Chromatographic examination of these cultures failed to show the presence of 6-APA but, did show benzylpenicilloic acid.

Destruction of benzylpenicillin in these serial-dilution tests was due therefore to penicillinase (β-lactamase) activity. In contrast, 6-APA was highly stable to these cultures of *E. coli*, there being little or no destruction of the compound in the serial-dilution tests (Table 4). Moreover, when these strains of *E. coli* were grown under conditions favourable for penicillin acylase production as described in the Methods, no trace of penicillin acylase activity was detected in reactions with benzylpenicillin. However, chromatographic examination of the reaction mixtures showed the presence of benzylpenicilloic acid, indicating β-lactamase activity.

As the three strains of *Escherichia coli* failed to show penicillin acylase activity in the tests described above, *E. coli* NCIB 9465 was cultured and tested under the conditions reported by Holt & Stewart (1964*b*). Thus, the organism was grown for 48 hr at 37° in nutrient broth containing benzylpenicillin 100 μg./ml., but after incubation of the 48-hr old culture with benzylpenicillin at 40°, pH 5·5, no trace of 6-APA was detected. Instead, chromatographic examination of the reaction mixtures at pH 5·5 showed the presence of large amounts of benzylpenicilloic acid and a small amount of benzylpenilloic acid, the the decarboxylation product of benzylpenicilloic acid (Pl. 1). Similarly, a dialysed, freeze-dried specimen of enzyme prepared from *E. coli* NCIB 9465 as described by Holt & Stewart (1964*b*) failed to show penicillin acylase activity, but destroyed benzylpenicillin as a result of β-lactamase activity.

DISCUSSION

In the experiments described here many of the strains of Gram-negative bacteria used were resistant to ampicillin and benzylpenicillin and were capable of destroying these penicillins in the serial-dilution sensitivity tests, but in only one case, a strain of *Escherichia coli* I 187, was there any evidence of penicillin acylase activity, namely the production of 6-APA from benzylpenicillin. In this series of tests, therefore, penicillin acylase activity was not a significant factor in the penicillin resistance shown by these cultures. Even with *E. coli* I 187, in which penicillin acylase was present, penicillin destruction was due primarily to β-lactamase activity.

It has been our previous experience in this laboratory that production of penicillin acylase by non-clinical strains of *Escherichia coli* is favoured by low temperature, vigorous aeration and the addition of phenylacetic acid to the medium. The results reported here indicate that these cultural conditions are also favourable for the production of penicillin acylase by bacteria of clinical origin. Even so, out of 148 cultures of clinical bacteria tested, only ten cultures, four strains of *E. coli* and six strains of *Proteus rettgeri*, converted ampicillin and benzylpenicillin to 6-APA under these conditions. It is evident, therefore, that only a relatively small number of Gram-negative bacteria of clinical origin are capable of producing penicillin acylase. Moreover, these results confirm that the conditions prevailing in sensitivity tests where the bacteria are cultured at alkaline pH at 37° in stationary culture, although favourable for the functioning of the enzyme (Rolinson *et al.* 1960), are most unfavourable for penicillin acylase formation. Hence, most strains of penicillin acylase-producing bacteria are unable to produce the enzyme in serial-dilution tests, but may instead bring about destruction of penicillins by β-lactamase activity.

The resistance of bacteria to penicillins as a result of penicillin acylase activity

has been described by English *et al.* (1960) but few details of experimental conditions or extent of occurrence were given. More recently, Holt & Stewart (1964*a*, *b*) have reported penicillin acylase activity to play a significant role in bacterial resistance to penicillins and have described the occurrence of penicillin acylase activity in 40% of clinical strains of coliform bacteria grown in normal nutrient media at 37°. However, we have been unable to demonstrate penicillin acylase activity with cultures of *Escherichia coli* NCIB 9464, NCIB 9465 and NCIB 9466 reported by Holt & Stewart (1964*a*, *b*) to be active penicillin acylase-producing cultures. On the contrary, we have found that the inactivation of penicillin by these strains was due solely to β-lactamase activity with the formation of the cognate penicilloic acid.

The reason for the striking discrepancy between the results reported here with *Escherichia coli* NCIB 9464, NCIB 9465 and NCIB 9466 and those reported by Holt & Stewart (1964*a*, *b*) would appear to be due to the fact that Holt & Stewart classified as penicillin acylase-producing bacteria, cultures which inactivated benzylpenicillin but not 6-APA. However, it is known that 6-APA is usually more stable to the penicillinases (β-lactamases) of bacteria than is benzylpenicillin (Percival, Brumfitt & de Louvois, 1963; Smith & Hamilton-Miller, 1963). Hence, β-lactamase producing strains of *E. coli* might well destroy benzylpenicillin much more rapidly than 6-APA and this is the case with *E. coli* NCIB 9464, NCIB 9465 and NCIB 9466. The most satisfactory way of detecting the presence of penicillin acylase is by demonstrating the product of the reaction, 6-APA. This is most conveniently done by detecting the 6-APA on chromatograms of reaction mixtures by making use of the unique property of conversion of 6-APA to a biologically-active substance after treatment with phenylacetyl chloride. The incidence of penicillin acylase among clinical strains of *E. coli* reported by Holt & Stewart (1964*a*, *b*) must be regarded as being erroneously high.

There seems little doubt that in typical antibiotic sensitivity tests with clinical strains of Gram-negative bacteria, penicillin acylase activity is seldom seen, not only because of the limited number of cultures capable of producing 6-APA from ampicillin or benzylpenicillin, but also because of the unfavourable cultural conditions in serial-dilution tests for the production of penicillin acylase by bacteria. Penicillin acylase activity is thus not a factor normally responsible for resistance of Gram-negative bacteria to ampicillin and benzylpenicillin.

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EXPLANATION OF PLATE

Chromatogram showing the degradation of benzylpenicillin by *Escherichia coli* NCIB 9465

Chromatography system: *n*-butanol + pyridine + water, 1 + 1 + 1 v/v. Descending. Whatman No. 1. Run overnight at 5°. Starch + iodine spray. Samples 1–5 applied at two spots/origin, i.e. 12–16 μl. Reaction mixtures 1–5 consisted of 1 vol. of sodium benzylpenicillin 3000 μg./ml. in 0·2 M-acetate buffer (pH 5·5) plus 5 vol. of one of the following, adjusted to pH 5·5 and incubated for 5 hr at 37°.

1. 40-hr culture of NCIB 9465 grown statically at 37° in nutrient broth containing benzylpenicillin 100 μg./ml.

2. *E. coli* centrifuged from 1 vol. of above culture and resuspended in 1 vol. of 0·2 M-acetate buffer (pH 5·5).

3. Filtrate obtained after centrifuging and membrane filtering above culture.

4. Freeze-dried material prepared as in 3 and reconstituted at double original concentration.

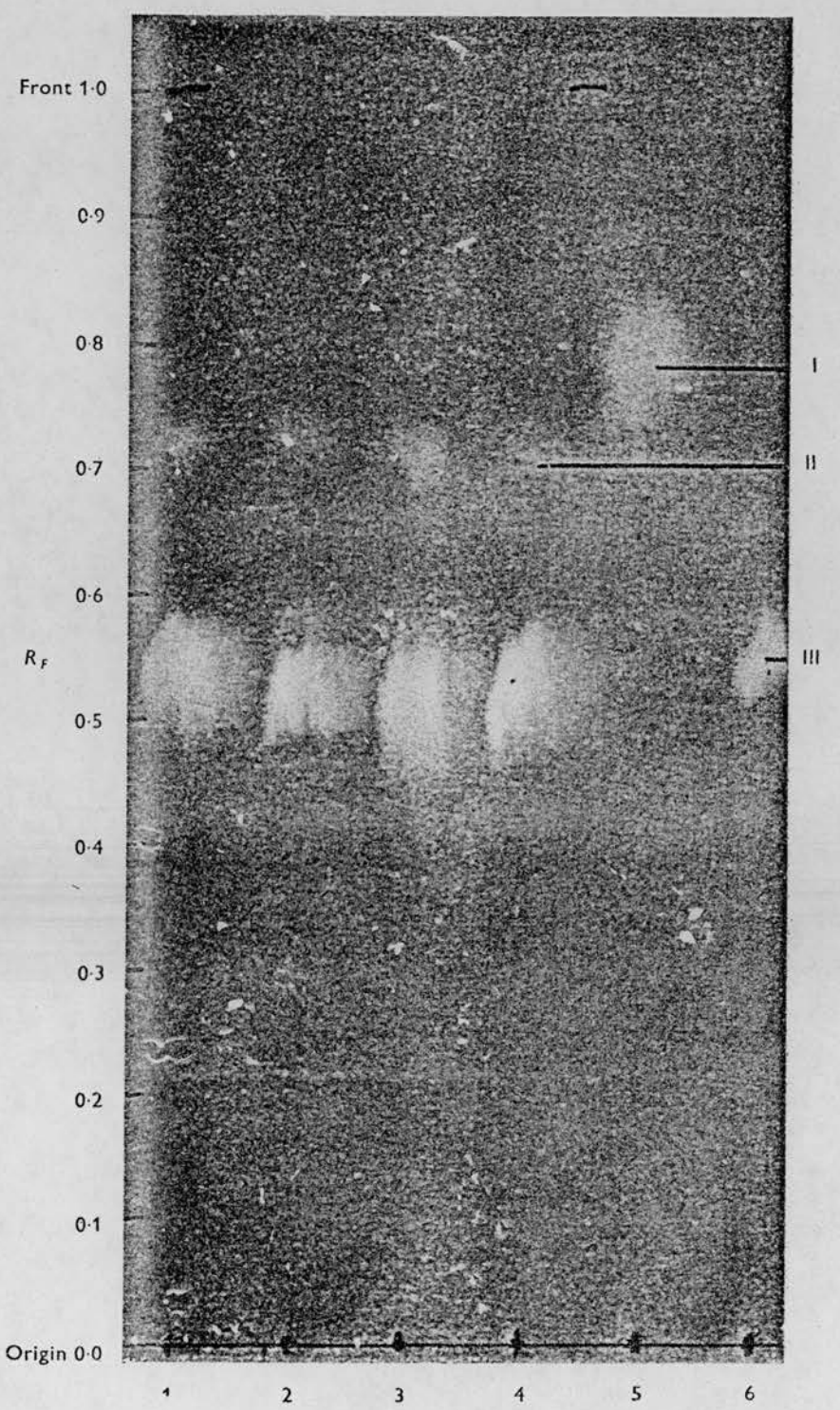
5. 0·2 M-acetate buffer (pH 5·5).

6. Benzylpenicilloic acid 500 μg./ml. prepared by treatment of benzylpenicillin with β-lactamase obtained from *B. cereus* (one spot/origin).

I = benzylpenicillin, II, = benzylpenilloic acid, III, = benzylpenicilloic acid.

Butanol + ethanol + water chromotograms of reaction mixtures 1–5 did not reveal any 6-APA after phenylacetylation and contacting with agar seeded with *B. subtilis*. A 6-APA zone was detected for a 5 μg./ml. 6-APA solution.

Journal of General Microbiology, Vol. 42, No. 3 Plate 1



M. COLE AND R. SUTHERLAND

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β-Lactamase Activity and Resistance to Ampicillin, Carbenicillin, and Cephaloridine of *Klebsiella*, *Enterobacter*, and *Citrobacter*

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Seventy-six strains of gram-negative bacilli were identified as *Klebsiella*, *Enterobacter*, or *Citrobacter*, and their susceptibility to the β-lactam antibiotics ampicillin, carbenicillin and cephaloridine was determined. In general, the majority of strains of *Klebsiella* were resistant to the penicillins but were susceptible to cephaloridine, whereas the *Enterobacter* and *Citrobacter* bacteria were resistant to both ampicillin and cephaloridine but were susceptible to carbenicillin. Most strains of *Klebsiella* inactivated ampicillin as a result of β-lactamase activity, but carbenicillin and cephaloridine were relatively stable to enzymatic destruction by *Klebsiella*. In contrast, *Enterobacter* and *Citrobacter* rapidly inactivated cephaloridine, but generally caused little destruction of ampicillin or carbenicillin. All bacterial strains tested displayed β-lactamase activity, but destruction of the β-lactam antibiotics was not the sole mechanism of resistance.

Early studies with ampicillin showed that the majority of strains belonging to the *Klebsiella-Enterobacter* (*Aerobacter*) group of bacteria were resistant to the action of this broad-spectrum penicillin (12, 17), and that the resistance to ampicillin appeared to be associated with the β-lactamase activities of these organisms (1, 16). In contrast, *Klebsiella* species have been found to be generally susceptible to cephalosporin antibiotics (2, 3), although the closely related *Enterobacter* group has been shown to have virtually uniform resistance to cephalosporins (3, 9, 18). Fleming and co-workers (5, 6) demonstrated that *Enterobacter* species inactivated cephalosporins by β-lactamase activity, and suggested that the resistance of *Enterobacter* to cephalosporins was associated with enzymatic destruction. These workers also observed that *Klebsiella* strains consistently failed to inactivate cephalosporins (5), but Medeiros and O'Brien (10) reported that a significant number of *Klebsiella* isolates produced a cephalosporinase, and that these strains were usually resistant to cephalothin. Carbenicillin, the most recent of the semisynthetic penicillins to be introduced into clinical practice, was found to be inactive against *Klebsiella* species (8, 13) but, unlike ampicillin, the resistance of this group of bacteria to carbenicillin was not associated with β-lactamase activity.

This study was designed to compare the degrees of susceptibility of *Klebsiella*, *Enterobacter*, and *Citrobacter* bacteria to ampicillin, carbenicillin, and cephaloridine, and to attempt to determine the role of β-lactamase production in the resistance of these organisms to the β-lactam antibiotics.

MATERIALS AND METHODS

Cultures. The bacteria used were isolated from clinical specimens (urine, sputum, wounds, and exudates) obtained from a number of hospitals in the United Kingdom. A small number of strains of type species were obtained from the National Collection of Type Cultures, London, England, and were used as taxonomic control strains.

Classification of bacteria. The cultures were identified by standard fermentation and biochemical reactions, and by motility tests, and were classified according to the scheme proposed by Cowan and Steel (4; Fig. 1).

Minimal inhibitory concentrations. The susceptibility of the organisms to ampicillin, carbenicillin, and cephaloridine was determined by twofold serial dilution of the

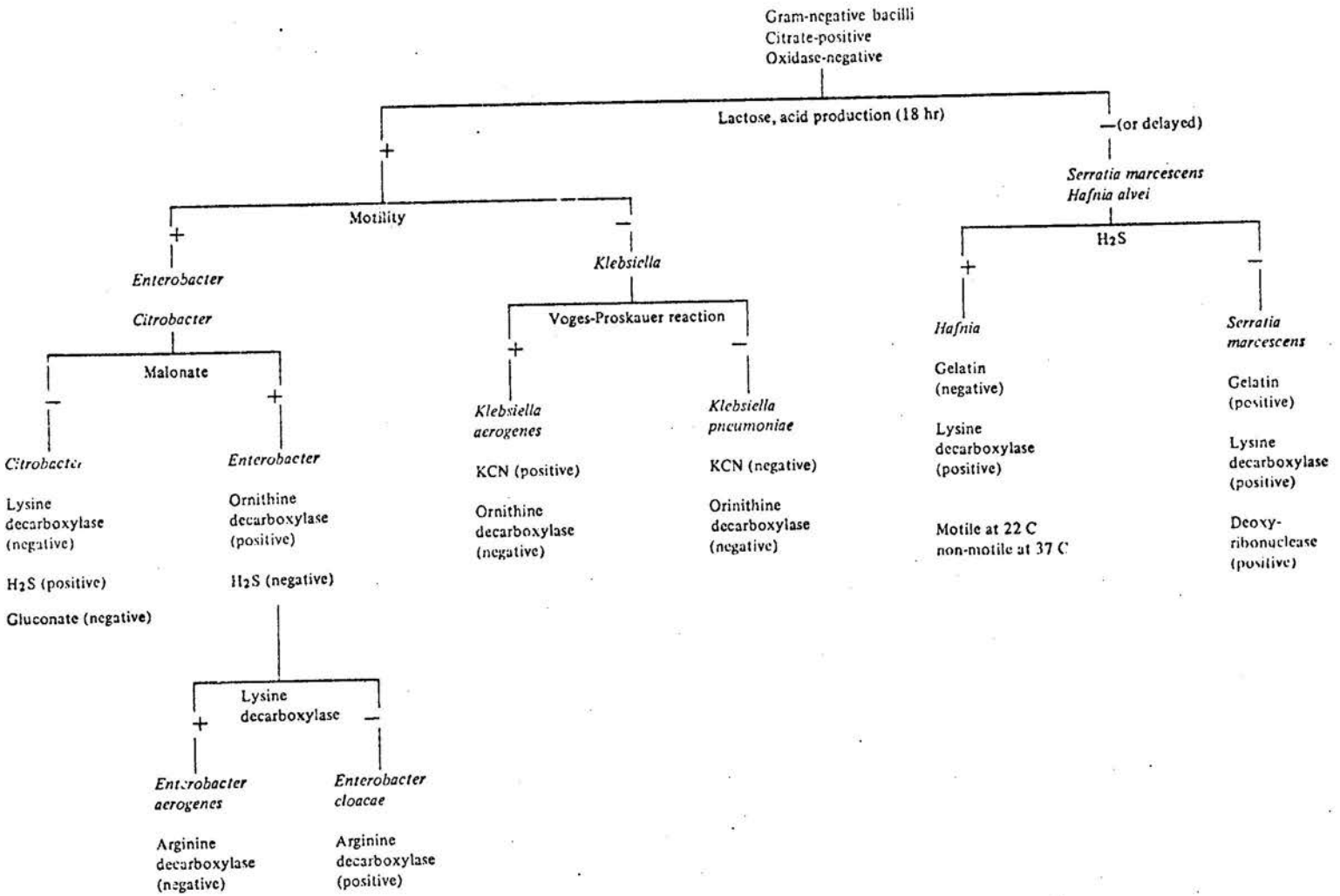

Fig. 1. *Schema for differentiation of citrate-utilizing, gram-negative bacilli [after Cowan and Steel (4)].*

antibiotics in 5-ml volumes of nutrient broth or 18-ml volumes of nutrient agar. For tests in liquid medium, the inoculum was 1 drop (0.03 ml) of an undiluted overnight broth culture, and for tests with solid medium, 1 drop (0.003 ml) of an overnight broth culture delivered with a multiple inoculating device. Minimal inhibitory concentrations were determined after 18 hr of incubation at 37 C.

Viable-count experiments. The test bacteria were incubated at 37 C in nutrient broth containing known concentrations of the antibiotics. Samples were taken at intervals, and 0.02-ml volumes of suitable dilutions of the cultures were pipetted onto nutrient agar plates. Colonies were counted after 18 hr incubation at 37 C, and the number of surviving bacteria was estimated.

Antibiotic assay. Ampicillin, carbenicillin, or cephaloridine concentrations in cultures from serial dilution tests or viable-count experiments were measured by microbiological assay. Samples were suitably diluted and plated in large rectangular plates seeded with *Bacillus subtilis* ATCC 6633 for assay of ampicillin and cephaloridine (16) or with *Pseudomonas aeruginosa* NCTC 10490 for assay of carbenicillin (8).

Transfer of antibiotic resistance. Antibiotic-resistant strains were grown for 6 hr at 37 C in Penassay broth (Oxoid CM287), and 0.2-ml volumes of these donor strains were added to 0.4-ml volumes of a 6-hr culture of the recipient strain of *Escherichia coli* K-12 711 Nx, a lactose-negative, antibiotic-susceptible, nalidixic acid-resistant strain. The mating mixture of donor and recipient bacteria was inoculated into 9-ml volumes of Penassay broth and, after overnight incubation at 37 C, 0.003 ml was plated on MacConkey agar (Oxoid CM3) containing the appropriate antibiotic (20 or 40 μg/ml) and nalidixic acid (20 μg/ml). Control plates containing antibiotic alone (20 or 40 μg/ml), or nalidixic acid alone (20 μg/ml), were also set up, and a control culture of the antibiotic-susceptible, nalidixic acid-resistant recipient strain was always included in the test. The selective media plates were incubated overnight at 37 C, and any lactose-negative colonies growing on the antibiotic-containing plates were subcultured into Penassay broth and incubated overnight at 37 C. These cultures were checked for purity and retested for antibiotic resistance on MacConkey agar containing the appropriate antibiotic and nalidixic acid. A number of tests were also carried out with other suitable recipient strains, namely, *Salmonella typhi* and *S. typhimurium*.

