TRANSFERABLE TRIMETHOPRIM RESISTANCE AND ITS EVOLUTION IN BACTERIA

by

HILARY-KAY YOUNG

Thesis presented for the degree of Doctor of Philosophy.

University of Edinburgh

1984.

ABSTRACT

An examination of the first trimethoprim (Tp) resistance plasmid (R-plasmid) containing strain isolated, Klebsiella sp. D770, revealed that the host dihydrofolate reductase (DHFR) produced by this strain was not the precursor of the plasmid enzymes. However, the introduction of the incompatibility W group plasmid Sa, into Klebsiella sp. D770 promoted its mutation to high level Tp resistance. The resistance mechanism was found to involve the overproduction of a less susceptible chromosomal DHFR.

Plasmid Sa was investigated for its role as a possible precursor of the Tp R-plasmid R388 by examining its ability to encode the enzyme DHFR, but no plasmid mediated DHFR could be detected. However, Escherichia coli strain J53, both with and without Sa, was found to mutate rapidly during serial subculture to high level Tp resistance. Detailed examination of the resistant strains revealed that the mutation had resulted in the increased production of an altered chromosomal DHFR showing several significant similarities to the R-plasmid enzymes.

The spread of the Tp resistance gene on R-plasmids and transposons was studied by detailed examination of a highly resistant bacterial population isolated during an epidemic of Tp resistance in Edenhall Hospital, Musselburgh. A high proportion of the Tp resistance genes were shown to reside on two different resistance transposons. Investigations of the transposon gene products showed that all encoded the production of a DHFR similar, biochemically, to the Type I plasmid enzyme. However, two separate enzymic entities, differing significantly in molecular size, could be distinguished amongst these DHFRs. The marked similarities between these transposon mediated DHFRs suggest an evolutionary relationship between them.

"We will now discuss in a little more detail
the struggle for existence."

Charles Robert Darwin
in "On the Origin of Species" (1859)

CONTENTS

DECLARATION

The experiments and composition of this thesis are entirely the work of the author.

ACKNOWLEDGEMENTS

I am indebted to my supervisor Dr. S.G.B. Amyes for his invaluable advice, guidance and encouragement throughout this work. I am also grateful to colleagues for useful discussions and to Professor J. G. Collee for his continued encouragement and help. I should also like to thank Miss Diana Dewar for her skilled and efficient preparation of the manuscript.

Finally, I should like to thank Edinburgh University Medical Faculty for the Davidson Research Scholarship that provided the financial support enabling me to study for this thesis.

ABBREVIATIONS

Ap	ampicillin
bp	base pairs
Ce	cephradine
Cm	chloramphenicol
DHF	dihydrofolate
DHFR	dihydrofolate reductase
DM	Davis-Mingioli
dTMP	Thymidylic acid
dUMP	Uridylic acid
F+	F-factor containing
F'	F-prime
F-factor	fertility factor
fi$^+$	fertility-inhibition positive
fi$^-$	fertility-inhibition negative
Gm	gentamicin
Hfr	high-frequency recombination
his	histidine
ID_{50}	dose giving 50% inhibition
IEF	isoelectric focusing
inc	incompatibility
Km	kanamycin
lac	lactose fermenting
mA	milli-amp
Mdal	megadaltons
met	methionine
MIC	minimum inhibitory concentration
MR+	methyl-red positive
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
nm	nano-metres
PABA	para-amino benzoic acid
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
pro	proline
psi	pounds per square inch
Rif	rifampicin
R-factor	resistance factor
R-plasmid	resistance plasmid
Sm	streptomycin
Sp	spectinomycin
Sx	sulphamethoxazole
Tc	tetracycline
THF	tetrahydrofolate
Tn	transposon
Tp	trimethoprim
trp	tryptophan
U	units of DHFR activity
VP+	Voges-Proskauer positive

Plasmid and transposon classification

The plasmids and transposons from this laboratory have recently been reclassified after consultation with Professor Ester Lederberg (Plasmid Reference Laboratory, Stanford University, USA). The plasmids have now been given a unique prefix of pUK and this supercedes the use of pSA in some of our previous publications. The plasmid numbers remain unchanged. Transposon designations have been changed considerably and will be identified in the text.

1. INTRODUCTION

Charles Darwin's remarkable book, "On the Origin of Species" (1859), in which he put forward the theory of evolution by natural selection, marked a turning point in biological thought. However, it was not until 1903, following the rediscovery of Mendel's work on heritability (1866), and the subsequent publication of the chromosome theory of heredity (Sutton, 1903), that an explanation was provided for the source of the inheritable variation, which was of fundamental importance to Darwin's argument. These discoveries ushered in the classical period of formal genetics.

In the 1940s it was established that genes must specify the structure of enzymes (Beadle and Tatum, 1941). Concurrent research revealed DNA to be the agent of genetic transformation (Avery et al., 1944). With the identification of DNA as the genetic material of a bacterial virus (Hershey and Chase, 1952) and elucidation of its biochemical structure (Watson and Crick, 1953) the concept of the gene was transformed from an element of formal genetic analysis to a physical entity.

The understanding of the molecular biology of DNA - its structure, replication mutation, recombination and expression - provided at one and the same time a material basis for what had once seemed irreconcilable - constancy and change: constancy deriving from the high fidelity of its replication and change arising from mutation and its recombination.

A. ANTIMICROBIAL CHEMOTHERAPY

HISTORICAL BACKGROUND

The concept of chemotherapy began with Paul Ehrlich's principles of selective drug action (1913). The therapy of bacterial infections, however, remained, for many years, an elusive and apparently unattainable goal. It was not until 1935, when Domagk (1935) reported the activity of Prontosil rubrum against infections in animals, that any real practical progress was made. Tréfouel *et al.* (1935) went on to show that Prontosil rubrum is broken down in the body to give sulphanilamide, the effective anitbacterial agent. Consequently many sulphonamide-containing compounds were synthesised and several were introduced for the treatment of infection.

Despite the success of the sulphonamides, the field of synthetic antibacterial agents has produced few other successes and today the only widely-used synthetic compounds apart from the sulphonamides, are nalidixic acid, the nitrofuran antibacterials, metronidazole and trimethoprim. The nalidixic acid series now shows considerable promise. The discovery of the sulphonamides, however stimulated a renewed interest in the chemotherapy of bacterial infections and the search for other antibacterial agents. The earlier work of Fleming (1929) with the mould *Penicillium notatum* was re-examined by Chain *et al.* (1940), who succeeded in isolating an impure solid preparation (penicillin) from this mould, which was found to be highly active against bacteria. The success of penicillin quickly attracted a

great deal of scientific effort towards the search for other antibiotics and extensive screening methods were developed. However, among the thousands of antibiotics isolated, only a few were found to be suitable for development into effective chemotherapeutic agents.

By the early 1950s, bacterial infection was being successfully treated with antibiotics and chemotherapeutic agents.... but there were already signs of trouble ahead.

THE EMERGENCE OF DRUG RESISTANCE

In 1955, Kitamoto *et al.* (1956) isolated a multiply resistant strain of *Shigella* from a patient with bacillary dysentery in Japan. During the next few years an increasing number of these strains isolated from cases of bacillary dysentery were found to be resistant to two or more of the commonly used chemotherapeutic agents - streptomycin, chloramphenicol, tetracycline and sulphanilamide. Until now, it had been assumed that the appearance of drug resistant bacteria was the result of a predictable process: the spontaneous mutation of a bacterium to drug resistance and the selective multiplication of the resistant strain in the presence of the drug. However, since the rate of spontaneous mutation was known to occur about once in every 10^7 cell divisions (Lederberg and Lederberg,1952), simultaneous resistance to all four of the above antibiotics would be predicted to occur once in 10^{28} divisions - an extremely low probability of occurrence. The familiar process of mutation and selection seemed, therefore, an improbable explanation

for the rapid increase in multiple resistance observed in Japan. Moreover, non-pathogenic strains of Escherichia coli obtained from these dysentery cases often carried a similar pattern of resistance to that of the infecting Shigella strain (Mitsuhashi et al., 1960). These and other observations, ultimately led to the discovery by Ochiai et al. (1959) and Akiba et al. (1960) that this multiple resistance is transferable en bloc to sensitive bacteria, not only among Shigella strains but among the Enterobacteriaceae as a whole, and that transfer depends on contact between living cells (ie. conjugation).

The discovery that resistance was transferred by conjugation suggested similarities to the F-factor of E. coli (Lederberg et al., 1952; Cavalli-Sforza et al., 1953; Hayes, 1953 a, b). There followed intensive studies by several groups of Japanese workers which culminated in the identification of an extrachromosomal element, the resistance factor - R-factor (Watanabe, 1967), responsible for infectious drug resistance. The R-factors were subsequently characterised and ultimately classified as plasmids, defined as extrachromosomal, autonomously replicating, stably inherited elements which are neither essential to the cell nor damaging to it (Lederberg, 1952). The term resistance plasmid (R-plasmid) - defined as a plasmid that carries genetic information to antibiotics and/or other antibacterial drugs was introduced in 1976 (Novick et al.) and now supersedes R-factor.

Shortly after the initial discovery of multiple drug resistance in Japan, various investigators demonstrated similar resistant organisms in countries as diverse as West Germany (Lebek, 1963), England (Datta, 1962) and America (Smith and Armour, 1966) and many subsequent reports have shown transfer to occur to such phylogenetically remote species as Vibrio cholerae, Pasteurella pestis, Serratia, Proteus, Pseudomonas and more recently Haemophilus and Neisseria.

RESISTANCE MECHANISMS

Due to the abrupt appearance of resistance to many drugs at once it was first thought that multiple resistance was attributable to a single genetic determinant, which conferred a non-specific permeability barrier on the host. This theory, however, was abandoned when R-plasmid segregants were found, showing that each drug resistance was specified by a separate gene (Watanabe et al., 1964a).

Several mechanisms exist by which a bacterial cell might be resistant to a particular drug (Goldstein et al., 1968). However, not all may be applicable to resistance conferred by R-plasmids and in many instances the plasmid resistance mechanism may differ from that determined by the chromosome. To be effective an R-plasmid resistance mechanism must be 'dominant' over the wild type inhibited function.

The most common R-plasmid resistance mechanism is drug

inactivation. The best studied system is the β-lactamase hydrolysis of penicillins and cephalosporins. Since their discovery (Abraham and Chain, 1940) β-lactamases have been found on plasmids of Staph. aureus (Novick, 1963), E. coli (Anderson and Datta, 1965) Haemophilus (Thomas et al., 1974; Elwell et al., 1975) and Neisseria (Ashford et al., 1976; Percival et al., 1976; Perine et al., 1977). The types of β-lactamase can, however, differ considerably from those chromosomally mediated (Dale and Smith, 1971).

In contrast to the "inactivating" β-lactamases, R-plasmid resistance to aminoglycoside antibiotics is due to the production of enzymes which modify the antibiotic molecule by either acetylation (Brzezinska et al., 1972), adenylation (Benveniste and Davies, 1971) or phosphorylation (Umezawa et al., 1967) thus rendering it inactive. This plasmid encoded resistance mechanism is quite distinct from chromosomally mediated streptomycin resistance which results from an alteration of the 30s ribosomal unit (Nomura, 1970) and subsequent inability of the drug to bind to it (Chang and Flaks, 1972).

Chromosomal resistance to many chemotherapeutic drugs may be due to decreased uptake of the drug (Chopra and Ball, 1982) but resistance to the tetracyclines, fusidic acid (Chopra, 1976) and chloramphenicol (Gaffney et al., 1981) provide the only examples of specific plasmid-determined resistance mechanisms resulting from decreased drug accumulation. Tetracycline resistance, however, is the only well documented example. Recent work indicates that the mechanism of resistance involves plasmid-mediated synthesis of new

membrane-located proteins (Levy and McMurray, 1978; Tait and Boyer, 1978; Coleman et al., 1983) which promote energy-dependent efflux of tetracycline across the cell membrane (Ball et al., 1980; McMurray et al., 1980; Hedstrom et al., 1982; McMurray et al., 1982).

Finally plasmids can direct the synthesis of enzymes capable of acting themselves as modified targets which are less susceptible to the action of antimicrobials. For instance, in the case of trimethoprim and sulphonamide resistance, plasmid-determined resistant dihydrofolate reductase and dihydropteroate synthetase are synthesised respectively (Amyes and Smith, 1974a; Wise and Abou-Donia 1975).

CLASSIFICATION OF RESISTANCE PLASMIDS

Due to the rapidly increasing numbers of R-plasmids which were being identified it became necessary to produce a suitable method of classification, in order that effective investigations into plasmid biology and epidemiology could be carried out. The initial method of classification was introduced by Watanabe and Fukasawa (1962) and was based on the interactions which were noted to occur between resistance plasmids and the F-factor of E. coli K-12. (Lederberg et al., 1952) These workers observed that the introduction of certain R-plasmids (later classified fi^+ (fertility inhibition positive) by Watanabe et al., 1964b) into F+ or intermediate F' donor bacteria enormously reduced their capacity to transfer the F factor, while the frequency of chromosome transfer by Hfr strains was reduced to 1% or

less of its normal value. Those R-plasmids which had no effect on fertility were termed $\underline{fi}^-$ plasmids.

It was originally thought that members of each group probably shared considerable homology but it soon became apparent that a certain amount of heterogenity existed in both the $\underline{fi}^+$ and $\underline{fi}^-$ classes. Further classification was therefore made on the basis of the interactions which occurred between R-plasmids themselves. Watanabe and Lyang (1962) noticed that bacteria carrying two $\underline{fi}^+$ R-plasmids with different drug resistance markers were genetically very unstable, resulting in either the loss of one of the two R-plasmids or, the occurrence of genetic recombination between them. This point was further investigated by Watanabe *et al.* (1964) employing many more R-plasmids of both $\underline{fi}^+$ and $\underline{fi}^-$ types of independent origins. Their findings confirmed that 'mutual exclusion' does not take place between $\underline{fi}^+$ and $\underline{fi}^-$ R-plasmids, whereas it does occur between two different $\underline{fi}^+$ R-plasmids. The presence of one $\underline{fi}^+$ plasmid within a cell seemed, therefore, to interfere with the entry and establishment of a second $\underline{fi}^+$ plasmid - a phenomenon now termed superinfection inhibition. These observations have subsequently led to the method of plasmid classification used most widely today - plasmid incompatibility, defined as the inability of two different plasmids to coexist stably in the absence of selective pressures (Novick *et al.*, 1976) ie. plasmids belonging to the same incompatibility group are unable to coexist stably within a cell.

The use of incompatibility as a criterion for the classification of bacterial plasmids has resulted in many investigations directed at determining whether incompatibility is an indication of molecular (and therefore probably phylogenetic) relatedness between plasmids. The use of the modern techniques of molecular weight estimations, DNA reassociation experiments and restriction endonuclease digest patterns, has shown that, in general, plasmids within an incompatibility group have much DNA in common (Grindley et al., 1973; Falkow et al., 1974) with highly conserved regions encoding replication and transfer functions (Gorai et al., 1979; Ward and Grinsted, 1981; Villarroel et al., 1983). This strongly suggests, therefore, that classification of plasmids by incompatibility does indeed appear to reflect true phylogenetic relationships.

Due to the lengthy and laborious experiments involved in classifying plasmids by their incompatibility it is now becoming more popular to distinguish plasmids by their resistance patterns, molecular weights and restriction endonuclease digest patterns (Richards et al., 1981; Tietze and Tschape., 1983) as these techniques become more standard.

Despite the similarities in transfer and replication genes, particular resistance genes do not show much correlation with the incompatibility group. In contrast, the same resistance genes, determining identical properties, are often carried by otherwise unrelated plasmids. This diversity of resistance genes has been partly explained by the discovery of drug resistance transposons

which are able to 'jump' from one piece of DNA to another.

MOVABLE GENETIC ELEMENTS

Movable genetic elements were first described by Barbara McClintock following her analysis of crosses between genetically marked corn plants (McClintock, 1951). Full understanding of her observations, however, was not achieved until almost 20 years later with the discovery of a new class of mutations in genes of a laboratory strain of Escherichia coli and of bacteriophage λ (Jordan et al., 1968, Shapiro, 1969; Fiandt et al., 1972; Hirsch et al., 1972).

These mutations, termed polar mutations (Jacob and Monod, 1961) were unusual in that their effects were detectable beyond the borders of the mutated genes themselves, a property which could not be explained by any known mutational mechanism. In addition most of the mutants could revert spontaneously to wild type, but the reversion rate was not enhanced by mutagens, indicating that the mutations were not the result of deletions and were unlikely to be point mutations. Subsequent investigations (Jordan et al., 1968; Shapiro, 1969) revealed that the mutations had been caused by the insertion of sizeable DNA fragments into the mutated gene.

These DNA elements were named insertion sequences or IS elements. Several unrelated IS sequences have since been described in E. coli and λ (Bukhari et al., 1977; Guyer, 1978) which can insert themselves within many different genes, interrupting the continuity of

the gene and turning off its activity. Further characterisation of these elements has revealed them to be of a defined length ie. 800-1400 base pairs (Fiandt et al., 1972). Extensive investigations by Saedler and Heiss (1973) could not reveal the presence of IS elements in the cytoplasm of E. coli and it is therefore assumed that these elements do not replicate autonomously. From molecular hybridization experiments Saedler and Heiss (1973) concluded that two such elements, IS1 and IS2, are present in the E. coli chromosome in about 8 and 5 copies respectively. The large number of insertion sites suggested that some type of non-homologous recombination was taking place as it seemed unlikely that an IS element could be homologous with the nucleotide sequences at so many different sites.

Studies by Nevers and Saedler (1978) showed that insertion of IS elements is indeed independent of the host recA gene product known to be necessary for homologous recombination. However, IS sequences have been shown to play a role in the formation of Hfr and F'13 (Hu et al., 1975) by recombination between pairs of homologous sequences present on the chromosome and F-factor. This process, in contrast to IS insertion, requires the host's recombination system. Thus IS sequences are involved in at least two kinds of recombination.

The mechanisms involved in IS translocation are still unclear. Grindley (1978) and Calos et al. (1978) have shown that insertion of IS1 results in the generation of two directly repeated sequences of 9 base pairs (bp) at either end of the inserted sequence. Similar repeated sequences of 5bp have been shown to occur following

integration of IS2 (Rosenberg et al., 1978). The explanation proposed is that integration involves the introduction of two nicks nine or five bases apart, and the covalent attachment of the 3' ends of the IS1 element to the free and protruding 5' ends that are created. The two single-stranded gaps of 9 or 5 bases would then be filled in by DNA synthesis (Grindley, 1978). Ohtsubo et al. (1980) studying the mechanism of insertion and cointegration mediated by IS1 demonstrated that mutations occurring inside IS1 markedly reduced or abolished the frequency of cointegration, suggesting that IS1 may code for a protein that is required for cointegration, and therefore for the insertion of IS1. However, genetic identification of functions specified by these elements is still not evaluated.

At the same time that IS elements were discovered other microbiologists and geneticists made observations that certain genes, known to be responsible for resistance to antibiotics by bacteria, were capable of transfer from one molecule of DNA to another (Dubnau and Stocker, 1964; Kondo and Mitsuhashi, 1964; Harada et al., 1967; Datta et al., 1971). The first direct evidence that such transfer is by a process analogous to the insertion of IS elements was published in 1974. Hedges and Jacob (1974) found that the transfer from one plasmid to another of the TEM-β-lactamase gene, conferring resistance to ampicillin, was always accompanied by an increase in the size of the recipient plasmid. The recipient could then donate the resistance trait to still other plasmids, which thereupon showed a similar increase in size. Hedges and Jacob (1974) postulated that the gene for ampicillin resistance was carried by a DNA element that

could be "transposed" or could move from one molecule of DNA to another, and they called such an element a transposon (Tn).

Originally transposons were distinguished from IS elements because they carry detectable genes, often conferring antibiotic resistance. However, as more information concerning the structure of transposons and the mechanism of transposition becomes available, it is clear that there is a very close relationship between the IS and Tn elements. Kopecko and Cohen (1975), also studying the acquisition of the TEM-β-lactamase gene by various plasmids, observed that such transfer could take place in mutated bacteria lacking the recA gene product. Thus the transposition event, like IS translocation, involves recA independent non-homologous recombination.

Electron microscopy of the plasmid DNA further revealed that the two ends of the transposable DNA segment had a unique feature: they consisted of nucleotide sequences that were complementary to each other but in the reverse order. This feature results in the formation of stem and loop structures following separation and reannealment of the DNA strands. These repeat segments appear to be a common feature of most of the transposons so far identified (Kleckner, 1977). They do, however, vary in length, some transposons terminating in very long (800-1500 base pair (bp)) inverted or direct repeats and others in very short (ca. 40bp) repeats. The long repeat segments of transposons are often IS or IS-like elements. Many transposons thus represent a segment of DNA that is mobile because it is flanked by IS units; for example Tn9 (MacHattie and Jackowski,

1977) and Tn1681 (So et al., 1979) are flanked by copies of IS1, which accounts for the mobilization of the intervening genetic material. Other transposons, notably Tn5, Tn10 and Tn903, also terminate in very long inverted repeats, which are IS-like but which have not been identified as normal constituents of E. coli (Berg and Drummond, 1978; Ross et al., 1979). The shorter inverted repeats of elements such as Tn3 may represent a vestige of what once were two full insertion sequences. Since the IS elements themselves terminate in short inverted repeats, a transposon that is flanked by two IS units, even in direct repeats as in Tn9, will also be flanked by inverted repeats. Virtually all the insertion sequences and transposons characterised at the sequence level have a terminal inverted repeat. The only exception to date is bacteriophage Mu. Analysis of the nucleotide sequences of bacteriophage Mu revealed only 2bp at either end to be repeated (Kahman and Kamp, 1979).

As indicated insertion of IS1 results in a 9bp sequence of target DNA being repeated on either side of the inserted element (Calos et al., 1978; Grindley, 1978). Nine base pair repeats are also formed during insertion of Tn5 (Schaller, 1979), Tn9 (Johnsrud et al., 1978), Tn10 (Kleckner, 1979) and Tn903 (Oka et al., 1978). Similarly, 5bp repeats result following insertion of Tn3 (Cohen et al., 1978, Ohtsubo et al., 1979) and phage Mu (Allet, 1979). Analysis of the distribution of transposons at various DNA sites and the different repeated sequences (Johnsrud et al., 1978; Kleckner, 1979; Ohtsubo et al., 1979) indicates that these repeated sequences are generated during the process of integration.

Over the last ten years much has been discovered concerning the mechanism and regulation of transposition. Genetic studies of the ampicillin resistance transposon Tn3, employing deletion mutants, suggested the existence of two or more trans-acting transposition functions encoded by the transposon (Heffron et al., 1977; Gill et al., 1978). Heffron et al., (1979) went on to identify three polypeptides encoded by this transposon: the transposase, a high MW polypeptide encoded by the tnpA gene; the Tn3-specific repressor, a low MW polypeptide encoded by the tnpR gene and the 286 amino acid β-lactamase. Mutation studies have demonstrated that the tnpA gene product is an absolute requirement for transposition and that the protein encoded by tnpR acts as a repressor to regulate expression of this tnpA encoded transposase, (Gill et al., 1979; Heffron et al., 1979). Arthur and Sherratt (1979) proposed that tnpR or some other gene enocded in this region is required for resolution. Subsequent studies by Heffron et al. (1980) confirmed that the tnpR gene of Tn3 encodes a site-specific recombination system that will efficiently resolve cointegrates independent of recA. The tnpR gene product is therefore responsible for both repression of tnpA gene function and resolution of cointegrate intermediates during transposition.

In addition to these two essential genes, three sites are required for Tn3 transposition. Both the right and left inverted repeats are absolutely required for the complete transposition event (Heffron et al., 1977). Deletion mutants of Tn3 lacking the 39bp inverted repeat, are transposition deficient and no complementation

by wild-type DNA is possible. The termini are therefore thought to function as possible recognition sites for an enzyme, presumably the transposase, required for transposition (Heffron et al., 1977). A third site, IRS (internal resolution site), located between the inverted repeats, is also required for resolution of cointegrates by the tnpR gene product resolvase (Heffron et al., 1979). However, if transposition occurs in recA$^+$ cells this site is not required as the host cell recombination system resolves the intermediate cointegrates.

In contrast to the functional map of Tn3 (Figure 1a), the enzymes required for transposition of the kanamycin resistance transposon, Tn5, are encoded by the long terminal inverted repeats (Figure 1b). Rothstein et al. (1980a) were able to study some of the transcription, translation and transposition properties of Tn5, by construction and analysis of deletion, insertion and substitution mutants of Tn5. From these and subsequent studies (Rothstein et al., 1980b) they concluded that five polypeptides are encoded by Tn5, two being translated by each inverted repeat and the fifth is the resistance enzyme, neomycin phosphotransferase (NPT II). The four enzymes coded by the inverted repeats are all different, the active polypeptides for transposition being encoded by the right inverted repeat. By construction of a transposon from Tn5 that is of the Tn3 'type' with short inverted repeats Rothstein et al. (1980b) were able to show that the only regions of the left repeat that appear to be absolutely necessary for the functions of the transposon are its two ends - the outside being required to provide a substrate for the

Figure 1a. The genetic organisation of Tn3 (adapted from Lee et al., 1983).

IR = inverted repeat sequence; tnpA = transposase gene; IRS = internal resolution site; tnpR = resolvase gene; bla = TEM-1 β-lactamase. Circles and arrows indicate origin and direction of transcription, respectively, of the appropriate genes.

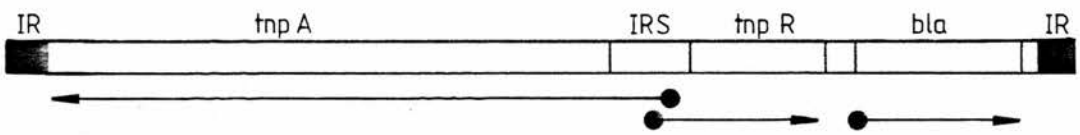
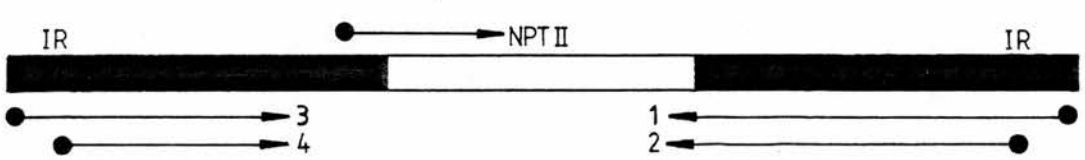


Figure 1b. The genetic organisation of Tn5 (adapted from Rothstein et al., 1980b).