RESULTS

Cultures. A total of 76 strains of bacteria were classified as *Klebsiella*, *Enterobacter*, or *Citrobacter* (Table 1), and the distribution of positive reactions in the basic biochemical tests is shown in Table 2. The motility tests and ornithine decarboxylase reactions showed good correlation for differentiation between *Klebsiella* and *Enterobacter* groups, and the Voges-Proskauer test and malonate utilization served in most cases to distinguish the *Enterobacter* and *Citrobacter* groups. Twelve strains of indole-positive bacteria, identical in most other respects to *K. aerogenes*, were classified as *K. oxytoca*.

Table 1. *Distribution of Klebsiella-Enterobacter-Citrobacter bacteria tested*

Species	No. of strains	
K. aerogenes	29	48
K. oxytoca	12	
K. pneumoniae	2	
K. ozaenae	2	
K. rhinoscleromatis	1	
K. edwardsii	2	
E. cloacae	8	15
E. aerogenes	4	
E. liquefaciens	1	
E. species	2	
Citrobacter freundii	13	
Total	76	

Antibiotic susceptibility. The results of serial dilution tests to determine the minimal inhibitory concentrations of ampicillin, carbenicillin, and cephaloridine against the 76 strains of bacteria tested are summarized in Fig. 2, which shows the cumulative percentage inhibition of the strains by each antibiotic. It can be seen that *Klebsiella* species were distinctly more susceptible to cephaloridine than to ampicillin or carbenicillin. Thus, 67% of the 48 strains tested were inhibited by 12.5 μg or less of cephaloridine per ml, whereas only 12.5 and 6% of these strains were inhibited by similar concentrations of ampicillin and carbenicillin, respectively. In contrast, *Enterobacter* species were almost uniformly resistant to cephaloridine, as well as ampicillin, and were generally susceptible to carbenicillin. Thus, 11 of 15 strains (74%) were inhibited by 12.5 μg or less of carbenicillin per ml, in contrast to 7% inhibition by ampicillin and cephaloridine. Similarly, *Citrobacter* bacteria were typically susceptible to carbenicillin (11 of 13 strains inhibited by 12.5 μg/ml) and resistant to ampicillin and cephaloridine.

Antibiotic susceptibility and β-lactamase activity. The relationship between the susceptibility of this group of bacteria and their ability

to destroy the β-lactam antibiotics as a result of β-lactamase activity was investigated by measurement of residual antibiotic activity of cultures incubated overnight in serial dilution tests. Typical results are shown in Table 3. In general, *Klebsiella* species were resistant to ampicillin, and there was complete destruction of ampicillin in the tubes showing visible growth after overnight incubation. These organisms were also resistant to carbenicillin, but there was usually only slight or partial destruction of the penicillin, and it was obvious that *Klebsiella* bacteria were capable of growing in the presence of relatively high concentrations of carbenicillin. Cephaloridine was active against *Klebsiella*, and there was evidence of destruction only at relatively low concentrations of the antibiotic. In minimal inhibitory concentration tests with cephaloridine against *Enterobacter* and *Citrobacter* species, growth was invariably accompanied by total destruction of the antibiotic, whereas there was relatively little destruction of either ampicillin or carbenicillin, even when the organism was resistant to the penicillin under test.

Examination by thin-layer chromatography of broth cultures of *Klebsiella*, *Enterobacter*, or *Citrobacter* bacteria for which minimal inhibi-

Table 2. *Biochemical reactions of 76 strains of gram-negative, oxidase-negative bacilli*

Test	Positive reactions					
	Klebsiella (48 strains)		*Enterobacter* (15 strains)		*Citrobacter* (13 strains)	
	No.	Per cent	No.	Per cent	No.	Per cent
Lactose fermentation (18 hr at 37 C)	44	91.7	15	100	13	100
Motility	0	0	15	100	12	92.3
Indole production	11	22.9	0	0	2	15.4
Methyl red	7	14.6	0	0	13	100
Voges-Proskauer reaction	44	91.7	15	100	0	0
Citrate utilization	47	97.7	15	100	13	100
Lysine decarboxylase	45	93.7	6	40.0	0	0
Ornithine decarboxylase	0	0	15	100	8	61.5
H_2S production	0	0	0	0	9	69.2
Gluconate oxidation	44	91.7	15	100	0	0
Malonate utilization	46	95.8	14	93.3	0	0

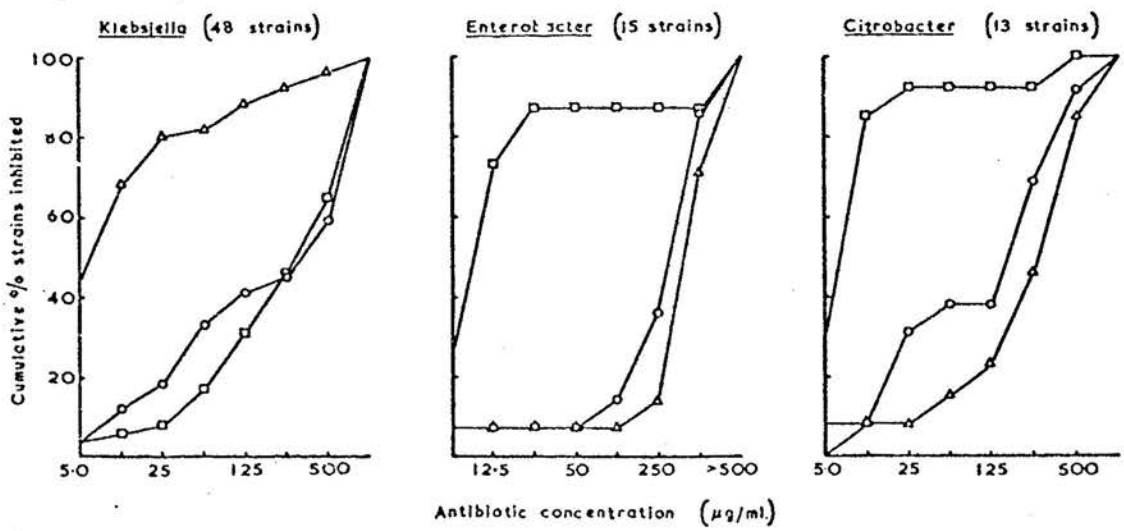

Fig. 2. *Antibiotic susceptibility of 76 strains of Klebsiella-Enterobacter-Citrobacter bacteria. Symbols:* ○, *ampicillin;* □, *carbenicillin;* ∧, *cephaloridine.*

tory concentration tests indicated a marked loss of ampicillin or carbenicillin activity, as judged by microbiological assay, showed the presence of the penicilloic acids of ampicillin or carbenicillin, confirming that inactivation was due to the β-lactamase activities of the bacteria.

Although the results of these experiments indicated the relative stabilities of the three β-lactam antibiotics to the β-lactamases produced by the *Klebsiella, Enterobacter,* and *Citrobacter* organisms, this type of test gave no indication as to whether growth occured as a result of destruction of the antibiotic, or whether destruction occured as a result of growth of the organism. Accordingly, viable-count experiments were carried out to measure growth of the test organism in the presence of β-lactam antibiotic, and the antibiotic contents of the cultures were assayed over the same period to attempt to elucidate the relationship between growth and destruction. The results of typical viable-count experiments with a strain of *K. aerogenes* and one of *E. aerogenes* are illustrated in Fig. 3 and 4. In Fig. 3, it can be seen that cephaloridine at a concentration of 5 μg/ml reduced the viable count to zero within 3 hr. Both ampicillin and carbenicillin showed significant bactericidal activity at concentrations of 50 and 100 μg/ml during the first 6 hr of the experiment, but failed to prevent growth from increasing to visible levels after 24 hr. With ampicillin, growth appeared to take place after there was marked destruction of the antibiotic, but with carbenicillin growth occurred when there was still a substantial amount of antibiotic remaining.

With the test strain of *E. aerogenes* (Fig. 4), cephaloridine produced a fall in the viable count in the initial 3-hr period while there was still antibiotic present, but growth followed destruction of the antibiotic. Both ampicillin and carbenicillin also showed bactericidal activity during the first 6 hr of the experiment. However, growth occurred after this period, even though 70% of the original ampicillin activity remained after 24 hr in the culture which had originally contained 500 μg of ampicillin per ml. There was no detectable loss of carbenicillin activity.

Table 3. *Relationship between resistance of Klebsiella-Enterobacter-Citrobacter bacteria to ampicillin, carbenicillin, and cephaloridine and the β-lactamase activities of these organisms*

Genus	Antibiotic	Growth[a] and antibiotic concn (μg/ml)						
		500[b]	250	125	50	25	12.5	5.0
K. aerogenes NCTC 8172	Ampicillin	±	+	+	+	+	+	+
		0[c]	0	0	0	0	0	0
	Carbenicillin	±	±	+	+	+	+	+
		>125	125	32	12.6	5.4	1.5	0
	Cephaloridine	–	–	–	–	±	±	+
		500	250	90	40	8.5	2.5	0
E. cloacae NCTC 10005	Ampicillin	±	±	+	+	+	+	+
		500	250	125	23	2.6	0	0
	Carbenicillin	–	+	+	+	+	+	+
		500	200	94	40	18	8	4
	Cephaloridine	+	+	+	+	+	+	+
		0	0	0	0	0	0	0
C. freundii NCTC 9750	Ampicillin	NT	NT	+	+	+	+	+
				43	21	12	0.8	0.3
	Carbenicillin	NT	NT	–	–	–	±	+
							6.6	2.7
	Cephaloridine	NT	NT	+	+	+	+	+
				0	0	0	0	0

[a] The antibiotics were serially diluted in nutrient broth, and the tubes were inoculated with one drop of an overnight broth culture of the test organism. Growth was determined by visual inspection after 24 hr of incubation at 37 C, and the residual antibiotic activities were measured by microbiological assay. Symbols: + = visible growth, ± = trace growth, – = no visible growth; NT = not tested.

[b] Initial antibiotic concentration.

[c] Residual antibiotic concentration at the conclusion of the minimal inhibitory concentration test.

Transfer of antibiotic resistance. A total of 62 strains of *Klebsiella, Enterobacter,* and *Citrobacter* were tested for their ability to transfer ampicillin resistance to *E. coli* K-12 711 Nx. Only one culture, a strain of *K. aerogenes* was found to transfer ampicillin resistance to this organism (Table 4). These strains also failed to transfer ampicillin resistance to recipient strains of *S. typhi* and *S. typhimurium.* Difficulty was experienced in selecting transcipients (ampicillin-resistant *E. coli*) when an undiluted culture was plated on the antibiotic-containing selective medium. Lactose-negative colonies were recovered at a high frequency, but when these were cultured and retested for ampicillin resistance they were found to be susceptible to the antibiotic. These "transcipients" were in fact colonies of the ampicillin-susceptible recipient strain of *E. coli* which had been able to grow as a result of destruction of ampicillin in the selective medium by the β-lactamase activity of the donor strain. This problem was overcome by use of an inoculum diluted 1:5 with sterile saline, or by increasing the ampicillin concentration from 20 to 40 μg/ml.

Thirty-one of these strains, resistant to ampicillin and one or more antibiotics, namely, cephaloridine, chloramphenicol, kanamycin, streptomycin, and tetracycline, were tested for frequency of R factor transfer. Eighteen strains transferred antibiotic resistance at a relatively high rate (Table 4), but none of the strains transferred resistance to cephaloridine, and only one strain (described above) transferred an ampicillin R factor.

In a separate series of experiments, the majority of test strains were examined for ability to transfer carbenicillin R factors, but transfer of carbenicillin resistance was not demonstrated.

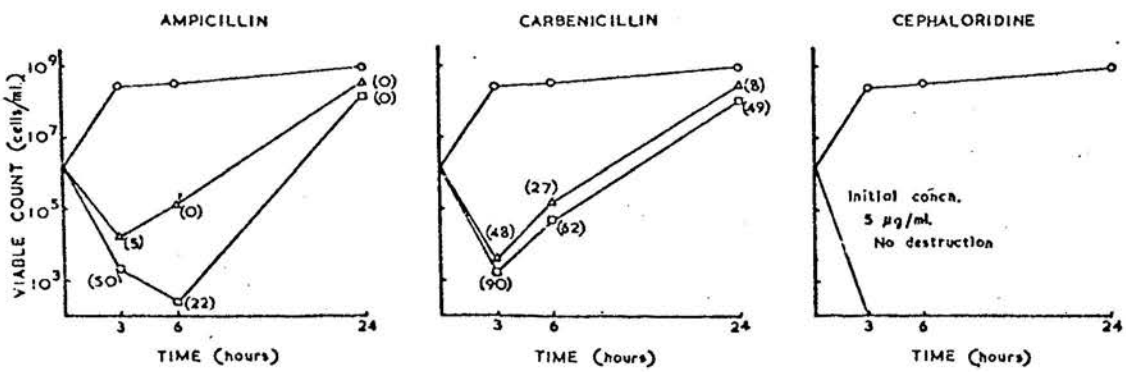

Fig. 3. *Bactericidal activities of ampicillin, carbenicillin, and cephaloridine against Klebsiella aerogenes. Symbols:* ○, *antibiotic-free control;* △, *50 μg/ml;* □, *100 μg/ml. Figures in brackets show residual antibiotic concentration (μg/ml).*

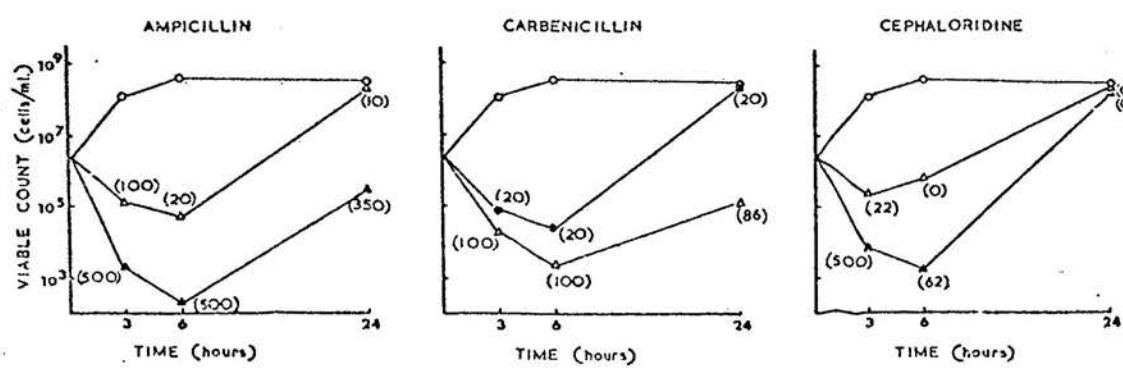

Fig. 4. *Bactericidal activities of ampicillin, carbenicillin, and cephaloridine against Enterobacter aerogenes. Symbols:* ○, *antibiotic-free control;* ●, *20 μg/ml;* △, *100 μg/ml;* ▲, *500 μg/ml. Figures in brackets show residual antibiotic concentrations (μg/ml).*

Table 4. *Incidence of transferable drug resistance in Klebsiella, Enterobacter, and Citrobacter bacteria*

Resistance of donor strain[a]	No. of strains tested			No. of strains transferring resistance			Resistance pattern transferred[a]
	Klebsiella	*Enterobacter*	*Citrobacter*	*Klebsiella*	*Enterobacter*	*Citrobacter*	
A	29	0	0	1	0	0	A
A Cp	0	5	4	0	0	0	–
Cp	0	0	2	0	0	0	–
A S Cp	0	1	0	0	1	0	S
A T	1	0	0	0	0	0	–
A T Cp	1	0	0	1	0	0	T
A T S	1	1	0	1	1	0	T S
A T C	4	0	0	3	0	0	T C (2) C (1)
A C Cp	0	1	0	0	1	0	C
A T C S	7	0	0	6	0	0	T C S
A T C S K	5	0	0	4	0	0	T C S K
Total	48	8	6	16	3	0	

[a] A = ampicillin; C = chloramphenicol; Cp = cephaloridine; K = kanamycin; S = streptomycin; T = tetracycline.

DISCUSSION

The results of this study with a relatively limited number of strains of *Klebsiella, Enterobacter,* and *Citrobacter* are in agreement with published reports showing the resistance of this group to ampicillin (12, 17). Our results also confirm the strikingly different susceptibility of *Klebsiella* species to cephaloridine compared with the high degree of resistance of *Enterobacter* and *Citrobacter* bacteria to this antibiotic (3, 5, 9). However, in this study a relatively high proportion of strains of *Klebsiella* were relatively resistant to cephaloridine. Thus, it would not seem realistic to attempt to use susceptibility to cephaloridine as a means of distinguishing *Klebsiella* species from *Enterobacter,* or conversely to assume that nonmotile strains of this taxonomic group will of necessity be susceptible to cephalosporins. However, P. C. Fleming (*personal communication*) has observed that cephalosporin C may be used with confidence to differentiate *Klebsiella* from *Enterobacter.*

The finding that the majority of strains of *Enterobacter* and *Citrobacter* were relatively susceptible to carbenicillin, as opposed to strains of *Klebsiella* which were almost invariably resistant to carbenicillin, was unexpected. Early studies with carbenicillin had indicated that the majority of strains of lactose-fermenting, citrate-utilizing bacteria were resistant to carbenicillin as well as to ampicillin (8, 13), but it now seems probable that most of the strains tested belonged to the genus *Klebsiella,* although this was not determined at the time. The susceptibility of the *Enterobacter* and *Citrobacter* bacteria to carbenicillin has obvious clinical implications, particularly in view of the resistance of this group to the cephalosporin and other antibiotics.

The similarity of the *Enterobacter* and *Citrobacter* organisms with regard to susceptibility to the β-lactam antibiotics, as well as on the basis of biochemical characteristics, would point to a close relationship between these genera and a distinct difference between these organisms and the *Klebsiella* genus.

The relationship between the resistance of the *Klebsiella, Enterobacter,* and *Citrobacter* groups of bacteria to β-lactam antibiotics and the β-lactamase activities of these organisms is not clear, as, for example, is the case with penicillin-resistant strains of staphylococci. All strains of bacteria tested in this study demonstrated β-lactamase activity, *Klebsiella* strains destroyed ampicillin and, on occasion, carbenicillin and cephaloridine; *Enterobacter* and *Citrobacter* bacteria inactivated cephaloridine invariably, ampicillin occasionally, and carbenicillin rarely.

The results reported here indicate that cephaloridine is usually stable to the β-lactamases produced by *Klebsiella* but is susceptible to the enzymes produced by *Enterobacter* and *Citrobacter* bacteria, in agreement with published reports (2, 5, 10). This suggests that the resistance of the latter group to cephaloridine may be attributable to enzymatic destruction. In

contrast, ampicillin is inactivated by *Klebsiella* species, but is relatively stable to destruction by *Enterobacter* and *Citrobacter* organisms. The resistance of the latter organisms to ampicillin is, therefore, not generally associated with β-lactamase activity, whereas the data presented here appear to support suggestions that *Klebsiella* strains are resistant largely as a result of production of β-lactamase (1, 16). However, Seligman (14) has produced evidence suggesting a mechanism of intrinsic resistance for *Klebsiella* in addition to β-lactamase activity.

With carbenicillin, the resistance of *Klebsiella* species was in most cases not associated with inactivation of the antibiotic, unlike ampicillin, and was obviously due to some other mechanism. The susceptibility of *Enterobacter* and *Citrobacter* organisms to carbenicillin and their relative insusceptibility to ampicillin was apparently not due to differences in stability to the β-lactamase activities of this group, as neither antibiotic appeared to be inactivated to any great extent. It must be assumed that the greater activity of carbenicillin against these bacteria is due to some other mechanism, as is the case with *P. aeruginosa*, which is relatively susceptible to carbenicillin but resistant to ampicillin.

The very low transfer frequency of ampicillin resistance (2%) and failure to transfer carbenicillin or cephaloridine resistance indicate that the insusceptibility of the *Klebsiella*, *Enterobacter*, and *Citrobacter* bacteria to the β-lactam antibiotics is not extrachromosomally mediated, in keeping with the findings of Medeiros and O'Brien (10, 11). However, the results of this study are at variance with those reported by Hinshaw and co-workers (7), who found that ampicillin resistance was transferred frequently as a unit in combination with resistance to other antibiotics, for example, streptomycin, chloramphenicol, and tetracycline. A possible explanation for the discrepancy between these two reports is that conditions in this study were not favorable for transfer of ampicillin, carbenicillin, or cephaloridine resistance. This does not seem likely, as under these conditions resistance to other antibiotics was transferred readily from these bacteria. Also, in other studies in which the same conditions were used, ampicillin and cephaloridine resistance has been transferred readily between other bacterial species (15). The role of transferable resistance and the resistance of the *Klebsiella*, *Enterobacter*, and *Citrobacter* bacteria to β-lactam antibiotics requires further investigation.

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Transferable Antibiotic Resistance in Enteropathogenic *Escherichia coli* Between 1948 and 1968

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Enteropathogenic strains of *Escherichia coli* which had been isolated in the United Kingdom during three periods between 1948 and 1968, namely 1948 to 1951, 1957 to 1960, and 1967 to 1968, were tested for susceptibility to ampicillin, streptomycin, tetracycline, chloramphenicol, and sulphonamides. Antibiotic-resistant strains were tested for their ability to transfer antibiotic resistance to an antibiotic-susceptible strain of *E. coli* K-12. A relatively high proportion of strains isolated between 1948 and 1951 was resistant to ampicillin, streptomycin, or sulphonamides. None of these strains transferred ampicillin or streptomycin resistance, but sulphonamide resistance was R-factor-mediated in three out of 14 sulphonamide-resistant strains. Resistance to tetracycline and chloramphenicol was rare before 1951 but had become common among enteropathogenic *E. coli* by 1957. Much of the antibiotic resistance of bacteria isolated between 1957 and 1960 was R-factor-mediated, and transferable resistance was about as prevalent among *E. coli* isolated between 1957 and 1960 as among strains isolated in 1967 and 1968. Nevertheless, there was no appreciable increase in the overall incidence of antibiotic resistance among these enteropathogenic strains of *E. coli* between 1957 and 1968, although transferable antibiotic resistance was common during this period. These results do not suggest that the emergence of transferable antibiotic resistance will inevitably lead to the rapid development of antibiotic resistance among this group of bacteria.

The discovery in 1959 of transferable antibiotic resistance among enteric bacteria (1, 17) resulted in an increased interest in the incidence of antibiotic resistance among these organisms and in speculation regarding the possibility of the rapid development of widespread antibiotic resistance among gram-negative bacilli. It is evident that there has been an increase in the resistance to antibiotics among certain groups of infective enteric bacteria in the past years, e.g., shigellae (6, 12, 17) and *Salmonella typhimurium* (2, 8), and that many of these bacteria possess transferable resistance factors. On the other hand, the evidence regarding other *Enterobacteriaceae* is much less clear, and although it has been established that bacteria capable of transferring antibiotic resistance are at present common among the intestinal flora of healthy subjects (4, 5, 11) as well as of hospital patients (4, 9, 10, 15), little is known about the emergence of bacteria possessing transferable R-factors and the development of resistance. Accordingly, we approached various sources in the United Kingdom with a view to obtaining gram-negative bacilli isolated before 1960 which could be tested for ability to transfer antibiotic resistance to other bacteria. We were fortunate in learning that Joan Taylor (former Director of the Salmonella Reference Laboratory, Central Public Health Laboratory, Colindale, London) had established a collection of cultures of enteropathogenic strains of *Escherichia coli* isolated from 1948 onwards. Dr. Taylor very kindly made cultures available to us, and the results reported here describe the antibiotic susceptibility patterns of enteropathogenic *E. coli* isolated at three different periods between 1948 and 1968, together with the incidence of transferable antibiotic resistance among these organisms.

MATERIALS AND METHODS

Cultures. A total of 512 strains of *E. coli* was obtained from the Salmonella Reference Laboratory, Colindale, London, and these bacteria were tested for susceptibility to ampicillin, tetracycline, strep-

tomycin, chloramphenicol, and sulphamethoxazole. The cultures had been isolated from clinical sources throughout the United Kingdom at three different periods of time between 1948 and 1968: (i) 1948–1951, 112 strains; (ii) 1957–1960, 200 strains; and (iii) 1967–1968, 200 strains. These strains had been stored on Dorset egg agar slopes in the dark at room temperature. Between 1948 and 1951, benzylpenicillin, streptomycin, and sulphonamides were the most widely used antibiotics; the broad-spectrum antibiotics, chloramphenicol and the tetracyclines, became available shortly afterwards, and ampicillin was introduced in 1961. Most of the strains (90%) from the two latter periods had been isolated from babies with diarrhea or gastroenteritis and comprised a wide range of serotypes. The remainder (10%) were from other sources (urine, blood, cerebrospinal fluid). Records were not available for the strains isolated between 1948 and 1951, but it is known that they were of human origin and it is believed that they were mainly from infantile gastroenteritis, as was the case for most of the other strains.

Minimal inhibitory concentrations. Twofold serial dilutions of the test antibiotics were prepared in 18-ml volumes of molten nutrient agar (Blood Agar Base, Oxoid) and poured in petri dishes. When set, the plates were dried at 37 C and inoculated with a drop (0.003 ml) of an undiluted overnight broth culture of the test organism by means of a replicating device delivering 20 cultures to each plate. Tests in liquid medium were done in 5-ml volumes of nutrient broth (Nutrient Broth no. 2, Oxoid) which were inoculated with a drop (0.03 ml) of an overnight broth culture of the test organism. The susceptibility of *E. coli* to sulphonamides was measured by serial dilution of sulphamethoxazole in antagonist-free agar (D.S.T. agar, Oxoid) containing 10% lysed horse blood, using as inoculum a drop of a 1:1,000 dilution of an overnight broth culture. Minimal inhibitory concentrations (MICs) were determined after overnight incubation at 37 C. The β-lactamase activity of ampicillin-resistant strains of *E. coli* was determined by measurement of the destruction of ampicillin during the course of serial dilution tests by using *Bacillus subtilis* ATCC 6633 to assay residual antibiotic activity.

Transfer of resistance. Antibiotic-resistant isolates of *E. coli* were grown overnight in Penassay broth (Antibiotic Medium no. 3, Oxoid), and 0.2-ml volumes of these donor strains were added to 0.4-ml volumes of an overnight culture of the recipient strain, *E. coli* K-12/711Nx, a non-lactose-fermenting, nalidixic acid-resistant mutant. The mixed cultures of donor and recipient bacteria were cultured in 9 ml of fresh, prewarmed Penassay broth, and after overnight incubation at 37 C they were plated on MacConkey agar, incorporating 20 μg of the appropriate test antibiotic per ml and 20 μg of nalidixic acid per ml; control plates containing either 20 μg of antibiotic or 20 μg of nalidixic acid per ml were also inoculated with the mating mixtures. A number of donor strains were resistant to nalidixic acid, and in these cases selection was made on agar containing 1,000 μg of streptomycin per ml by using a streptomycin-resistant strain of *E. coli* K-12/711 as recipient. Inoculation of the plates was carried out with a multipoint inoculating device delivering approximately 0.003 ml of culture. After overnight incubation at 37 C, the plates were examined for non-lactose-fermenting colonies growing in the presence of 20 μg of the antibiotic and 20 μg of nalidixic acid per ml. Lactose-negative colonies were subcultured into Penassay broth and retested for antibiotic resistance. The transfer of sulphonamide resistance was determined by using D.S.T. agar (Oxoid) containing 10% lysed horse blood, incorporating sulphamethoxazole (200 μg/ml) and nalidixic acid (20 μg/ml). Neither the donor nor recipient bacteria grew on the selective medium. Colonies which developed from the mixed cultures were purified and identified as *E. coli* K-12/711Nx and retested for sulphonamide and antibiotic resistance.

In cases where the donor strain inhibited the growth of *E. coli* K-12/711Nx as a result of bactericcin activity, appropriate colicin-tolerant mutants were selected and used as recipient strains. A number of antibiotic-resistant cultures were filtered through membrane filters (0.22 μm pore size; Millipore Corp.), and the sterile filtrates were incubated with *E. coli* K-12/711Nx as described above. None of the cell-free filtrates conferred resistance on the recipient strain.

Elimination of extrachromosomal antibiotic resistance. Antibiotic-resistant strains of *E. coli* were cultured on nutrient agar containing ethidium bromide (50 to 500 μg/ml), and colonies growing on the plates containing the highest concentrations of ethidium bromide which supported normal growth were subcultured in drug-free medium and tested for antibiotic susceptibility (3).

RESULTS

Results in Table 1 show the distribution of the MICs of ampicillin, tetracycline, streptomycin, and chloramphenicol against *E. coli* isolated between 1948 and 1951, 1957 and 1960, and 1967 and 1968. Data expressed in Fig. 1 show the percentage inhibition of strains inhibited by serial concentrations of the antibiotics. Results in Table 2 show the incidence of transferable resistance among the antibiotic-resistant strains of *E. coli*, and the antibiotic resistance patterns transferred are described in Table 3.

Resistance to ampicillin. Ampicillin inhibited 66% of the 112 strains of *E. coli* isolated between 1948 and 1951 at a concentration of 12.5 μg/ml or less; the remainder (34%) required inhibitory concentrations of from 25 to 500 μg of ampicillin per ml. Of the 200 strains isolated in the period 1957 to 1960, 95% were susceptible to 12.5 μg of ampicillin per ml, and the remaining 5% of the strains were susceptible to concentrations of 25 to 250 μg of ampicillin per ml. Most (85%) of the 200 strains isolated in 1967 and 1968 were susceptible to 12.5 μg of ampicillin

TABLE 1. *Antibiotic susceptibility of 512 strains of Escherichia coli isolated between 1948 and 1968*

Period	Antibiotic	No. of strains	No. of strains with MIC[a] (μg/ml) of									
			>500	500	250	125	50	25	12.5	5.0	2.5	1.25
1948–1951	Ampicillin	112		1	23	10	1	3	15	38	20	1
	Chloramphenicol	112				1				36	74	1
	Streptomycin	112	28						1	22	45	16
	Tetracycline	112							1	75	36	
1957–1960	Ampicillin	200			2	3	4	1	21	92	75	2
	Chloramphenicol	200	1	13			1		12	85	85	3
	Streptomycin	200	6	3	2	7	5	5	8	24	35	105
	Tetracycline	200			17	14	1	3	30	112	23	
1967–1968	Ampicillin	200	20	1	1	4	3	1	3	74	90	4
	Chloramphenicol	200	4	2	3			1		113	75	2
	Streptomycin	200	3	2	3	13	14	9	1	4	21	130
	Tetracycline	200			17	21	3	3	32	116	8	

[a] Serial dilution in agar.

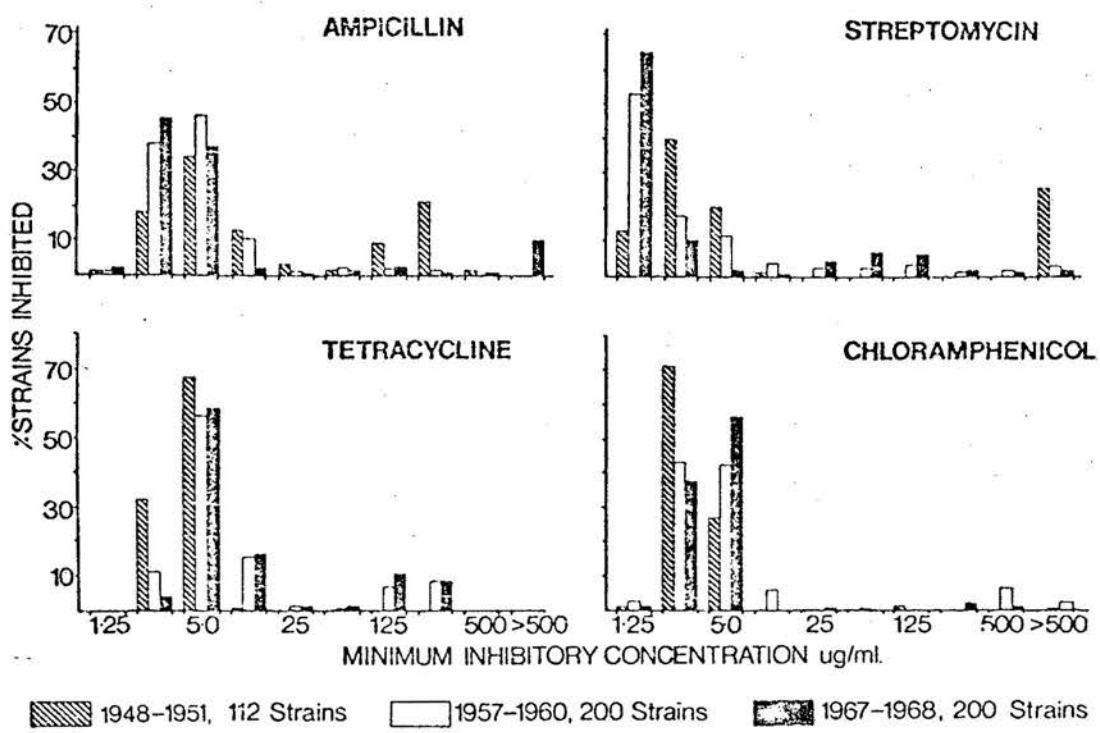

FIG. 1. *Susceptibility of 512 enteropathogenic strains of Escherichia coli isolated between 1948 and 1968 to four antibiotics.*

per ml, but two-thirds of the resistant strains were insusceptible to 500 μg of ampicillin per ml. The incidence of ampicillin resistance was highest in the group of bacteria isolated between 1948 and 1951, but the resistant strains isolated in 1967 and 1968 were generally more insusceptible to ampicillin. Thus, the ampicillin-resistant strains isolated between 1948 and 1951 and between 1957 and 1960 were inhibited by concentrations of 500 μg or less of ampicillin per ml, whereas most of the resistant strains isolated in 1967 and 1968 were not susceptible to

TABLE 2. *Transferable antibiotic resistance among Escherichia coli isolated between 1948-1968*

Antibiotic	Time of isolation	No. of strains	Resistant strains[a]		Transferable resistance		
			No.	% of total	No. of strains	% of resistant strains	% of total strains
Ampicillin	1948-1951	112	38	34	0	0	0
	1957-1960	200	10	5.0	0	0	0
	1967-1968	200	30	15	21	70	10.5
Chloramphenicol	1948-1951	112	1	1.0	0	0	0
	1957-1960	200	15	7.5	13	87	6.5
	1967-1968	200	10	5.0	10	100	5.0
Streptomycin	1948-1951	112	28	25	0	0	0
	1957-1960	200	28	14	19	68	9.5
	1967-1968	200	44	22	29	66	14.5
Tetracycline	1948-1951	112	0	0	0	0	0
	1957-1960	200	35	17.5	21	60	10.5
	1967-1968	200	44	22	30	68	15
Sulphamethoxazole	1948-1951	112	14[b]	12.5	3	21	2.7
	1957-1960	200	43[b]	21.5	17	40	8.5
	1967-1968	200	42[b]	21	17	40	8.5

[a] Not inhibited by 12.5 μg of test antibiotic per ml.
[b] Resistant to 200 μg of sulphamethoxazole per ml.

500 μg of ampicillin per ml. Examination of a number of the ampicillin-resistant strains from the three periods showed that these strains were able to destroy ampicillin during the course of the antibacterial tests, indicating that the resistance to ampicillin was associated with β-lactamase activity.

None of the ampicillin-resistant strains isolated in the periods 1948 to 1951 or 1957 to 1960 transferred ampicillin resistance to the selected recipient strain, *E. coli* K-12/711Nx (Table 2). Similarly, those ampicillin-resistant strains isolated in 1967 and 1968, which like earlier strains had ampicillin MIC values in the range 25 to 250 μg/ml, also failed to transfer ampicillin resistance. On the other hand, all strains isolated between 1967 and 1968 which were resistant to 500 μg of ampicillin per ml transferred ampicillin resistance associated with β-lactamase activity. Treatment with ethidium bromide had no effect on the susceptibility to ampicillin of ampicillin-resistant strains of *E. coli* isolated before 1960, but resulted in the elimination or reduction of the resistance of strains with transferable ampicillin resistance isolated in 1967 and 1968.

Resistance to streptomycin. In general, there was little difference in the streptomycin susceptibility patterns of the three groups of *E. coli* (75% susceptible to 12.5 μg/ml in 1948-1951, 86% susceptible in 1957-1960, and 78% susceptible in 1967-1968). However, all resistant organisms isolated between 1948 and 1951 were insusceptible to 500 μg of streptomycin per ml, whereas most of the resistant strains isolated in the two more recent periods were less resistant and had MIC values in the range of 25 to 500 μg of streptomycin per ml. None of the strains isolated between 1948 and 1951 transferred streptomycin resistance, but 68% of the streptomycin-resistant strains isolated between 1957 and 1960 and 66% of the most recently isolated strains were able to transfer streptomycin resistance to *E. coli* K-12/711Nx. R-factor-mediated streptomycin resistance was eliminated by treatment with ethidium bromide, but the streptomycin resistance of strains isolated before 1951 was unaffected by this treatment.

Resistance to tetracycline. All strains of *E. coli* isolated between 1948 and 1951 were susceptible to tetracycline (MIC = 5 μg/ml or less), but resistance to tetracycline was common among bacteria isolated from 1957 onwards. Thus, 17.5% of the strains isolated between 1957 and 1960, and 22% of those isolated between 1967 and 1968, were not inhibited by 12.5 μg of tetracycline per ml. The distribution of susceptibility to tetracycline of these two groups of *E. coli* was very similar (Fig. 1). Transfer of tetracycline resistance was readily demon-

TABLE 3. *Antibiotic resistance patterns in Escherichia coli between 1948 and 1968*

Resistance pattern[a]	No. of strains					
	1948–1951 (112)		1957–1960 (200)		1967–1968 (200)	
	Resistant	Transferring resistance	Resistant	Transferring resistance	Resistant	Transferring resistance
Su	2	0	16	2	10	3
S	4	0	3	0	6	4
SSu			3	2	6	2(S-1)
T			9	6	10	8
TSu			6	4(T-2)	1	0
TS			1	1	7	7(S-5)
TSSu			4	3(TSu-1)	7	6(TS-1)
TCS			1	0		
CSSu			3	3(CS-1)	1	1
TSKSu					1	1(Su-1)
TCSSu			10	10 (TC-1), (TCS-2), (CSSu-4)		
A	7	0	5	0	9	4
AC			1	0	1	1
AK					1	1
AS	19	0	1	1(S-1)		
AT			1	0	2	2(A-1)
ASu	6	3(Su-3)				
ACSu	1	0				
ASK					1	0
ATS			2	1(T-1)	1	1(A-1)
ASSu	5	0			1	1(AS-1)
ATCSu					1	1(ATC-1)
ATSSu					2	2 (T-1), (ATS-1)
ATCSSu					5	4(ACTS-3)
ATSKSu					3	3(ATKSu-1)
ATCKSSu					1	1
ATCKSuNx					1	1(ATCKSu-1)

[a] Abbreviations: A, ampicillin; C, chloramphenicol; K, kanamycin; Nx, nalidixic acid; S, streptomycin; Su, sulphamethoxazole; T, tetracycline.

strated with organisms isolated between 1957 and 1968, and 60% of the tetracycline-resistant strains isolated between 1957 and 1960 transferred tetracycline resistance, compared with 68% of the tetracycline-resistant strains isolated between 1967 and 1968 (Table 2).

Resistance to chloramphenicol. Only one of the strains of *E. coli* isolated between 1948 and 1951 was resistant to chloramphenicol, and this strain failed to transfer its chloramphenicol resistance. A small proportion of strains isolated in the two later periods were resistant to chloramphenicol (7.5% in 1957–1960 and 5% in 1967–1968), and virtually all of these strains transferred their chloramphenicol resistance to *E. coli* K-12/711Nx (Table 2).