IR = inverted repeat sequence; 1, 2, 3 and 4 = proteins encoded by Tn5; NPT-II = neomycin phosphotransferase. Circles and arrows indicate origin and direction of transcription, respectively, of the appropriate genes.

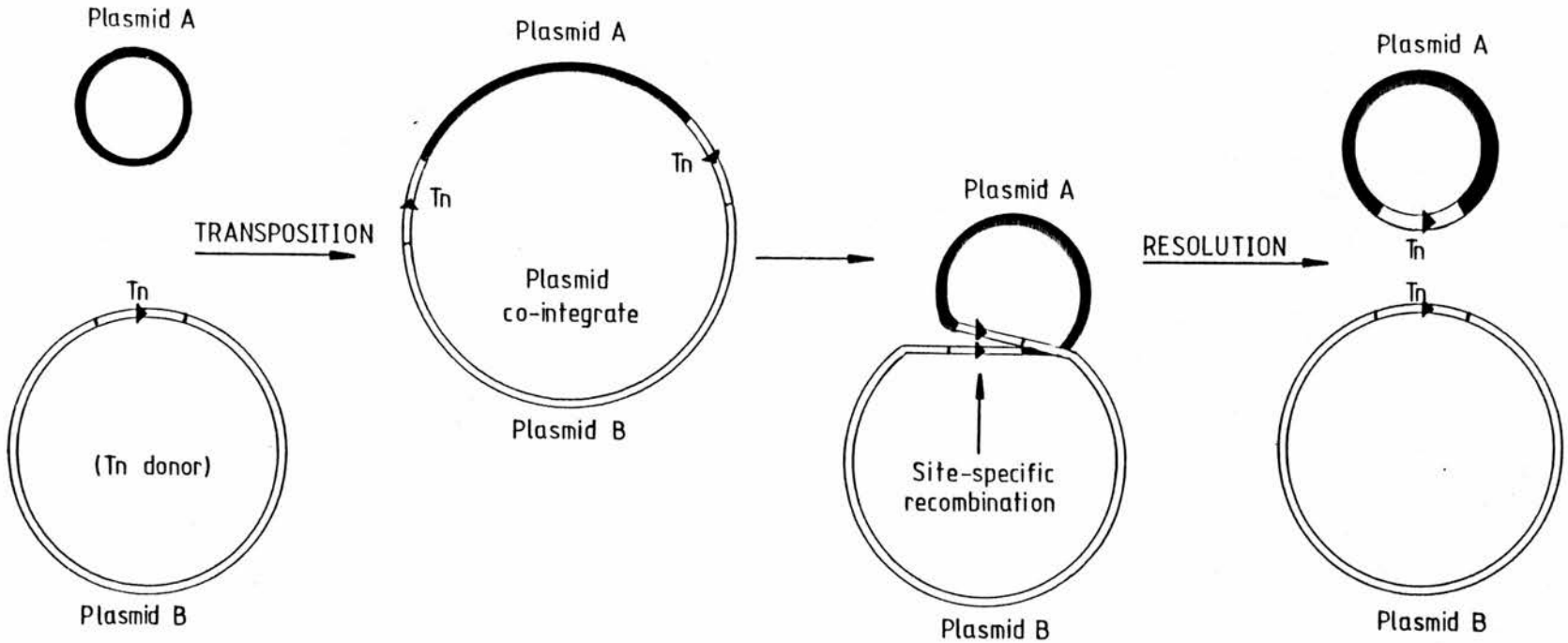


transposase enzyme and the inside end which carries the promoter for the kanamycin resistance gene.

Several models have been proposed for the mechanism of transposition (Arthur and Sherratt, 1979; Shapiro, 1979; Galas and Chandler, 1981; Harshey and Bukhari, 1981). In general it is believed transposition involves three principle steps (Figure 2). The first stage involves cleavage of the DNA strands of the transposon and the recipient replicon at a specific site. The specificity of insertion differs markedly between different transposons. Some, such as Tn10 (Kleckner et al., 1979) and Tn5 (Miller et al., 1980), show a tendency to insert repeatedly at particular preferred positions or 'hot-spots'. Other transposons such as Mu, Tn3 and Tn9 insert efficiently at a great many different sites even within a region as short as a single gene (Bukhari and Zipser, 1972; Weinstock et al., 1979; Miller et al., 1980). Such transposons are said to be regionally specific but locally non-specific. The endonucleases responsible for these cleavage events are probably specified by the transposon and the bacterial cell respectively (Grindley and Sherratt, 1978). Next the donor and target strands are ligated and formation of a cointegrate intermediate follows with the semi-conservative replication of the transposable element. Formation of the cointegrate is mediated by the transposase enzyme. Finally, resolution of the cointegrate takes place restoring the original donor and giving rise to a recipient replicon containing the transposon. Resolution results from recombination mediated by the hosts own recombination system, or by

Figure 2. Model for transposition (adapted from Saunders, 1984).

Tn = transposon; ▶ = IRS site. The diagram shows the formation and resolution of plasmid co-integrates during transposition.



site-specific recombination mediated by the tnpR gene product resolvase.

Recently it has been proven that formation of cointegrates are not obligatory in the transposition of certain transposons (Galas and Chandler, 1981; Harshey and Bukhari, 1981; Bennet et al., 1983). It is suggested that if only one strand of the element is cut, or cut more efficiently than the other, direct transposition would occur as opposed to formation of intermediate cointegrates (Bennet et al., 1983). In any event transposition results in the transfer of a segment of DNA from one replicon to another without concomittant loss from the original donor.

Many different insertion sequences and transposons have now been described. Transposons encoding resistance to a wide variety of antimicrobials have been identified. The ability of these elements to transpose efficiently between different replicons, provides not only an explanation for the diversity of identical resistance genes among plasmids of different incompatibility groups but also provides valuable insights into the evolution of R-plasmids and bacterial resistance.

B. TRIMETHOPRIM

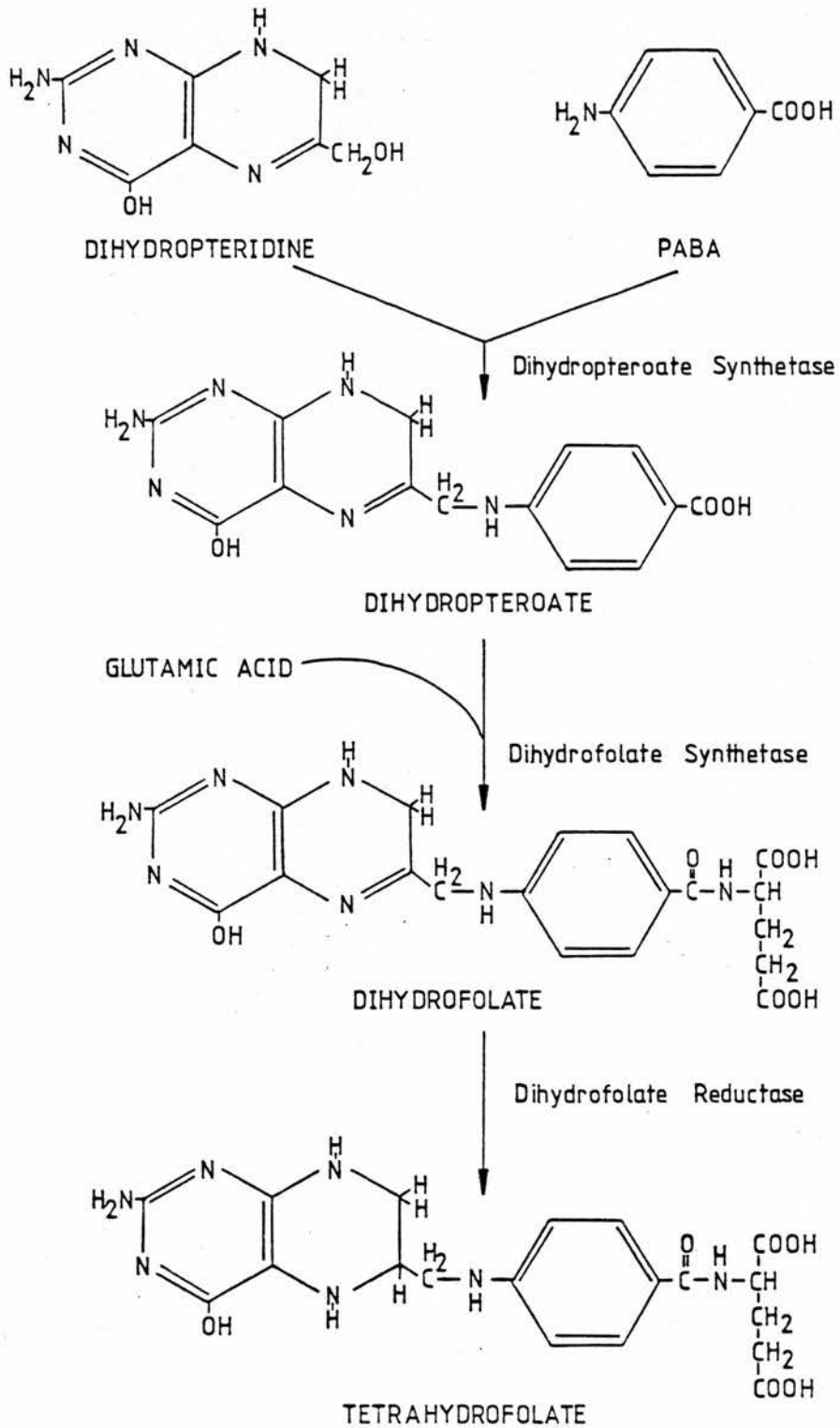
FOLATE METABOLISM

The past two decades of the chemotherapeutic era have been dominated as much by transferable drug resistance as by the introduction of new and potent drugs or combinations such as trimethoprim/sulphamethoxazole (cotrimoxazole, Bactrim, Septrin, Septra). Both trimethoprim and sulphonamides are inhibitors of bacterial folic acid metabolism.

Tetrahydrofolate derivatives of the vitamin folic acid are of paramount importance in both mammalian and bacterial cells, serving as essential cofactors in the biosynthesis of several amino acids, purines and pyrimidines (Blakely, 1969). Folic acid itself is a widely distributed group B vitamin. It was first isolated in crystalline form from liver by Pfiffner *et al.* (1943) and its structure established and confirmed by total chemical synthesis as pteroylglutamic acid (Angier *et al.*, 1946).

The biochemical pathway for folate biosynthesis was worked out with extracts of *Escherichia coli* by Brown (1971) (Figure 3). The first step involves the formation of dihydropteroate by the condensation of dihydropteridines with p-amino benzoic acid (Brown *et al.*, 1961), catalysed by the enzyme dihydropteroate synthetase (Weisman and Brown, 1964). One or more glutamic acid residues are then added to dihydropteroate, in the presence of a condensing enzyme, dihydrofolate synthetase (Griffin and Brown, 1964), to form

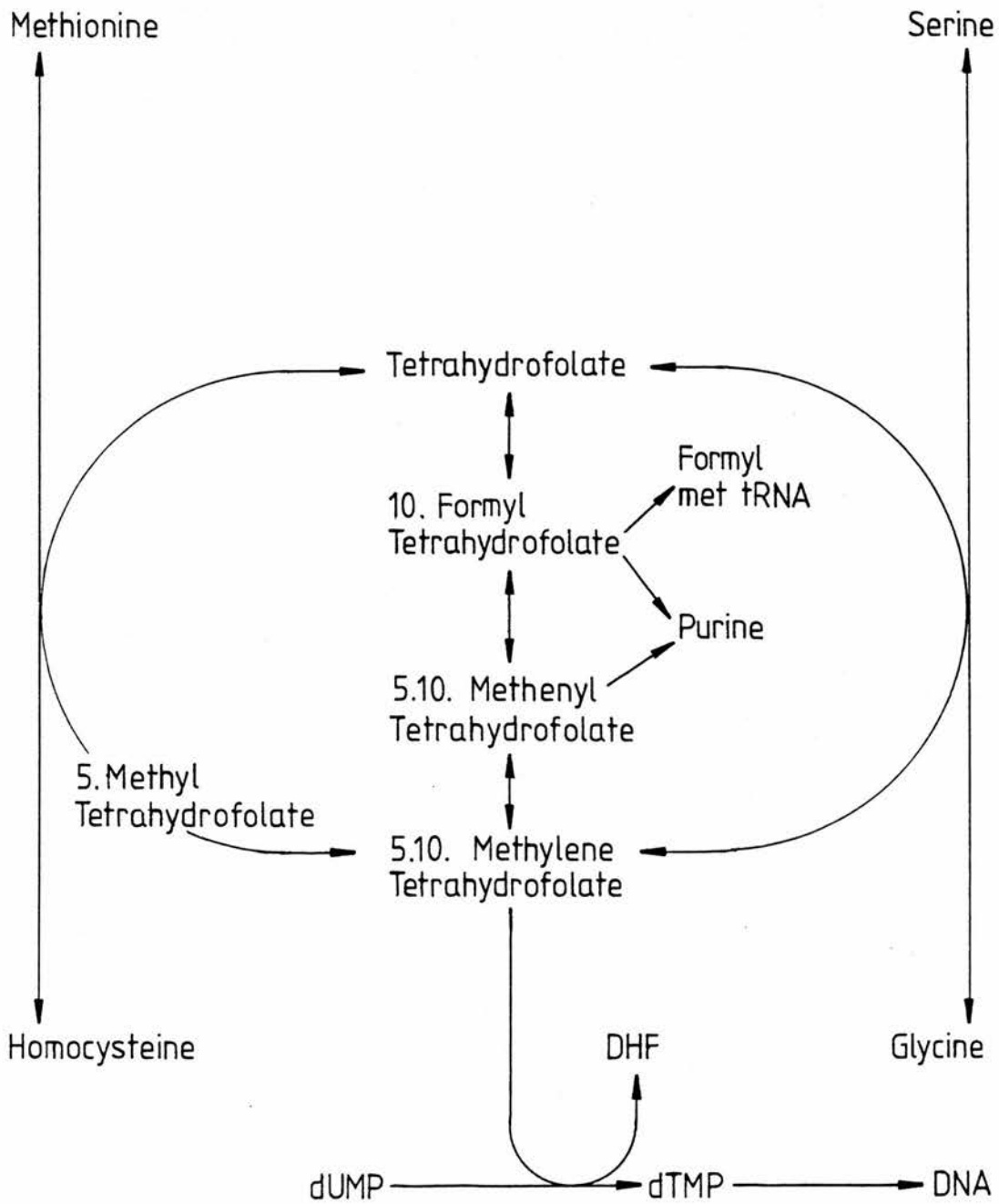
Figure 3. The biosynthetic pathway of tetrahydrofolate employed by bacteria.



dihydrofolate (DHF) (Brown et al., 1961). These two enzymic reactions are absent in mammalian cells which in contrast utilize pre-formed folates, obtained as the vitamin folic acid, from the diet. This ability to use exogenous folates is confined to higher species and a few unusual bacteria such as Streptococcus faecalis and Lactobacillus casei, which possess an active transport system for the uptake of folic and folinic acid which is dependent on glucose (Wood and Hitchings, 1959a). However, with the exception of some streptococci these unusual bacteria are not generally pathogens. It is these two essential differences, the absence of the initial step in folate biosynthesis in mammalian cells and the inability of bacterial cells to utilize exogenous folates, that have been exploited with the sulphonamide group of drugs. As structural analogues of p-amino benzoic acid, the sulphonamides competitively inhibit dihydropteroate synthetase (Brown, 1962).

Reduction of dihydrofolate to its active form tetrahydrofolate (THF) by the enzyme dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NAD^+ oxidoreductase EC.1.5.1.3.) occurs in both mammalian and bacterial cells. Tetrahydrofolate serves as a carrier of one carbon units (Rabinowitz and Himes, 1960) by the formation of a number of cofactors, each one of which is formed enzymatically from a suitable one-carbon donor and tetrahydrofolate. Each substituted THF is a cofactor in a specific reaction (Figure 4). After reaction with a suitable precursor, again catalysed by a specific enzyme, a new product and unsubstituted THF are formed. In most reactions THF is just a carrier and the result of the reaction is the release of

Figure 4. The interconversion of tetrahydrofolate intermediates and their relationship with purine and protein synthesis (taken from Amyes and Smith, 1974b).



free THF. Thymidylate synthetase (EC.2.1.1.b.) however, requires two atoms of hydrogen, in addition to the methylene group of deoxythymidylate. These are taken from the THF carrier itself, producing dihydrofolate. Thus dihydrofolate reductase (DHFR) is again required, to regenerate the system by returning the folate to the tetrahydrofolate state.

The enzyme DHFR therefore plays a key role in folate metabolism and subsequent biosynthetic reactions. Partial purification of a chicken liver DHFR enzyme was the first to be achieved (Futterman 1957, Futterman and Silverman 1957) following observations by Greenberg (1954) that reduction of DHF to THF in chicken liver required NADH. This system performed both a NADPH-dependent reduction of folate to DHF and a NADH- or NADPH-reduction of DHF to THF. The direct reduction of DHF by pyridine nucleotides was established by Osborn and Huennekens (1958) following their partial purification of a NADPH-linked DHFR from acetone powder extracts of chicken liver. Similar systems were soon found in S. faecalis (Blakely and McDougal, 1961) and E. coli B (Mathews and Sutherland, 1965). Like the avian liver enzymes, these bacterial DHFRs were specific for NADPH and DHF but in contrast showed no reaction with folate. More sophisticated purification procedures subsequently led to the establishment of the molecular weight of both the avian liver and bacterial enzymes as about 20,000 (Kaufman and Gardiner, 1966, Mathews and Sutherland, 1965). Since then this enzyme has been shown to be widely present, both in microbial and mammalian systems.

ANTIFOLATE DRUGS

The earliest reported small molecule 'antifolates' were the 2,4-diaminopteridines of Daniel and Norris (1947) (Daniel et al., 1947). They had been synthesised to simulate the pteridine portion of the folate molecule, and the authors were puzzled to find that the 2,4-diamino derivatives were much more potent inhibitors of lactic acid bacteria than the chemically more nearly related 2-amino-4-hydroxy-pteridines. Similarly, Hitchings et al. in 1945 started investigations with 2,4-diamino-5-methylpyrimidine followed by a family of inhibitors which evolved through elaboration of the 5-methyl group. By 1948 (Hitchings et al.) it was asserted that all derivatives of 2,4-diaminopyrimidine are antagonists of folic acid, and shortly a variety of substances of this general structure were in hand as a result of a program directed toward antimalarials (Falco et al., 1951).

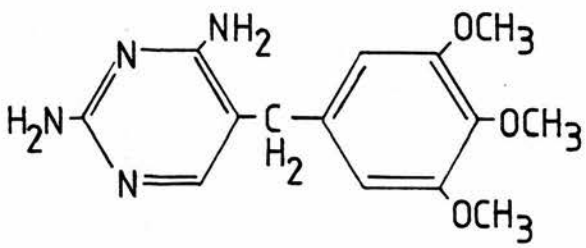
The more these small molecule antifolates were studied, the more varied and selective their acitvities appeared to be. There was no reason to doubt, however, that although these compounds were originally designated antithymine compounds their principal mode of action was as anitfolates. The inhibitory effects could be overcome in vivo by leucovorin indicating a blockage in folate reduction (Wood and Hitchings, 1959b; Wood et al., 1960) similar to the mechanism which had been shown by Nichol and Welch (1950) for the large molecule inhibitor aminopterin, in rat liver slices and intact animals. In 1965, Hitchings and Burchall confirmed that the target

site of 2,4-diaminopyrimidines was indeed the oxidoreductase enzyme dihydrofolate reductase.

The effort to optimise the selective nature of these compounds resulted in the development of the antibacterial drug trimethoprim (2,4-diamino-5-(3'4'5'-trimethoxybenzyl)pyrimidine) (Figure 5) (Hitchings and Roth, 1959; Hitchings and Bushby, 1961; Roth et al., 1962) which was shown to exhibit a tremendous specificity for bacterial dihydrofolate reductases over the mammalian enzyme (Burchall and Hitchings, 1965). The selectivity of the small molecule inhibitors of DHFR is the basis for their chemotherapeutic utility and extensive studies of both mammalian and bacterial DHFRs have ensued in an effort to determine the mechanism of this specificity. Studies of pH profiles, molecular weight estimations, isoelectric points and the enzyme kinetics all fail to reveal a significant difference between DHFRs from mammalian and bacterial sources although these characteristics do show that variations occur confirming that each species has one (or more) type of reductase that is unique (Hitchings and Smith, 1980). Inhibitor binding analysis, on the other hand, reveals that trimethoprim (Tp) binds very much more tightly to the bacterial DHFR than the mammalian enzyme (Burchall and Hitchings, 1965), but the difference between host and parasite enzymes that give rise to these selective effects are still being determined.

Burchall (1969, 1971), in an attempt to explain the differential binding of trimethoprim, suggested that the bacterial and animal DHFRs have evolved independently. Thus the more sophisticated

Figure 5. The structure of trimethoprim.



TRIMETHOPRIM

mammalian enzyme is able to distinguish between the pteridine substrate and the small molecule inhibitors (2,4-diaminopyrimidines). On the other hand, the large molecule inhibitors (2,4 diaminopteridines) are bound to both DHFRs at their pteridine binding site. Thus the 2,4-diaminopyrimidines are the more selective drugs against micro-organisms.

It is now widely accepted that all diaminopteridine and diaminopyrimidine derivatives, from methotrexate to trimethoprim, have the same mode and locus of binding of the 2,4-diaminopyrimidine moiety. For this reason Hitchings and Smith (1980) speculate that the special affinity of Tp for bacterial DHFRs must be the result of the conformation of the molecule and the fit of the trimethoxybenzyl moiety into some hydrophobic pocket. Perkins and Bertino (1966) suggested that some inhibitors may bind more tightly in the ternary complex than to the enzyme alone. The structure of the ternary complex of E. coli DHFR with the co-enzyme NADPH and an inhibitor is not as yet known. However, the structure of the ternary complex of L. casei DHFR with NADPH and methotrexate was described in 1978 (Mathews et al.). These authors have compared the L. casei DHFR ternary complex with methotrexate to the E. coli DHFR binary structure. They suggest that the large cooperative effect in the binding of methotrexate and analogues to the ternary, as opposed to the binary complex of DHFR enzymes, which has been observed in several laboratories (Williams et al., 1973; Birdshall et al., 1980) is caused by conformational changes in the DHFR which occur on binding of the NADPH co-factor.

A recent study (Baccanari et al., 1982) of Tp, its parent benzylpyrimidine and the monomethoxy and dimethoxy analogues in interaction with DHFR from E. coli and mouse lymphoma showed there to be an increasing cooperativity in binding in the ternary complex, as well as an increased specificity in binding to the bacterial enzyme on increasing the meta- or para-methoxylation ie. monomethoxy derivatives were about ten fold more active than the unsubstituted benzylpyrimidine, the dimethyl analogues another ten fold more active and Tp roughly six fold more active again. These authors conclude that cooperativity in binding with the ternary complex is largely responsible for the high affinity of Tp for E. coli DHFR as cooperativity is not as prominent with DHFR of mouse lymphoma.

Studies by Stuart et al. (1983) involving molecular models of newly developed ortho-substituted derivatives of the 2,4-diamino-5-benzylpyrimidines in the active site of dihydrofolate reductase, has provided a rational explanation for their activities, relative to Tp. So far all the new analogues developed have exhibited lower antibacterial activity than Tp and, in most cases, very much reduced specificity. It is interesting, however, that the new dihydro-benzofuran derivative, compound 46, developed by Burroughs Wellcome (Stuart et al., 1983) shows relatively good activity against the gonococcal enzyme, compared to Tp. The inherent resistance to Tp of the Neisseriaceae is known to be the result of a decreased affinity of their DHFR for the drug (Then, 1979). This is a similar, if somewhat less pronounced, mechanism to R-plasmid determined resistance (Amyes and Smith, 1974a; Sköld and Widh, 1974).

ANTIFOLATE CHEMOTHERAPY - ONE DRUG OR TWO?

Trimethoprim has been used widely since 1969 in the UK, initially in combination with sulphamethoxazole (Sx) as co-trimoxazole and, since 1979, also as a single agent. Its main uses are in the treatment of urinary tract and lower respiratory tract infections. When co-trimoxazole was introduced in 1969, the two components were thought to present a unique combination for three principal reasons: (i) remarkable synergy between them, (ii) the ability to suppress the development of resistance and (iii) the bactericidal effect of the combination in contrast to the individual bacteriostasis observed with each drug individually (Bushby and Hitchings, 1968). Thus although Tp was known to possess potent antibacterial activity in its own right (Hitchings, 1969) it was considered a sulphonamide potentiator (Bushby and Hitchings, 1968; Hitchings, 1970) and there was little evaluation of its use clinically as a single substance. Since its introduction, however, there have been many reports questioning these claims, and much evidence to suggest that Tp alone may be as clinically effective and as safe as the combination.

The demonstration of synergy between Tp and Sx has often been reported *in vitro* (Darrell *et al.*, 1968; Bushby, 1969, 1973) but has never been shown *in vivo*. Two different mechanisms have been suggested to explain the *in vitro* synergy which occurs. As a structural analogue of the pteridine portion of DHF, Tp serves as a competitive inhibitor in the reduction of DHF to THF thus depleting

the cells concentration of THF (Burchall, 1969, 1979). This biological effect can be minimised by inclusion of a sulphonamide which blocks the synthesis of DHF, and it is this sequential blockade which is thought to result in potentiation of the combination of Tp and Sx over the action of each component alone (synergy) (Darrell et al., 1968). On the other hand, Poe (1977) has suggested that synergy results from the simultaneous binding of Tp and Sx to DHFR, when the drugs are present in sub-inhibitory concentrations.

However, whilst synergy between Tp and Sx readily occurs in vitro, this does not seem to be a requirement for success in treating many acute infections. Models of urinary tract infections (UTI) using concentrations of each agent that occur during therapy have shown that the activity of Tp is so dominant over the Sx that the potential for synergy between them does not occur (Anderson et al., 1974; Greenwood and O'Grady, 1976; Greenwood, 1979; Lacey et al., 1980b). In addition the concentration of Tp in the body during therapy is sufficient to inhibit most of the pathogens in question (Hansen et al., 1973, Wikinson and Reeves, 1979). Clinical trials have indeed shown that Tp alone is as effective as the combination in treating acute urinary tract and acute respiratory tract infections (Brumfitt and Pursell, 1972; Koch et al., 1973, Kasanen et al., 1978, Lacey et al., 1980a, Mabek and Vejlsgaard, 1980).

The claim that the administration of a sulphonamide simultaneously with Tp would prevent bacteria from acquiring resistance to Tp has been more difficult to prove. Darrell et al.

(1968) studied serial transfers of four cultures of the Enterobacteriaceae on ditch plates containing low concentrations of Tp. After 25 subcultures of a heavy inoculum each culture yielded resistant variants. These variants were not, however, detected if a light inoculum was used. When a sulphonamide was incorporated into the medium in addition to Tp, the development of this resistance was inhibited (Darrell et al., 1968; Grunberg and Beskid, 1977). However, these manipulations may not represent events within patients treated with Tp and the nature of Tp resistance found in clinical isolates may differ from that in cultures exposed to Tp in vitro. Bacteria in vivo are not exposed to gradually increasing concentrations of Tp over weeks and moreover, if Tp is added in therapeutic concentrations to E. coli suspended in serum, urine or nutrient broth, resistant variants of E. coli do not emerge (Lacey et al., 1980b).

Several studies have been carried out comparing the use of Tp with Tp/Sx in patients with acute, recurrent or chronic urinary infections and more recently, in the treatment of patients with chest infections (Lacey, 1982). If the use of Tp alone is particularly liable to select resistance in vivo trials, where it was prescribed as a single agent, should show evidence of this. However, in each of the studies there was no report of a higher incidence of Tp resistance after the use of Tp alone compared with Tp/Sx.

A recent survey, concluding in October in 1982, of Tp resistance in Gram negative urinary pathogens isolated from hospital and general

practitioner patients, indicates a sudden rise, during 1982, in the percentage of organisms resistant to Tp but sensitive to Sx (Maskell, 1983). It is suggested that the use of Tp alone has promoted this increase in the level of resistance from 12% to 19%. These results, however, are not comparable with those published by Lacey (1982) who reported no change in the level of resistance of E. coli to Tp in the hospitals of King's Lynn Health District, where co-trimoxazole was completely replaced by Tp in 1980. His study concluded in January 1982 and did not include Gram negative pathogens other than E. coli. Nevertheless, if the use of Tp alone is now resulting in increased resistance it is important that this should be recognised.

Two surveys have shown that Tp resistance is rare in Haemophilus influenzae and has not increased during 1974-1978 (Williams and Andrews, 1974; Howard et al., 1978). This is significant since during therapy with co-trimoxazole, the sulphonamide concentration in lung tissue and sputum may be too low to inhibit the growth of Haemophilus (Wilkinson and Reeves, 1979). In effect, therefore, this organism may have been exposed to Tp alone. If Tp did have a particular predilection to select resistance during clinical use, the incidence of Tp resistant Haemophilus influenzae would be expected to be much higher than has been reported.

Finally a comparison of the levels of Tp resistance in countries such as Finland, where Tp has been used extensively without Sx, shows that the incidence of Tp resistance among enterobacteria is no higher than in other countries, where only the combination has been given

(Kasanen et al., 1978; Hamilton-Miller, 1979; Amyes et al., 1981).

The third reason for issuing the combination was based on the observation that individually the drugs are bacteriostatic, whereas in combination they are bactericidal (Bushby and Hitchings, 1968). In minimal medium the effect of Tp on the bacterial cell is indeed bacteriostatic. However, if methionine, glycine and a purine are present in the medium the organisms die rapidly and Tp is bactericidal (Amyes and Smith, 1974b). In complex media, and in the urine, these metabolites are usually present and have the effect of overcoming the inhibitory action of Tp on protein synthesis. This results in sustained protein synthesis, which has been shown to be essential for a maximal bactericidal response (Angehern and Then, 1973), and produces an effect similar to "thymineless death" (Amyes and Smith, 1974b). The bactericidal action of Tp can, however, be reversed completely if both thymine and lysine are present in the medium (Amyes and Smith, 1974b). In addition thymidine has been shown to antagonise the antibacterial action of Tp (Miovic and Pizer, 1971) and thus media lacking these compounds should be employed for accurate Tp sensitivity testing (Amyes and Smith, 1974b).

At low concentrations of Tp (less than $0.2mgL^{-1}$) in the presence of methionine, glycine and adenine, Tp is either bacteriostatic or has no effect on viability of sensitive organisms. Addition of Sx, however, produces a bactericidal response even when the individual drugs used alone are bacteriostatic (Amyes, 1982). Since the presence of methionine, glycine and adenine has no rapid effect on Sx

action, the bactericidal effect is presumed to result from potentiation of Tp activity by Sx. This Sx enhanced Tp bactericidal activity has also been observed in vitro with E. coli strains carrying the Sx R-plasmid R1 and strains carrying the Tp R-plasmids R483 and R751. But the ability of Sx to promote Tp activity only occurs at Tp concentrations less than $0.2 mgL^{-1}$ and is therefore not applicable to the clinical situation where the expected level of Tp, in the urinary tract, after normal dosage, is between 30 and 150 mgL^{-1} (Brumfitt and Pursell, 1973).

Taken together, these findings concerning the reported synergy between Tp and Sx and the emergence of Tp resistant strains would suggest that Tp is probably as active and as safe as the combination in the treatment of acute infections. Nevertheless, resistance to Tp is still a major problem and, as with all antibacterial agents, resistance is likely to be most effectively minimised by prescribing Tp only for those patients for whom it is indicated.

CHROMOSOMAL RESISTANCE TO TRIMETHOPRIM

Resistance to trimethoprim is defined as a minimum inhibitory concentration (MIC) greater than $2 mgL^{-1}$ (Grey et al., 1979). Acquired resistance to Tp can be related to chromosome mutations or to the acquisition of Tp resistance plasmids. Since the action of Tp is to cause a metabolic block by competitive inhibition with the substrate dihydrofolate for reaction with the enzyme dihydrofolate reductase (DHFR), resistance may be achieved by one or a combination

of three mechanisms. First, the nature of the inhibited enzyme ie. DHFR, can be changed qualitatively, so that the affinity of the inhibitor is decreased. Second, a greater amount of DHFR may be produced resulting in greater overall activity of the blocked system without changing the percentage inhibition. Thirdly, resistance may be achieved by some unusual bacteria by their ability, under certain circumstances, to use exogenous folates, thus by-passing the inhibited pathway, eg. Streptococcus faecalis (Bushby and Hitchings, 1968).

Chromosomal resistance is generally due to mutations in the structural or regulating genes for dihydrofolate reductase resulting in decreased susceptibility and/or overproduction of the enzyme (conditions one and two above).

Although much of the work done on mechanisms of resistance to antifolate agents in bacteria has involved methotrexate resistance, such mutants serve to illustrate the range of changes possible in DHFR. Hyperproduction of chromosomal DHFR, between 3-80 fold, has been reported in pneumococci (Sirotnak, 1971; 1973; McCuen and Sirotnak, 1974) and in E. coli K-12 (Burchall and Hitchings, 1968; Breeze et al., 1975). The enzyme is still susceptible to Tp, but the amount of Tp necessary to inhibit the increased amount is much higher. In some strains of E. coli or Klebsiella, resistance has been shown to be due to production of an altered DHFR enzyme with decreased affinity for Tp (Poe et al., 1972; Grey et al., 1979; Sheldon, 1977). The fol mutant of E.coli studied by Sheldon (1977)

was also hyperproduced (10 fold). The combination of these two mutations, therefore, explain the unusually high level of resistance (MIC $>1000 mgL^{-1}$) observed in these strains.

For many chromosomally resistant strains, which have been selected in the laboratory by passage of heavy inocula of bacteria in media containing increasing concentrations of Tp, the biochemical mechanism for their resistance remains unknown (Darrell et al., 1968; Freisheim et al., 1972). However the clinical significance of these mutants seems to be minimal now since the discovery of Tp R-plasmids (Lacey et al., 1972; Fruensgaard and Komer, 1974; Grey et al., 1979).

An infrequent (less than 1% of Tp resistant isolates) but, nonetheless, important type of resistance is the example of thymidine-dependent organisms. Thymineless (thy$^-$) mutants, lack the enzyme thymidylate synthetase required for the synthesis of thymidine (Barner and Cohen, 1959). To overcome this defect, these organisms have developed a mechanism by which they are able to utilize exogenous thymine or thymidine. These thymineless strains are thus highly resistant to Tp, providing ample thymidine is available, since the site of action to Tp is by-passed by the direct incorporation of thymidine by the bacteria (Stacey and Simson, 1965; Amyes and Smith, 1975).

Intrinsic resistance to Tp exhibited by certain species has been explained in two ways. In Pseudomonas aeruginosa, which has MICs for Tp in the range 100-200mgL^{-1} (Grey and Hamilton-Miller, 1977), insensitivity may relate to a relative permeability block to the drug

which cannot penetrate the bacterial cell to work (Hitchings, 1973). On the other hand, some resistant species (Neisseria meningiditis, Clostridium perfringens and Bacteroides fragilis) have DHFR which is 100-1000 times less susceptible to inhibition by Tp than corresponding enzymes from sensitive species (Burchall, 1971; Then and Angehern, 1978). These DHFRs are not, however, as insusceptible as the R-plasmid mediated DHFR which in general confer a high degree of resistance to Tp (MIC $>1000 mgL^{-1}$).

THE EMERGENCE OF TRIMETHOPRIM R-PLASMIDS

The introduction of each new group of anitbiotics used on the Enterobacteria has led to the emergence of R-plasmids conferring resistance to these drugs (Anderson, 1968). When co-trimoxazole was introduced there was already a high percentage of bacteria harbouring R-plasmids within the clinical population. Most of these R-plasmids contained determinants for sulphonamide resistance (Datta, 1969).

The initial resistance to the combination remained low, although Tp resistant bacteria especially Klebsiella, had already been isolated long before 1969 (Hamilton-Miller and Grey, 1975). Two years after the introduction of co-trimoxazole the percentage of E. coli isolated from urinary tract infections, that were resistant to the combination, remained less than 3% (Grüneberg, 1976). The only organisms, isolated from the urinary tract, which were consistently resistant at that time were the Pseudomonas species (Amyes, 1974). However, in 1972, only three years after its introduction, bacteria

harbouring R-plamsids conferring Tp resistance, were isolated in London (Fleming et al., 1972).

This R-plasmid mediated resistance had two important distinctive features. Firstly, the level of Tp resistance conferred by the plasmids was extremely high, resistant bacteria had a minimum inhibitory concentration greater than 1000mgL^{-1}. Secondly, the resistance was able to transfer between bacterial strains.

Following the initial discovery of Tp R-plasmids, Datta and Hedges (1972) began an examination of trimethoprim resistant Enterobacteria isolated from hospitals around the UK. A total of 50 bacteria were isolated which conferred resistance to Tp at a concentration of 8mgL^{-1} or more. Transfer of Tp resistance to E. coli K-12 was obtained with 19 of these 50 bacteria and the presence of R-plasmids inferred. All 19 bacteria had been isolated from three hospitals in a small area of North London. None of the bacteria isolated from hospitals in West London, Bristol or Glasgow possessed Tp R-plasmids.

Already a pattern was beginning to emerge that has, in general, remained with Tp R-plasmids ever since. ~~Each~~ All bacteria harbouring the plasmid had an MIC of greater than 1000mgL^{-1} Tp. In most cases the bacteria that did not show transferable Tp resistance possessed a far lower MIC. Recent reports, however, indicate that Tp R-plasmids are not always associated with high levels of resistance (Amyes et al., 1982). Strains of Proteus harbouring Tp R-plasmids have been isolated in which the MIC of the host organism is only 100mgL^{-1}. The

R-plasmids, however, when transferred to E. coli K-12 confer the usual high level of Tp resistance on this host (MIC >1000mgL^{-1}).

Each of these early Tp R-plasmids conferred the same pattern of resistance to Tp and sulphamethoxazole (Sx) and, in addition, each was shown to belong to the W incompatibility group (Datta and Hedges, 1972) suggesting that they were all related to one another. It appeared, therefore, that the emergence and spread of one type of Tp R-plasmid resistance was being observed within these three North London Hospitals. Extensive examination of mainly urine samples in Bristol (Lacey et al., 1972) and faecal samples in Dublin, Eire (Moorhouse and Farrell, 1972) revealed no emergence of Tp R-plasmids during the same period although some Tp resistance, mediated by the bacterial chromosome, was emerging.

Jobanputra and Datta (1974), extending the survey to include more hospitals in the London area, went on to show that Tp resistance was not restricted to plasmids of the W inc group, but was also present on plasmids belonging to incompatibility groups I and P. Further clinical surveys carried out at the Whittington Hospital, London, between 1975 and 1977 by Amyes et al. (1978) showed that R-plasmid resistance was increasing rapidly in London. A similar explosive increase in Tp resistance was also being observed in areas as far removed from the south east of England as Italy (Romero and Perduca, 1977) and France (Acar et al., 1977). The character of the resistant population was also changing. The Tp resistance due to R-plasmids was increasing at a greater rate than Tp resistance as a

whole. The increase in the number of R-plasmids was accompanied by a diversification of their incompatibility groups and by 1975 many more incompatibility groups were represented including N, M, N-M, S and F_{II} (Romero and Perduca, 1977).

Most of the early reports on Tp resistance concern enteric Gram negative bacteria. However, over the last few years Tp resistant strains of Haemophilus have been reported (Comere and Menzies, 1974; Howard et al., 1978; Kirven and Thornsberry, 1978). The level of Tp resistance in these strains, however, has never been greater than $64mgL^{-1}$ suggesting the resistance is not plasmid mediated. Recently the methods of sensitivity testing of Haemophilus have been questioned. The fastidious growth requirements of the organisms and the subsequent variation in sensitivity test medium employed is thought to be responsible for many inaccurate reports of Tp resistance within this species (Philpott-Howard et al., 1983). The validity of recorded levels and incidence of Tp resistance within the H. influenzae are, therefore often questionable.

Trimethoprim resistant Gram negative bacteria have been isolated not only from humans but also from animals. As transmission of R-plasmids from animals to humans has been demonstrated or strongly suggested (Anderson et al., 1975; Amyes, 1983) it is important to keep track of the Tp resistant strains wherever they occur.

Before 1977 there were only two reports of Tp resistant strains isolated from animal sources (Fleming, 1973; Cooke, 1976). Isolation of Tp resistant strains in faecal specimens from pigs has increased

very sharply since their initial isolation in 1977. Ninety-five percent of the resistant strains had plasmid mediated Tp resistance and at least 12 different resistance patterns have been isolated, suggesting that resistance, in this survey, was not related to the spread of one plasmid (Smith, 1980).

In Melbourne, Australia, E. coli harbouring Tp R-plasmids have been isolated from frozen chicken carcasses. The plasmids belonged to the incompatibility group F_I, not described anywhere else in association with Tp (Caudry and Stanisich, 1979). Multi-resistant strains of Salmonella typhimurium appeared in calves and then in cattle in 1977 in Great Britian. The Tp resistance in these strains was also plasmid mediated; four different plasmids harbouring the Tp resistance marker could be identified belonging to incompatibility groups I_1, I_2, and H_2 (Threlfall et al., 1980).

The results of these studies show clearly that, since its introduction, the levels of trimethoprim resistance have gradually increased. Initially Tp resistance was of a low level and, in general, chromosome determined. From 1972, an explosive increase in the numbers of Tp resistant organisms was observed, accompanied by raised MICs to the drug. This spread of resistance was being manifested by resistance plasmids carrying the Tp resistance determinant. During the last three years the percentage of resistant organisms has remained fairly stable. However, the nature of the resistant population is again changing - from a population in which the level of resistance was high and transferable to one in which the

level of resistance remains high but is no longer transferable. This phenomenon has largely been explained by the identification of transposable elements carrying the Tp resistance determinant.

THE PROMISCUITY OF THE TRIMETHOPRIM RESISTANCE GENES

The diversity of the Tp resistance gene among plasmids of many different incompatibility groups, suggested a similarity to the promiscuous nature of the TEM β-lactamase gene carried by the transposon Tn3. It was not therefore unexpected when a similar genetic element carrying resistance to Tp and Sm was identified (Barth et al., 1976). This transposon, Tn7, was first detected in the plasmid R483 when a piece of DNA of about 9 Megadaltons (Mdal), carrying genes conferring resistance to streptomycin (Sm) and Tp was found to transpose from R483 to other replicons, including the bacterial chromosome, from where further migration could then occur. As with other transposons, the attachment of Tn7 is site specific and occurs independent of the recA gene product.

Since its discovery Tn7 has been found to be widespread among R-plasmids isolated from both human infections and animal sources (Richards et al., 1978; Richards and Datta, 1981). The location of Tn7 has not, however, been restricted to R-plasmids. Increased incidence of this transposon in the chromosome of clinical strains and strains from animals is now being reported (Towner, 1981; Amyes et al., 1982; Towner et al., 1982; Amyes, 1983). Recent reports indicate that Tn7 is unusual, among transposable elements, in that it

inserts into a particular site of the E. coli chromosome with high efficiency (Barth et al., 1976; Lichtenstein and Brenner, 1981), the specificity of attachment being determined by specific DNA sequences and possibly by the involvement of secondary structures (Lichtenstein and Brenner, 1982). This would account for the change in recent years to bacteria possessing high level non-transferable Tp resistance.

Datta et al. (1979) reported the spread of Tp resistance in bacteria, isolated during an outbreak of hospital infection, to be mediated by the spread of TpSm resistance transposons indistinguishable from Tn7. During this survey, these authors identified another transposon, Tn78, which conferred resistance to Tp alone. This transposon, however, had a MW of about 9Mdal, similar to Tn7. Mutation back to Sm resistance was not possible, but restriction enzyme analysis revealed no difference between this transposon and Tn7. By these criteria it was concluded that Tn7, by means of a small deletion, had given rise to the new transposon Tn78. Towner et al. (1982) have since suggested the presence of similar transposons in the chromosomes of clinical isolates of E. coli.

Shapiro and Sporn (1977) reported the presence of a 5Mdal transposon, Tn402, which confers sole resistance to Tp, in the plasmid R751. However, this transposon has only been shown to integrate at low frequency into the bacteriophage lambda genome, and together with the knowledge that the Tp resistant dihydrofolate reductase of R751 is as yet, unique to this plasmid, it seems

unlikely that transposition of Tn402 occurs in the clinical situation.

PLASMID DETERMINED TRIMETHOPRIM RESISTANCE MECHANISMS

The mechanism of R-plasmid determined resistance to trimethoprim remained obscure for some years. Extensive investigations with bacteria harbouring Tp R-plasmids failed to reveal an inactivation enzyme or a permeability barrier to the drug (Amyes, 1974). Trimethoprim resistant strains of E. coli had however, been isolated which owed their resistance to a chromosomal mutation resulting in an alteration of the target enzyme, dihydrofolate reductase (Poe et al., 1972). For this reason, E. coli 114, harbouring the first Tp R-plasmid, R388 (Datta and Hedges, 1972), was investigated for an alteration in DHFR activity (Amyes, 1974).

Gel filtration of cell extracts of the R-plasmid containing strain, when assayed in the presence and absence of 4×10^{-6}M Tp, revealed a Tp resistant enzyme. Repetition of the gel filtration in the presence of standard marker proteins showed that the plasmid enzyme has a molecular weight of 35000, compared with the E. coli chromosomal enzyme of MW 21000. In addition, the resistant enzyme was found to be 20,000 times more resistant to Tp than the sensitive enzyme (Amyes and Smith, 1974a).

This was the first example of an R-plasmid encoding the synthesis of an additional insusceptible target site enzyme that by-passed an inhibited step in the bacterium. It has since been

shown that some R-plasmid sulphamethoxazole resistance employs this type of mechanism (Wise and Abou-Donia, 1975).

Subsequent to the discovery of the DHFR of R388, another plasmid conferring Tp resistance, R483, was also shown to mediate the synthesis of an additional Tp resistant DHFR (Sköld and Widh, 1974). This enzyme, like the R388 DHFR, has a MW of 35000, but in contrast is produced in far greater quantities, the specific activity of the plasmid containing strain being about ten times greater than that of the strain lacking the plasmid. In addition, the R483 enzyme was shown to be very sensitive to heat, again in contrast to the R388 encoded enzyme.

A distinguishing feature of the plasmid mediated enzymes was their inability to utilize NADH as a hydrogen donor whilst the sensitive enzyme could utilize this co-enzyme at 16% of the efficiency of NADPH. In addition, unlike both the mammalian and sensitive bacterial DHFRs, the plasmid encoded enzymes are resistant to the 2,4-diaminopteridine, methotrexate (Amyes and Smith, 1976). This would suggest that the R-plasmid enzymes have evolved differently from both the mammalian and bacterial DHFRs developing the ability to differentiate between the substrate, dihydrofolate, and its closest structural analogue.

PROPERTIES OF PLASMID DIHYDROFOLATE REDUCTASES

Since the initial discoveries of the R388 and R483 enzymes, many plasmid encoded Tp resistant DHFRs have been isolated. Initially the

properties of the enzymes from different plasmids seemed to be similar; for example their MW, molecular charges and resistance profiles revealed few differences (Amyes and Smith, 1978). However, the yields of enzyme associated with the different plasmids varied considerably and, in 1977, Pattishall et al. proposed the division of R-plasmid DHFRs into two groups, Type I and Type II. Those of Type I (exemplified by the DHFR from R483) are produced in large quantities, are heat sensitive and are 10,000 times less susceptible to Tp than the chromosomal enzyme. In contrast, those of the Type II enzyme group, (exemplified by R67 (Pattishall et al., 1977)) are produced in low quantities, are heat resistant and are virtually insusceptible to Tp.

However, some of the enzymes did not conform strictly to one group or the other. Re-examination of the properties of DHFRs encoded by three plasmids, R751, R388 and R483, by polyacrylamide gel electrophoresis showed each enzyme had slightly different migratory properties (Tennhammar-Ekman and Skold, 1979). These authors concluded that the three enzymes were distinct from one another.

By 1980, it had been shown that the Type I and Type II DHFRs have different subunit structures. Zolg et al. (1978), by excision of the Type II, R388 DHFR gene by restriction endonuclease digestion, and subsequent integration of the DNA fragment in a multiple copy derivative of plasmid ColE1, showed that the size of the DNA fragment encoding the R388 DHFR was 1770 bp. This fragment, at most, could encode for a protein of 13000 daltons whereas the MW estimation of

the plasmid enzyme was 35000 when estimated by gel filtration. This R-plasmid DHFR must therefore be comprised of subunits which were later shown to be four identical of MW 8300 (Zolg and Hänggi, 1981). Other Tp resistant DHFRs related to the R388 have also been shown to be tetrameric (Smith et al., 1979; Fling and Elwell, 1980). In contrast, the Type I enzyme from R483 is not tetrameric, but consists of two identical subunits of MW 18000 (Fling and Elwell, 1980).

The different subunit structures of the enzymes appeared to clarify the distinction between Type I and Type II DHFRs. Nevertheless, confusion persisted with respect to the other biochemical properties of the enzymes. Classification of these plasmid determined DHFRs therefore required a more exacting technique. Broad and Smith (1982) examined DHFRs of Type I and Type II as well as those from R388 and R751, by isoelectric focussing (IEF). By this method three different groups could be distinguished. Type I enzymes were found to exhibit isoelectric points of 6.4 while Type II enzymes were of pI 5.5. The DHFR of R388 also exhibited a pI of 5.5 and was therefore clearly identified as a Type II enzyme. The enzyme mediated by R751, however, was found to exhibit a unique isoelectric point (pI 7.2) and hence constituted a third group. Nevertheless, as the enzymes of R751 and R388 are known to be serologically related (Fling and Elwell, 1980), it is suggested that the R751 enzyme should perhaps be regarded as a sub-group of Type II enzymes rather than being of a completely separate type.

In November 1982, a new type of plasmid mediated DHFR was

detected in a non-self-transmissable plasmid (p669) which conferred resistance to modest levels of Tp ($64mgL^{-1}$) (Fling et al., 1982). This enzyme differs markedly from both Type I and Type II DHFRs. The concentration of Tp required to give 50% inhibition (ID_{50}) of the enzyme was only 1.5μM compared with 60μM for Type I enzymes and 70000μM for Type II enzymes. In addition the MW of the p669 enzyme was unusually low for a resistant DHFR at only 16000-18000. A resistant DHFR of similar MW has previously been reported by Poe et al. (1979). However, unlike the DHFR of Fling et al. this enzyme was chromosomally mediated.

THE EVOLUTION OF THE TRIMETHOPRIM RESISTANCE GENE

Since their discovery, the origins of the resistance determinants of R-plasmids have been a topic of much speculation. Despite many years of research and profound advances in DNA technology, very little conclusive evidence of their origin has been forthcoming. Several lines of investigation have been pursued the most lucrative being an examination of soil bacteria for antibiotic inactivating enzymes. Benveniste and Davies (1973), with the knowledge that most antibiotics are produced by soil bacteria, reasoned that synthesis of antibiotic inactivating enzymes by species of actinomycetes might be advantageous in protecting the organism against anitbiotics produced by itself or by a competitor in the soil environment. In their search for such enzymes they succeeded in finding a kanamycin-acetylating enzyme in Streptomyces kanamyceticus which produces kanamycins, and a gentamicin acetyltransferase in S.

spectabilis which produces spectinomycin. More recently, the amino acid sequence of the aph gene (aminoglycoside 3'-phosphotransferase) of S. fradiae has been determined, (Thomson and Grey, 1983) and its comparison with the phosphotransferases encoded by two transposons Tn5 and Tn903, show all three enzymes to share 36-40% homology. The three enzymes exhibit an area of high conservation at the carboxy terminus which may be involved with the binding of one of the two substrates (aminoglycoside antibiotic or ATP). The level of homology observed between the enzymes is significant according to Doolittle (1981) suggesting that the aph genes of antibiotic producing bacteria and R-plasmids may share a common ancestor.

Amino acid and nucleotide sequence studies of plasmid-specified penicillinases expressed in E. coli show these enzymes to share marked sequence homology with the chromosomal enzymes of a number of Gram positive organisms, indicating a common evolutionary origin (Ambler and Scott, 1978; Sutcliffe, 1978). The SHV-1 β-lactamase found on some plasmids is indistinguishable by kinetic analysis and IEF from a chromosomal β-lactamase found in many isolates of K. pneumoniae. Nugent and Hedges (1979) suggest that the SHV-1 β-lactamase gene evolved as a chromosomal gene in Klebsiella and was later incorporated into a plasmid.