Resistance to sulphonamides. Most (98 of 112) of the strains of *E. coli* isolated between 1948 and 1951 were susceptible to 20 μg of sulphamethoxazole per ml, but 14 strains were not inhibited by 200 μg of sulphamethoxazole per ml. Three of these 14 resistant strains transferred sulphonamide resistance to *E. coli* K-12/711Nx. These three R-factor strains, which had been isolated in 1951, were resistant also to ampicillin, but ampicillin resistance was not transmissible. The incidence of sulphonamide-resistant strains was higher in the two later periods, and 40% of the sulphonamide-resistant strains transferred sulphonamide resistance, usually in association with streptomycin or other resistance (Table 2).

DISCUSSION

An inherent weakness of a retrospective study of this kind is the possibility that the characteristics of the test organisms may have altered during the course of storage in the laboratory. It is possible that some of the strains examined

which now appear to be susceptible to antibiotics may have possessed R-determinants at the time of isolation, or that antibiotic-resistant strains which now fail to transfer resistance may once have possessed transfer factors. In practice, the data obtained suggest that the enteropathogenic strains of *E. coli* described here may not have altered greatly on storage, and it seems possible that the antibiotic resistance patterns reported may well be a true reflection of those prevailing at the time of isolation of the cultures.

This supposition is based upon the following facts. (i) The strains of *E. coli* isolated in 1967 and 1968 were examined relatively soon after isolation when it was unlikely that there would have been any significant change in the antibiotic resistance patterns of this group. (ii) The overall incidence of resistance and the frequency of bacteria possessing transmissible R-factors were similar for strains isolated between 1957 and 1960 and for strains isolated in 1967 and 1968, with the exception of resistance to ampicillin. It seems unlikely, therefore, that there was any great loss of resistance from the former group of bacteria as a result of storage, with the possible exception of ampicillin. (iii) There was a relatively high incidence of resistance to ampicillin, streptomycin, and sulphonamides among *E. coli* isolated between 1948 and 1951, and so it is less likely that there was any significant loss of resistance on storage. These bacteria were isolated before chloramphenicol and the tetracyclines became freely available in the United Kingdom, and so the lack of resistant organisms is not unexpected. (iv) The data suggest that the antibiotic resistance of *E. coli* isolated between 1948 and 1951 is not extrachromosomally mediated, and therefore it seems reasonable to assume that failure to demonstrate transferable antibiotic resistance among bacteria from this period is due to the fact that these bacteria never possessed transfer factors, rather than to loss during storage.

Another potential failing of this type of study is that the strains examined may not represent a true sample of the bacterial populations predominant at the time of isolation. This could apply particularly to the enteropathogenic bacteria isolated before 1951, for which precise records are unavailable, but less so to the strains isolated between 1957 and 1968, since they were isolated from various areas of the United Kingdom and were of numerous different serotypes. Nevertheless, despite these reservations, the results presented here appear to be meaningful in tracing the development of transferable antibiotic resistance among enteropathogenic *E. coli* isolated in the United Kingdom between 1948 and 1968.

The relatively high incidence of ampicillin-resistant strains of *E. coli* isolated before 1951 may be associated with the general use of benzylpenicillin during this period, which could have resulted in the selection of ampicillin-resistant mutants. The resistance of ampicillin-resistant strains isolated between 1948 and 1951 was associated with β-lactamase activity, but the β-lactamase produced by these strains differed from that produced by the majority of strains isolated 20 years later, in 1967 and 1968. The former is probably chromosomally mediated, since it is not possible to demonstrate transfer of the β-lactamase, it is not eliminated by treatment with ethidium bromide, and the substrate and inhibition profiles of the cell-free enzyme are different from those of the extrachromosomally mediated β-lactamase possessed by the majority of the ampicillin-resistant strains isolated in 1967 and 1968 (S. Elson, personal communication). These facts suggest that R-factor-mediated ampicillin resistance was not common among enteropathogenic *E. coli* between 1948 and 1960. However, it is possible that some of the strains which are now susceptible to ampicillin may have possessed ampicillin R-factors which were subsequently lost during storage. For example, R-factor-mediated ampicillin resistance has been described among *E. coli* isolated in Mexico in 1956 (7) and among *E. coli* isolated in London in 1961 (13). In the latter study, three out of seven ampicillin-resistant strains isolated in 1961 had lost their resistance to ampicillin when retested in 1968; the remaining four strains possessed transferable ampicillin resistance. Moreover, since transferable streptomycin and sulphonamide resistance became relatively common among enteropathogenic *E. coli* between 1951 and 1960, it would not be unexpected to find transferable penicillin resistance appearing during this period. One third of the ampicillin-resistant strains of *E. coli* isolated in 1967 and 1968 had a nontransferable β-lactamase determinant similar to that produced by strains isolated in the earlier periods, whereas the remainder of the ampicillin-resistant strains possessed a transferable extrachromosomally mediated β-lactamase.

The pattern of streptomycin resistance of the *E. coli* examined is in some ways analogous to that of the ampicillin resistance of these strains. Thus, there was a relatively high incidence of streptomycin-resistant strains of *E. coli* isolated

before 1951, which is not entirely unexpected since streptomycin was freely available at this time. It seems probable too that the resistance of these particular strains is chromosomally mediated since, apart from the fact it is non-transferable and is not "cured" by ethidium bromide, the level of resistance of strains isolated before 1951 is very high, a characteristic of streptomycin-resistant mutants, in contrast to the lower levels of resistance found in the R-factor-mediated streptomycin-resistant strains isolated from 1957 onwards. In addition, most of the streptomycin-resistant strains isolated before 1951 were susceptible to sulphonamides, whereas most of the streptomycin-resistant strains isolated from 1957 onwards which transferred streptomycin resistance were also resistant to sulphonamides. It is unlikely, therefore, that the streptomycin-resistant strains isolated before 1951 are R-factor bacteria lacking transfer factors. These facts suggest that transferable streptomycin resistance was not a feature of the streptomycin resistance of *E. coli* isolated before 1951 but became common relatively soon afterwards, so that by 1957 the proportion of strains carrying streptomycin R-factors was as high as that observed in strains isolated in 1968.

It is perhaps not unexpected that three of the 14 sulphonamide-resistant strains isolated between 1948 and 1951 were able to transfer sulphonamide resistance, since this class of compounds had been widely used for more than 10 years before this, although, in fact, the incidence of sulphonamide resistance of the strains isolated before 1951 was relatively low. It is probable that the three strains isolated in 1951 ~~1957~~ capable of transferring sulphonamide resistance came from the same source, and it is of interest that the sulphonamide resistance of these strains was not linked with transferable streptomycin resistance as was generally the case for strains isolated in later periods. By 1957 ~~1951~~, sulphonamide resistance had become more common and was often R-factor mediated, associated with streptomycin or multiple resistance. It seems likely, therefore, that there was a marked increase in transferable sulphonamide resistance between 1951 and 1957.

The data relating to chloramphenicol and tetracycline resistance show that resistance to these two antibiotics, largely R-factor mediated, appeared sometime after 1951, coincident with the increasing usage of these antibiotics. By 1957 most of the chloramphenicol- and tetracycline-resistant strains were capable of transferring antibiotic resistance, and as with streptomycin, the incidence of transferable chloramphenicol and tetracycline resistance apparently remained unchanged between 1957 and 1968.

Although it is known that R-factors were present in bacteria prior to 1959 (7, 14, 16), when the phenomenon of transferable antibiotic resistance was first reported, data on the frequency of such strains are lacking. Our results with a relatively large number of homogenous bacteria isolated between 1948 and 1968 show that R-factor-mediated sulphonamide resistance was present in *E. coli* as long ago as 1951, and that transferable antibiotic resistance became established among enteropathogenic strains of *E. coli* in the United Kingdom at some time between 1951 and 1957. It was unexpected, however, that the incidence of R-factor-mediated resistance was about as high among antibiotic-resistant strains isolated between 1957 and 1960 as in strains collected in 1967 and 1968. Moreover, although strains capable of transferring antibiotic resistance to susceptible bacteria were widespread between 1957 and 1968, there was apparently no marked increase in the overall incidence of resistance of these strains of *E. coli* to the four antibiotics tested, or to sulphonamides, during this period. These results show that the phenomenon of transferable antibiotic resistance has been common among *E. coli* for a longer period of time than has hitherto been supposed and do not support the view that the emergence of transferable antibiotic resistance among enteric bacteria will inevitably lead to a rapid or general increase in the antibiotic resistance of this group of bacteria.

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DRUG-INACTIVATING ENZYMES AND ANTIBIOTIC RESISTANCE

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ANTIBIOTIC RESISTANCE AND β-LACTAMASES OF *ESCHERICHIA COLI* BETWEEN 1948 AND 1968

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It was first reported from Japan in 1959 (Ochiai et al., 1959; Akiba et al., 1960), that multiple antibiotic resistance could be transferred readily between strains of *E. coli* and *Shigella* and it was shown that R-factor bacteria constituted a high proportion of the antibiotic-resistant isolates of enteric bacteria in Japan at that time. The original report was followed within a fairly short time by other similar reports from Europe, Israel, Taiwan and the U.S.A., (Nakaya, 1960; Datta, 1962; Lebek, 1963; Kabins and Cohen, 1966), and it was evident that transferable antibiotic resistance was common and world-wide in the early 1960's. Little is known however, of the origin or spread of R-factors among bacteria prior to the original report from Japan in 1959 and this study is concerned with the development, in the United Kingdom, of transferable antibiotic resistance among enteropathogenic strains of *E. coli* between 1948 and 1968. (Slocombe and Sutherland, 1973).

A total of 512 strains of *E. coli* was obtained from the Salmonella Reference Laboratory, Colindale, through the courtesy of its former Director, Dr. Joan Taylor. The cultures examined had been isolated from clinical sources throughout the United Kingdom at three different periods of time between 1948 and 1968, namely 1948 to 1951, (112 strains) 1957 to 1960, (200 strains) and 1967 to 1968 (200 strains). Bacteria from these three periods were selected for study as different groups of antibiotics were available during each period. Thus, benzylpenicillin, streptomycin and sulphonamides were widely used between 1948 and 1951, chloramphenicol and the tetracyclines became available in the United Kingdom soon afterwards, and ampicillin was introduced in 1961. Other antibiotics used in 1967 and 1968 for the treatment of infections due to Gram-negative bacteria included cephaloridine and kanamycin, and carbenicillin and gentamicin first became available during this period.

Most of the strains of *E. coli* isolated during the two later periods had been isolated from cases of infantile diarrhoea throughout the country and represented a wide range of serotypes. The origin of the strains isolated between 1948 and 1951 is less certain, but they are known to be of human origin and are thought to be enteropathogenic strains of *E. coli* responsible for infantile gastroenteritis.

Prior to the tests described here, the cultures had been stored in the dark at room temperature on antibiotic-free Dorset egg-agar slopes. The 512 strains were tested for sensitivity to ampicillin, chloramphenicol, streptomycin, tetracyclines and sulphonamides and Minimum Inhibitory Concentrations were determined by serial-dilution tests in agar. Strains of *E. coli* found to be resistant to one or more of the antibacterial agents were tested for ability to transfer resistance to *E. coli* K12/711Nx (Slocombe and Sutherland, 1973).

ANTIBIOTIC RESISTANCE

Results in Fig. 1 compare the sensitivities of the strains of *E. coli* to each of the four antibiotics for the three different time periods.

Ampicillin resistance

Ampicillin inhibited 66% of the 112 strains of *E. coli* isolated between 1948 and 1951 at a concentration of 12.5 μg/ml or less; the remainder (34%) required inhibitory concentrations of from 25 to 250 μg/ml of ampicillin. Of the 200 strains isolated between 1957 and 1960, 94% of the strains were sensitive to ampicillin and the remainder were sensitive to concentrations of 25 to 250 μg/ml of ampicillin. Most (85%) of the strains isolated in 1967 and 1968 were sensitive to ampicillin, but only one-third of the resistant strains were inhibited by 250 μg/ml of ampicillin. The remainder of the resistant strains (10% of the total tested) were insensitive to 1000 μg/ml ampicillin.

Ampicillin-resistant strains destroyed ampicillin during the course of serial-dilution tests showing that resistance was associated with β-lactamase activity.

Streptomycin resistance

In general, the incidence of resistance to streptomycin was similar for the three periods (74% sensitive to 12.5 μg/ml in 1948–1951, 85% sensitive in 1957–1960, and 78% sensi-

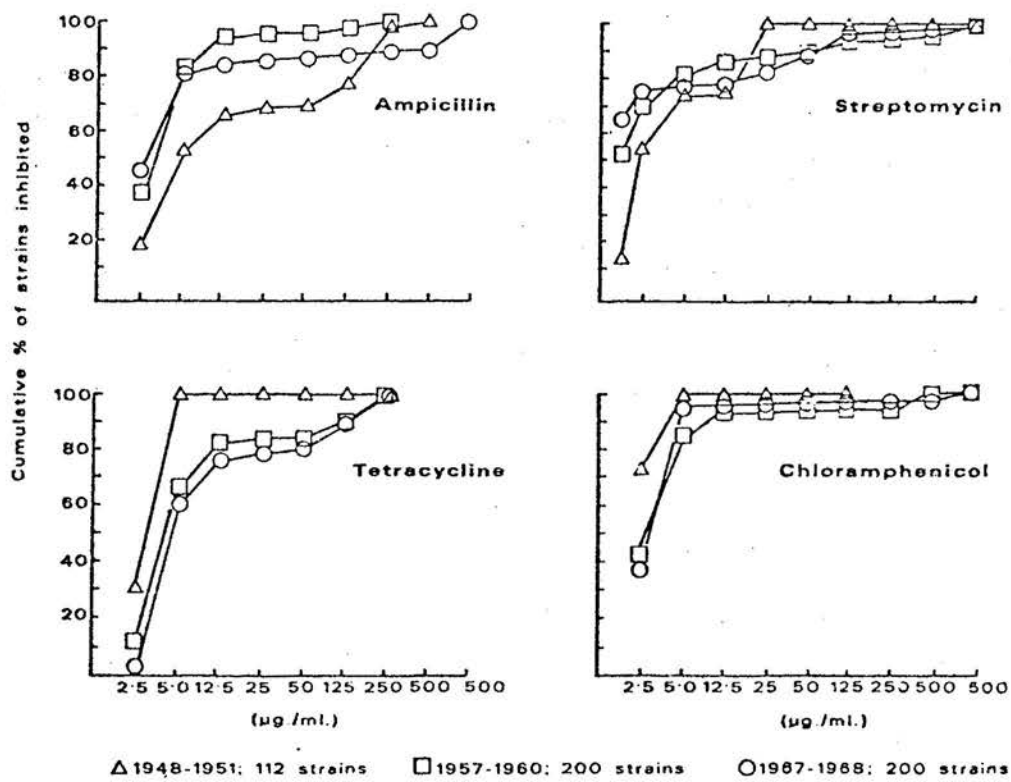

Figure 1. Antibiotic sensitivity of *Escherichia coli* isolated between 1948–1951; 1957–1960; 1967–1968.

tive in 1967–1968). However, whereas all of the resistant strains isolated between 1948 and 1951 were insensitive to 500 μg/ml to streptomycin only a small proportion of the resistant strains isolated in the two later periods were resistant to this concentration of streptomycin.

Chloramphenicol resistance

Only one of the strains of *E. coli* isolated between 1948 and 1951 was resistant to chloramphenicol, and only a small proportion of strains isolated in the two later periods were insensitive to 12.5 μg/ml of chloramphenicol (7.5% in 1957 to 1960 and 5% in 1967 to 1968).

Tetracycline resistance

None of the strains of *E. coli* isolated between 1948 and 1951 was resistant to a concentration of 5 μg/ml of tetracycline but resistance was common among bacteria isolated between 1957 and 1968. Thus, 17.5% of the strains isolated in 1957–1960 and 22% of those isolated in 1967–1968 were resistant to 12.5 μg/ml of tetracycline. The distribution of resistance to tetracycline among these two groups of *E. coli* was very similar.

Sulphonamide resistance

Most (87.5%) of the strains of *E. coli* isolated between 1948 and 1951 were sensitive to 20 μg/ml of sulphamethoxazole. The incidence of sulphonamide-resistant strains was higher in the two later periods when 21% of strains tested were found to be insensitive to 20 μg/ml of sulphamethoxazole.

These results show that resistance to penicillin (or ampicillin) streptomycin and sulphonamides was relatively common between 1948 and 1951, and is presumably a reflection of the selective pressures of the usage of these antibiotics. Resistance to chloramphenicol and tetracycline was rare before 1951, but after their introduction into clinical use resistance to these two antibiotics became more common. It is striking that with the exception of ampicillin resistance, the antibiotic resistance-patterns for the period 1957–1960 were very similar to those of the later period, 1967–1968.

TRANSFERABLE RESISTANCE

Strains shown to be resistant to any of the four antibiotics, or to the sulphonamide, wer tested for their ability to transfer antibiotic resistance to *E. coli* K12/711Nx and the results of these tests are shown in Table 1.

None of the ampicillin-resistant strains isolated between 1948 to 1960 (with ampicillin MIC values of 25–250 μg/ml) was able to transfer ampicillin resistance to the recipient strain, as was the case for strains isolated in 1967–1968 with ampicillin MIC values in the same range. In contrast, all of the ampicillin-resistant strains isolated in 1967–1968 with ampicillin MIC values of greater than 1000 μg/ml transferred ampicillin resistance associated with β-lactamase. Treatment with ethidium bromide had no effect on the resistance to ampicillin of strains that failed to transfer ampicillin resistance but resulted in the elimination of the ampicillin-resistance of the R-factor strains isolated in 1967–1968.

None of the streptomycin-resistant strains of *E. coli* isolated between 1948 and 1951 transferred streptomycin-resistance, but two-thirds of the streptomycin-resistant strains isolated between 1957 and 1968 were capable of transferring streptomycin-resistance to the recipient strain. R-factor mediated streptomycin resistance was eliminated by treatment with ethidium bromide but the streptomycin resistance of strains isolated before 1951 was unaffected by this treatment.

TABLE 1

Transferable antibiotic resistance among Escherichia coli isolated between 1948–1968

Antibiotic	Time of isolation	No. of strains	Resistant strains*		Transferable resistance		
			number	% of total	No. of strains	% of resistant strains	% of total strains
Ampicillin	1948–1951	112	38	34.	0	0	0
	1957–1960	200	10	5.0	0	0	0
	1967–1968	200	30	15	21	70	10.5
Chloramphenicol	1948–1951	112	1	1.0	0	0	0
	1957–1960	200	15	7.5	13	87	6.5
	1967–1968	200	10	5.0	10	100	5.0
Streptomycin	1948–1951	112	28	25	0	0	0
	1957–1960	200	28	14	19	68	9.5
	1967–1968	200	44	22	29	66	14.5
Tetracycline	1948–1951	112	0	0	0	0	0
	1957–1960	200	35	17.5	21	60	10.5
	1967–1968	200	44	22	30	68	15
Sulphamethoxazole	1948–1951	112	14**	12.5	3	21	2.7
	1957–1960	200	43**	21.5	17	40	8.5
	1967–1968	200	42**	21	17	40	8.5

* Not inhibited by 12.5 μg/ml of test antibiotic
** Resistant to 200 μg/ml of sulphamethoxazole

Only one strain of *E. coli* isolated in 1948–1951 was resistant to chloramphenicol and this strain failed to transfer its resistance. In contrast, virtually all of the chloramphenicol-resistant strains isolated between 1957 and 1968 transferred chloramphenicol resistance to *E. coli* K12/711Nx. Similarly, tetracycline resistance was readily transferred from resistant strains isolated in this period (60% in 1957–1960 and 68% in 1967–1968).

Three of the fourteen sulphonamide-resistant strains of *E. coli* that were isolated between 1948 and 1951 were found to be capable of transferring sulphonamide resistance of *E. coli* K12/711Nx. These three R-factor strains were isolated in 1951 and probably had a common source. The strains were resistant also to ampicillin, but the ampicillin resistance was not transferred. In the two later periods, 40% of the sulphonamide-resistant strains transferred sulphonamide resistance, usually in association with streptomycin or other resistance.

These results show that R-factor-mediated resistance became prevalent among enteropathogenic strains of *E. coli* in the United Kingdom at some time between 1951 and 1957 although transferable sulphonamide resistance was demonstrated in a small number of strains isolated in 1951. It is of interest, too, that the incidence of R factor-mediated resistance was about as high among antibiotic-resistant strains isolated between 1957–1960 as in strains collected in 1967 and 1968.

Phenotype resistance to β-lactam antibiotics

All ampicillin-resistant strains tested were found to destroy ampicillin in serial-dilution tests to measure Minimum Inhibitory Concentrations of ampicillin, showing that resistance was associated with β-lactamase activity. It was apparent, too, that the ampicillin-resistant bacteria could be separated into two groups on the basis of their resistance to β-lactam antibiotics.

Thus, ampicillin-resistant strains isolated between 1948 and 1968 that had ampicillin MIC values in the range 25–250 μg/ml were sensitive to carbenicillin and to combinations of ampicillin and cloxacillin but were resistant to cephalosporin antibiotics (Table 2). These strains failed to transfer ampicillin resistance, their ampicillin resistance was

TABLE 2

Phenotypic resistance to β-lactam antibiotics

Ampicillin M.I.C. (ug/ml)	Sensitivity to			Resistance	
	Carbenicillin	Ampicillin/ Cloxacillin	Cephalosporins	transferable	effect of curing agents
25—250	S	S	R	—	—
>1000	R	R	variable	+	+

S = Sensitive R = Resistant

unaffected by treatment with ethidium bromide or sodium dodecyl sulphate, and efforts to mobilise or displace the ampicillin resistance by the introduction of plasmids of different compatibilities (RTEM, RA3, S-a, K64, N3, "wild type") were unsuccessful. In contrast, strains isolated in 1967–1968 that were resistant to 1000 μg/ml of ampicillin, were resistant also to carbenicillin and ampicillin/cloxacillin, but were generally more sensitive to the cephalosporins (Table 2). These strains were capable of transferring their ampicillin-resistance and treatment with curing agents resulted in the loss of ampicillin resistance.

It seemed likely, therefore, on the basis of these properties that the strains of *E. coli* that did not transfer ampicillin resistance possessed a chromosomally-mediated β-lactamase that differed from the extrachromosomally-mediated β-lactamase of the R factor strains.

Properties of β-lactamases

Crude, cell-free, β-lactamase preparations were prepared by ultrasonication of selected cultures (Cole, Elson, and Fullbrook, 1972) and these enzyme preparations were tested for electrophoretic mobility and substrate and inhibition profiles. The R-factor strains investigated were transferred to a common host, *E. coli* K12 711/Nx, and the recombinants, enriched by selection in the presence of carbenicillin, were used as the sources of extrachromosomal β-lactamases. For comparison, β-lactamase preparations were also made from the following reference strains:

a) Chromosomally-mediated, *E. coli* D31 (Lindstrom, Boman and Steele, 1970).

b) extrachromosomally-mediated 1. *E. coli R-TEM* (*R6K*)
(Datta and Kontomichalou, 1965;
Datta and Richmond, 1966.)

2. *E. coli R-GN238*
(Egawa, Sawai and Mitsuhashi, 1967.)

3. *E. coli R-1818* (*R46*)
(Dale and Smith, 1971).

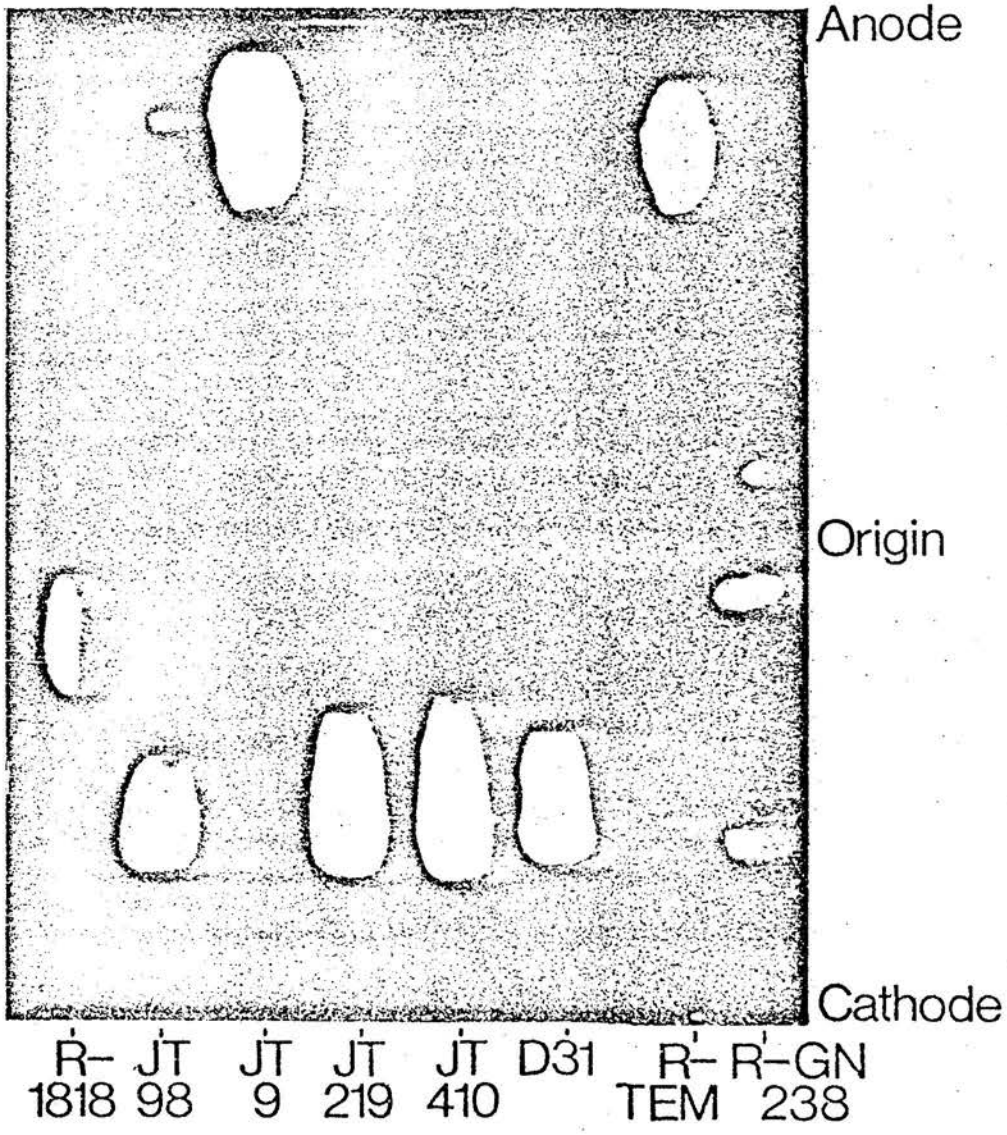

Figure 2. Electrophoretic mobilities of β-lactamases of *E. coli*.

Electrophoretic mobility

The electrophoretic mobilities of the β-lactamases were determined on starch-agar gel (360v, pH 8.5) by a modification of the method described by Jack and Richmond (1970) and results of a typical experiment are illustrated in Fig. 2. The enzyme from the R factor strain of *E. coli* (JT9) moved towards the anode at the same rate as the R-TEM preparation (—2.5 cm/hr) whereas the enzymes from the strains of *E. coli* with non-transferable ampicillin resistance (JT 219, JT 415) migrated towards the cathode (+2.1 cm/hr), slightly faster than the chromosomally-mediated β-lactamase from *E. coli* D31 (+1.8 cm/hr). The reference oxacillin-hydrolising β-lactamases, R-GN238, R-1818, migrated with low positive mobilities (+0.3 cm/hr, +1.1 cm/hr respectively). One strain JT 98, possessed two electrophoretically distinct enzymes, one with positive mobility (+2.0 cm hr) the other with negative mobility (—2.5 cm/hr).

Substrate and inhibition profiles

Substrate and inhibition profiles of the β-lactamase preparations are shown in Table 3, where the hydroxylamine method described by Dale and Smith (1971) was used for assay of β-lactamase activity. The non-transferable enzymes, JT163 and JT415, resembled the

TABLE 3

Substrate and Inhibition profiles of β-lactamases from ampicillin-resistant E. coli

Enzyme	Relative rates of hydrolysis: Penicillin G	Ampicillin	Carbenicillin	Cephaloridine	Cephalexin	Cloxacillin	Inhibition by: Cloxacillin	Cl⁻ (%)
JT 163	100	0	18	600	32	0	>60	0
JT 415	100	0	0	321	38	0	>60	0
JT 219	100	0	0	335	67	0	>60	0
D-31	100	0	5	350	37	0	>60	0
JT 9	100	87	11	76	0	3	>60	0
JT 20	100	74	13	65	0	5	>60	0
R-TEM	100	86	18	72	0	3	>60	0
R-GN238	100	453	86	24	0	90	—*	60
R-1818	100	150	48	26	0	186	—*	80

* Cloxacillin was a substrate.

chromosomally-mediated β-lactamase, D31, and were more active against the cephalosporins than against ampicillin and carbenicillin. The β-lactamase from the R factor strain JT9, was very similar to that of R-TEM and inactivated the penicillins more readily than the cephalosporins. The oxacillin-hydrolising enzymes, R-GN238 and R-1818 represented a third group of β-lactamases.

One strain, JT98, isolated in 1968, was found to possess both a chromosomally-mediated β-lactamase and an extrachromosomal-mediated β-lactamase, both enzymes having characteristics similar to those described above. This strain was insensitive to both penicillins and cephalosporins. It is perhaps surprising that more strains with both β-lactamases were not found in this series of tests as strains possessing a chromosomal β-lactamase were good recipients of R-factor mediated β-lactamases in transfer experiments.

These results show that the β-lactamases of ampicillin-resistant strains of *E. coli* isolated between 1948 and 1968 fell into two groups according to their biochemical characteristics. Thus, strains that did not transfer ampicillin resistance possessed a β-lactamase similar to the chromosomally-mediated Type Ib enzyme (Richmond and Sykes, 1973) of *E. coli* D31, while the extrachromosomal β-lactamases of R factor strains of *E. coli* were similar to R TEM, a Type IIIa enzyme (Richmond and Sykes, 1973). These facts suggest that the extrachromosomally-mediated β-lactamases of R-factor bacteria were not derived from the chromosomally-mediated β-lactamase found in ampicillin-resistant strains of *E. coli* between 1948 and 1966.

CONCLUSION

This study of a homogenous group of enteropathogenic strains of *E. coli* isolated in the United Kingdom between 1948 and 1968 has shown that transferable antibiotic resistance became prevalent among these bacteria between 1951 and 1957, although a small number of strains with R factor sulphonamide-mediated resistance were isolated before this (in 1951). The incidence of R factor-mediated antibiotic resistance among this group of bacteria was similar in 1957–1960 as it was in 1967–1968 showing that transferable antibiotic resistance was well-established in the United Kingdom at this earlier period of time.

The ampicillin-resistant strains of *E. coli* isolated between 1948 and 1960 did not transfer ampicillin-resistance and appeared to possess a chromosomally-mediated β-lactamase which was quite distinct from the extrachromosomally-mediated β-lactamase of R factor strains of *E. coli* isolated in 1967–1968. R factor mediated β-lactamase woud not appear therefore to have its origin in β-lactamase producing strains of *E. coli*.

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Activity of ampicillin *in vitro* compared with other antibiotics

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SYNOPSIS Comparative tests *in vitro* for antibacterial activity were carried out with ampicillin, tetracycline, and chloramphenicol using 673 clinical isolates of Gram-negative bacilli and *Streptococcus faecalis*. Further comparative tests were also carried out with ampicillin, chloramphenicol, colistin sulphate, colistin methane sulphonate, cycloserine, kanamycin, nitrofurantoin, polymyxin, streptomycin, and tetracycline, using groups of 20 strains of each of the main species selected at random from the total number of isolates. Of the total number of isolates a higher percentage was inhibited by ampicillin than by tetracycline or chloramphenicol. Ampicillin showed particularly high activity against certain species of bacteria and displayed an antibacterial spectrum not shown by any of the other antibiotics tested.

Ampicillin(6[D(-)α-aminophenylacetamido]penicillanic acid) is a penicillin with broad-spectrum activity (Rolinson and Stevens, 1961). The compound is acid stable and gives satisfactory serum concentrations after oral administration (Knudsen, Rolinson, and Stevens, 1961). Ampicillin shows a very low order of toxicity (Acred, Brown, Turner, and Wilson, 1962) and the early clinical experience with ampicillin was reported by Stewart, Coles, Nixon, and Holt (1961); Bunn (1961); Rutenberg, Greenberg, Schweinburg, and Perreault (1961); Brumfitt, Percival, and Carter (1962); and Trafford, Maclaren, Lillicrap, Barnes, Houston, and Knox (1962).

The antibacterial spectrum of ampicillin includes the pyogenic cocci, enterococci, *Neisseria* species including the gonococcus, *Haemophilus influenzae*, *Salmonella* species, and strains of *Escherichia coli*, *Shigella* and *Proteus*. Not all strains of these organisms are uniformly sensitive to ampicillin, however, and conflicting statements regarding the activity of this penicillin have appeared in the literature, particularly against coliforms and *Proteus* species.

METHODS

A total of 673 strains of bacteria was obtained from the Middlesex Hospital, the West Middlesex Hospital, and St. Thomas's Hospital, London. Nearly all the cultures had been associated with urinary tract infections and were received as isolated routinely without selection of any kind.

CLASSIFICATION The cultures were supplied on agar slopes and were cultured on MacConkey's agar, from which mixed cultures were separated into pure cultures.

The *E. coli* group was defined as lactose-fermenting, non-citrate utilizing bacteria; this group included indole-positive (type I) and indole-negative (intermediate coliforms) organisms. Lactose-fermenting, citrate-utilizing, indole-negative bacteria were classified as *Aerobacter aerogenes*.

Proteus species were identified with some considerable care in view of the wide variation in sensitivity of these organisms to ampicillin, and classification was based on the biochemical tests as reported by Mackie and McCartney (1962). The majority of Proteus strains encountered in this study belonged to the species *Proteus mirabilis;* other *Proteus* species were rare.

The remainder of the non-lactose fermenting, non-urea-hydrolysing organisms were tested for phenylpyruvic acid production by the method of Stewart (1961) in order to isolate providencia (*Proteus inconstans*) organisms; these strains were also tested for β-(D)-galactosidase production (Lowe, 1962) to isolate late-lactose fermenting cultures which would be classified as paracolon bacteria. Pseudomonas was distinguished by the characteristic odour and production of green pigment on MacConkey's medium, although in some instances weak reactions were confirmed by culture in Hugh and Leifson's medium, and by tests to demonstrate utilization of arginine (Thornley, 1960).

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Streptococcus faecalis was identified by colonial appearance on MacConkey's agar and Gram-stain reaction.

ANTIBIOTIC SENSITIVITIES Minimum inhibitory concentrations were determined by serial dilution in agar. A loop of an overnight broth culture was used as inoculum and the plates were incubated at 37° C. overnight.

RESULTS

COMPARATIVE ACTIVITIES OF AMPICILLIN, CHLORAMPHENICOL, AND TETRACYCLINE Of the 673 isolates tested for sensitivity to ampicillin, chloramphenicol, and tetracycline, the predominant organism was *E. coli*. This accounted for 40% of the total number. *Proteus mirabilis* was the next most common pathogen (24%); other species of *Proteus*, namely *P. vulgaris* and *P. morganii*, comprised only 2% of the total. The remainder was accounted for by *Aerobacter aerogenes* (12%), *Streptococcus faecalis* (10%), *Pseudomonas pyocyanea* (7%), and miscellaneous organisms, comprising mainly paracolon and some achromobacter bacteria, (6%). The antibiotic sensitivities of the bacteria are shown in Table I. Of the total of 269 strains of *E. coli*, 78% were inhibited by 5 μg./ml. or less of ampicillin. The corresponding figures for chloramphenicol and tetracycline were 52% and 64%, respectively. At this concentration, therefore, ampicillin was more effective than either chloramphenicol or tetracycline. At the lower concentration of 2·5 μg./ml., however, only 19% were inhibited by ampicillin whereas with tetracycline the corresponding figure was 45% and for chloramphenicol only 8%.

Resistance of *E. coli* to ampicillin was not associated with resistance to tetracycline or chloramphenicol. The strains resistant to tetracycline or chloramphenicol were as sensitive to ampicillin as was the total number of isolates tested.

The strains of *P. mirabilis* were generally highly sensitive to ampicillin, 83% being inhibited by a concentration of 5·0 μg./ml. and 74% by 2·5 μg./ml. Both chloramphenicol and tetracycline were considerably less active at these concentrations. In contrast with *P. mirabilis*, all the strains of *P. morganii* and *P. vulgaris* were relatively resistant to ampicillin.

Ampicillin was highly active against the isolates of *Streptococcus faecalis*, 95% being inhibited by 2·5 μg./ml. whereas chloramphenicol and tetracycline were relatively ineffective. Against strains of *Aerobacter aerogenes*, ampicillin generally showed a low order of activity. Of a total of 80 strains, however, 20% were inhibited by 5·0 μg./ml. or less of ampicillin. Similarly, not all paracolon organisms were resistant to ampicillin, about half of the strains tested being inhibited by 5·0 μg./ml. ampicillin.

TABLE I

ACTIVITY OF AMPICILLIN, CHLORAMPHENICOL, AND TETRACYCLINE AGAINST 673 STRAINS OF GRAM-NEGATIVE BACTERIA AND ENTEROCOCCI

Organism	*Antibiotic*	*Number of Strains*	*Percentage of Total*	*Minimum Inhibitory Concentrations (μg./ml.) and No. of Strains*										
				>250	250	125	50	25	12·5	5·0	2·5	1·25	0·5	0·25
E. coli	Ampicillin	269	40·0	8	2	4	3	1	39	161	46	5		
	Chloramphenicol	270		15	3	2	4	6	101	116	12	9	2	
	Tetracycline	259		11	20	13	11	23	13	50	59	54	3	2
Proteus mirabilis	Ampicillin	156	23·2	11		3	2	5	5	14	80	36		
	Chloramphenicol	156		1	3	6	5	6	89	41	4	1		
	Tetracycline	156		16	3	17	58	14	1	1	4	2		
Streptococcus faecalis	Ampicillin	67	10·0	1		1			1		21	37	3	3
	Chloramphenicol	66				3	8	2	34	12	6	1		
	Tetracycline	67		1	10	17	3	4	3	5	7	13	4	
Aerobacter aerogenes	Ampicillin	80	11·9	25	7	5	13	5	9	11	2	3		
	Chloramphenicol	80		10	3	3	4	2	18	26	5	8	1	
	Tetracycline	80		5	8	6	6	15	5	6	9	17	2	1
Pseudomonas pyocyanea	Ampicillin	47	7·0	40	4		2			1				
	Chloramphenicol	47		4	4	25	10	1	2	1				
	Tetracycline	47		15	9	6	9	7			1			
Miscellaneous[1]	Ampicillin	40	6·0	5	3	3	1	4	5	12	6	1		
	Chloramphenicol	40		1	1	2	1	3	15	12	5			
	Tetracycline	40		2	6	1	3	2	1	12	8	5		
Proteus species[2]	Ampicillin	13	1·9	5	2	2	2		1	1				
	Chloramphenicol	13				2	1		3	6	1			
	Tetracycline	13		3	2	1	1		4			1	1	

[1]Mainly paracolon organisms but including achromobacter sp. and *Proteus inconstans*.
[2]*Proteus morganii*, 10 strains; *Proteus vulgaris*, three strains.