Unlike the aminoglycosides and penicillins, trimethoprim is a chemically synthesised antibacterial substance, which inhibits the activity of the enzyme dihydrofolate reductase (DHFR). Since no naturally occurring substance has yet been discovered that mimics

this action it seems unlikely that a resistance mechanism to trimethoprim evolved specifically in the past. It is more probable that trimethoprim resistance has developed during the clinical use of this drug. In addition, the introduction of trimethoprim has followed the discovery of R-plasmids and hence a unique opportunity exists to investigate the de novo pathway of drug resistance evolution.

High level resistance to trimethoprim is achieved by the production of plasmid determined Tp resistant DHFRs which provide an additional insusceptible target site thus by-passing the inhibited target site of the host. Dihydrofolate reductases are common to all living cells. It is therefore thought, that the plasmid enzymes may have arisen either by mobilization of a DHFR gene from elsewhere or alternatively by mutation of a sensitive enzyme.

As previously indicated, plasmid-determined DHFRs may be classified into at least two different groups - Type I and Type II. The enzymes of both groups have molecular weights (MW) of 35000 but have different sub-unit composition, Type I enzymes consisting of 2 sub-units MW 18000 and Type II enzymes consisting of 4 sub-units MW 8500. The amino acid sequences of the R67 and R388 Type II enzymes and the R483 Type I enzyme have been determined and compared. The R67 and R388 enzymes are closely homologous (Swift et al., 1981) but show little homology with other prokaryotic and eukaryotic DHFRs (Smith et al., 1979; Zolg and Hänggi, 1981). This has led to the suggestion that these plasmid DHFRs are not 'classical' reductases

but may have evolved from non-reductase proteins which happen to have reductase activities (Smith and Burchall, 1980; Zolg and Hänggi, 1981). In contrast the R483 Type I enzyme shares 22-29% homology with sensitive bacterial and eukaryotic DHFRs (Fling and Richards, 1983; Simonsen et al., 1983), indicating a genuine relationship (Doolittle, 1981). Seventy percent of this homology occurs in the hydrophobic binding site of the enzyme. This sequence appears to have been conserved in DHFRs isolated from many bacterial strains (Bitar et al., 1977).

Dihydrofolate reductases from many different sources have been extensively investigated and compared with those from R-plasmids. Studies on inherently resistant organisms such as Neisseria, Clostridium and Bacteroides have indicated that resistance is due principly to production of altered, less sensitive DHFRs (Then, 1979; Then and Angehern, 1979). Amethopterin and trimethoprim resistant mutants of S. faecium (Gleisner et al., 1974), L. casei (Bately and Morris, 1977) and E. coli B strain (MB1428) (Bennett et al., 1978) have also been examined and the mutant DHFRs produced by them compared. In all cases there appears to be a significant region of homology at the amino terminal although each enzyme remains distinct from the plasmid resistance enzymes.

An examination of DHFRs produced by the T-even bacteriophages has led many authors to speculate about their relationship to the R-plasmid enzymes (Mosher et al., 1977; Pattishall et al., 1977; Purohit et al., 1981). Varying reports concerning the MW of the

phage DHFR have resulted in some confusion. Erickson and Mathews (1971, 1973) reported the DHFR of bacteriophage T4 to be a single polypeptide with a MW of 29000, determined by SDS-PAGE. Mosher and Mathews (1979) subsequently published the MW to be 22800. However, recently Purohit et al. (1981) found that the T4 phage coded DHFR is in fact a dimer comprised of two subunits of about 23000 daltons each. In any event, it is evident that phage DHFRs are quite distinct from other bacterial DHFRs (MW 18000) and are perhaps more similar in MW to the plasmid enzymes.

In addition to being a larger MW, the T-even DHFRs are moderately resistant to trimethoprim (Purohit et al., 1981), heat sensitive (Mathews and Sutherland, 1965; Purohit et al., 1981) and exhibit a definite pH optimum at about pH7.0. These properties are very different from E. coli chromosomal DHFRs but reveal a distinct similarity to the Type I plasmid enzymes. The phage and plasmid enzymes are however, distinct. Precipitation of the phage enzyme by ammonium sulphate occurs at 0-40% saturation (Mathews and Sutherland, 1965), in contrast to the plasmid enzymes which are precipitated at 50-80% saturation (Amyes and Smith, 1978). In addition the phage enzyme is as sensitive to aminopterin as the chromosomal enzyme (Mathews, 1967) and has been shown to be immunologically distinct from the plasmid DHFRs (Mosher et al., 1977). Nevertheless, the similarities plus the probable ecological juxtaposition of phage and plasmids would seem to justify further comparisons at the level of primary structure of the enzymes and/or their controlling genes.

Detailed analysis of DHFRs from mammalian sources show strong homology between the enzymes isolated from mice, chicken, cattle and man (Kumar et al., 1980; Masters and Attardi, 1983). This strong homology is not observed when comparisons are made with enzymes from bacterial sources (Then and Riggenbach, 1978; Stone et al., 1979; Kumar et al., 1980). Thus although mammalian and type I plasmid DHFRs have similar affinities for trimethoprim, their lack of sequence homology (Stone and Smith, 1979; Kumar et al., 1980; Novak et al., 1983) coupled with their different triazine sensitivities (Hitchings and Burchall, 1965) would suggest distinct evolutionary origins.

Two recent reports have identified the existence of Tp R-plasmids pUN212, isolated from E. coli (Towner and Pinn, 1981), and p699, isolated from Salmonella typhimurium (Fling et al., 1982) which confer only moderate levels of resistance to trimethoprim (MICs of 256mgL^{-1} and 64mgL^{-1} respectively) compared with the level of resistance usually associated with R-plasmids (MIC $>$ 1000mgL^{-1}). Towner and Pinn (1981) speculate that the R-plasmid pUN212 specifies an 'intermediate' type of DHFR more sensitive to Tp than the 'normal' plasmid-encoded reductases. However, this enzyme has not yet been characterised. On the other hand, Fling et al. (1982) isolated and partially characterised the DHFR of plasmid p699. The reductase was found to differ significantly from other plasmid DHFRs. It has a MW of only 16-18000, is methotrexate sensitive and is inhibited by 50% with a concentration of trimethoprim of 1.5μM. In addition it is antigenically distinct from the DHFRs specified by plasmids R67 and

R483 and from the chromosomal DHFR of E. coli RT500 (Baccanari et al., 1975; Fling et al., 1982). This enzyme would therefore not appear to be a precursor of the more usual plasmid DHFRs.

2. MATERIALS AND METHODS

A. Bacterial Strains, Plasmids and Phage

The bacteria, plasmids and phage used are listed in table 1. All bacterial strains were stored at -70°C in nutrient broth containing 10% (v/v) sterile glycerol.

B. Media

Complex media

The complex media used were Nutrient Broth No. 2 (CM67), Diagnostic Sensitivity Test Agar (CM216), Columbia agar base (CM331) and MacConkey agar (CM7b), (Oxoid, Basingstoke, Hants).

Minimal medium

Double strength minimal salts medium (DM) was made up as described by Davis and Mingioli (1950) (table 2). Single strength minimal medium DM base was prepared by diluting double strength DM base with an equal volume of distilled water prior to autoclaving. For the preparation of diluents single strength DM base was distributed in 9.9 ml and 4.5 ml aliquots which were autoclaved at 15 psi for 15 minutes.

Supplement solutions for incorporation into minimal medium were as shown in Table 3.

Table 1 Bacterial Strains, Plasmids and Phage

	Markers	Reference
Bacterial Strain		
Escherichia coli K-12 J62	*pro*⁻ *his*⁻ *trp*⁻ *lac*⁻	Clowes and Rowley (1954)
Escherichia coli K-12 J62-2	*pro*⁻ *his*⁻ *trp*⁻ *lac*⁻ Rif^R	Bachmann (1972)
Escherichia coli K-12 J53	*pro*⁻ *met*⁻	Bachmann (1972)
Klebsiella sp. D770*	Ap^R	Datta and Hedges (1972)
Plasmids		
R6K	*incX* $Ap^R Sm^R$	Kontamichalou *et al.*(1970)
RP4	*incP* $Ap^R Km^R Tc^R$	Datta *et al.* (1971)
R15	*incN* $Sm^R Su^R$	Datta and Hedges (1971)
Sa	*incW* $Cm^R Km^R Sm^R Su^R$	Watanabe *et al.* (1968)
R1	$incF_{II}$ $Ap^R Cm^R Km^R Sm^R Su^R$	Meynell and Datta (1966)
R389	*incW* $Cm^R Sm^R Su^R Tc^R Tp^R$	Datta and Hedges (1972)
R1E	$incF_{II}$ Cm^R	This plasmid was kindly supplied by Prof. J. T. Smith
Phage		
PR4	Specific for *inc* P, N and W	Stanisich (1974)

* This strain is MR+ VP+ malonate−ve ie. is not *K. aerogenes* or *K. pneumoniae*.

Table 2 Preparation of Double Strength Davis Mingioli Basal Medium (DM)

K_2HPO_4	14.0g
KH_2PO_4	6.0g
Tri-sodium citrate.$2H_2O$	0.9g
$MgSO_4.7H_2O$	0.2g
$(NH_4)_2SO_4$	2.0g

These ingredients were dissolved in a litre of distilled water in the order given. 50ml quantities were distributed and autoclaved at 15 psi for 15 minutes.

Table 3 Supplement Solutions

Solution	Source	Strength Prepared	Final Concentration	Mode of Sterilization
D-glucose	B.D.H.	200mg/ml	2.8mg/ml	Autoclaving
L-proline	B.D.H.	5mg/ml	50µg/ml	Steaming
L-methionine	Sigma	5mg/ml	50µg/ml	Steaming
L-histidine	B.D.H.	5mg/ml	50µg/ml	Steaming
L-trytophan	B.D.H.	2mg/ml	50µg/ml	Steaming

Autoclaving was performed for 15 minutes at 15 psi.
Steaming was for 30 minutes.

Preparation of plates

All laboratory media were made up according to the manufacturer's instructions and were autoclaved at 15 psi for 15 minutes. The plates were poured while the agar was still molten, each containing approximately 15 ml. After setting, the plates were dried, inverted at 50°C for 20 - 30 minutes.

Preparation of DM medium plates

The supplements were added aseptically to 50 ml double-strength DM base as shown in table 3. Antibiotic and chemotherapeutic drug solutions were added aseptically to give the requisite concentration. 1.4 ml of a 20% glucose solution was then added and the volume made up to 60 ml by the addition of sterile distilled water. This was mixed and added to 40 ml water containing 1.5g bacteriological agar (Oxoid) which was previously autoclaved at 15 psi for 15 minutes. After rolling gently to mix and avoid frothing, the plates were poured. They were allowed to set and dried as above. The plates were freshly made and used within one week of manufacture.

C. Materials

Chemotherapeutic drugs

The chemotherapeutic drugs used are listed in table 4. They were kindly donated by each of the suppliers listed. All were supplied sterile and were prepared aseptically with sterile distilled water.

Table 4 Chemotherapeutic Drugs

Chemotherapeutic drug	Supplier
Trimethoprim lactate*	Wellcome Foundation Ltd., Beckenham, Kent
Sulphamethoxazole+	Wellcome Foundation Ltd., Beckenham, Kent
Streptomycin sulphate	Glaxo Ltd., Greenford, Middlesex
Oxytetracycline hydrochloride	Glaxo Ltd., Greenford, Middlesex
Spectinomycin	Upjohn Ltd., Crawley, Sussex
Rifampicin**	Le petite, Milan, Italy
Kanamycin	Bristol Laboratories, Slough, Middlesex
Ampicillin	Beechams Ltd., Brentford, Middlesex
Cephradine	E. R. Squibb and Sons Ltd., Hounslow, Middlesex
Chloramphenicol**	Parke Davis, Pontypool, Gwent
Gentamicin	Roussel Laboratories Ltd., Wembley, Middlesex
Methotrexate+	Lederle Laboratories, Gosport, Hants

* 1.3g of Trimethoprim lactate is equivalent to 1.0g of trimethoprim base and all results are expressed in terms of the base.

\+ These drugs were dissolved with the aid of N/10 NaOH.

** These drugs were dissolved with the aid of absolute alcohol.

Reagents

NADPH tetrasodium salt and NADH disodium salt were purchased from Sigma Chemical Company. Both compounds were stored desiccated at -20°C and solutions were freshly prepared on the day of use. Dihydrofolic acid (DHF), from the Sigma Chemical Company, was prepared by dissolving 25mg of DHF in 0.05M sodium phosphate buffer pH 7.4 containing 0.05M β-mercaptoethanol. This solution was distributed into aliquots which were stored in total darkness at -20°C. No aliquot was refrozen after thawing.

Lysozyme (stored desiccated at -20°C) and β-mercaptoethanol were also purchased from the Sigma Chemical Company.

Triton X-100 and agarose 15 'Electron' were obtained from BDH Chemicals Ltd., Poole and caesium chloride and restriction endonucleases from the Boehringer Corporation (London) Ltd.

Buffers

Sodium phosphate, sodium acetate, Tris-HCl and Tris-glycine buffers were made according to the Data for Biochemical Research, (Oxford University Press). Restriction endonuclease digestion buffers were those recommended by the Boehringer Corporation.

D. Methods

Viable counts

Serial dilutions were made using 1 in 10 and 1 in 100 dilutions in DM base - that is 0.5 ml of culture and 4.5 ml diluent or 0.1 ml culture and 9.9 ml diluent, respectively. Suspensions, appropriately diluted, were mixed on a Rotamixer (Hook and Tucker Ltd.) and 0.1 ml amounts spread with a sterile glass spreader onto solid medium. The spread plates were incubated, inverted, at 37°C. Incubation for all plates, except DM medium, was 18 hours and those of DM medium were incubated for 66 hours.

Minimum inhibitory concentration (MIC) determinations on solid media

4.5 ml amounts of Oxoid No. 2 nutrient broth were seeded with an inoculum from a fresh nutrient agar plate, and grown overnight at 37°C. A 1 in 10^4 dilution was prepared by serial dilution in DM base lacking glucose. A drop of this suspension (2µl) was spotted onto media containing varying concentrations of the drug (Miles and Misra, 1938). All plates, except DM medium, were incubated, inverted, for 18 hours while DM medium plates were incubated for 66 hours. Controls were suitably supplemented media lacking any drug. Concentrations of the drug were usually increased by a factor of two and the MIC was expressed as the first concentration permitting no visible growth.

Sensitivity testing against chemotherapeutic agents

Overnight cultures were grown in Oxoid No. 2 nutrient broth as

above. A 1 in 10^4 dilution was prepared by serial dilution in DM base lacking glucose. Drops of this suspension were placed on Diagnostic Sensitivity Test Agar (DSTA) plates containing various drugs at a concentration of 10 mgL^{-1} with the exception of Sx and Sp which were at a concentration of 100 mgL^{-1}. A Denley multipoint inoculator A400 was used for delivering approximately 2µl. The plates were incubated, inverted, for 18 hours at 37°C. Growth at the spot of application on the plates containing drugs, indicated resistance.

Replica Plating

Overnight cultures were grown in nutrient broth as above. A 1 in 10^7 dilution was prepared by serial dilution in DM base lacking glucose. 0.1 ml aliquots of this suspension were spread on nutrient agar plates using a sterile glass spreader and the plates were incubated inverted for 12 hours at 37°C. The initial plates, bearing between 100 - 150 colonies, were then replica plated by the methods of Lederberg and Lederberg (1952). The plates were inverted over a square of sterile velvet, placed pile upwards on a cylindrical rubber block of diameter 8 cm, the material being held in position by a perspex collar. The agar surface was pressed gently against the pile and the plate was then carefully removed. Replica plates, marked with a reference point for subsequent orientation, were then inverted in turn over the fabric, pressed gently against it so as to pick up a sample of the bacteria adhering to the pile, and then removed and incubated, inverted, at 37°C for 12 hours.

Transfer of R-plasmids

Overnight cultures were grown at 37°C in nutrient broth from a fresh plate inoculum. After a viable count of a 10^{-6} dilution on MacConkey agar was performed, 0.1 ml of the donor culture was mixed with 1.0 ml of the recipient culture in 4.5 ml prewarmed broth. The resultant mating mixture was incubated at 37°C for the time specified in the results. 19.8 ml of prewarmed DM base was added to the mating mixture - a process equivalent to washing, to remove excess broth - and the suspension was mixed and then spun for 20 minutes at 4000 rpm on a Heraeus Christ Bactifuge at room temperature. The supernatant was decanted and the pellet was resuspended in a volume of prewarmed DM base equal to that of the original mating mixture, which was 5.6 ml. After mixing thoroughly to resuspend, the "washed" mating mixture was diluted serially to 10^{-4}, as described for viable counts, and 0.1ml of each dilution was plated on DM medium plates supplemented only for the recipients, and containing antibiotic at a concentration or concentrations which allowed only those recipients containing the R-plasmid to grow. MacConkey plates were incubated, for 18 hours at 37°C and DM medium plates for 66 hours at 37°C, all plates being inverted. Isolates were purified by streaking out for single colonies on identical DM selective plates.

Enzyme assays

DHF reductase (by the method of Osborn and Huennekens, 1958).

Stock solutions: DHF was made up to 1mM and stored as described in the Materials. NADPH was made up to 1mM in distilled water and used that day only.

Method 1: The assays were performed in a Pye Unicam Sp8000 spectrophotometer, at 30°C. 1 ml quartz cuvettes were used and contained the following reagents:-

40 mM buffer (usually sodium phosphate pH 6.0) 0·4ml of 100mM buffer
10 mM β-mercaptoethanol 0·1ml of 100mM β-mercapto-ethanol.
0.08 mM NADPH 0·08 ml of 1mM NADPH
enzyme
distilled water to a volume of 0.95 ml

The blank cuvette contained all these constituents except NADPH. Both cuvettes were placed in the spectrophotometer for four minutes to allow for temperature equilibration. To start the reaction 0.05 ml of 1 mM DHF was added to both the blank and the test cuvettes and the decrease in absorbancy at 340 nm followed for 10 minutes. When a decrease in absorbance occurred before the addition of DHF to the test cuvette, this was taken to be due to a DHF-independent NADPH oxidase. This rate was subtracted form the total rate observed in the presence of DHF, to give the level of DHFR activity. The decrease in absorbance at 340 nm is due to reduced absorbancy both by oxidation of NADPH to NADP and by the reduction of DHF to THF. 52% of the combined decrease in absorbance is due to DHF to THF, when both substrates are being consumed stoichiometrically (Mathews et al., 1963), and hence, with this proviso, the rate of enzymic activity was expressed in molar terms of DHF being converted to THF. Controls were performed routinely in the absence of any enzyme to ensure that the addition of the two substrates together did not result in a change of absorbance.

Method 2a: The DHF-independent NADPH oxidase activity was so

high in some crude extracts that it was found necessary to assay enzymic activities by placing all the reagents including DHF, but excluding the enzyme, in the test cuvette for temperature equilibration. The blank cuvette contained all the constituents except NADPH and the enzyme. The reaction was then started by the addition of the enzyme to both the test and blank cuvettes and the initial rate was taken. When method 2a was used a separate assay was performed to estimate the NADPH oxidase activity.

Method 2b: In this, the test cuvette was set up to contain buffer, β-mercaptoethanol, NADPH and water. The blank cuvette contained all these reagents, but lacked NADPH and the enzyme. The reaction was started by the addition of the enzyme to both the test and blank cuvettes. The NADPH oxidase rate so found, was subtracted from the total rate determined in the assay containing DHF (2a), to give the rate of DHFR activity.

Note: It is uncommon to assay enzymes spectrophotometrically with the enzyme in the blank cuvette as a routine. However, DHFR is a special case because some strains of E. coli can exhibit an NADPH-independent DHF monooxygenase activity (Poe, 1973), which is corrected for by the methods used.

One unit of DHFR activity was taken as the amount of enzyme causing the conversion of 1 n mole of DHF to THF per minute using the assumption of Mathews et al., (1963).

Preparation of cell extracts for enzyme estimation

100 ml of an overnight culture, in suitably supplemented DM

medium, was subcultured into 900ml of prewarmed DM medium. The culture was grown aerobically in a Gallenkamp rotary shaker at 37°C to logarithmic phase. The cells were harvested by centrifugation at 4,000g for 60 minutes at 4°C in the 6 x 1L head of the MSE Mistral 6L centrifuge. The cells were resuspended to 5ml with 50mM sodium phosphate buffer pH 7.4 containing 10mM β-mercaptoethanol and 1mM EDTA at 4°C. The bacteria were lysed in the French Press (Aminco) at a pressure of 7 tons/in^2 and the disrupted suspension was centrifuged at 40,000g for 1 hour, at 4°C in a Du Pont Sorvall RC-5B. The supernatant was decanted and used as the crude extract for the determination of enzymic activities.

Preparation of cell extracts for enzyme purification

One litre of an overnight culture grown in suitably supplemented DM medium at 37°C was inoculated into 9 litres of DM medium. The culture was grown aerobically to logarithmic phase by shaking in the rotary shaker at 37°C. The cells were harvested by centrifugation at 4,000g for 60 minutes at 4°C as before. The pellet was resuspended in about 20ml of 50mM sodium phosphate buffer pH 7.4 containing 10mM β-mercaptoethanol and 1mM EDTA (buffer A) at 4°C. All subsequent operations were performed at 4°C. The bacteria were lysed with the French Press (Aminco) at a pressure of 7 tons/in^2 and the disrupted suspension centrifuged at 40,000g for 1 hour in the 8 x 25ml head of a Sorval RC-5B centrifuge. The supernatant fluid was carefully decanted and stored at 4°C for further purification.

Purification of dihydrofolate reductase

The crude enzyme preparation, prepared from a 10 litre culture, as described above, was further purified by a modification of the method of Amyes and Smith (1974a). Nucleic acids were precipitated by the gradual addition of 0.1 volume 10% streptomycin sulphate. The precipitate was removed by centrifugation at 12,000g for 30 minutes in a Sorvall RC-5B centrifuge. The supernatant was collected and dialysed against a 50% saturated solution of ammonium sulphate (made up in buffer A) for four hours. Following centrifugation at 12,000g for 30 minutes the pellet was discarded and the resulting supernatant was then dialysed against an 80% saturated solution of ammonium sulphate, again made up in buffer A, for a further four hours. The pellet was collected by centrifugation at 12,000g for 30 minutes, resuspended in a minimum volume (about 2 ml) of buffer A, and stored frozen at -20°C for further purification.

Gel filtration

After being allowed to swell for 3 hours at 100°C in 50 mM sodium phosphate buffer pH 7.4 containing 10 mM β-mercaptoethanol and 1 mM EDTA (buffer A), a sufficient quantity of Sephadex G-75 superfine (Pharmacia, Uppsala, Sweden) was shaken and allowed to settle overnight in a flask. The "fines" were carefully decanted off, then more buffer A added and the process repeated.

The Sephadex slurry was poured carefully into a Wright acrylic column (2 cm^2 x 90 cm) which was maintained at 4°C in an LKB minicold lab. When the column was full, the top was connected and the flow changed to an upward direction using an LKB peristaltic pump.

The flow rate was adjusted to between 6 and 8 ml per hour and the column washed continually with buffer A for 48 hours before use.

The samples for separation, usually 2-3 ml, were applied slowly at the bottom of the column by syringe and eluted with buffer A. 2 ml fractions were collected and maintained at 4°C using an LKB ultrorac fraction collector. Elution was continued until a volume of buffer A, equivalent to the total volume (180 ml) of the column, had passed through. The column was washed for twelve hours between each run with buffer A.

Enzyme molecular weight determinations
(by the method of Andrews, 1964)

Chymotrypsinogen, ovalbumin and cytochrome c were dissolved in the sample to be tested, which was applied to the Sephadex G-75 superfine column as before. The DHFR activities were determined as described earlier. The position of the peaks of the three additional protein markers were found by a protein estimation of each fraction by the method of Waddell (1956). The position of the cytochrome c peak was confirmed by measuring the absorbance at 410 nm.

Protein estimations

All protein estimations were performed by the method of Waddell (1956). A standard curve was prepared using bovine serum albumin dissolved in 20 mM sodium phosphate buffer pH 7.4. The absorbance of various dilutions of this protein was estimated at 215 nm and 225 nm on a Pye Unicam SP6-550 UV/VIS spectrophotometer. The difference between the two values was plotted against protein concentration to

construct the standard curve.

All samples to be tested were suitably diluted in 50 mM sodium phosphate buffer pH 7.4 and estimated in the same manner as the standard protein, bovine serum albumin. The protein concentrations of the test samples were read off the standard curve.

This method has been shown to be as precise and more sensitive than the protein estimation of Lowry et al. (1951), (Hesslewood, 1973). It has the additional advantage that it may be employed in the presence of ammonium sulphate, whereas the Lowry method cannot.

Polyacrylamide gel electrophoresis

The stock solutions were made up according to the method of Davis (1964). The tubes for running the gel were sealed at one end and placed in a vertical stand. The polymerization mixture for the "small pore" running gel was added to within 2 cm of the top of the tube. A layer of distilled water was very carefully placed on the gel to prevent the formation of a meniscus. The gel was allowed to polymerize for 45-60 minutes, after which time the top water layer was tipped off and the polymerization mixture for the "large pore" gel added to within 1cm of the top of the tube. A water layer was carefully placed on the top of this gel and photopolymerization continued for 45-60 minutes. The water layer was tipped off and the sample, mixed with an equal volume of 80% sucrose solution, was added to the top of the tube.

The seals at the bottom of the tubes were removed and the tubes were placed in a vertical Shandon disc electrophoresis apparatus. Tris-glycine buffer pH 8.6 was added to the lower reservoir, care

being taken to ensure that no air bubbles lodged at the bottom of the gels. The same buffer was added to the upper reservoir, but in addition it contained 1 ml of 0.001% bromophenol blue. The anode was connected to the lower reservoir and the cathode to the upper. The current was switched on and maintained at 5mA per tube throughout the run.

When the bromophenol blue band had nearly reached the bottom of the tubes the current was switched off and the tubes disconnected. The gels were removed by "teasing" them out with water from a syringe and needle. DHFR activity was detected by either zymographic staining or by elution of the enzyme activity from segments of the gel and the subsequent assay of the eluate by the method of Osborn and Huennekens (1958).

Zymography

Bands of DHFR activity were located on polyacrylamide gels using the zymographic stain described by Tennhammar-Ekman and Skold (1979). The tetrazolium salt MTT is reduced by tetrahydrofolic acid (which is produced by DHFR from the substrate dihydrofolic acid) to form an insoluble blue precipitate at the site of DHFR activity.

Immediately after the electrophoretic run, the gels were transferred into glass tubes containing 40 mM sodium phosphate buffer pH 6.0, 1.2 mM NADPH and 0.8 mM dihydrofolic acid. After 5 minutes of incubation at 37°C, 0.6 mM tetrazolium salt was added. The gels were incubated in the dark for another 30-45 minutes at 37°C and finally washed in 40 mM sodium phosphate buffer, pH 6.0. Non-specific reduction of the tetrazolium salt was identified on control gels,

stained as described, but in the absence of dihydrofolic acid.

Preparation of DNA for agarose gel electrophoresis

Plasmid and chromosomal DNA were isolated from bacteria employing a modification of the method of Meyers et al. (1976). 10 ml of an overnight culture grown in Oxoid nutrient broth No. 2 at 37°C was inoculated into 1 litre of nutrient broth. The culture was grown aerobically in a Gallenkamp rotary shaker at 37°C overnight. The cells were harvested by centrifugation at 4,000g for 60 minutes at 4°C and the pellet resuspended in 2.0 ml of 25% sucrose in 0.05M Tris-HCl (pH 8.0). Gentle lysis of the cells was begun by adding 0.3 ml of lysozyme (50mg/ml in water) and mixing gently at 37°C for 5 minutes. This was followed by the addition of 2 ml of 0.25M EDTA (pH 8.0) and the mixture was shaken gently on ice for 5 minutes. Finally 3.0 ml of Triton mixture (2% Triton X-100 in 0.05M Tris-HCl (pH 8.0) containing 0.0625M Na_2EDTA) was added and the mixture kept at room temperature, swirling occasionally, until viscous. The pellet was removed by centrifugation at 40,000g for 1 hour in a Sorvall RC-5B centrifuge. The supernatant was carefully decanted and retained. Proteins and some carbohydrates were then removed by extraction with phenol. An equal volume of distilled phenol equilibrated against TES buffer (10mM Tris, 10mM NaCl, 0.0316mM EDTA) was added to the cleared lysate and the two phases mixed gently. The phenol and aqueous phases were then separated by centrifugation at 4,000rpm for 15 minutes in a Heraeus Christ Bactifuge. The upper aqueous phase was removed using a wide bore pasteur pipette and retained. Following a second phenol extraction, DNA was precipitated from the aqueous

phase, by the addition of two volumes of cold absolute ethanol, the tube being kept at -70°C for 30 minutes. The precipitated DNA was recovered by centrifugation at 2,500rpm for 5 minutes and resuspended in 0.5 ml TES buffer. The DNA sample was then analysed immediately by agarose gel electrophoresis or stored at 4°C until ready for use. DNA preparations of standard molecular weight plasmids were freshly made and stored at 4°C for no longer than 1 week.

Agarose gel electrophoresis

Horizontal slab gel electrophoresis was performed by the method of Meyers et al. (1976). For analysis of plasmid DNA 0.5% or 0.4% agarose gels, of dimension 14cm x 25cm x 0.5cm, were made in a Tris-borate buffer containing 89 mM Tris base, 2.5 mM disodium EDTA and 89 mM boric acid (pH 8.2). The agarose was dissolved in the Tris-borate buffer by steaming for 20 minutes and then cooled to 50°C before pouring. Sample wells were made by use of a perspex slot former with thirteen teeth, each 5 mm wide and spaced by 3 mm.

10µl of a dye solution consisting of 0.025% bromophenol blue and 50% glycerol in water was mixed with 30µl of ethanol precipitated DNA obtained from cleared lysates. The samples (total volume 40µl) were loaded into the wells using a Gilson automatic pipettman (P200). Electrophoresis was carried out in a plastic trough, of dimension 43cm x 25cm x 7cm. Tris-borate running buffer pH 8.2 was carefully poured into the trough until both ends of the agarose gel were in contact with the buffer being careful not to submerge the gel. The samples were run from cathode to anode. An initial voltage of 200V was applied for 15 minutes to allow the DNA to enter the gel. The

power was then switched off and enough running buffer was added to completely cover the gel. The apparatus was then maintained at 4°C and a voltage of 80V applied for 16 hours. Power was supplied by a Vokam 500-150 power pack.

Following electrophoresis the gel was carefully removed, placed on a glass sheet, and immersed in distilled water containing ethidium bromide at a concentration of 0.5 $\mu g ml^{-1}$ for 30 minutes. The gel was then counterstained in pure distilled water for 30 minutes, placed on a long-wave UV light source (model Blak-Ray C-62, UV Products Ltd.) and examined in the dark for fluorescence. The distance travelled by the DNA through the gel is inversely proportional to the logarithm of its molecular weight and plasmid molecular weights were calculated accordingly.

Ethidium bromide/caesium chloride density centrifugation
(by the method of Heffron et al., 1975)

3.64g of caesium chloride was dissolved in 3 ml sterile distilled water containing 0.5 $mg ml^{-1}$ ethidium bromide. Alcohol precipitated DNA, dissolved in 0.5 ml TES buffer, was mixed with the CsCl solution. The refractive index of the resulting solution was measured using a refractometer (Bellingham and Stanley Ltd.) and adjusted to 1.391 by the addition of sterile distilled water. The gradient was formed by centrifugation in a Beckman ultracentrifuge at 96,000g for 65 hours at 18°C. After centrifugation, the tubes were placed on a long wave UV light source and examined in the dark for fluoresence.

Preparation of DNA for restriction endonuclease digestion

Plasmid DNA for restriction endonuclease digestion was extracted from bacteria by the method of Holmes and Quigley (1981). 4.5 ml of nutrient broth was inoculated with a single bacterial colony taken from a fresh agar plate. The culture was grown aerobically with vigorous agitation in a Gallenkamp rotary shaker at 37°C overnight. 1.5ml of this overnight broth culture was poured into an Eppendorf tube and centrifuged for 1 minute at 11,500g in an MSE Micro Centaur. The remainder of the overnight culture was stored at 4°C. Following centrifugation, the medium was removed with a pasteur pipette, leaving the bacterial pellet as dry as possible. The cell pellet was then resuspended in 0.35 ml of 8% sucrose in 10mM Tris-HCl (pH 8.0) containing 50mM EDTA and 0.5% Triton X-100. 25µl of a freshly prepared solution of lysozyme (10mg/ml in 10mM Tris-HCl pH 8.0) was added and the mixture vortexed for 3 seconds. The tube was then placed in a boiling water bath for 40 seconds, to remove chromosomal DNA, and immediately centrifuged at 11,500g for 10 minutes at room temperature in an MSE Micro Centaur. Following centrifugation the pellet was removed from the Eppendorf tube with a toothpick and discarded. 40µl of 2.5M sodium acetate and 0.42 ml of isopropanol were then added to the supernatant. The resulting solution was mixed by vortexing and stored for 15 minutes at -70°C. The precipitated nucleic acids were then collected by centrifugation at 11,500g for 15 minutes at 4°C in an MSE Micro Centaur. The supernatant was discarded and the pellet resuspended in 50µl of TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) containing DNase-free RNase (50 $\mu g ml^{-1}$)

(Sigma Chemical Co.). The tube was then incubated for 10 minutes at 37°C to allow the RNase to act, resulting in the elimination of RNA that can mask small fragments of DNA in agarose gels. This mini-preparation containing 2-3 µg of plasmid DNA was stored at -20°C until ready for use.

Restriction endonuclease cleavage of DNA

Twenty microlitres of the plasmid mini-preparation was removed to a sterile Eppendorf tube. To this tube was added 2.2µl of the appropriate enzyme buffer and 2 units of the desired restriction enzyme. The tube was incubated for 1 hour at 37°C. Following incubation the reaction was terminated by cooling to 4°C and the addition of 15µl of a solution containing 4M urea, 50% (w/v) sucrose, 50mM EDTA and 0.1% bromophenol blue (pH 7.0). The mixture was then analysed on an agarose gel using as molecular weight markers, λ DNA treated with Hind III (Murray and Murray, 1975).

3. RESULTS

A. Investigations into the source of the Tp resistance gene

1. The role of the genus Klebsiella

Prior to the isolation of Tp R-plasmids, a significant number of Klebsiella species were isolated which were resistant to low levels of Tp (Fruensgaard and Komer, 1974; Jobanputra and Datta, 1974; Hamilton-Miller and Grey, 1975). In addition, a high proportion of the first organisms isolated which harboured Tp R-plasmids were Klebsiella spp. (Datta and Hedges, 1974), suggesting that this genus might provide the origin of the plasmid Tp resistance gene and a suitable environment for its evolution.

In order to test this hypothesis, the dihydrofolate reductase (DHFR) produced by the original plasmid-containing Klebsiella strain was investigated. As the original strain without a plasmid was never found, the Klebsiella strain D770 containing the Tp R-plasmid, R389, was studied. This strain was isolated in 1971, from a patient in the St. Pancras Branch of University College Hospital, London (Fleming et al., 1972) and was kindly supplied by Professor N. Datta. In addition to Tp resistance, strain D770 possesses transferable resistance to streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc) and sulphamethoxazole (Sx), and non-transferable resistance to ampicillin (Ap) (Datta and Hedges, 1972).

(i) Elimination of plasmid R389 from Klebsiella sp. D770

In order to investigate the host dihydrofolate reductase, it was first necessary to remove the Tp R-plasmid. Datta and Hedges (1972) reported that the introduction of the inc W group R-plasmid, Sa, into Tp resistant E. coli J62-2 transconjugant strains, harbouring plasmid R389, resulted in the simultaneous loss of resistance to Tp, Sm, Sx and Cm, while resistance to Tc was retained. Therefore R389 was classified as an inc W group plasmid carrying resistance determinants to Tp, Sx, Sm and Cm. Elimination of R389 from the original plasmid-bearing Klebsiella sp. D770, designated Strain A, was attempted by replica plating to select for colonies which had spontaneously lost Cm resistance, and therefore, by definition, resistance to Tp. Trimethoprim resistance was not used initially to test for the loss of the plasmid as the prevalence of this resistance gene on a transposon might result in just the loss of the transposon.

Strain A was grown overnight at 37°C in nutrient broth and diluted 1 in 10^6. 0.1 ml aliquots of the diluted culture were spread on nutrient agar plates which were subsequently incubated overnight to obtain single colonies. The single colonies were then replica plated by the method of Lederberg and Lederberg (1952) onto Diagnostic Sensitivity Test Agar (DSTA) plates and DSTA plates containing Cm at a concentration of 10 mgL^{-1}. Following overnight incubation at 37°C the plates were examined for colonies which had lost their resistance to Cm. Loss of Cm resistance occurred at the very high frequency of 1×10^{-2} per cell, suggesting the spontaneous

loss of an R-plasmid. Following purification on nutrient agar, the Cm sensitive derivatives (D770Cm^S) were tested for simultaneous loss of Tp resistance by streaking for single colonies on DSTA plates containing Tp at a concentration of 1000 mgL^{-1}. The results showed, however, that all of the D770Cm^S derivatives had retained high level resistance to Tp.

(ii) The plasmid profile of Klebsiella sp. D770

Since Cm resistance was lost at high frequency from Strain A without the simultaneous loss of Tp resistance, the possibility exists that these resistance determinants are not present on the same replicon within the original Strain A.

This theory was tested by conjugating Strain A for five hours with the rifampicin resistant E. coli strain J62-2, selection being made for the transfer of Cm and Tp resistance genes both separately and together.

Table 5 Transfer of Tp and Cm resistance determinants from Strain A (Klebsiella sp. D770(R389)) into E. coli J62-2

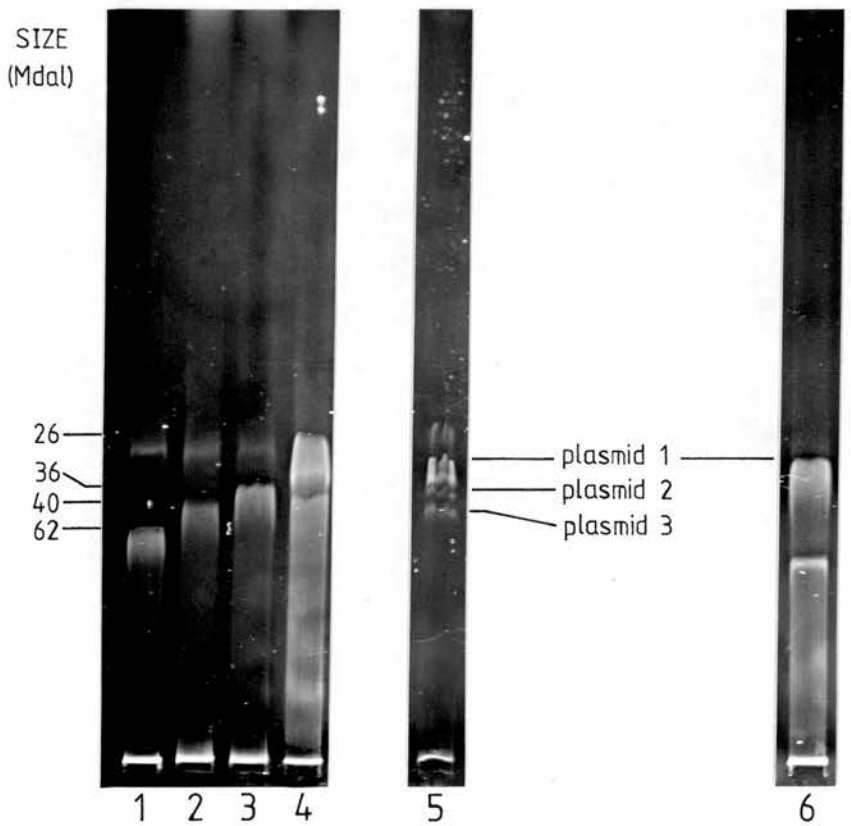
Donor	Recipient	Selection	Transfer frequency/ donor cell
Strain A	J62-2	Tp	3.74×10^{-4}
		Cm	3.28×10^{-1}
		TpCm	1.95×10^{-4}

The results (table 5) show that the Cm resistance determinant transfers at a much greater frequency than the Tp resistance determinant, but that the Tp resistance gene is always accompanied by the transfer of the Cm resistance gene. This would support the Datta and Hedges (1972) hypothesis that R389 is a non-self-transferable Tp R-plasmid which is mobilised by a second plasmid, shown here to carry the Cm resistance determinant.

In order to confirm that two R-plasmids are present within the original donor (Strain A) and the Tp resistant transconjugants, the DNA of these strains was extracted and analysed by agarose gel electrophoresis by the method of Meyers et al. (1976). One litre stationary phase cultures were harvested and lysed gently by treatment with lysozyme, EDTA and Triton X-100. Proteins and some carbohydrates were removed by phenol extraction of the cleared lysates and alcohol precipitated DNA was then separated by electrophoresis in 0.4% agarose. The results (figure 6) show that both the donor and recipient strains possess several plasmids of various molecular weights (MW). Unfortunately the plasmid bands obtained for the original Klebsiella D770 strain (figure 6, track 5) are not as clear as those obtained for the E. coli J62-2 recipient (figure 6, track 6). This is a result of the mucoid nature of D770 and the inherent problems often encountered during lysis of this organism. However, both strains can be seen to possess a plasmid of 30 Mdal (plasmid 1). Strain A also appears to possess two more plasmids of 37 and 42 Mdal (plasmids 2 and 3). An additional faint band of DNA at 33 Mdal is also present in this strain and represents

Figure 6. Agarose gel electrophoresis of plasmids extracted from Strain A (Klebsiella sp. D770(R389)) and E. coli J62-2 transconjugants selected on Tp containing plates.

Track 1, R1; track 2, R15; track 3, RP4; track 4, R6K; track 5, plasmid profile of Strain A; track 6, plasmid profile of J62-2 transconjugant strains.
Electrophoresis was in 0.4% agarose at 80V for 16 hours.



open circular forms of plasmid 1. The transconjugant E. coli J62-2 on the other hand, possesses a single very large plasmid of approximately 70 Mdal in size. The size of this plasmid could only be estimated approximately because of the inaccuracies which occur at molecular weights greater than 60 million.

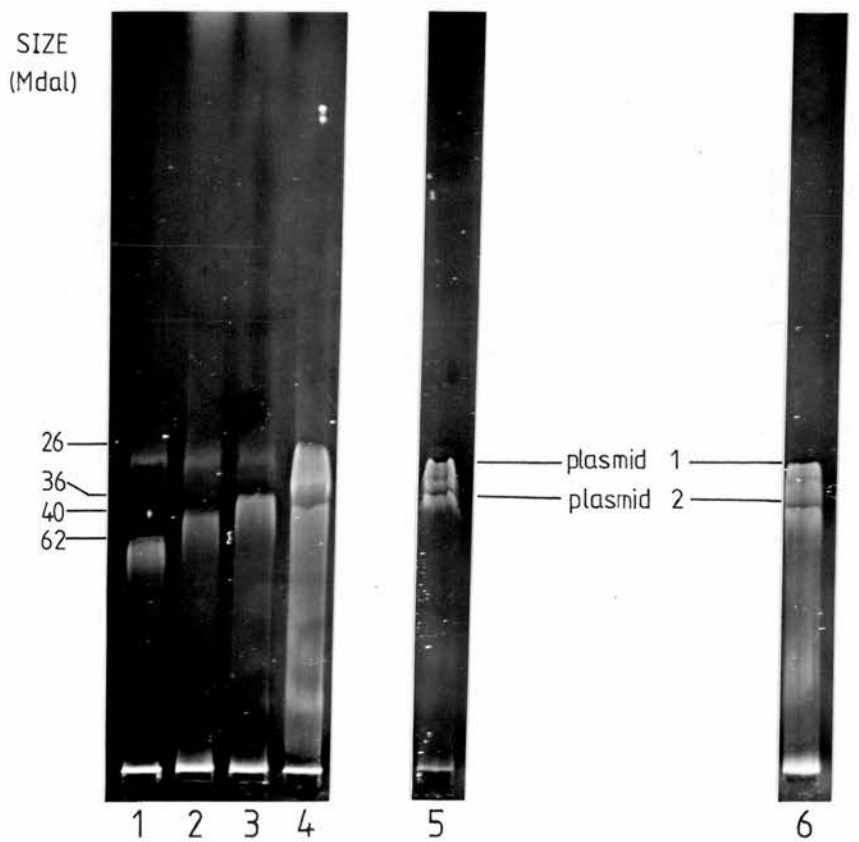
Determination of the resistance pattern of the Tp resistant transconjugant revealed that Tc, Sm, Sx and Cm resistance genes had also been transferred, suggesting that the 70 Mdal plasmid present within this strain has probably resulted from recombination between plasmids 2 and 3, present within the original host.

(iii) Physical analysis of the Cm sensitive derivative of Klebsiella sp. D770

The high frequency of loss of Cm resistance from strain D770 suggests that loss of this resistance determinant may be due to either the spontaneous loss of an R-plasmid, or the loss of a resistance transposon. This was investigated by an examination of the DNA of a purified D770Cm^S derivative. Cellular DNA was extracted and analysed by agarose gel electrophoresis as before. The results (figure 7, track 5) show that the bands of DNA corresponding to plasmids 1 and 2 are still present in this strain. However, no band was observed corresponding to the 42 Mdal plasmid 3. The less well defined open-circular form of plasmid 1 is again present. Loss of Cm resistance appears, therefore, to be due to the spontaneous loss of the 42 Mdal plasmid 3.

Figure 7. Agarose gel electrophoresis of plasmids extracted from Cm sensitive derivatives of Strain A (Klebsiella sp. D770Cm^S) and E. coli J62-2 transconjugants selected on Tp containing plates.

Track 1, R1; track 2, R15; track 3, RP4; track 4, R6K; track 5, plasmid profile of Klebsiella sp. D770Cm^S; track 6, plasmid profile of J62-2 transconjugant strains. Electrophoresis was in 0.4% agarose at 80V for 16 hours.



In order to determine whether plasmid 3 encodes any of the other D770 resistances, Cm sensitive derivatives of D770 were tested for resistance to Sm, Sx, Tp and Tc by streaking onto DSTA plates containing these drugs. The results indicated that Tc resistance was the only other resistance determinant carried by this plasmid since all Cm sensitive derivatives were now Tc sensitive but had retained their resistances to Tp, Sm and Sx.

Since the Tp R-plasmid had been shown previously to be mobilised by the Cm R-plasmid, the Cm sensitive derivative (D770Cm^S) was tested for its ability to transfer Tp resistance. Following 18 hours incubation of the D770Cm^S donor with an E. coli J62-2 recipient transfer of the Tp resistance determinant was measured and shown to occur at a frequency of 2.6×10^{-4} per donor cell. Thus it would appear that the Cm R-plasmid is not an absolute requirement for the mobilisation of R389, although in its presence the transfer frequency of the Tp resistance determinant is seen to increase 10^4 fold to 2.96 per donor cell, during an 18 hour mating.

Analysis of the DNA of this J62-2 Tp resistant, Cm sensitive transconjugant by agarose gel electrophoresis revealed the presence of only two DNA bands corresponding to the covalently closed circular plasmids 1 and 2 (figure 7, track 6). Comparison of these results with those previously obtained for the E. coli Tp resistant, Cm resistant transconjugant indicate that the large MW plasmid observed in the Cm resistant strain is no longer present when Cm resistance and the 42 Mdal plasmid (plasmid 3) are lost. Hence, it is likely

that this large plasmid is indeed formed as a result of recombination between plasmids 2 and 3.

(iv) Selection of Tp sensitive derivatives of Klebsiella sp. D770

A 10^{-6} dilution of an overnight broth culture of Klebsiella strain D770Cm^S was spread on nutrient agar plates as described previously. Single colonies were subsequently replica plated onto DSTA plates and DSTA plates containing Tp at a concentration of 1000 mgL^{-1}. Loss of Tp resistance now occurred at a frequency of 3×10^{-3} per cell which was similar to the spontaneous loss of a plasmid. Following purification on DSTA, three of the spontaneous Tp sensitive derivatives were tested for the simultaneous loss of resistance to Sm and Sx. In addition, the minimum inhibitory concentration (MIC) of Tp was determined as described in the Materials and Methods. The results showed that all three Tp sensitive derivatives had retained resistance to both Sm and Sx but had reduced their MIC of Tp to 1 mgL^{-1}.

The transfer frequencies of the Sm and Sx resistance determinants were calculated following an 18 hour mating of the Tp sensitive D770 derivative with E. coli J62-2. The transfer frequencies at 4.2×10^{-4} per donor cell for the Sm resistance gene and 3.7×10^{-4} per donor cell for the Sx resistance gene were similar to those obtained for the Tp resistance determinant of R389 when the D770Cm^S derivative was used as a donor in a similar mating.

Since Sm and Sx resistances had been retained and were still

transferable the DNA of the Tp sensitive derivatives was analysed by the method of Meyers et al. (1976) in order to determine the plasmid content of the cell. After separation of the cellular DNA by electrophoresis in 0.4% agarose two bands of plasmid DNA were found to be present corresponding to MWs of 30 million (plasmid 1) and 32 million (figure 8, track 5). These results suggest therefore, that loss of Tp resistance has resulted in the loss of a 5 Mdal fragment of DNA from the original 37 Mdal of plasmid 2. The high frequency of loss of Tp resistance coupled with the reduction in MW of plasmid 2 suggests that resistance to Tp may have been lost from this strain as a result of the spontaneous loss of a Tp resistance transposon, confirming the original uncertainty about the use of Tp resistance loss as a marker for the loss of the plasmid.

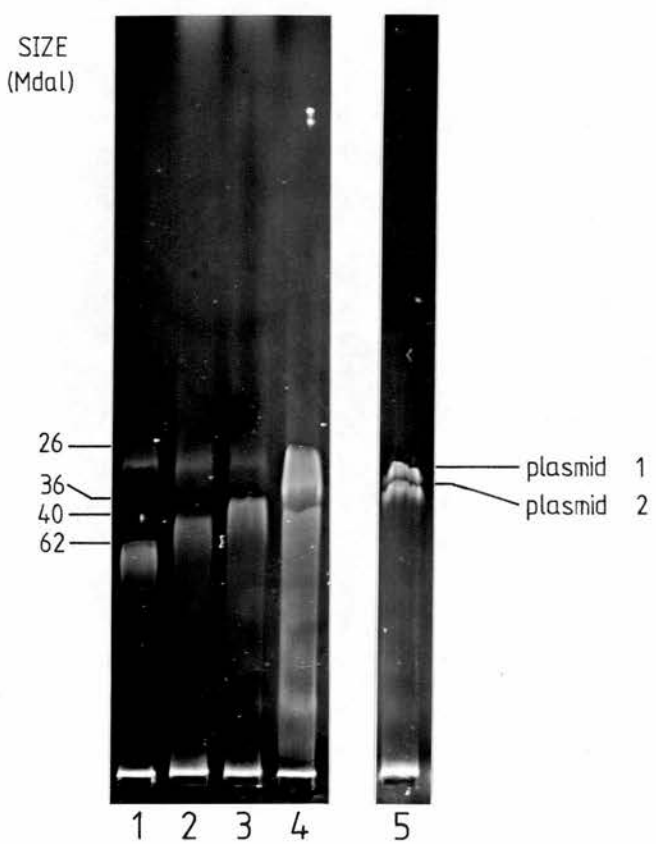
Therefore, a Tp sensitive derivative of Klebsiella strain D770 had now been prepared and was taken to represent the original strain lacking the Tp resistance determinant. This strain, designated Strain B, was employed in further investigations.

(v) Mutation to Tp resistance of the Tp sensitive derivative of Klebsiella sp. D770

In order to determine whether the Klebsiella D770 chromosomal DHFR is a precursor of the Tp R-plasmid DHFR it was necessary to mutate the Tp sensitive derivative, Strain B, to high level Tp resistance. An overnight culture of Strain B, grown in nutrient broth, was harvested and resuspended in 0.1 volume of DM base. 0.1 ml of this ten times suspension was then spread onto DM plates

Figure 8. Agarose gel electrophoresis of plasmids extracted from Cm sensitive, Tp sensitive derivatives of Strain A (Klebsiella sp. D770$Cm^{S}Tp^{S}$).

Track 1, R1; track 2, R15; track 3, RP4; track 4, R6K; track 5, plasmid profile of Klebsiella sp. D770$Cm^{S}Tp^{S}$. Electrophoresis was in 0.4% agarose at 80V for 16 hours.