TABLE II

COMPARISON OF AMPICILLIN WITH NINE ANTIBIOTICS AGAINST GRAM-NEGATIVE BACTERIA AND ENTEROCOCCI

Organism	*Number of Strains*	*Antibiotic*	*Minimum Inhibitory Concentrations (μg./ml.) and No. of Strains*										
			>500	*500*	*250*	*125*	*50*	*25*	*12·5*	*5·0*	*2·5*	*1·25*	*0·5*
E. coli	20	Ampicillin	2				1		2	9	6		
		Chloramphenicol		2			1		4	13			
		Colistin sulphate	1			1				3	15		
		Colistin methane sulphonate	1			1			17	1			
		Cycloserine				4	12	4					
		Kanamycin						1		3		16	
		Nitrofurantoin				3	3	2	10	2			
		Polymyxin	1							2	10	7	
		Streptomycin	2					1		1	6	9	1
		Tetracycline		1	1	2					14	2	
Proteus mirabilis	20	Ampicillin	1								2	15	2
		Chloramphenicol						2	16	2			
		Colistin sulphate	19							1			
		Colistin methane sulphonate	18	1				1					
		Cycloserine			1	19							
		Kanamycin							6	14			
		Nitrofurantoin				8	12						
		Polymyxin	19						1				
		Streptomycin	1						11	8			
		Tetracycline		2	18								
Streptococcus faecalis	20	Ampicillin										20	
		Chloramphenicol				5	1	3	11				
		Colistin sulphate	20										
		Colistin methane sulphonate	20										
		Cycloserine			2	18							
		Kanamycin						5	15				
		Nitrofurantoin				1		19					
		Polymyxin	5	15									
		Streptomycin	10				8	2					
		Tetracycline			11	1	1			1	6		
Aerobacter aerogenes	10	Ampicillin	2	2	1		3	1	1				
		Chloramphenicol	1						2	3	2	2	
		Colistin sulphate								4	4	2	
		Colistin methane sulphonate						2	8				
		Cycloserine			2	6	2						
		Kanamycin						1	1		2	6	
		Nitrofurantoin				5	2	2	1				
		Polymyxin								6	4		
		Streptomycin	1	1							1	7	
		Tetracycline			2				1	3	1	3	
Pseudomonas pyocyanea	20	Ampicillin	19							1			
		Chloramphenicol			1	18			1				
		Colistin sulphate								19		1	
		Colistin methane sulphonate					18	1	1				
		Cycloserine		1	11	8							
		Kanamycin			10	6	4						
		Nitrofurantoin			18	2							
		Polymyxin								19	1		
		Streptomycin	7			2	2	8	1				
		Tetracycline				15	4				1		
Mixed *proteus*[1]	9	Ampicillin	1		1		1		2		2	2	
		Chloramphenicol		1		1			4	3			
		Colistin sulphate	6						1	2			
		Colistin methane sulphonate	5	1				2	1				
		Cycloserine	1			8							
		Kanamycin								2	3	4	
		Nitrofurantoin				6	2	1					
		Polymyxin	7							1	1		
		Streptomycin	3							3	1	2	
		Tetracycline			1	1	3	1	1			2	

[1]Three of *P. morganii*, one of *P. inconstans*, four of *P. vulgaris*, and one of *P. rettgeri*.

With the strains of *Pseudomonas pyocyanea*, ampicillin, and also chloramphenicol and tetracycline, showed a very low order of activity.

ACTIVITY OF AMPICILLIN AND NINE OTHER ANTIBIOTICS

Groups of 20 strains of each of the main species were selected at random from the total number of isolates and tested for antibiotic sensitivity to ampicillin, chloramphenicol, colistin sulphate, colistin methane sulphonate, cycloserine, kanamycin, nitrofurantoin, polymyxin, streptomycin and tetracycline. The minimum inhibitory concentration values are shown in Table II. Against the group of *E. coli*, colistin sulphate, kanamycin, polymyxin, streptomycin and tetracycline were all more active than ampicillin. Colistin methane sulphonate, however, was less active and cycloserine and nitrofurantoin were relatively ineffective.

Against *P. mirabilis*, ampicillin was markedly more active than any of the other antibiotics tested. *Proteus mirabilis* was tested as a separate group because this was the species of proteus most frequently encountered clinically. Results for a mixed group of nine proteus cultures comprising *P. morganii*, *P. inconstans*, *P. vulgaris*, and *P. rettgeri* are also shown in Table II. The minimum inhibitory concentration values were generally very variable for most of the antibiotics tested. At a level of 5·0 μg./ml. only two strains were inhibited by tetracycline and three by chloramphenicol. Four strains were inhibited by ampicillin at this level. Kanamycin and streptomycin were the most effective of the antibiotics tested against this group of organisms.

Ampicillin was outstandingly active against *Streptococcus faecalis* in comparison with the other antibiotics. Ampicillin, however, showed little activity against 10 strains of *Aerobacter aerogenes*, and chloramphenicol, colistin sulphate, kanamycin, polymyxin, and streptomycin all showed greater activity although resistant strains were observed with most of these antibiotics. Against *Pseudomonas pyocyanea* only polymyxin and colistin sulphate were active at concentrations of 5 μg./ml. Colistin methane sulphonate showed a considerably lower order of activity against this organism and none of the other antibiotics demonstrated significant activity.

DISCUSSION

The results obtained in this report with a fairly large number of clinical isolates from three different hospitals give some indication of the general sensitivity of Gram-negative bacilli and enterococci to ampicillin. Some comparisons can also be drawn between the activity of ampicillin and that of other antibiotics in current use.

Taking the group of 673 isolates tested as a whole, 448 (67%) were inhibited by ampicillin at a concentration of 5 μg./ml. The corresponding figures for chloramphenicol and tetracycline were 42% and 38%, respectively. Among the coliforms, strains of *E. coli* were fairly sensitive to ampicillin whereas strains of aerobacter were mostly resistant. Similarly, with *Proteus* species, strains of *Proteus mirabilis* were mostly sensitive to ampicillin whereas strains of other *Proteus* species, such as *P. morganii*, *P. vulgaris* and *P. rettgeri* were generally resistant. Ampicillin was notably active against *Strep. faecalis*. Strains of *E. coli*, *Proteus mirabilis*, and *Strep. faecalis* appear as the organisms most sensitive to ampicillin and these account for 75% of the total number of isolates tested in this work. They are also reported by Brumfitt *et al.* (1962) and Garrod, Shooter, and Curwen (1954) to be the organisms most frequently encountered in urinary tract infections.

Resistance to ampicillin was encountered, particularly among strains of *Aerobacter* and *Proteus* species other than *P. mirabilis*. Unlike the resistance of staphylococci to penicillin G, the resistance of Gram-negative bacilli to ampicillin can be due in certain cases to penicillinase production, and in other cases to intrinsic resistance, and some bacteria exhibit both of these characteristics. Penicillinase production, however, does not always result in a high degree of resistance to ampicillin. This is because ampicillin is significantly more stable than penicillin G to the penicillinase produced by certain bacteria, and in particular certain strains of *E. coli* (Auhagen, Gloxhuber, Hecht, Knott, Otten, Rauenbusch, Risse, Schmid, Scholtan, and Walter, 1962; Ayliffe, 1963; Percival, Brumfitt, and de Louvois, 1963; Smith and Hamilton-Miller, 1963; Sutherland, 1964). On the other hand ampicillin is generally less stable than penicillin G to the penicillinase produced by aerobacter strains (Smith and Hamilton-Miller, 1963) and the resistance which these strains generally show to ampicillin is usually associated with inactivation of the drug (Ayliffe, 1963; Sutherland, 1964). In contrast, the resistance of *Pseudomonas pyocyanea* to ampicillin is due entirely to intrinsic insensitivity, ampicillin being extremely stable to the penicillinase produced by these organisms (Sutherland, 1964).

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Sensitivity of Gram-negative bacilli to ampicillin after six years' clinical use

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SYNOPSIS A total of 1,102 clinical isolates of Gram-negative bacilli was obtained from four hospitals during 1967 and these cultures were tested for sensitivity to ampicillin. Approximately 80% of the strains of *Escherichia coli* and 90% of the strains of *Proteus mirabilis*, the two organisms most frequently isolated, were sensitive to ampicillin. Klebsiella-Enterobacter species and *Pseudomonas aeruginosa* were generally insensitive. Comparison of these results with data obtained in an earlier study with Gram-negative organisms isolated in 1961 showed that there had been no significant increase in the incidence of resistance of Gram-negative bacilli to ampicillin during the period 1961-67.

The majority of ampicillin-resistant strains of *E. coli* isolated in 1967 transferred ampicillin resistance to a sensitive strain of *E. coli* K12. Only four ampicillin-resistant strains of *E. coli* isolated in 1961 were available for transferable resistance tests but all four strains transferred ampicillin resistance. Infective or transferable resistance was therefore a feature of ampicillin resistance of certain Gram-negative bacteria before ampicillin became generally available for clinical use.

The relatively recent demonstration of transferable antibiotic resistance among bacteria (Watanabe, 1963) has led to renewed interest in the incidence of antibiotic resistance among Gram-negative bacilli, particularly as these organisms appear to be responsible for an increasing proportion of serious infections within hospitals (Watt and Okubadejo, 1967; Barrett, Casey, and Finland, 1968).

Shortly after the introduction of ampicillin into clinical use in mid-1961 several hundred freshly isolated cultures of Gram-negative bacilli were obtained from three London hospitals, and these organisms were tested for sensitivity to ampicillin, chloramphenicol, and tetracycline (Sutherland and Rolinson, 1964). During 1967 approximately similar numbers of isolates were obtained from the same three London hospitals as in 1961 and these were tested for sensitivity to ampicillin. In addition, a fourth hospital situated just outside the London area supplied approximately 300 cultures which were tested for sensitivity to ampicillin.

The data presented here show the incidence of resistance to ampicillin among Gram-negative bacilli isolated in 1967, and this is compared with the incidence of resistance among organisms isolated in 1961.

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METHODS

CULTURES The Gram-negative bacilli used in this study were fresh clinical cultures isolated routinely in the laboratories of the four hospitals concerned in this study and were sent to this laboratory without selection of any kind. The majority of cultures were isolated from urine specimens but a substantial number of organisms from sputum, wound and pus swabs, and faecal specimens were also tested.

CLASSIFICATION The methods used to identify and classify the bacteria were essentially the same as those described in the previous study (Sutherland and Rolinson, 1964) and were based on criteria described by Cowan and Steel (1961).

SENSITIVITY TESTING Minimum inhibitory concentrations were determined by serial dilution of ampicillin in nutrient agar. Plates were inoculated by means of an inoculating device with a drop (approximately 0·003 ml) of an overnight broth culture of the test organism and inhibition of growth was determined after overnight incubation at 37°C.

MEASUREMENT OF INFECTIVE RESISTANCE (TRANSFERABLE RESISTANCE) Ampicillin-resistant isolates of *E. coli* were grown overnight in Penassay broth (Oxoid, antibiotic medium no. 3), and 0·2 ml volumes of these donor strains were added to 0·4 ml volumes of an overnight culture of

the recipient strain *E. coli* K12/711 NX, a lactose-negative variant resistant to nalidixic acid. The mixed cultures of donor and recipient bacteria were cultured in 9 ml of Penassay broth, and, after overnight incubation at 37°C. were plated on MacConkey agar containing 25 μg/ml ampicillin and 20 μg/ml nalidixic acid; control plates containing either 25 μg/ml ampicillin or 20 μg/ml nalidixic acid were also inoculated with the mating mixtures. Inoculation of the plates was carried out with a multipoint inoculating device delivering approximately 0·003 ml of culture. After overnight incubation at 37°C the plates were examined for lactose-negative colonies growing in the presence of 25 μg/ml ampicillin and 20 μg/ml nalidixic acid. Lactose-negative colonies were subcultured into Penassay broth and retested for resistance to ampicillin.

RESULTS

SENSITIVITY TO AMPICILLIN OF ORGANISMS ISOLATED IN 1967 In all, a total of 1,102 strains of Gram-negative bacilli was tested, 233 from hospital 1, 240 from hospital 2, 342 from hospital 3, and 287 from hospital 4. The predominant organisms were *E. coli* (45·1%), *P. mirabilis* (22·1%) and Klebsiella-Enterobacter species (18·3%). The remainder comprised *Ps. aeruginosa* (9·7%), Proteus species (2·3%), and miscellaneous organisms (2·5%) such as paracolon bacteria, Achromobacter species, and Alkaligenes species (Table I).

Of the total of 497 strains of *E. coli*, 80% (397 strains) were sensitive to 5 μg/ml ampicillin or less. There was some variation between the four hospitals, ranging from 90·3% sensitivity at hospital 2 to 66% sensitivity at hospital 1. At the latter hospital the majority of resistant organisms were received at this laboratory in a single batch of cultures, and it is possible that this group of resistant organisms was associated with cross-infection occurring at the time these cultures were isolated.

The second most common pathogen, *P. mirabilis* (22% of isolates), was also predominantly sensitive, 90% (217 strains) being inhibited by 5 μg/ml ampicillin or less. In fact, 208 out of these 217 strains were sensitive to 2·5 μg/ml ampicillin and more than half of the strains isolated were sensitive to 1·25 μg/ml ampicillin.

In contrast, Klebsiella-Enterobacter species, representing 18% of the total bacteria, were very much more resistant to ampicillin (6·4% sensitive to 5 μg/ml, 11·9% sensitive to 12·5 μg/ml), and strains of *Ps. aeruginosa* were uniformly insensitive. Proteus species (other than *P. mirabilis*) tended to be resistant to ampicillin, and the majority of the paracolon bacteria tested were also insensitive.

A number of strains of enterococci (*Streptococcus faecalis*), one of the more common of the pathogens associated with urinary tract infections, were also tested for sensitivity to ampicillin, but these results are not included in the tables as isolates were sent by only two of the four hospitals. Out of 39 strains tested only one strain was resistant to 5 μg/ml ampicillin; the majority (37/39 strains, 95%) were inhibited by 2·5 μg/ml ampicillin or less.

COMPARISON OF AMPICILLIN SENSITIVITIES OF GRAM-NEGATIVE BACILLI ISOLATED IN 1961 AND 1967 The cumulative percentage inhibition by ampicillin of the three most common species, namely, *E. coli*, *P. mirabilis*, and Klebsiella-Enterobacter, isolated at the three London hospitals in 1961 and in 1967, is illustrated in Figure 1. Details of the incidence of ampicillin sensitivity in 1961 and 1967 are presented in Table II.

The proportion of strains of *E. coli* isolated in 1967 (42·5%) was very similar to that of 1961 (44·5%) and the same was true for *P. mirabilis* (25·8% in 1961 and 23·4% in 1967). In general, the proportions of strains of these genera sensitive to ampicillin were similar for both studies. For example, 80·9% of *E. coli* isolated in 1967 were inhibited by 5 μg/ml ampicillin or less; the corresponding figure

TABLE I

AMPICILLIN SENSITIVITY OF 1,102 STRAINS OF GRAM-NEGATIVE BACILLI ISOLATED AT FOUR HOSPITALS IN 1967

Organism	*No. of Strains*	*Percentage of Total*	*Number of Strains Inhibited by Ampicillin (μg/ml)*								
			>250	250	125	50	25	12·5	5·0	2·5	1·25 or less
Escherichia coli	497	45·1	49 (100%)[3]	3 (90·1%)	8 (89·5%)	4 (87·9%)	5 (87·1%)	31 (86·1%)	260 (79·9%)	127 (27·6%)	10 (2·0%)
Proteus mirabilis	244	22·1	17 (100%)	4 (93·0%)	1 (91·4%)	— (91·0%)	2 (91·0%)	3 (90·2%)	9 (88·9%)	65 (85·2%)	143 (55·5%)
Klebsiella-Enterobacter	202	18·3	88 (100%)	15 (56·4%)	29 (49·0%)	28 (34·7%)	18 (20·1%)	11 (11·9%)	8 (6·4%)	3 (2·1%)	2 (1·0%)
Pseudomonas aeruginosa	107	9·7	103 (100%)	2 (3·7%)	2 (1·9%)	—	—	—	—	—	—
Proteus species[1]	25	2·3	10 (100%)	5 (60·0%)	3 (40·0%)	5 (28·0%)	1 (8·0%)	1 (4·0%)	—	—	—
Miscellaneous[2]	27	2·5	9 (100%)	1 (66·7%)	— (63·0%)	1 (63·0%)	— (59·3%)	4 (59·3%)	10 (25·5%)	2 (7·4%)	—
Total	1,102										

[1] *P. morganii*, *P. rettgeri*, *P. vulgaris*
[2] Mainly Paracolon, Achromobacter, and Alkaligenes
[3] (% cumulative inhibition shown in brackets)

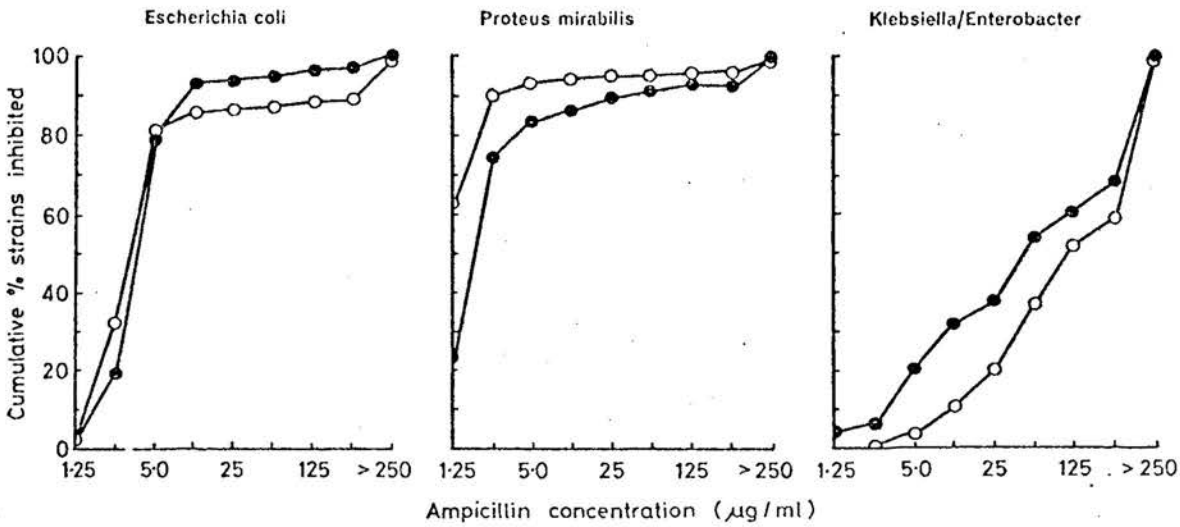

FIG. 1. *Susceptibility to ampicillin of Gram-negative bacilli isolated in 1961 (●) and 1967 (○). Escherichia coli 1961, 269 strains; 1967, 346 strains; Proteus mirabilis, 1961, 156 strains; 1967, 191 strains; Klebsiella-Enterobacter 1961, 80 strains; 1967, 156 strains.*

TABLE II

SUSCEPTIBILITY TO 5 μG/ML AMPICILLIN OF GRAM-NEGATIVE BACILLI ISOLATED IN 1967 AND IN 1961[1]

	1967		*1961*	
Organism	*No. Strains Tested*	*No. Inhibited by 5 μg/ml Ampicillin*	*No. Strains Tested*	*No. Inhibited by 5 μg/ml Ampicillin*
Escherichia coli	346 (42·5%)	280 (80·9%)	269 (44·5%)	212 (78·8%)
Proteus mirabilis	191 (23·4%)	178 (93·2%)	156 (25·8%)	130 (83·3%)
Klebsiella-Enterobacter	156 (19·1%)	6 (3·8%)	80 (13·2%)	16 (20·0%)
Pseudomonas aeruginosa	92 (11·3%)	0 0	47 (7·8%)	1 (2·1%)
Proteus species	19 (2·3%)	0 0	13 (2·1%)	1 (7·7%)
Miscellaneous	11 (1·3%)	3 (27·3%)	40 (6·6%)	19 (47·5%)
Total	815		605	

[1]Organisms isolated at the same three London hospitals

for *E. coli* isolated in 1961 was 78·8% (Table II). It is true that 11% of the strains of *E. coli* isolated in 1967 were not inhibited by 250 μg/ml ampicillin, compared with a figure of 3% for 1961, but in part this may be due to cross-infection at one of the hospitals in 1967 as described previously. With *P. mirabilis* the proportion of ampicillin-sensitive strains isolated in 1967 (93·2%) was similar to that of 1961 (83·3%). If anything, this genus was more sensitive in 1967 than in 1961.