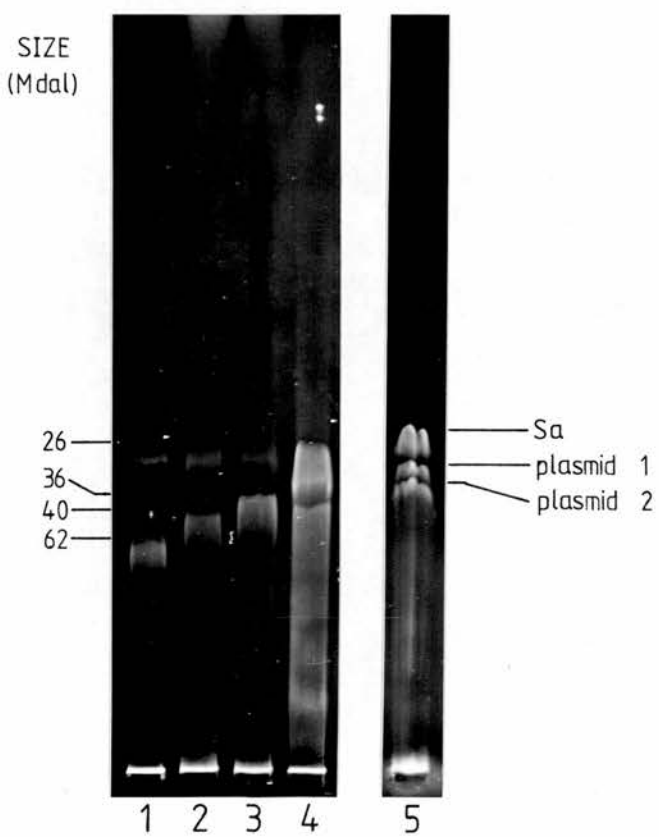
containing Tp at a concentration of 100 mgL^{-1}. However, no Tp resistant colonies were found on any of the plates, even after incubation at 37°C for three days.

Since mutation to high level Tp resistance could not be demonstrated in Strain B, there remained the possibility that the acquisition of Tp resistance required the presence of an additional factor. In view of the fact that most of the early Tp R-plasmids isolated belonged to incompatibility group W, it is conceivable that the presence of an incompatibility W group plasmid within strain B may provide the additional information required to promote mutation of this strain to Tp resistance. The inc W plasmid Sa, which does not carry the Tp resistance gene but possesses resistance to Sm, Sx, Cm and kanamycin (Km), was therefore, chosen as a suitable candidate to test as the promoter of mutation to Tp resistance.

Plasmid Sa was introduced into Strain B by mating with E. coli J53(Sa). The transfer frequency was 2.9×10^{-7} per donor cell in an 18 hour mating. This frequency was low so the identity of the recipient was checked thoroughly by purifying on DM plates devoid of added amino acids but containing either Cm or Km, resistance to both of which are carried by Sa but were not present in Strain B. The recipients grew well on these plates whereas no growth of the E. coli J53(Sa) donor strain was observed. In addition, the physical presence of Sa was confirmed by extracting the DNA from the trans-conjugant and subsequently analysing it on a 0.4% agarose gel by electrophoresis. The results (figure 9, track 5) show the presence

Figure 9. Agarose gel electrophoresis of plasmids extracted from Strain B (Klebsiella sp. D770(Sa)Tp^S).

Track 1, R1; track 2, R15; track 3, RP4; track 4, R6K; track 5, plasmid profile of Strain B.
Electrophoresis was in 0.4% agarose at 80V for 16 hours.



of an additional plasmid band in this strain corresponding to a MW of 23 million, similar to that reported for Sa (Gorai et al. 1979).

Tp resistant mutants of Strain B, harbouring Sa, were again selected for on DM plates containing Tp at concentrations of 100 mgL^{-1} and 1000 mgL^{-1} as described previously. This time colonies resistant to 100 mgL^{-1} Tp were detected. The mutation frequency was calculated by dividing the viable count obtained from an overnight broth culture on MacConkey medium by the number of resistant colonies which grew on DM medium containing Tp, and found to be 1.56×10^{-8}. However, no resistant colonies grew on plates containing 1000 mgL^{-1} Tp. Following purification of the Tp resistant colonies on DM plates containing Tp at 100 mgL^{-1}, a further selection was made for mutation to 1000 mgL^{-1} Tp resistance. Mutation to this level of Tp occurred at a very low frequency of 7.07×10^{-11}. These high-level Tp resistant mutants (Strain C) were then purified and tested for their ability to transfer Tp resistance.

Strain C was employed as a donor in an 18 hour mating with E. coli strain J62-2. Selection was made for the transfer of Km and Tp resistance determinants both separately and together in order to determine whether the mutation to Tp resistance had occurred on plasmid Sa. The Km resistance gene was found to transfer at a frequency of 2.8×10^{-6} per donor cell. However, no transfer of the Tp resistance determinant was detected suggesting that plasmid Sa had promoted a chromosomal mutation to high-level Tp resistance.

(vi) Isolation and purification of Klebsiella DHFRs

In order to determine whether mutation of the Tp sensitive Klebsiella, Strain B, to Tp resistance was due to the production of a 'new' dihydrofolate reductase (DHFR), the enzyme from the resistant mutant, Strain C, was isolated and purified by a modified method of Amyes and Smith (1974a) as described in the Materials and Methods. The DHFRs from the original Tp R-plasmid containing strain, Strain A, and the Tp sensitive derivative Strain B were also isolated and purified, in order that the DHFR of Strain C could be compared with both the R-plasmid enzyme (ie. Strain A enzyme) and the original chromosomal enzyme (ie. Strain B enzyme).

Ten litre exponential phase cultures of each organism, grown in DM minimal medium, were harvested by centrifugation and lysed at 4°C in the French Press. Nucleic acids were removed by precipitation with streptomycin sulphate. NADPH oxidase activity was then precipitated by dialysis against a 50% saturated solution of ammonium sulphate. The pellet was removed by centrifugation. DHFR activity was then precipitated by further dialysis against an 80% saturated solution of ammonium sulphate. Following centrifugation, the supernatant was discarded. The pellet containing the DHFR activity was then resuspended in a minimum volume of 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and 10 mM β-mercaptoethanol (buffer A). Further purification of the DHFRs was then achieved by Sephadex exclusion chromatography by the method of Pattishall et al. (1977).

The 50-80% pellet, resuspended in buffer A, was applied to a Sephadex G-75 superfine column (2 cm^2 x 90 cm). Sephadex G-75 superfine was employed, as opposed to ordinary Sephadex, to obtain better separation of the resistant and sensitive enzyme peaks. In contrast to the observations of Amyes and Smith (1976), Pattishall et al. (1977) reported the complete separation of R-plasmid and chromosomal encoded enzymes by gel filtration on Sephadex G-75. This was achieved by reducing the flow rate of the column buffer from 30 mlh^{-1} to less than 5 mlh^{-1}. The flow rate of the Sephadex G-75 superfine column was therefore adjusted to between 6 and 8 mlh^{-1}. DHFR activity was eluted with buffer A at 4°C. 2 ml fractions were collected and then assayed for DHFR activity by the method of Osborn and Huennekens (1958), in the presence and absence of 4 x 10^{-6}M Tp, a concentration sufficient to abolish the activity of the chromosomal enzyme. This allowed the identification of peaks of Tp-resistant and Tp-sensitive DHFR.

DHFR activities and protein concentrations were assayed throughout the purification procedure. The purification tables for each enzyme show that about ten times more DHFR was recovered from both the Tp R-plasmid containing Strain A (Table 6) and the Tp resistant mutant Strain C (Table 8) than with the Tp sensitive Strain B (Table 7) following gel filtration. In addition the level of purification of the DHFR from Strain B was about 5 times lower than that achieved for the other two enzymes.

The enzyme profiles, obtained following gel filtration, for the

Table 6 Purification of the dihydrofolate reductase activity from Strain A (Klebsiella sp. D770(R389)

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	34.5	21.50	41.35	1.92	1426.60	100	1
STREPTOMYCIN SULPHATE	32.0	23.86	115.78	4.85	3704.96	259.7	2.53
50-80% $(NH_4)_2SO_4$ SUPERNATANT	7.8	7.74	41.35	5.34	322.53	22.6	2.78
SEPHADEX FRACTION 43	2.1	1.43	25.64	17.93	53.84	3.8	9.34

Table 7 Purification of the dihydrofolate reductase activity from Strain B (*Klebsiella sp.* D770(Sa)Tp^S

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	42.5	36.98	90.97	2.46	3866.22	100	1
STREPTOMYCIN SULPHATE	39.5	38.48	41.35	1.07	1633.32	42.2	0.44
50-80% $(NH_4)_2SO_4$ SUPERNATANT	7.5	22.36	3.31	0.15	24.82	0.64	0.06
SEPHADEX FRACTION 52	2.1	0.66	3.14	4.76	6.60	0.17	1.94

Table 8 Purification of the dihydrofolate reductase activity from Strain C (*Klebsiella sp.* D770(Sa)Tp^R

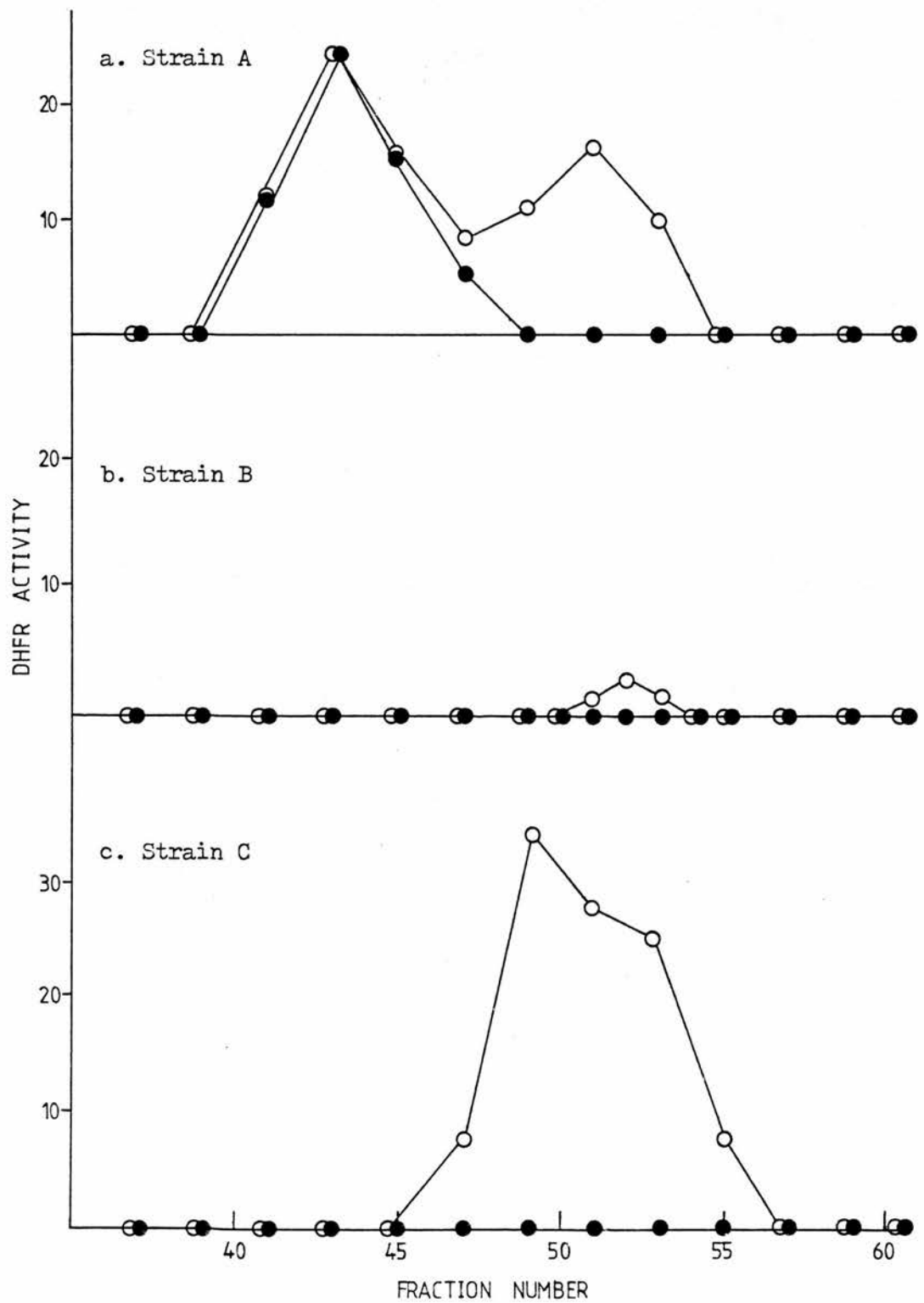
The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	36.0	35.48	165.40	4.66	5954.40	100	1
STREPTOMYCIN SULPHATE	33.0	36.76	44.66	1.21	1473.71	24.8	0.26
50-80% $(NH_4)_2SO_4$ SUPERNATANT	10.5	14.84	33.08	2.23	347.34	5.8	0.48
SEPHADEX FRACTION 49	2.1	0.92	34.73	37.75	72.93	1.2	8.09

DHFRs encoded by each of the three Klebsiella strains (figure 10) show marked differences. The Tp R-plasmid containing Strain A was found to produce two separate enzyme peaks eluting at fractions 43 and 51 (figure 10a). When assayed in the presence of 4×10^{-6}M Tp the peak eluting at fraction 43 was found to be resistant and thus represents the plasmid encoded enzyme. On the other hand, the enzyme eluting at fraction 51 was abolished at this concentration of Tp and must therefore represent the Tp sensitive chromosomal DHFR. In addition the enzyme profiles indicate that the chromosomal determined enzyme of Strain A is produced at two-thirds of the level of the R-plasmid mediated enzyme. The Tp sensitive derivative, Strain B, on the other hand, showed a single peak of DHFR activity eluting at fraction 52 (figure 10b). This peak represents the Tp sensitive chromosomally mediated enzyme of this strain since DHFR activity was completely abolished when assayed in the presence of 4×10^{-6}M Tp. Analysis of the enzyme profile of Strain C again shows marked differences to the Strain A and Strain B enzymes. A large prominant peak of Tp sensitive activity was found to elute at fraction 49 (figure 10c). In addition a slight shoulder of Tp sensitive activity was also observed at fraction 51. The relative quantities of Tp sensitive DHFR, represented by the areas under each enzyme curve, reveals that Strain C produces approximately two and a half times more Tp sensitive DHFR than Strain A, which in turn produces about ten times more than Strain B.

Figure 10. Elution of DHFR activities of Klebsiella Strains A, B and C on Sephadex G-75 superfine gel filtration.

a = Strain A (Klebsiella sp. D770(R389)); b = Strain B (Klebsiella sp. D770(Sa)Tp^S); c = Strain C (Klebsiella sp. D770(Sa)Tp^R).
Total DHFR activity, O; DHFR activity in the presence of 4×10^{-6}M Tp, ● . Enzyme activity refers to DHFR level in units/ml.



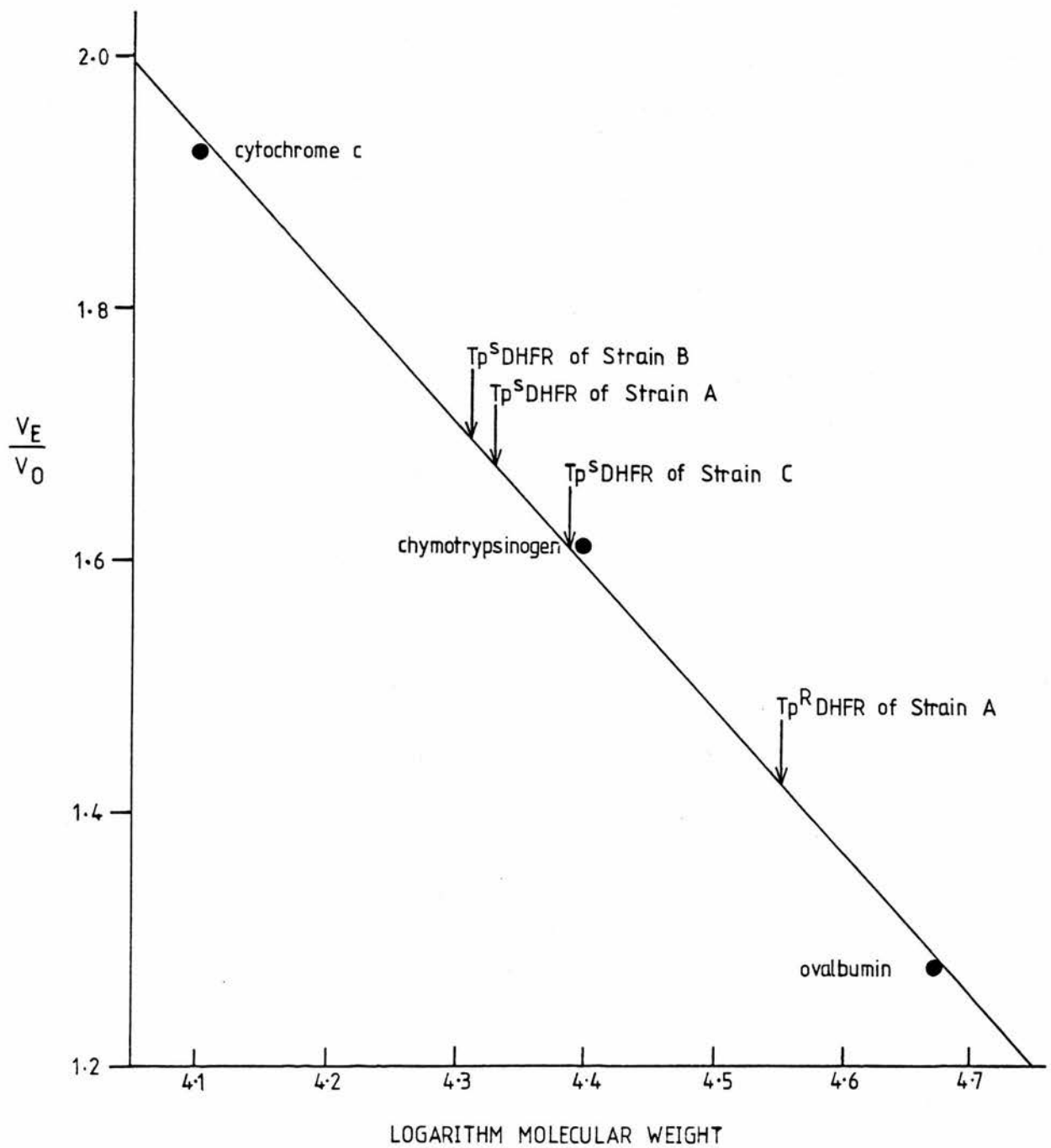
(vii) Molecular Weight of DHFR coded by Klebsiella spp.

The molecular weight (MW) of each enzyme was estimated by repetition of Sephadex gel filtration in the presence of standard MW marker proteins. Crude enzyme preparations were prepared as before. The 50-80% ammonium sulphate pellet was resuspended in buffer A and mixed with 50 mg quantities of cytochrome c, chymotrypsinogen and ovalbumin, before applying to the Sephadex G-75 superfine column. The logarithm of the MW is proportional to the ratio of the elution volume (Ve) to the void volume (Vo) ie. Ve/Vo (Andrews, 1964). The ratio was found for each of the three molecular weight marker proteins and this value was plotted against the logarithm of the MW. The results (figure 11) show that each strain produces a chromosomal, Tp sensitive DHFR of slightly varied MW. The chromosomal enzymes from Strains A and B have MWs of 21400 and 20000 respectively. These MWs agree, within experimental error, with those found previously for E. coli chromosomal DHFRs (Amyes and Smith, 1976). On the other hand, the enzyme produced by Strain C has a MW of 24300. This value is somewhat higher than those previously reported for chromosomal DHFRs produced by bacteria. However, the enzyme profile of Strain C also showed a slight shoulder of activity which corresponds to a MW of 21300, a similar value to that obtained for Strain A. It is therefore possible that Strain C may be producing a second DHFR of 24300 daltons in addition to the normal host DHFR of 21300 daltons.

The Tp resistant enzyme of Strain A, eluting at fraction 43, corresponds to a MW of 35500 which agrees well with previous reports

Figure 11. The molecular weight of DHFR as measured by gel filtration on Sephadex G-75 superfine.

Strain A = Klebsiella sp. D770(R389); Strain B = Klebsiella sp. D770(Sa)Tp^S; Strain C = Klebsiella sp. D770(Sa)Tp^R.



for the Type I and Type II R-plasmid determined enzymes (Amyes and Smith, 1976; Pattishall et al., 1977).

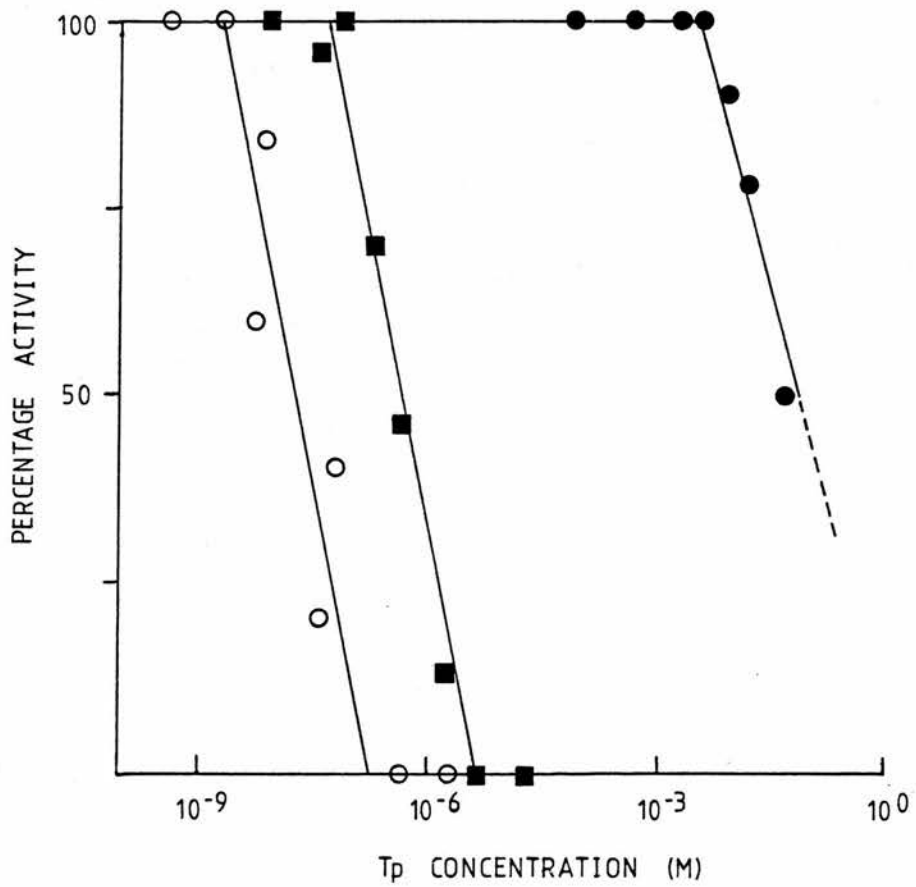
(viii) Inhibition of DHFR activity by trimethoprim

The most distinguishing feature between the chromosomally mediated DHFRs and the Type I and Type II R-plasmid enzymes is their susceptibility to Tp. The concentration of Tp required to achieve 50% inhibition of the activity (ID_{50}) of the R-plasmid encoded enzyme of Strain A and the two chromosomal enzymes of Strains B and C was measured. Partially purified enzyme preparations obtained from gel filtration were assayed for DHFR activity in the presence of increasing concentrations of Tp. The results (figure 12) show that the enzyme encoded by R389 (ie. plasmid 2 of Strain A) is highly resistant to Tp with an ID_{50} of 80,000μM (obtained by extrapolation). This value is similar to those previously reported for Type II DHFRs (Pattishall et al., 1977). Thus R389 appears to encode a Type II DHFR.

The drug-sensitive chromosomal enzyme of Strain B is inhibited by 50% in the presence of 0.02μM Tp. Again this result is in agreement with the published values for Tp sensitive E. coli chromosomal enzymes (Broad and Smith, 1982). However, the enzyme produced by Strain C has an ID_{50} for Tp of 0.5μM. This enzyme is hence 25 times more resistant to Tp than the original host chromosomal enzyme of Strain B.

Figure 12. Trimethoprim inhibition of partially purified DHFR from Klebsiella Strains A, B and C.

Strain A (Klebsiella sp. D770(R389)), ● ; Strain B (Klebsiella sp. D770(Sa)Tp^S), ○ ; Strain C (Klebsiella sp. D770(Sa)Tp^R), ■ .



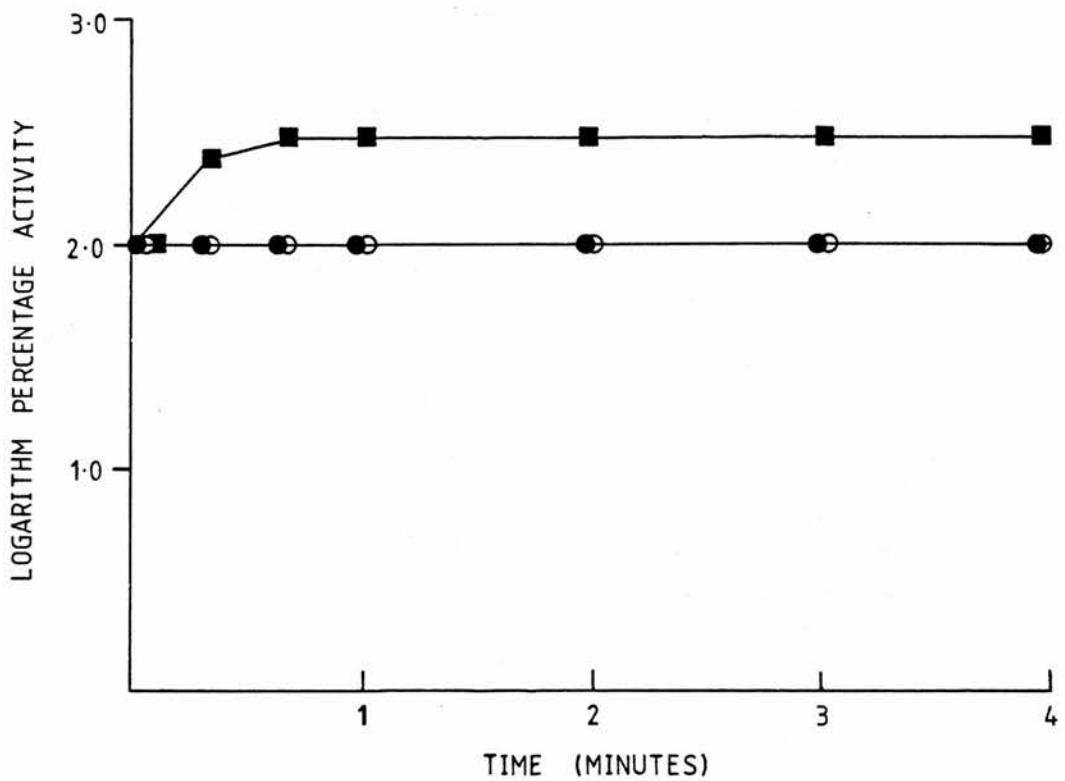
(ix) Inhibition of DHFR activity at 45°C

Although the relative insusceptibility to Tp of the R389 mediated enzyme indicates that it should be classified as a Type II enzyme, the level of production of this enzyme suggests a similarity to Type I DHFRs (Pattishall et al., 1977). In order to distinguish the R389 encoded enzyme further, its heat sensitivity was measured. The partially purified enzyme preparation, described earlier, was maintained at 45°C. At various time intervals samples were withdrawn and assayed for DHFR activity as described. The temperature inactivation curve (figure 13) shows that the R389 enzyme retains 100% of its activity after 4 minutes of incubation at 45°C. This result is in agreement with those obtained previously for Type II enzymes (Tennhammar-Ekman and Sköld, 1979; Broad and Smith, 1982).