Strains of Klebsiella-Enterobacter were apparently more common in 1967 than in 1961 (19·1% compared with 13·2%); in part this may have been due to stricter classification in the later study, *ie*, some of the organisms classified in 1967 as Klebsiella-Enterobacter species were probably classified as miscellaneous bacteria in 1961. In both years, this group of organisms was largely resistant to ampicillin, possibly more so in 1967 than in 1961. The numbers of *Ps. aeruginosa* were slightly higher in 1967 (11·3%) than in 1961 (7·8%), and in both years these organisms were uniformly resistant to ampicillin. The incidence of Proteus species (*P. morganii*, *P. rettgeri*, and *P. vulgaris*) remained the same (approximately 2%), and fewer cultures were classified as miscellaneous bacteria in 1967 (1·3%) than in 1961 (6·6%) for the reason given above.

TETRACYCLINE RESISTANCE AT HOSPITAL 3 IN 1961 AND 1967 Most of the cultures isolated in 1967 at hospital 3 were also tested for sensitivity to tetracycline. Figure 2 shows the percentage cumulative inhibition by tetracycline of *E. coli*, *P. mirabilis*, and Klebsiella-Enterobacter isolated in 1961 and 1967. As was the case for ampicillin there was no great difference in the incidence of resistance to tetracycline of cultures isolated in 1961 and in 1967. Thus 71% of the strains of *E. coli* isolated in 1967 were inhibited by 5 μg/ml tetracycline; the corresponding figure for 1961 was 72%. Similarly, 71% of the Klebsiella-Enterobacter isolated in 1967 were sensitive to tetracycline compared with 50% of 16

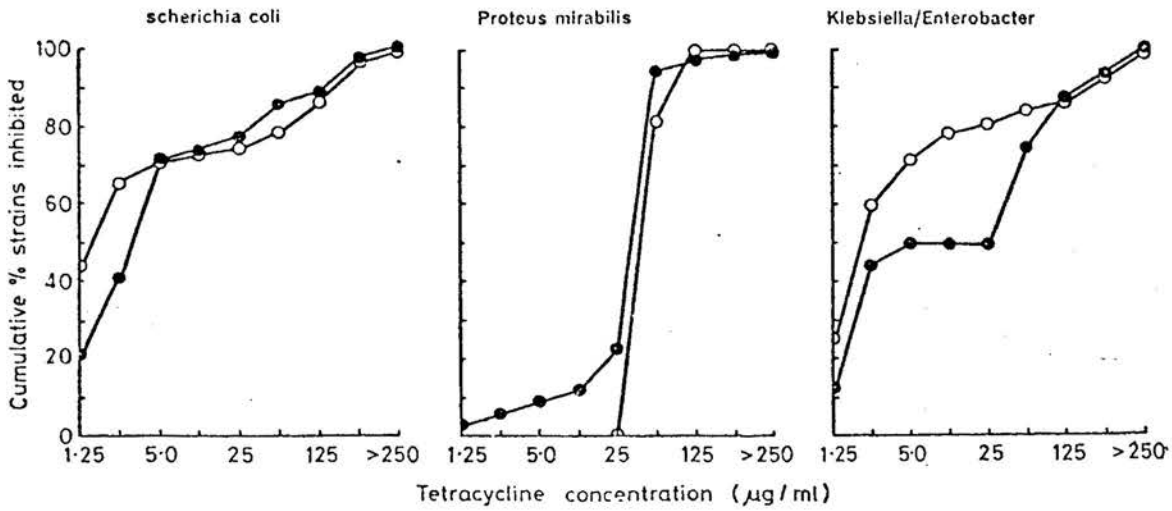

FIG. 2. *Susceptibility to tetracycline of Gram-negative bacilli isolated in 1961 (●) and 1967 (O). Escherichia coli 1961, 88 strains; 1967, 137 strains; Proteus mirabilis 1961, 36 strains; 1967, 58 strains; Klebsiella-Enterobacter 1961, 16 strains; 1967, 52 strains.*

strains isolated in 1961. The majority of strains of *P. mirabilis* and *Ps. aeruginosa* were insensitive to tetracycline in 1967 as was the case in 1961.

INFECTIVE RESISTANCE AMONG AMPICILLIN-RESISTANT STRAINS OF *E. coli* A total of 33 strains (out of 49 strains) isolated in 1967 resistant to 250 μg/ml ampicillin were tested for ability to transfer ampicillin resistance to the ampicillin-sensitive organism *E. coli* K12/711 NX. Of these strains, 23 (70%) transferred ampicillin resistance to the recipient strain.

Only seven of the strains of *E. coli* isolated in 1961 and found to be resistant to ampicillin were available for tests of transferable resistance. Three of these cultures had lost their resistance and were sensitive to ampicillin. The remaining four cultures were insensitive to ampicillin and transferred their resistance to *E. coli* K12/711.

DISCUSSION

The results presented here show that there has been no marked increase in the incidence of ampicillin resistance among Gram-negative bacilli in the hospitals in question since the introduction of this antibiotic into clinical use in 1961. In this study of bacteria isolated in 1967, and in the earlier study of organisms isolated in 1961, the majority of strains of the two most commonly isolated pathogens, namely, *E. coli* and *P. mirabilis*, were sensitive to ampicillin, while Klebsiella-Enterobacter species and *Ps. aeruginosa* were generally insensitive. A recent study in the United States involving over 4,000 isolates of Gram-negative bacteria recorded a similar distribution of sensitivity to ampicillin (Turck, 1967).

It is also of interest that the limited study of the incidence of tetracycline resistance among bacteria isolated at one hospital in 1961 and in 1967 indicated that there had been no significant change in resistance to tetracycline between 1961 and 1967. Recent studies in the USA (Bulger, Roberts, and Sherris, 1967; Petersdorf and Turck, 1966) have also reported no appreciable increase in the resistance of Gram-negative bacilli to a variety of antibiotics between 1959 and 1965. These studies did not include ampicillin, but the results reported here indicate that ampicillin appears to be no different from other antibiotics in this respect.

These findings may appear surprising in the light of the relatively recent awareness of the ability of Gram-negative bacteria to transfer antibiotic resistance to other bacteria, and subsequent speculation regarding the possibility of a rapid increase in the incidence of antibiotic resistance among Gram-negative bacteria. Our results confirm that the majority of resistant strains of *E. coli* isolated in 1967 were able to transfer ampicillin resistance *in vitro* to a suitable ampicillin-sensitive recipient strain. However, we were also able to demonstrate transfer of ampicillin resistance from four strains of *E. coli* isolated in 1961 soon after ampicillin became available for clinical use. It is a possibility therefore that the ampicillin resistance of certain Gram-negative bacilli has always been largely associated with transferable resistance, and that it is not

necessarily a relatively new phenomenon. Unfortunately, only a limited number of bacteria isolated in the early 1960s were available to us, so we can draw no conclusions as to whether there has been any change in the incidence of strains of *E. coli* carrying R factors.

As R factors have evidently been present in Gram-negative bacilli over a relatively long period of time, and as ampicillin resistance can be readily transferred *in vitro*, it may be asked why there has not been an obvious increase in the incidence of ampicillin resistance among Gram-negative bacteria. There are various possibilities, including factors *in vivo* which affect the efficiency of transfer, incompatability between bacteria, and spontaneous loss of R factors. Whatever the reason, it is encouraging that the results reported here show no evidence of increased resistance to ampicillin among Gram-negative bacilli after six years' clinical usage.

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SENSITIVITY OF HOSPITAL BACTERIA TO AMPICILLIN IN 1961, 1967 and 1975

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It is often assumed that the incidence of antibiotic-resistant bacteria will of necessity increase significantly as a result of the selective pressures exerted by antibiotic usage. In this way not only would antibiotic-resistant strains of sensitive bacteria become more common but also the more naturally-resistant Gram-negative bacteria, such as Proteus spp., Klebsiella spp., Enterobacter spp., Serratia spp., Providencia spp. and Pseudomonas spp. might be expected to become more prevalent, particularly in hospitals. Specifically, it has been predicted that the process of selection of resistant organisms, many with the ability to transmit genetically-mediated resistance determinants to the sensitive population will result in the establishment of a predominantly antibiotic-resistant population within a relatively short period of time.

In recent years there has been a number of studies on the relative susceptibilities of Gram-negative bacteria to ampicillin and other antibiotics. Some of these studies appear to show little change in sensitivity; for example, Goodwin showed a high degree of sensitivity to ampicillin among urinary E. coli (82%) over a three year period 1971-1974 (2). On the other hand, Gruneberg showed data which indicated an increase in resistance to ampicillin during

the same period among E. coli causing urinary tract infections in hospitalized patients, from 26% resistance in 1971 to 39% resistance in 1974, although strains derived from general practice were not significantly changed, 86% to 81% strains being sensitive to ampicillin (3). The deficiency of most of the reported studies of ampicillin resistance has been that they have either been a single study, and perhaps within one hospital, or that they have been conducted over relatively short periods of time. It is the purpose of this paper to present data accumulated in our laboratories over a period of 15 years derived from three contemporary surveys of antibiotic susceptibilities to Gram-negative bacilli, in 1961, 1967 and in 1975.

In all three studies, consecutive clinical isolates of Gram-negative bacilli were collected from the routine laboratories of four large hospitals (Table 1). Hospitals A, B and C were situated in the London Area and were the basis of the first survey in mid-1961, shortly after the introduction of ampicillin (5). The same three hospitals participated in the second study in 1967 and a fourth hospital, Hospital D, outside the London area was included (4). Unfortunately it was not possible to obtain cultures from Hospital C for the latest study in 1975, but Hospital A, B and D once again ccoperated.

Table 1

Numbers of bacteria

Hospital	Location	Number of cultures		
		1961	1967	1975
A	London	176	342	222
B	London	212	240	249
C	London	286	233	-
D	Surrey	-	287	302
		674	1102	773

Data in Table 2 show the frequency of the bacteria isolated in each study. E. coli was the most common organism, in agreement with the literature, and in each study the majority of strains were isolated from urinary tract infections. There was no evidence to show that organisms intrinsically resistant to ampicillin, such as klebsiellae, indole-positive Proteae or Ps. aeruginosa, have increased significantly in frequency over the period studied, despite the undoubted increase in ampicillin usage during this time.

Table 2

Frequency of isolates

Organism	1961 (ABC)		1967 (ABCD)		1975 (ABD)	
	No.	%	No.	%	No.	%
E. coli	269	40	497	45	419	54
P. mirabilis	156	23	244	22	102	13
K. aerogenes, Enterobacter, Citrobacter	80	12	202	19	120	15
Proteus spp.	13	2	25	1	27	3
S. faecalis	67	10			32	4
P. aeruginosa	49	8	107	11	73	9
Others	40	6	27	1	6	1
Total	674		1102		773	

The antibiotic sensitivity of the organisms to selected antibiotics was measured by the serial dilution method. In the first two studies minimum inhibitory concentrations of the antibiotic were determined by serial dilution in nutrient agar. The plates were inoculated by means of an inoculating device, with a drop (approximately 0.003 ml) of an overnight broth culture of the organism and the inhibition of growth determined after overnight incubation at 37°C. An organism was regarded as being sensitive to ampicillin if inhibited by 5 μg/ml. In the latest study the minimum inhibitory concentrations were determined by serial dilution in broth using an automated microtitre system. The plates were inoculated with 0.001 ml of an overnight broth diluted 1/10, which gave a final inoculum equivalent to 10^6 cells/ml. The inhibition of growth was determined, as previously, after overnight incubation at 37°C.

The organism was judged to be susceptible if inhibited by 16 µg/ml ampicillin in this system, which was shown, by prior experimentation, to be equivalent to the end-point of 5 µg/ml in the agar tests.

The characterisation of the strains in 1975 was confirmed by biochemical tests performed in microtitre wells which were inoculated automatically with an inoculating device at the same time as the antibiotic susceptibility test. The system comprised ten tests and the organisms were classified according to criteria described by Cowan and Steele (1). The strains were characterised on a more basic conventional system in the studies in 1961 and 1967 which did not permit such accurate classification.

In general, it may be seen that the proportions of strains of E. coli and P. mirabilis sensitive to ampicillin in all three studies were similar (Table 3). For example, 79% of E. coli isolated from all sources in 1961 were inhibited by 5 µg/ml ampicillin or less, compared to 80% of strains in 1967 and 74% in 1975.

Table 3

Susceptibility to ampicillin

	1961 (ABC)		1967 (ABCD)		1975 (ABD)	
	No.	% sens	No.	% sens	No.	% sens
E. coli	269	79	497	80	419	74
P. mirabilis	156	83	244	89	102	97
K. aerogenes*	80	20	202	11	120	8
Proteus spp.	13	8	25	24	27	0
P. aeruginosa	49	0	107	0	73	0
S. faecalis	67	100	-	-	32	100
Miscellaneous	40	50	27	25	-	-
Total	674	57	1102	58	773	59

* Includes Enterobacter spp. and Citrobacter spp.

With strains of P. mirabilis the incidence of sensitivity to ampicillin rose from 83% of strains sensitive in 1961 to almost 100% susceptibility in 1975. It is obvious that ampicillin-resistant strains of P. mirabilis were uncommon in these hospitals at the time of the survey in 1975.

Strains classified as Klebsiella/Enterobacter species were apparently more common in latter years, 1967 and 1975, but this probably reflects the stricter taxonomic classifications of these latter studies, as was reflected in Table 3, in the smaller numbers of unclassified bacteria reported. For instance, in 1961 there was a relatively large number of unidentified bacteria which probably included some Enterobacter/Citrobacter spp. In all studies these organisms were resistant to ampicillin (80% in 1961 to 92% in 1975).

Examination of the data on an individual hospital basis shows that in Hospital A there had, in fact, been some increase in the resistance to Gram-negative bacilli to ampicillin over the years, from 39% resistant strains isolated in 1961 rising to 55% resistant strains in 1975 (Table 4). The same trend is seen with Hospital B, 34% resistant strains in 1961 to 49% resistant in 1975. This trend towards increased resistance in these two hospitals is offset, however, by the high incidence of ampicillin-sensitive strains isolated from Hospital D, especially in the latter study.

Table 4

Susceptibility to ampicillin

Individual hospitals

Hospital	1961		1967		1975	
	No.	% sens	No.	% sens	No.	% sens
A	159	61	342	64	222	45
B	259	66	240	56	249	51
C	256	63	233	52	-	-
D	-	-	287	60	302	75
Total	674	63	1102	58	773	59

Table 5

Susceptibility to ampicillin

Isolates from Hospital A and Hospital B

Organism	1961		1967		1975	
	No.	% sens	No.	% sens	No.	% sens
E. coli	130	83	108	87	214	65
P. mirabilis	107	80	86	93	64	98
K. aerogenes*	25	12	118	2	99	8
S. faecalis	38	100	-	-	16	100
Proteus spp.	6	17	25	1	18	0

* Including Enterobacter spp. and Citrobacter spp.

It has been possible to examine in greater detail the data from the two hospitals A and B which had contributed clinical isolates to the three studies (Table 5). The results show that for E. coli and P. mirabilis the level of sensitivity to ampicillin was high in 1961 and 1967 (greater than 80% sensitive). In 1975, the proportion of E. coli strains sensitive to ampicillin (in these two hospitals)

had fallen to 65%. On the other hand, more strains of P. mirabilis appeared to be sensitive to ampicillin in 1975 than in 1961, 98% sensitive in the latter period compared to 80% sensitivity in the earlier study.

A further collection of E. coli from Hospital A was taken approximately 9 months after the 1975 study to detect any bias in the original results, which might have arisen as a result of selection of an unrepresentative sample of isolates, by cross-infection or repeat specimens. The results on the second sample show a similar degree of sensitivity to all antibiotics tested at the two time periods (Table 6).

Table 6

Sensitivity of E. coli isolated from Hospital A in 1975 and 1976

Antibiotic	Aug/Sept 1975		May/June 1976	
	Total	% sens	Total	% sens
Ampicillin	94	63	94	66
Carbenicillin	94	66	94	68
Cefazolin	94	87	94	82
Tetracycline	94	63	94	65

Comparative data were also available for the sensitivity to tetracycline of Gram-negative bacilli isolated at Hospital A between 1961 and 1975 (Table 7). As with ampicillin there appears to be a trend to increased resistance among E. coli (71% sensitive strains in 1967 compared to 63% sensitive in 1975) but this decrease in sensitivity is not notable. Moreover, there was an increase in sensitivity of

strains of Klebsiella-Enterobacter over this period and these data may reflect the limited usage of this antibiotic in this hospital over the period under study.

Table 7

Susceptibility to tetracycline in Hospital A

Organism	1961		1967		1975	
	No.	% sens	No.	% sens	No.	% sens
E. coli	88	72	137	71	94	63
P. mirabilis	36	8	58	0	33	3
K. aerogenes	16	50	52	71	46	76

In conclusion, although it is undoubtedly true that certain antibiotic-resistant bacteria may present problems from time to time in specific hospitals or hospital units - Klebsiella, Pseudomonas aeruginosa, Serratia marcescens and Providencia spp. are all examples - these opportunistic pathogens represent only a small proportion of the total isolates received in the routine laboratory. The data presented here show that the distribution of organisms showed little variation between 1961 and 1975 and the frequency of isolation is consistent with that reported in other studies. In view of the concern felt about the reduction in the efficacy of antimicrobial agents in the face of plasmid-mediated resistance it is interesting to note that overall there appears to have been relatively small changes in susceptibility to ampicillin. There may be a trend towards increased ampicillin resistance among E. coli isolated from hospitalized patients, but this reduction in sensitivity is relatively small, which is of interest as those bacteria almost all harbour transmissible antibiotic resistance determinants.

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ANTIBACTERIAL ACTIVITY OF ANTIBIOTICS IN ACRYLIC BONE CEMENT

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ANTIBACTERIAL ACTIVITY OF ANTIBIOTICS IN ACRYLIC BONE CEMENT

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The release of various penicillins and other antibiotics from two brands of polymerised bone cement has been studied *in vitro* and *in vivo* in mice. Bone cement plugs containing antibiotics demonstrated antibacterial activity as a result of diffusion of antibiotic from the plugs into the surrounding medium. With all antibiotics tested, from 2·5 to 10 per cent of the antibiotic in the cement was released *in vitro* in active form within twenty-four hours. Most of the activity appeared within three hours of the start of the test, but in some cases low levels of activity were detected after four days. Antibiotic cement plugs implanted in mice and rats produced low concentrations of antibiotic in the blood up to two hours after implantation, but activity was seldom detected subsequently. In general, penicillins and non-penicillin antibiotics showed similar diffusion characteristics, and the pattern of release *in vitro* and *in vivo* was consistent with the leaching of antibiotic from, or near, the surface of the bone cement.

Incorporation of antibiotics in acrylic bone cement has been shown to have reduced the incidence of infection in patients after total joint replacement (Buchholz and Engelbrecht 1970; Wahlig and Buchholz 1972; Hermann 1973; Voorhoeve and Stöhr 1973). Experimental findings show that antibiotic incorporated into acrylic bone cement diffuses *in vitro* and *in vivo* into the surrounding environment (Buchholz and Engelbrecht 1970; Wahlig and Buchholz 1972; Medcraft and Gardner 1974; Levin 1975; Chapman and Hadley 1976), and it is assumed that, in patients, sufficient antibiotic is released from the artificial joint *in situ* to prevent wound infection.

The experiments reported here were designed to compare the extent of diffusion of various penicillins and other antibiotics from bone cement *in vitro* and *in vivo*, and to measure the antibacterial effects produced.

MATERIALS AND METHODS

Bone cement—Three brands of acrylic bone cement were tested: Palacos bone cement (Kulzer and Co. GMBH, Bad Homburg, West Germany); Surgical Simplex plain bone cement (P) (Howmedica U.K. Ltd., London); and Surgical Simplex radiopaque bone cement (RO) (Howmedica U.K. Ltd.).

Antibiotics—Five groups were used. 1) Penicillins: amoxycillin trihydrate, ampicillin trihydrate, carbenicillin, cloxacillin, flucloxacillin, methicillin and ticarcillin (Beecham Pharmaceuticals); 2) cephaloridine (Glaxo Laboratories Ltd.); 3) clindamycin (Upjohn Ltd.); 4) sodium fusidate (Leo Laboratories Ltd.); and 5) gentamicin (Roussel Laboratories Ltd.).

Preparation of bone cement plugs—One gramme of dry antibiotic powder was mixed thoroughly with 40 grammes of polymethyl methacrylate powder, and 20 millilitres of liq monomer were added with stirring. The mass was stir thoroughly according to instructions and kneaded by hand t dough-like consistency. Standard size plugs of cylindr shape (7 millimetres high × 7 millimetres diameter, m weight approximately 275 milligrammes) were prepared pressing the hardening cement into sterile glass mou These plugs contained a mean weight of about 4·5 m grammes of antibiotic. A limited number of smaller-si bone cement plugs (mean weight 135 milligrammes) w prepared in a similar manner for certain *in vivo* studies. plugs were stored at 4 degrees Celsius until use.

In vitro studies

Diffusion tests—Bone cement plugs containing the test a biotics were added to screw-capped bottles containing millilitres of sterile distilled water (one plug per bottle), the bottles were placed on a roller apparatus installed in incubator at 37 degrees Celsius. Three plugs were tested v each antibiotic in each test. Samples of solution were take three, six and twenty-four hours and stored at −20 degr Celsius until assayed for antibiotic content by agar-diffus microbiological assay. In the study with Palacos bone cem the plugs were also incubated at 37 degrees Celsius for f days and samples for assay were taken daily. Samples solutions from the diffusion tests were assayed by stand large plate microbiological assay with the following bacteri assay organisms: Bacillus subtilis ATCC 6633 for the p cillins (except carbenicillin) and for cephaloridine and ge micin; Pseudomonas aeruginosa NCTC 10701 for carb cillin; and Staphylococcus aureus NCTC 6571 for clindamy and sodium fusidate.

Antibacterial activity—First, one plug was added to each t tube containing ten millilitres of nutrient broth (Nutr broth, No. 2 Oxoid). The tube was inoculated with millilitres of an overnight culture of test organism, inoculum of about 2×10^7 organisms. The tubes were i bated at 37 degrees Celsius and examined daily for fourt

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days for turbidity. At the end of fourteen days a loopful of each culture was subcultured on to antibiotic-free agar. Secondly, in a separate series of experiments cement plugs were added to ten millilitres of nutrient broth, and each tube was inoculated with the test organism as above. After overnight incubation at 37 degrees Celsius the tubes were examined for growth, the plugs were removed, washed with sterile distilled water and transferred to fresh tubes containing ten millilitres of nutrient broth. The tubes were inoculated with an overnight culture of the test organism as before, and incubated at 37 degrees Celsius. This process was repeated daily for five days.

In vivo studies

Diffusion studies—Small bone cement plugs (Surgical Simplex plain; approximate weight 135 milligrammes) were implanted subcutaneously under halothane anaesthesia into male albino mice (Olac strain, 18–22 grammes). One plug was implanted in each mouse. Each plug contained approximately 2 milligrammes of test antibiotic, amoxycillin or flucloxacillin, equivalent to a dose to each mouse of approximately 100 milligrammes of antibiotic per kilogramme body weight. Groups of two mice each were killed at five, ten, thirty and sixty minutes, and at two and four hours after dosing, and blood was sampled from the axilla. Blood antibiotic concentrations were measured by large plate microbiological assay with Sarcina lutea NCTC 8340 as the assay organism.

In similar studies in Sprague-Dawley rats (100–150 grammes) a bone cement plug weighing approximately 275 milligrammes and containing about 4 milligrammes of antibiotic was implanted subcutaneously, equivalent to a dose of approximately 40 milligrammes per kilogramme. Samples of blood were taken from the tail vein at fifteen and thirty minutes and at one, two and four hours after dosing, and assayed for antibiotic content. Assay organisms were Corynebacterium xerosis NCTC 9755 for gentamicin and sodium fusidate, and Sarcina lutea NCTC 8340 for amoxycillin.

Infection studies—Mice (Olac, 18–22 grammes) were infected intraperitoneally with 0·5 millilitre of a mucin suspension of Staphylococcus aureus (Smith). One bone cement plug (Simplex; weight 135 milligrammes) containing 2·0 milligrammes of amoxycillin was implanted subcutaneously in each mouse, either one hour or immediately before infection. A control group of infected mice was dosed subcutaneously with amoxycillin immediately after infection. The animals were observed daily for four days.

RESULTS

In vitro studies

Diffusion studies—The diffusion of antibiotics from Palacos acrylic bone cement is illustrated in Table I, which shows the antibiotic activity released from bone cement plugs shaken in aqueous solution at 37 degrees Celsius. All the antibiotics tested showed a similar pattern of diffusion; an initial rapid release of antibiotic was followed by a more gradual diffusion. From 60 to 80 per cent of the activity measured was recovered during the first three hours after the start of the test. A very similar pattern of release of antibiotics was obtained with both formulations of Simplex cement. The amount of antibiotic released from the bone cement plugs ranged from 2·2 per cent for methicillin to 9·2 per cent for cloxacillin. A greater proportion of the isoxazolyl penicillins, cloxacillin (9·2 per cent) and flucloxacillin (7·7 per cent) was released from the cement than of the other penicillins—carbenicillin (5·8 per cent), amoxycillin (3·1 per cent) and methicillin (2·2 per cent). The non-β-lactam antibiotics clindamycin (7·7 per cent), gentamicin (7·1 per cent) and sodium fusidate (3·1 per cent) demonstrated a similar pattern of diffusion, with a rapid initial release of antibiotic from the bone cement plugs. The amounts appearing in the twenty-four-hour period were of the same order as those obtained with the penicillins.

TABLE I

DIFFUSION OF ANTIBIOTICS FROM PALACOS CEMENT PLUGS CONTAINING APPROXIMATELY 4·5 MILLIGRAMMES OF ANTIBIOTIC DURING INCUBATION IN AQUEOUS SOLUTION AT 37° CELSIUS

Antibiotic	Rate ($\mu g/hour$) Hours			Total diffused in 24 hours	
	0–3	3–6	6–24	μg	Percentage of dose
Amoxycillin	27·7	11·0	0·3	122	2·8
Carbenicillin	55·6	13·3	5·0	298	5·8
Cloxacillin	81·1	24·4	4·7	402	9·2
Flucloxacillin	71·4	19·3	4·3	34·9	7·7
Methicillin	24·9	3·6	0·7	98	2·2
Clindamycin	92·8	20·4	1·5	36·7	7·7
Sodium fusidate	39·7	0	1·1	139	3·1
Gentamicin	100·5	19·1	0	359	7·1

The diffusion of a number of penicillins and non-penicillin antibiotics from bone cement plugs over a four-day incubation period is shown in Table II. It can be seen that the amount diffusing during the first day was

TABLE II

RELEASE OF ANTIBIOTICS FROM PALACOS CEMENT DURING INCUBATION AT 37° CELSIUS FOR 4 DAYS IN AQUEOUS SOLUTION

Compound	Minimum detectable level (μg./ml)	Diffusion (as percentage of dose) after					Diffusion on Day 1 as percentage of total
		Day 1	Day 2	Day 3	Day 4	Total	
Amoxycillin	0·06	2·8	0·4	0·16	0·12	3·5	83
Carbenicillin	1·6	5·8	0·7	0*	0	6·5	89
Cloxacillin	3·0	9·2	1·0	0	0	10·2	90
Flucloxacillin	3·0	7·7	1·0	0	0	8·7	89
Methicillin	0·6	2·2	0	0	0	2·2	100
Clindamycin	0·6	7·7	1·7	1·1	0·9	11·4	68
Sodium fusidate	0·6	3·1	0·4	0·7	0·8	5·0	62
Gentamicin	3·0	7·1	0·6	0	0	7·7	92

*No antibiotic activity detected.

TABLE III

DIFFUSION OF ANTIBIOTICS FROM PLUGS OF TWO BRANDS OF BONE CEMENT (1 GRAMME OF ANTIBIOTIC TO 60 GRAMMES OF CEMENT) AFTER INCUBATION AT 37° CELSIUS FOR 24 HOURS IN AQUEOUS SOLUTION

Compound	Number of tests	Simplex (plain and radiopaque) Percentage of diffusion mean (and range)	Palacos Percentage of diffusion (single test)
Amoxycillin	3	2·9 (2·2– 3·6)	2·8
Ampicillin	2	2·8 (2·8– 2·9)	–
Carbenicillin	4	5·1 (2·8– 6·5)	5·8
Cloxacillin	4	11·0 (8·3–13·4)	9·2
Flucloxacillin	2	8·5 (7·9– 9·0)	7·7
Methicillin	–	–	2·2
Ticarcillin	2	2·6 (2·1– 3·0)	–
Cephaloridine	3	2·6 (1·6– 4·0)	–
Clindamycin	–	–	7·7
Sodium fusidate	–	–	3·1
Gentamicin	3	3·7 (2·9– 4·5)	7·1

high with the penicillins (from 83 to 100 per cent of the amount measured over the four-day period), and also with gentamicin (92 per cent), but was lower with sodium fusidate (62 per cent) and clindamycin (68 per cent). No activity was measured with the methicillin plugs on the second day, or with carbenicillin, cloxacillin, flucloxacillin and gentamicin on the third day, but this may have been due to the relatively low sensitivity of the assays for the latter compounds. Low levels of activity were measured with amoxycillin, clindamycin and sodium fusidate after four days. The proportion diffusing over the four-day period varied from 2·2 p cent of the total for methicillin to 11·4 per cent w clindamycin.

A comparison of the diffusion of antibiotics fr two different brands of acrylic bone cement, Surgi Simplex and Palacos, is shown in Tab'e III. There w little difference between the two preparations w regard to the amounts of antibiotic released over twenty-four-hour period, except for gentamicin, which 7·1 per cent diffusion was measured from Palac cement, compared with 3·7 per cent from the Surgi

TABLE IV

ANTIBACTERIAL ACTIVITY IN VITRO OF SIMPLEX BONE CEMENT PLUGS CONTAINING ANTIBIOTIC (4·5 MILLIGRAMMES PER PLUG)

Antibiotic	Organism	MIC ($\mu g/ml$)	Growth on day 1	7	14	Growth after subculture for day 14
Cloxacillin Flucloxacillin Clindamycin Sodium fusidate	S. aureus NCTC 6571 S. aureus 1555‡	0·1/0·25 0·1/0·25	–	–	–	–
Methicillin	S. aureus NCTC 6571	1·25	–	–	–	–
	S. aureus 1555	2·5	–	–	+	+
Ampicillin	S. aureus NCTC 6571	0·1	–	–	–	–
Amoxycillin	E. coli NCTC 10418	2·5	–	–	–	–
Carbenicillin	E. coli NCTC 10418	2·5	–	–	–	–
	Ps. aeruginosa NCTC 10662	50	+	+	+	+
Gentamicin	E. coli NCTC 10418	0·5	–	–	–	–
	Ps. aeruginosa NCTC 10662	2·5	–	–	–	–
Ampicillin* + Cloxacillin	E. coli NCTC 10418	–	–	–	–	–
	S. aureus 1555	–	–	–	–	–
Carbenicillin* + Cloxacillin	E. coli NCTC 10418	–	–	–	–	–
	Ps. aeruginosa NCTC 10662	–	+	+	+	+
	S. aureus 1555	–	–	–	–	–

*Mixture of equal parts of each penicillin.
‡β-lactamase-producing strain.

Simplex brand. The isoxazolyl penicillins, cloxacillin and flucloxacillin, showed the highest diffusion from both brands, and this was notably higher than that of the other penicillins tested, or of the other antibiotics, save for clindamycin (7·7 per cent).

Antibacterial activity—Nutrient broth incubated with antibiotic-free bone cement plugs remained sterile for fourteen days, but these plugs were not inhibitory when the nutrient broth was inoculated with the test organisms. In contrast, antibiotic-impregnated plugs inhibited the growth of the cultures over a fourteen-day period except in the cases of carbenicillin and methicillin (Table IV) and no growth was observed in subcultures taken on the fourteenth day into fresh sterile nutrient broth. Carbenicillin inhibited the growth of a strain of Escherichia coli NCTC 10418 during the test period, but was not inhibitory for Pseudomonas aeruginosa NCTC 10662. With methicillin, the penicillin-sensitive strain of Staphylococcus aureus NCTC 6571 was inhibited over the entire period, but growth of the β-lactamase-producing strain (1555) became evident on the fourteenth day.

Very different results were observed in the tests in which the plugs were transferred daily to fresh sterile broth which was re-inoculated with the test organisms. All of the compounds inhibited growth of the test bacteria for twenty-four hours, but after two days the staphylococcus grew in the presence of the methicillin plug and the gram-negative bacilli were not inhibited by amoxycillin, carbenicillin or gentamicin (Table V). After three days incubation, the clindamycin plug failed to inhibit the staphylococcus, and only flucloxacillin and sodium fusidate were active after four days. None of the antibiotics showed activity after five days.

In vivo studies

Only low antibiotic blood concentrations were measured in mice after implantation of Simplex bone cement plugs containing amoxycillin or flucloxacillin. Peak levels of less than 1·0 microgramme per millilitre were measured at ten minutes; activity was not detected with flucloxacillin at thirty minutes, but low levels of amoxycillin (0·03 milligramme per millilitre) were measured at two hours after implantation (Table VI). Similarly, low antibiotic levels were detected with

TABLE V

ANTIBACTERIAL ACTIVITY OF BONE CEMENT PLUGS AFTER DAILY TRANSFER IN NUTRIENT BROTH

Organism	Compound	Growth after day* 1	2	3	4	5
S. aureus 1555	Cloxacillin	−	−	−	+	+
	Flucloxacillin	−	−	−	−	+
	Methicillin	−	+	+	+	+
	Clindamycin	−	−	+	+	+
	Sodium fusidate	−	−	−	−	+
E. coli NCTC 10418	Amoxycillin	−	+	+	+	+
	Carbenicillin	−	+	+	+	+
Ps. aeruginosa NCTC 10662	Gentamicin	−	+	+	+	+

*Visual growth as determined by turbidity.

TABLE VI

ANTIBIOTIC BLOOD LEVELS AFTER SUBCUTANEOUS IMPLANTATION OF SIMPLEX BONE CEMENT PLUGS IN MICE AND RATS

Species	Compound	Dose (mg/kg)	Antibiotic blood concentration (μg/ml) Minutes 5	10	15	30	60	120	240
Mouse	Amoxycillin	100	NT	0·7	NT	NT	0·05	0·03	<0·01
	Flucloxacillin	100	0·40	0·55	0·16	<0·2	<0·2	<0·2	<0·2
Rat	Amoxycillin	40	NT	NT	0·06	0·34	0·20	0·19	0·01
	Sodium fusidate	40	NT	NT	<0·1	<0·1	<0·1	<0·1	<0·1
	Gentamicin	a* 40	NT	NT	0·56	0·84	0·44	0·16	<0·06
		b	NT	NT	0·10	0·08	0·05	<0·06	<0·06

*Palacos cement. NT = Not tested.

amoxycillin and gentamicin in rats, and no activity was observed in animals after implantation of plugs incorporating sodium fusidate. When the bone cement plugs were removed from the rats six hours after implantation all plugs produced low antibiotic concentrations after incubation in aqueous solution *in vitro*; but the amounts diffusing from the plugs removed from the animals were only one-thirtieth to one-fortieth of the quantities released from fresh unused plugs under the same conditions.

Although only low antibiotic blood levels were observed in mice in which amoxycillin-impregnated cement plugs had been implanted, significant therapeutic effects were produced in animals infected with Staphylococcus aureus (Smith). Thus, all mice implanted with plugs immediately before infection survived throughout the four-day observation period, and 80 per cent of mice infected one hour after implantation were protected. All untreated mice died within twenty four to forty-eight hours and the median curative dos (CD/50) of subcutaneous amoxycillin against this i fection was 0·19 milligramme of amoxycillin per kil gramme.

DISCUSSION

The results described here confirm reports in th literature that antibiotics incorporated in acrylic bon cement retain antibacterial activity and are able t diffuse from hardened bone cement, producing ant bacterial effects *in vitro* and *in vivo* (Buchholz an Engelbrecht 1970; Hermann 1973; Voorhoeve an Stöhr 1973; Levin 1975). The release of antibiotic occu rapidly after immersion of bone cement plugs i solution, and moderately high concentrations of ant biotic are produced in solution within a few hours; bu the amounts diffusing from the cement fall quickly t low levels. In the tests reported here 70 to 90 per cent

the antibiotic released appeared in solution within three hours, but low concentrations of antibiotic were still diffusing from the bone cement up to four days after the start of the test. These findings of rapid initial diffusion and prolonged low levels are in agreement with the results of published studies with gentamicin and sodium fusidate. Buchholz and Engelbrecht (1970) reported low levels of gentamicin being released for up to two months and Voorhoeve and Stöhr (1973) measured continued release of sodium fusidate for up to five weeks.

The amount of antibiotic released *in vitro* from bone cement was of about the same order for all antibiotics tested, namely up to 10 per cent of the antibiotic incorporated in the cement. The lowest value was recorded with methicillin (2·3 per cent) and the highest with cloxacillin (11·0 per cent); there was very little difference in the amounts released between the penicillins and the non-penicillin antibiotics. This is of interest because of the possibility that the heat evolved during the polymerisation process, which can cause the hardening material to reach temperatures of up to 80 degrees Celsius, might result in significant inactivation of the heat-labile β-lactam antibiotics. However, this seems not to be the case, as there was little difference between the penicillins and a heat-stable compound such as gentamicin. The values found for gentamicin in these studies, ranging from 2·9 to 7·1 per cent of the amount incorporated, were of the same order as those reported by others (Buchholz and Engelbrecht 1970; Levin 1975).

The fact that bone cement plugs inhibited growth of bacteria for up to fourteen days was not unexpected in view of the relatively high antibiotic concentrations produced initially in solution, but the results of the antibacterial tests in which the medium was changed daily confirmed that the amounts diffusing from the bone cement plugs fell rapidly. Thus, all the antibiotic plugs inhibited growth on the first day, but only flucloxacillin and clindamycin demonstrated activity by the fourth day, and neither inhibited growth of the test strain of staphylococcus aureus by the fifth day.

Implantation of bone cement plugs in mice and rats might have been expected to produce the effects of a depot preparation, leading to low prolonged antibiotic levels in blood, but this was not the case: only low antibiotic blood levels were measured and these were generally not detectable after two hours. It seems likely, therefore, that small quantities of antibiotic are released rapidly *in vivo*, producing low antibiotic blood concentrations initially, and that antibiotic continues to be released from the bone cement for a period of time, albeit not in sufficient amounts to produce detectable blood levels. The pattern of diffusion *in vitro* and *in vivo* is consistent with the release of antibiotic from or near the surface of the acrylic cement, with most of the antibiotic being sequestered within the plug.

The efficacy of implanted amoxycillin-impregnated bone cement plugs in animals infected with staphylococcus aureus is of interest in view of the relatively low transient blood concentrations measured in the infected animals. It seems that the small quantities of amoxycillin released from the plugs were adequate to ensure protection against an infection.

The significance of the results of these various *in vitro* and *in vivo* tests is uncertain, and depends upon the relevance of the experimental test to the clinical situation—namely the diffusion of the antibiotic from the cement around the artificial joint in the patient. Studies in patients have shown that only low levels of antibiotic (gentamicin) have been found in blood and urine after operation, and it has been suggested that the antibiotics are effective mainly in the boundary layer between the bone and the endoprosthesis (Voorhoeve and Stöhr 1973). However, this appears to be largely speculation, and there is as yet no simple test method for assessing the relative merits of different antibiotics for incorporation in acrylic bone cement. The results here suggest that antibiotics generally have similar characteristics in acrylic cement, and that there is little to choose between any of them with regard to release of antibiotic *in vitro* from polymerised bone cement. Consequently, the choice of antibiotic for use in joint replacement can be made on the basis of factors other than diffusion from acrylic cement—for example, antibacterial spectrum, mode of action, toxicity and cost. In addition, it would be essential to know that incorporation of any particular antibiotic would have no deleterious effects on the physical properties of the acrylic bone cement.

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