Similar heat inactivation curves were plotted for the enzymes produced by Strains B and C. The chromosomal enzyme of Strain B was also found to be heat stable, a characteristic also associated with E. coli Tp sensitive, chromosomal enzymes (Amyes and Smith, 1976; Tennhammar-Ekman and Sköld, 1979; Broad and Smith, 1982). However a significant difference in the behaviour of the chromosomal enzyme of Strain C was observed. In contrast to previous reports for bacterial DHFRs, the activity of this enzyme was increased over three fold on heating at 45°C. Thus, the mutant chromosomal DHFR appears not only to be heat stable but in addition is activated by heating at 45°C.

Figure 13. Heat sensitivity of partially purified DHFR from Klebsiella Strains A, B and C.

Strain A (Klebsiella sp. D770(R389)), ● ; Strain B (Klebsiella sp. D770(Sa)Tp^S), O ; Strain C (Klebsiella sp. D770(Sa)Tp^R), ■ .



2. The role of Escherichia coli and incompatibility W group plasmids

The inc W group plasmid R388 was the first R-plasmid found to confer Tp resistance. It was detected in a pathogenic E. coli strain isolated from a patient in St. Pancras Branch, University College Hospital, London (Fleming et al., 1972). Prior to the isolation of R388, only two other widely used inc W plasmids had been isolated, Sa conferring resistance to Cm, Sm, Sx and Km (Watanbe et al., 1968) and R7K conferring resistance to Sm and Ap (Coetzee et al., 1972). Electron microscope heteroduplex studies (Gorai et al., 1979) and physical and genetic analysis (Ward and Grinsted, 1982) of these three inc W plasmids have shown them to exhibit a high degree of genetic conservation in their replication and transfer regions, while their antibiotic resistance genes appear to have been acquired either by transposition or by some other mechanism. However, restriction enzyme maps of Sa and R388 (Ward and Grinsted, 1982) show that these two plasmids share some degree of homology within their resistance genes. Therefore, the relationship between Sa and R388 was examined to determine whether R388 is directly derived from Sa.

(i) Purification of DHFR from E. coli J53(Sa)

The enzyme dihydrofolate reductase (DHFR) indirectly plays an important role in many biosynthetic pathways including the synthesis of nucleic acids. Since plasmids are autonomously replicating DNA molecules it seems possible that even those not carrying Tp resistance determinants may encode the production of DHFRs.

Therefore, plasmid Sa was investigated for its ability to produce a DHFR which may be related, in some way, to the Tp resistant R388 encoded enzyme.

E. coli strain J53(Sa) was grown in 10 litres of DM medium to logarithmic phase. Enzyme extracts were prepared, in buffer A, as described in the Materials and Methods. Partial purification of the enzyme was performed by a modification of the method of Amyes and Smith (1974a) as described previously. Further purification was achieved by Sephadex exclusion chromatography by the method of Pattishall et al, (1977). Fractions were collected and assayed both in the presence and absence of 4×10^{-6}M Tp to determine whether an additional Tp resistant DHFR had been produced. DHFR activities and protein concentrations were assayed throughout the purification procedure. Following gel filtration 8.7% of the enzyme was recovered and the level of purification was 30.95 (Table 9). These values are similar to those obtained by Amyes and Smith (1976) for the E. coli 114 enzyme.

The enzyme profile following gel filtration (figure 14) shows that a single peak of DHFR activity eluted which was completely abolished when assayed in the presence of Tp. The molecular weight of this enzyme was determined by repetition of gel filtration in the presence of the standard molecular weight markers, cytochrome c, chymotrypsinogen and ovalbumin. The size of the DHFR was then estimated and found to be 23400 daltons. This value of 23400 daltons is slightly greater than those previously reported for Tp sensitive

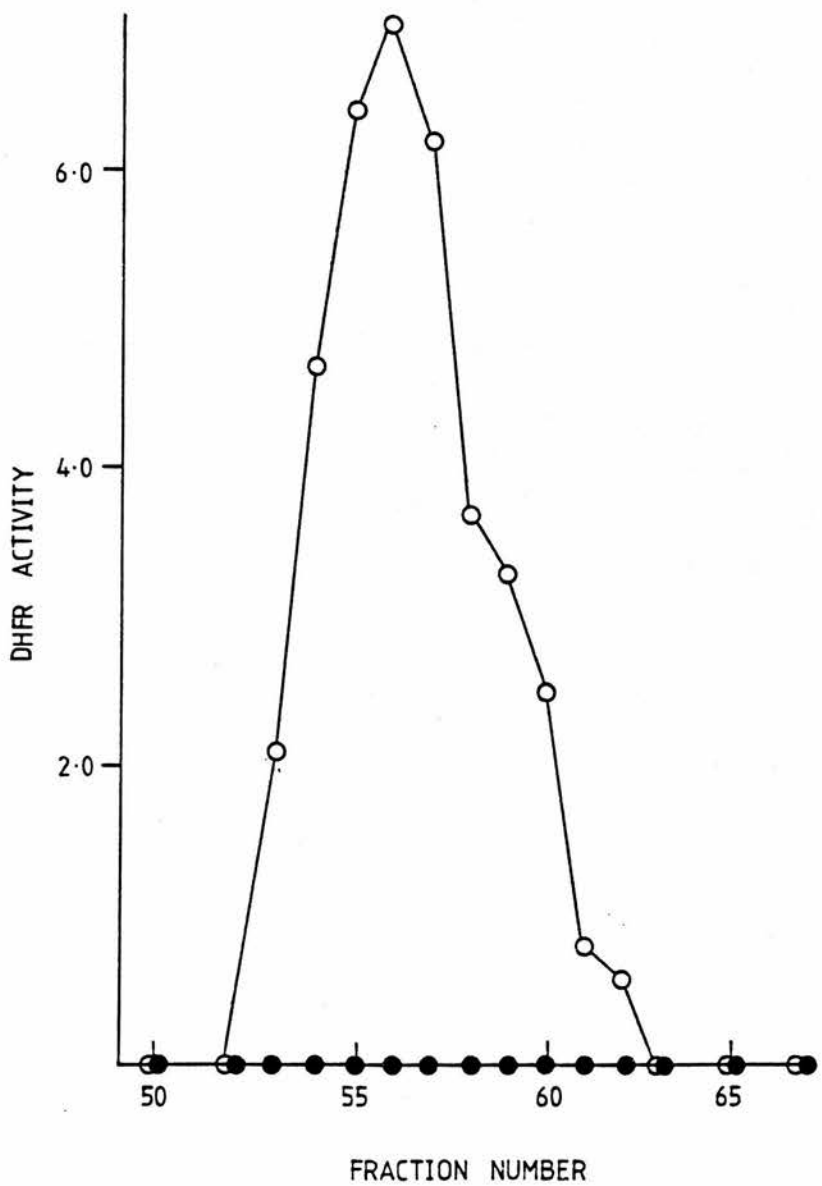
Table 9 Purification of the dihydrofolate reductase activity from E. coli J53(Sa)

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	14.0	48.37	11.58	0.24	162.09	100	1
STREPTOMYCIN SULPHATE	13.7	34.40	13.23	0.38	181.25	111.8	1.61
50-80% $(NH_4)_2SO_4$ SUPERNATANT	6.4	29.02	10.34	0.36	66.16	40.8	1.49
SEPHADEX FRACTION 57	2.0	0.95	7.03	7.40	14.06	8.7	30.95

Figure 14. Elution of DHFR activity of E. coli J53(Sa) on Sephadex G-75 superfine gel filtration.

Total DHFR activity, ○ ; DHFR activity in the presence of 4×10^{-6}M Tp, ● . Enzyme activity refers to DHFR level in units/ml.



E. coli chromosomal enzymes (Amyes and Smith, 1978). However, this discrepancy may be due to the use of Sephadex G-75 superfine which permits a finer resolution of proteins compared with ordinary Sephadex G-75.

In order to confirm that this 23400 dalton DHFR is indeed the chromosomally encoded enzyme of strain J53 the enzyme from an E. coli J53 strain devoid of plasmid Sa was similarly purified and applied to Sephadex G-75 superfine along with standard molecular weight marker proteins. Again a single peak of Tp sensitive DHFR activity was found to elute (figure 15) corresponding to a MW of 22400. Strain J53 therefore encodes a DHFR of MW approximately 23000.

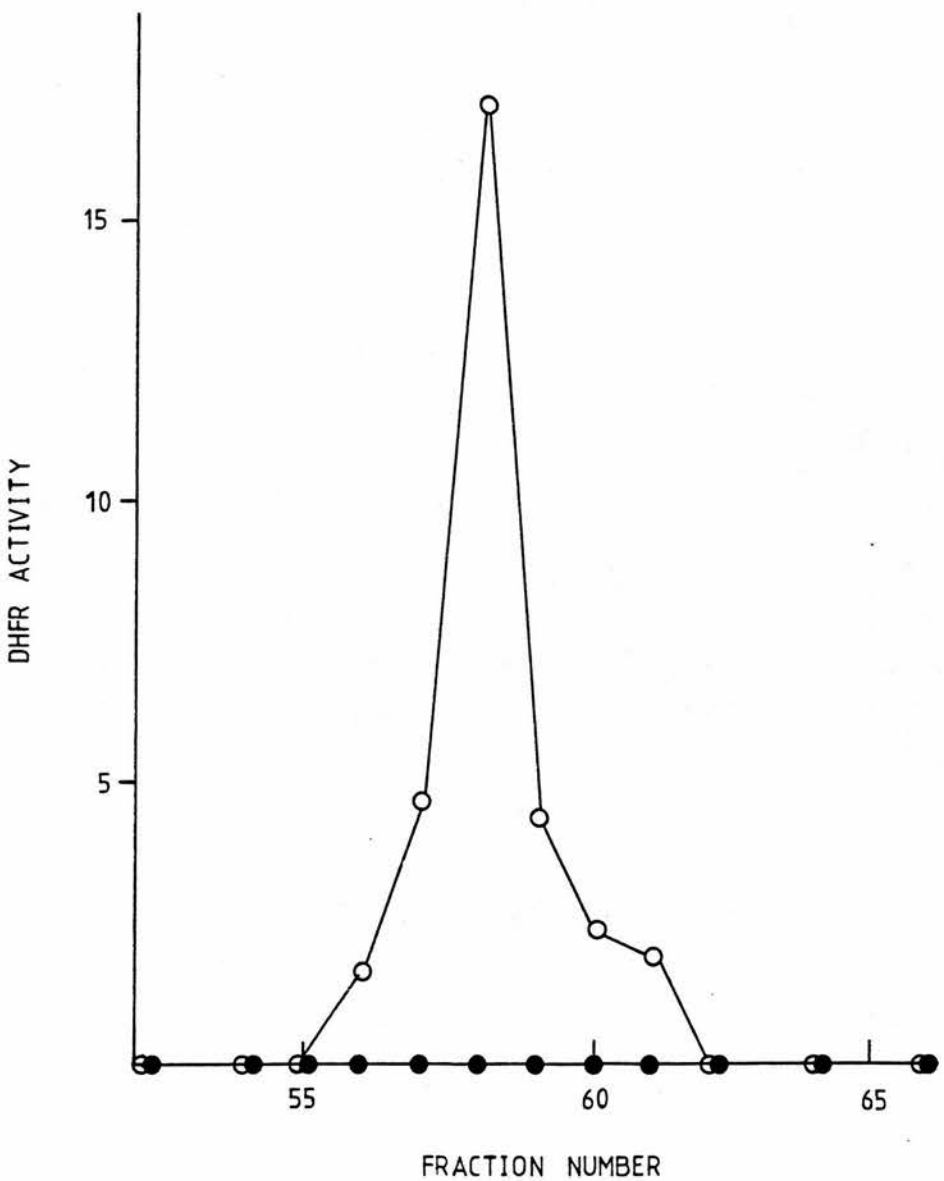
The results, so far, suggest that plasmid Sa does not encode its own DHFR. However, it is still possible that an enzyme of similar MW to the chromosomal enzyme may be being produced in very low amounts, its presence remaining undetected due to the presence of the chromosomal enzyme. A second attempt was therefore made to detect a plasmid encoded enzyme by mutating the J53(Sa) strain to Tp resistance in the hope of inducing the production of a Tp resistant, Sa encoded, DHFR.

(ii) Mutation to Tp resistance of E. coli J53(Sa)

Before mutation of strain J53(Sa) to Tp resistance was attempted the minimum inhibitory concentration (MIC) of Tp on DM medium for this strain was determined and found to be 0.1 mgL^{-1}. Mutation of J53(Sa) to high level Tp resistance was then achieved by sequential

Figure 15. Elution of DHFR activity of *E. coli* J53 on Sephadex G-75 superfine gel filtration.

Total DHFR activity, O ; DHFR activity in the presence of 4×10^{-6} M Tp, ● . Enzyme activity refers to DHFR level in units/ml.



subcultures of J53(Sa) on DM plates containing increasing concentrations of Tp.

Strain J53(Sa) was grown to stationary phase at 37°C in nutrient broth. The cells were harvested, washed in DM base and resuspended in the same volume of DM base. 0.1 ml amounts of the undiluted culture were then spread on suitably supplemented DM plates containing Tp at a concentration of 1.0, 5.0 and 10.0 mgL^{-1}. A viable count of the overnight broth culture was also made on MacConkey agar so that the mutation frequency to Tp resistance could be determined. The results (table 10) show that mutation to 5.0 mgL^{-1} Tp resistance occurred at a frequency of 5.4×10^{-10}.

Table 10 Tp resistance mutation frequency by E. coli J53(Sa)

The mutation frequency shown is the viable count obtained from an overnight culture on MacConkey agar divided by that obtained on DM medium containing Tp at the concentrations listed.

Bacterial Strain	Conc. Tp mgL^{-1}	Mutational frequency
J53(Sa)	5	5.40×10^{-10}
J53(Sa)$Tp5^R$	40	7.23×10^{-9}
J53(Sa)$Tp40^R$	1000	2.91×10^{-9}

Mutant colonies were purified on DM plates containing 5.0 mgL^{-1} Tp. These mutants (J53(Sa)Tp5^R) were then grown as before in nutrient broth, harvested, washed and resuspended in DM base. 0.1 ml amounts of the undiluted culture were spread on DM plates containing Tp at concentrations of 10, 20, 40 and 100 mgL^{-1}. Colonies resistant to 40 mgL^{-1} Tp occurred at a frequency of 7.23×10^{-9} (table 10), but no colonies grew on the Tp 100 mgL^{-1} plate. Following purification, the J53(Sa)Tp40^R mutants were further subcultured as before onto DM plates containing Tp at concentrations of 100, 200, 400, 600, 800 and 1000 mgL^{-1}. Mutation to Tp 1000 mgL^{-1} was found to occur at a frequency of 2.91×10^{-9} (table 10). Thus J53(Sa) appears to mutate easily to high level Tp resistance (ie. MIC >1000 mgL^{-1}), the mutations occurring during three sequential subcultures.

The final mutant, designated J53(Sa)Tp1K^R, was then checked both for the continued presence of plasmid Sa, and for its auxotrophic requirements. A 1 in 10^4 dilution of an overnight culture was used to inoculate DSTA plates containing Sm, Cm, Sx and Km (the four resistance determinants carried by Sa) and DM plates lacking either proline or methionine (the two J53 auxotrophic requirements). Good growth occurred on plates containing each of the drugs listed but no growth was observed on plates lacking either of the two amino acids. Thus J53(Sa)Tp1K^R has retained both Sa and its requirements for proline and methionine.

In order to determine whether the mutation to Tp resistance has occurred on plasmid Sa or within the bacterial chromosome, strain

J53(Sa)Tp1K^R was conjugated with E. coli strain J62 for five hours and selection was made for the transfer of Tp and Cm resistance determinants both separately and together. The results showed that Cm resistance transferred at a frequency of 1.13×10^{-2} per donor cell but that Tp resistance was non-transferable. Thus the mutation to Tp resistance appears to have occurred within the chromosome of J53.

(iii) Mutation to Tp resistance of E. coli J53

The results of the experiments with Klebsiella sp. D770 suggest that the presence of Sa within this strain promotes a chromosomal mutation to high level Tp resistance. In order to determine whether Sa is promoting a similar mutation in E. coli J53 the above mutation procedure was repeated with a J53 strain devoid of plasmid Sa.

The MIC of Tp on DM medium for E. coli J53 was determined initially and found to be 0.1 mgL^{-1}, a value similar to that obtained for J53(Sa). E. coli J53 was then subcultured in the same way as strain J53(Sa), on suitably supplemented DM plates containing increasing concentrations of Tp. The results (table 11) show that like J53(Sa) strain J53 also mutates to high level Tp resistance in three sequential steps. In addition the mutation frequencies at each stage are similar to those obtained with the plasmid containing strain. Therefore, the presence of plasmid Sa in strain J53 does not appear to be a prerequisite for the mutation of this strain to Tp resistance.

Table 11 Tp resistance mutation frequency by E. coli J53

The mutation frequency shown is the viable count obtained from an overnight culture on MacConkey agar divided by that obtained on DM medium containing Tp at the concentrations listed.

Bacterial Strain	Conc. Tp mgL^{-1}	Mutational frequency
J53	5	6.00×10^{-10}
J53Tp5^R	40	8.26×10^{-9}
J53Tp40^R	1000	9.71×10^{-9}

(iv) Mutation to Tp resistance of E. coli J62 and E. coli J62(Sa)

The results of the previous two sections indicate that E. coli J53 is able to mutate easily to high-level Tp resistance. In order to determine whether this phenomenon is unique to strain J53 or is applicable to other E. coli K-12 strains, the ability of strain J62 to mutate to Tp resistance was investigated. In addition a J62 strain containing plasmid Sa was also examined. The MIC of Tp on DM medium for both the original J62 strain and a plasmid containing transconjugant was determined as before and found to be 0.8 mgL^{-1}, slightly higher than the J53 strains. Each J62 strain was then grown in nutrient broth, harvested, washed and spread on suitably supplemented DM plates containing increasing concentrations of Tp. However, no growth occurred on plates containing Tp at concentrations greater than 0.4 mgL^{-1}, the mutation frequency of both strains to Tp

5.0 mgL^{-1} resistance being less than 10^{-11}. Thus the ease with which J53 is able to mutate to Tp resistance is associated with this strain only and is not a property of E. coli K-12 strains in general.

(v) The resistance mechanism of E. coli J53TplK^R and E. coli J53(Sa)TplK^R

Previous reports of chromosomal mutations to Tp resistance in E. coli strains have indicated that the most common mechanism involves the over-production of the normal Tp sensitive DHFR (Burchall and Hitchings, 1968; Breeze et al., 1975; Sheldon and Brenner, 1976). However, several reports have suggested that some Tp resistant mutants of E. coli K-12 may owe their resistance to the production of a mutant DHFR which has a slightly decreased affinity for Tp (Sheldon, 1977; Poe et al., 1979). In order to determine which mechanism was responsible for the high levels of resistance observed here, the DHFRs from both the J53 Tp resistant mutants (J53TplK^R and J53(Sa)TplK^R) and the original Tp sensitive strain, J53(Sa), were isolated and characterised.

Ten litre exponential phase cultures of each organism, grown in DM medium, were harvested and lysed in the French Press. The lysate was cleared by high speed centrifugation and the specific activity of DHFR in all three extracts measured in sodium phosphate buffer pH 6.0. The results (table 12) show that the DHFR of both the Tp resistant mutants is produced in much greater quantity, approximately ten times the amount, than the original Tp sensitive DHFR. However, despite the increased production of DHFR in these mutant strains, the

level of enzyme produced is not sufficient to explain the increase in MIC of Tp of the organism from 0.1 mgL^{-1} to greater than 1000 mgL^{-1}.

Table 12 The specific activity of DHFR in crude extracts of E. coli J53(Sa), E. coli J53(Sa)TplK^R and E. coli J53 TplK^R

The specific activity is expressed in nmoles dihydrofolate being converted per minute per mg protein in a 1.0 ml volume assay.

Strain	Specific Activity	Relative Specific Activity
J53(Sa)	0.24	1.00
J53(Sa)TplK^R	1.93	8.04
J53TplK^R	3.29	13.71

In order to characterise the DHFRs produced by the resistant mutants, partially purified enzyme preparations were made by the modified method of Amyes and Smith (1974a). Further purification was achieved by sephadex gel filtration on Sephadex G-75 superfine by the method of Pattishall et al. (1977). The purification tables for each enzyme (tables 13 and 14) show that the levels of recovery and purification of these resistant DHFRs were slightly lower than those obtained for the original J53(Sa) chromosomal enzyme (table 9).

Following elution of the enzymes with buffer A, fractions were

Table 13 Purification of the dihydrofolate reductase activity from E. coli J53TplK^R

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	15.0	40.20	132.32	3.29	1984.80	100	1
STREPTOMYCIN SULPHATE	14.7	35.48	124.05	3.50	1823.54	91.9	1.06
50-80% $(NH_4)_2SO_4$ SUPERNATANT	6.5	34.40	190.21	5.53	1236.36	62.3	1.69
SEPHADEX FRACTION 59	2.0	1.13	39.70	35.13	79.40	4.0	10.67

Table 14 Purification of the dihydrofolate reductase activity from E. coli J53(Sa)TplK^R

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	14.0	42.78	82.70	1.93	1157.80	100	1
STREPTOMYCIN SULPHATE	13.0	14.84	24.81	1.67	322.53	27.8	0.86
50-80% $(NH_4)_2SO_4$ SUPERNATANT	6.0	17.84	41.35	2.32	248.10	21.4	1.20
SEPHADEX FRACTION 58	2.0	0.72	16.54	22.97	33.08	2.9	11.90

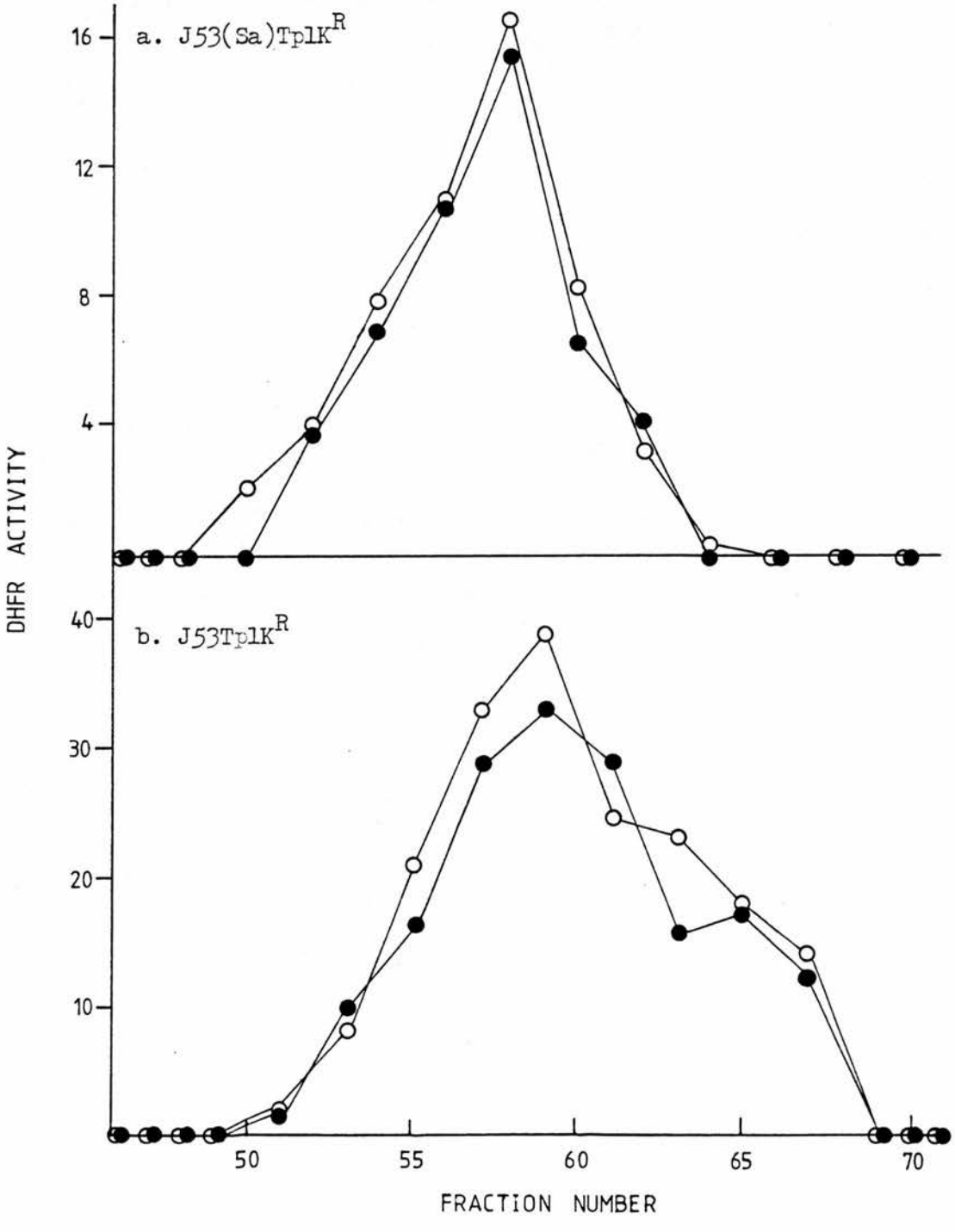
assayed for DHFR activity both in the presence and absence of 4 x 10^{-6}M Tp. Both strains showed the presence of a single enzyme peak (figure 16). However, in contrast to the original J53 Tp sensitive enzyme, the enzymes produced by J53Tp1K^R and J53(Sa)Tp1K^R were resistant to 4 x 10^{-6}M Tp and showed maximum activity when assayed in its presence (figure 16). The enzyme from J53Tp1K^R also showed an additional shoulder of activity eluting in fraction 65 (figure 16b).

(vi) Molecular weight of DHFR coded by E. coli J53Tp1K^R and E. coli J53(Sa)Tp1K^R

The molecular weights of the two resistant enzymes were then estimated. Further ten litre exponential cultures of the resistant strains were prepared as before. Partially purified samples were then mixed with standard molecular weight marker proteins and applied to a Sephadex G-75 superfine column as before. The molecular weights of the Tp resistant DHFRs were then estimated and found to be 22400 for the J53Tp1K^R enzyme and 22600 for the J53(Sa)Tp1K^R enzyme. These values are similar to those obtained for the original Tp sensitive chromosomal enzymes of strains J53 and J53(Sa). The additional shoulder of Tp resistant DHFR activity from strain J53Tp1K^R was present again and corresponds to a MW of 16000.

Thus it would appear that both J53Tp1K^R and J53(Sa)Tp1K^R owe their resistance to the over production of a DHFR encoded by the bacterial chromosome, which has a significantly reduced affinity for Tp.

Figure 16. Elution of DHFR activities of *E. coli* J53(Sa)Tp1K^R and *E. coli* J53Tp1K^R on Sephadex G-75 superfine gel filtration.
a = *E. coli* J53(Sa)Tp1K^R; b = *E. coli* J53Tp1K^R.
Total DHFR activity, O ; DHFR activity in the presence of 4 x 10^{-6}M Tp, ● . Enzyme activity refers to DHFR level in units/ml.



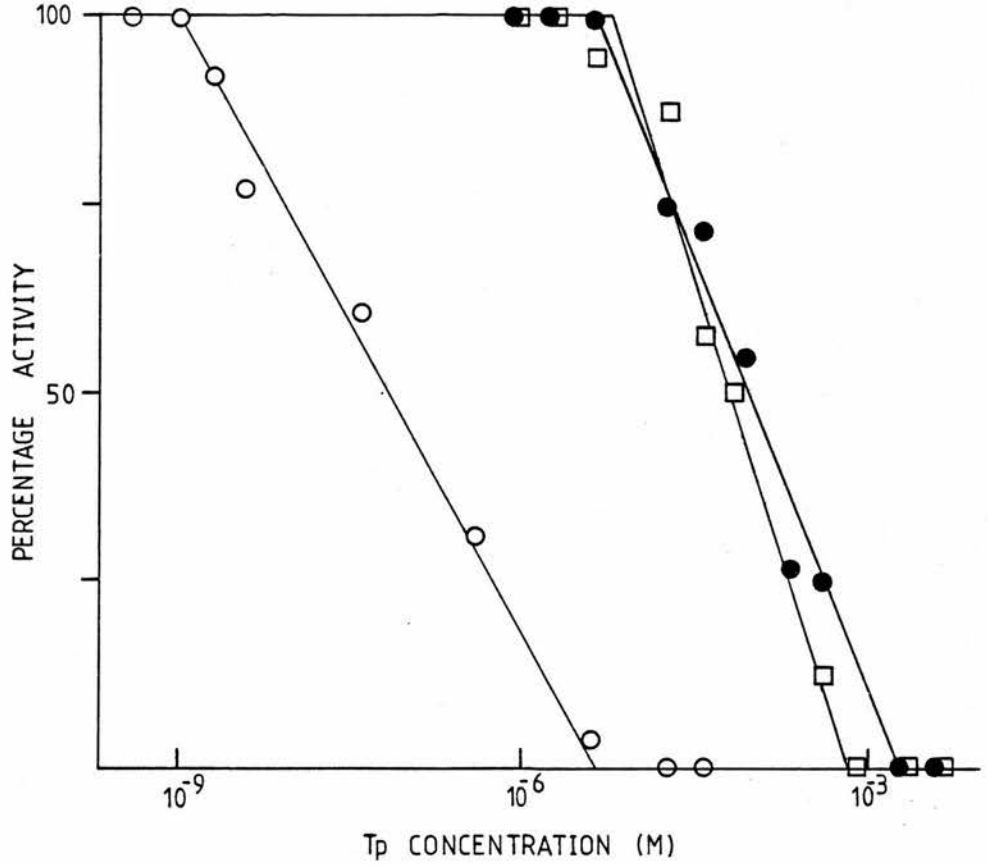
(vii) Inhibition of DHFR activity by trimethoprim

In order to characterise and compare the resistant and sensitive enzymes further, the extent of their resistance to Tp was determined. The enzyme produced by strain J53(Sa) was taken to represent the Tp sensitive chromosomal mediated DHFR of E. coli J53 and was used for further characterisation. This enzyme will be referred to as the J53(Sa)Tp^S enzyme.

Following sephadex gel filtration, fractions containing DHFR activity were pooled. The pooled fractions were than assayed in increasing concentrations of Tp at pH 6.0 and the concentration required to give 50% inhibition (ID_{50}) of each enzyme was determined. The inhibitor profiles of the two Tp resistant mutant enzymes were almost identical (figure 17), the activity being directly proportional to the logarithm of the Tp concentration. The ID_{50} for the J53Tp1K^R DHFR was 89 μM and for the J53(Sa)Tp1K^R enzyme 63 μM. These values are similar to those reported by Pattishall et al., (1977) for the Type I plasmid encoded DHFRs. The Tp sensitive chromosomal enzyme on the other hand has an ID_{50} of 0.08 μM. This value is similar to those obtained by Amyes and Smith (1978) for E. coli K-12 chromosomal enzymes. Thus it would appear that the mutant enzymes are 1000 times more resistant to Tp compared with the original J53 enzyme.

Since the enzymes from both resistant mutants appear to be produced in similar amounts and are equally resistant to Tp it was

Figure 17. Trimethoprim inhibition of partially purified DHFR from *E. coli* J53(Sa), *E. coli* J53TplK^R and *E. coli* J53(Sa)TplK^R.
E. coli J53(Sa), ○ ; *E. coli* J53TplK^R, ● ;
E. coli J53(Sa)TplK^R, □ .



assumed that the resistance mechanism of both mutants was similar. Therefore, further characterisation of only one of the resistant enzymes was performed, namely the DHFR produced by strain J53TplK^R.

(viii) Inhibition of DHFR activity by methotrexate

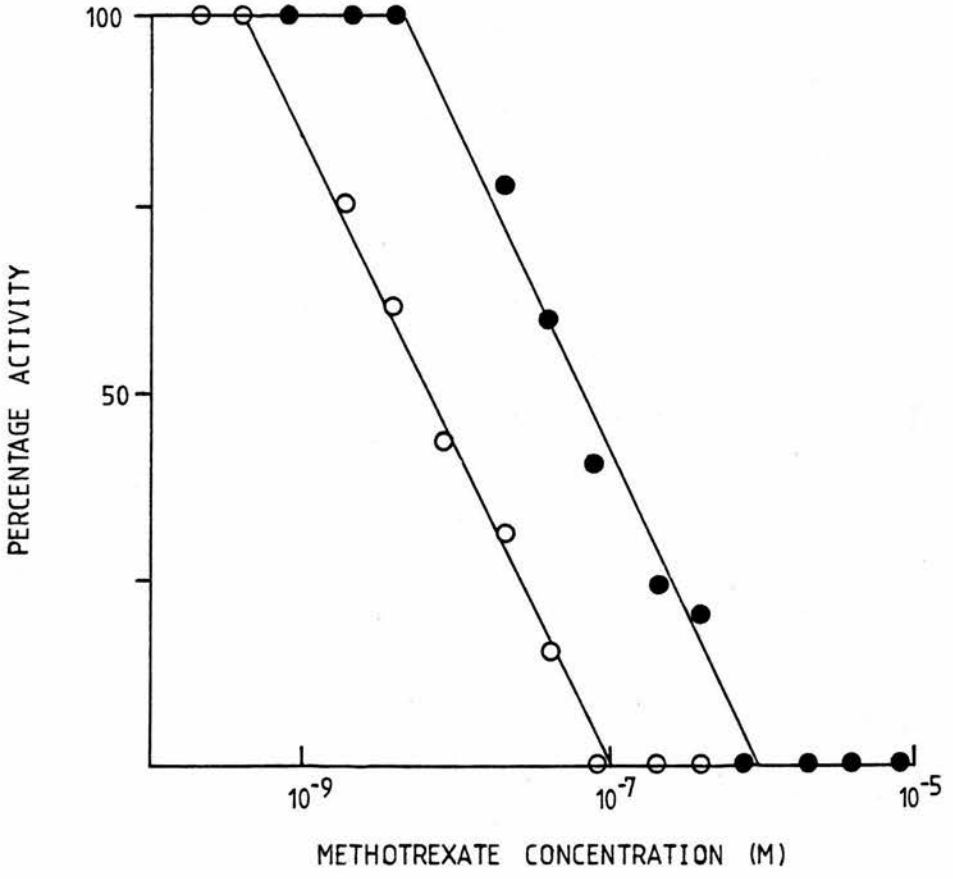
The activity of the J53(Sa)Tp^S and J53TplK^R DHFRs was reassayed in the presence of increasing concentrations of methotrexate. This was done to determine whether the mutation in strain J53TplK^R to resistance to Tp - a 2,4-diaminopyrimidine - also manifested resistance to 2,4-diaminopteridine derivatives. The inhibition of both the Tp sensitive and Tp resistant enzymes by methotrexate gave linear responses when activity was plotted against the logarithm of methotrexate concentration (figure 18). The ID_{50} of the Tp sensitive enzyme with methotrexate was 6.6 nM which is in good agreement with the published values for E. coli chromosomal enzymes (Amyes and Smith, 1976, 1978; Pattishall et al., 1977). The ID_{50} of methotrexate with the Tp resistant enzyme of J53TplK^R on the other hand was 71 nM indicating that this enzyme is about ten times less susceptible to the 2,4-diaminopteridine. Therefore, the mutation in the DHFR gene has resulted not only in a greatly decreased affinity of the enzyme for Tp, but also a slight decrease in its affinity for methotrexate.

(ix) Michaelis-Menten kinetics of DHFRs

Previous reports have shown that Tp R-plasmid determined DHFRs

Figure 18. Methotrexate inhibition of partially purified DHFR from *E. coli* J53(Sa) and *E. coli* J53Tp1K^R.

E. coli J53(Sa) Tp^S DHFR, ○ ; *E. coli* J53Tp1K^R DHFR, ● .



have a slightly higher affinity for the primary substrate (dihydrofolate) than E. coli Tp sensitive, chromosomally encoded enzymes (Amyes and Smith, 1976, 1978). From the ID_{50} experiments the Tp resistant enzyme of mutant J53Tp1K^R appears to be behaving in a similar way to Type I R-plasmid determined enzymes. Therefore, this enzyme was examined to determine if it, too, showed a similar higher affinity for dihydrofolate (DHF).

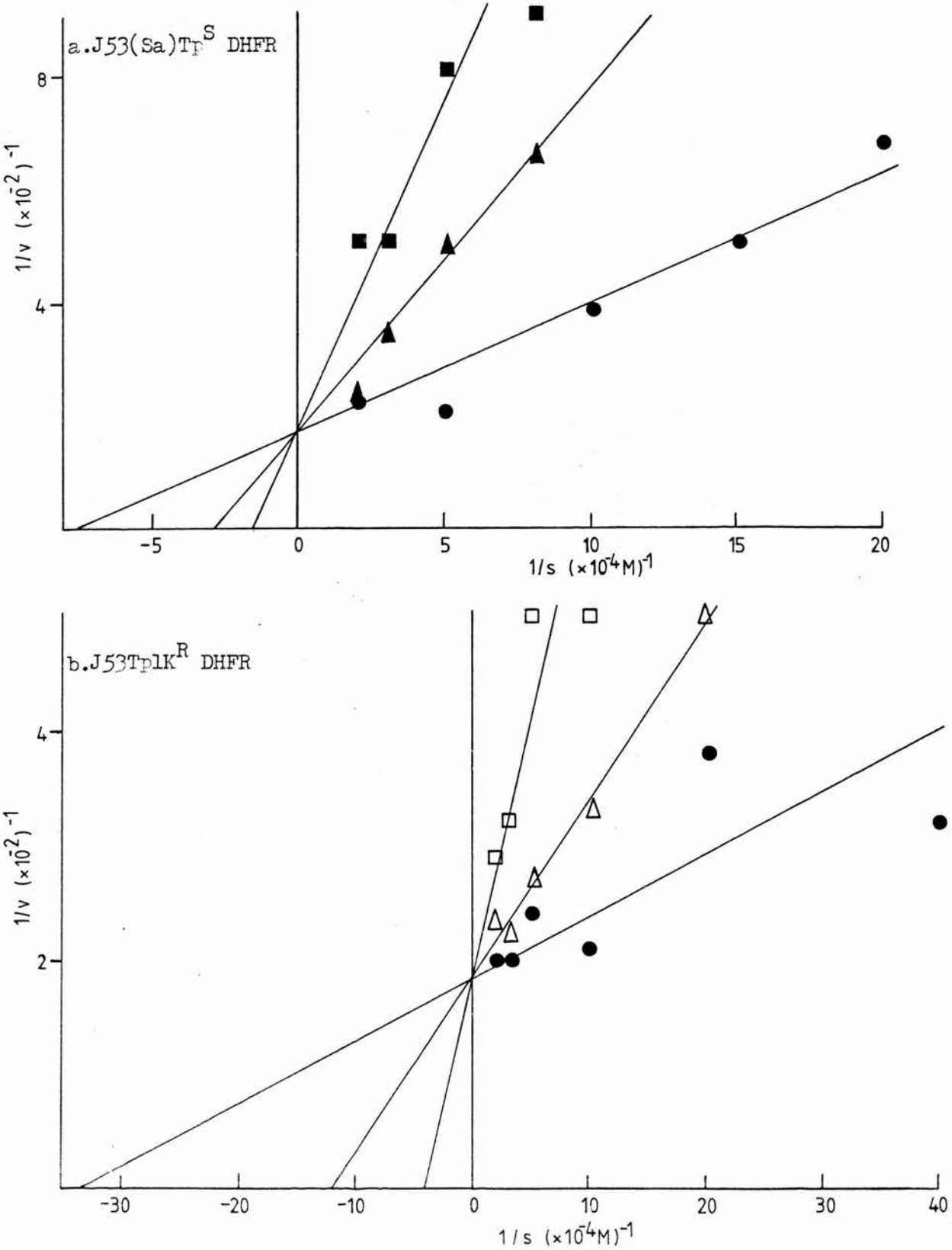
The partially purified J53Tp1K^R and J53(Sa)Tp^S enzymes were reassayed under conditions of partial saturation with DHF and the results analysed by the method of Lineweaver and Burk (1934) to determine the Km valves.

The results (figure 19) show that, in the absence of Tp, a linear response was obtained by plotting the reciprocal of the substrate concentration (1/s) against the reciprocal of the rate of the enzymic reaction (1/v) and hence the enzymes obey Michaelis-Menten kinetics at pH 6.0 and 30°C. The intercept on the abscissa gives the negative of the reciprocal of the Michaelis constant (Km), the Km value being the substrate concentration at which half the maximal velocity occurs (Dixon and Webb, 1958). The Km values for the two enzymes were determined and found to be 1.3 x 10^{-5}M with DHF for the Tp sensitive J53 enzyme and 3.0 x 10^{-6}M with DHF for the Tp resistant J53Tp1K^R enzyme. Thus the Tp resistant enzyme does have a slightly higher affinity for DHF than the Tp sensitive enzyme.

So far the most striking difference between these two enzymes

Figure 19. Lineweaver-Burk plots of the Tp^S and Tp^R DHFRs from *E. coli* J53(Sa) and *E. coli* J53Tp1K^R.

The reciprocal of the substrate concentration (DHF)(1/s) is plotted against the reciprocal of the DHFR activity (1/v). Tp concentrations: No Tp, ● ; 4 x 10^{-9} M Tp, ▲ ; 4 x 10^{-8}M Tp, ■ ; 2 x 10^{-5}M Tp, △ ; 4 x 10^{-5}M Tp, □ .



appears to be in their affinity for trimethoprim. This would suggest, therefore, that the inhibitor constants (Ki) of the enzymes should differ substantially. Hence, the activity of the Tp sensitive DHFR was assayed as before in the presence of 4×10^{-8}M and 4×10^{-9}M Tp, to determine the kinetic effects of the drug. The double reciprocal plots of 1/v against 1/s, in the presence of Tp, both intercept the ordinate at the same position (figure 19a). This intercept was also that obtained in the absence of Tp. This indicates that Tp inhibition of this enzyme is competitive with respect to DHF as the maximum rate is unaffected. However, the plots in the presence of Tp intercept the abscissa nearer the origin than the uninhibited slope. If the distance to the origin from the intercept is - 1/Kp:

$$\text{then } Ki = \frac{i}{\frac{Kp}{Km} - 1}$$

where i is the concentration of the inhibitor (Dixon and Webb, 1958). The Ki values obtained for the two Tp concentrations used were 1.05×10^{-8}M and 2.3×10^{-9}M with a mean value of 6.4×10^{-9}M, indicating that the inhibitor bound firmly to the enzyme.

The purified Tp resistant J53TplK^R DHFR was also assayed at various limiting DHF concentrations with two relatively high concentrations of Tp. The results (figure 19b) show that a linear response was obtained and these intercepted the ordinate at the same position observed in the absence of Tp. Thus this enzyme, though resistant to Tp, is also inhibited competitively by the drug. The Ki

values at 2.0 x 10^{-5}M and 4.0 x 10^{-5}M Tp were calculated as before and values of 1.13 x 10^{-5}M and 6.32 x 10^{-6}M were obtained respectively. The mean Ki value of Tp was hence 8.81 x 10^{-6} for the J53TplK^{R} Tp resistant enzyme. Thus this enzyme has a very low affinity for Tp with a mean Ki value approximately 1000 times greater than the Tp sensitive J53 enzyme. This matches the difference between the respective ID_{50}s for Tp.

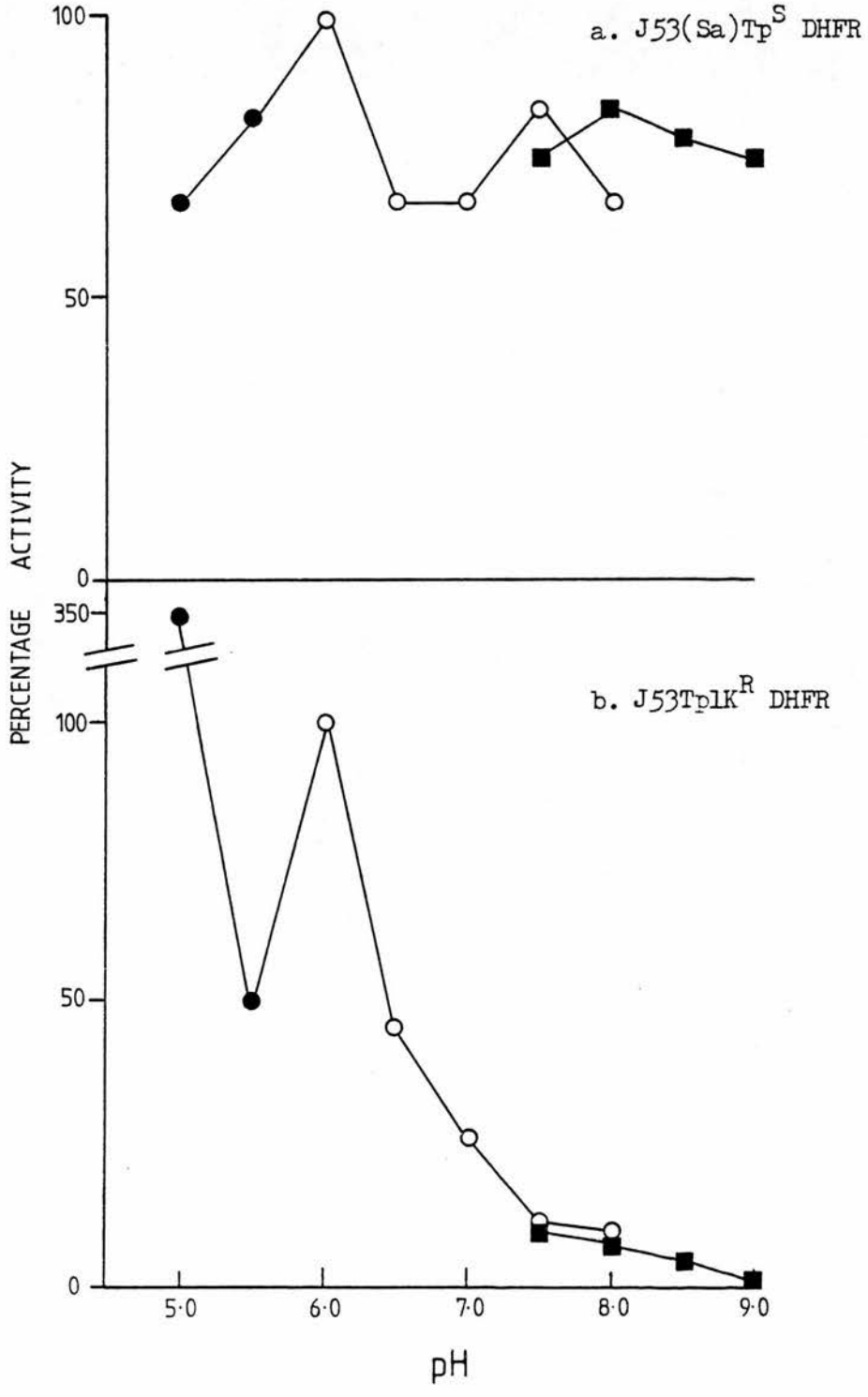
(x) pH profile of DHFRs

Pattishall et al. (1977) and Amyes and Smith (1978) have shown that there are significant differences between the pH profiles of E. coli chromosomal DHFRs and the Type I and Type II R-plasmid encoded enzymes. Whereas the R-plasmid Tp resistant enzymes exhibit distinct peaks of activity between pH 5.5 and 6.5, the Tp sensitive DHFRs show a broad spectrum of activity over almost the complete pH range of 5.0 to 9.0.

The pH profiles of both the Tp sensitive and Tp resistant J53 enzymes were determined. The pooled fractions of each enzyme after gel filtration were assayed at pH values between 5.0 and 9.0. Sodium acetate buffer was employed for pH's from 5.0 to 5.5, sodium phosphate buffer for pH's from 6.0 to 8.0 and Tris-HCl buffer for pH's 7.5 to 9.0. Controls were performed at each pH value tested to correct for decreases in the absence of the enzyme. The results (figure 20) show that the two enzymes differ significantly in their pH profiles. The Tp sensitive enzyme exhibits a broad range of

Figure 20. The pH activity curve for the Tp^S and Tp^R DHFRs from E. coli J53(Sa) and E. coli J53Tp1K^R.

The activity of DHFR is expressed as a percentage of the maximum activity at pH 6.0.
The buffers employed were sodium acetate, ● ; sodium phosphate, ○ ; Tris-HCl, ■ .



activity over the complete pH range tested (figure 20a). When assayed in the presence of sodium phosphate buffer there appear to be two small peaks of enzyme activity at pH 6.0 and 7.5. However, the percentage activity over the entire pH range remained above 65% which is similar to the profiles obtained for other chromosomal DHFRs (Baccanari et al., 1975; Pattishall et al., 1977; Amyes and Smith, 1978).

The pH profile of the Tp resistant J53 DHFR, on the other hand, was very different. A sharp peak of activity was observed at pH 6.0 (figure 20b) which is characteristic of the Type II plasmid encoded enzymes (Pattishall et al., 1977). However, an anomalously high result was obtained when this enzyme was assayed in sodium acetate buffer pH 5.0. Over three times the activity was recorded at this pH compared with pH 6.0. Nevertheless, with the exception of this anomaly, the pH profile of this Tp resistant enzyme bears considerable resemblance to those previously reported for plasmid encoded DHFRs.

(xi) Substrate profile of DHFRs

The natural substrates for E. coli DHFR have been reported to be NADPH and DHF (Burchall and Hitchings, 1965). However, these authors also showed that NADH was about 25% as efficient as NADPH, as the reductant for DHF. They found that folic acid could not, however, be substituted for DHF, in the presence of either NADH or NADPH. Subsequently Amyes and Smith (1978) showed that R-plasmid mediated

enzymes exhibit no detectable activity when NADH is substituted for NADPH, or when folic acid is substituted for DHF.

The two DHFRs from E. coli J53 were therefore, assayed using NADH instead of NADPH and substituting folic acid for DHF. The enzyme preparations used were the partially purified DHFRs following gel filtration. Unlike the previous reports, no DHFR activity could be detected when NADH or folic acid were substituted for the natural substrates. Hence, both the Tp resistant and Tp sensitive enzymes of J53 appear to be specific for the substrates DHF and NADPH.

(xii) Inhibition of DHFR activity at 45°C

The temperature sensitivity of each of the partially purified enzyme preparations was measured. Each preparation was maintained at 45°C. At various time intervals samples were withdrawn and assayed for DHFR activity as described. Both enzymes were found to be relatively stable on heating at 45°C, each retaining 60% of its activity following 4 minutes of incubation at this temperature. These results are typical of both the Type II R-plasmid encoded enzymes and the Tp sensitive chromosomally mediated DHFRs of E. coli strains (Amyes and Smith, 1978; Tennhammar-Ekman and Sköld, 1979; Broad and Smith, 1982).

B. The continuing evolution of the Tp resistance gene

During routine isolation of strains from the Orthopaedic Unit of Edenhall Hospital, near Edinburgh, it was revealed that a surge of Tp resistance was occurring within the Gram negative rods isolated from urine specimens. This highly resistant bacterial population, when further investigated, was shown to consist of both strains which possessed R-plasmids and were able to transfer their Tp resistance and strains which appeared to be devoid of R-plasmids and were unable to transfer their resistance (Amyes et al., 1982). The diversity of Tp resistance exhibited by this bacterial population suggested that a detailed examination of these isolates might serve to illustrate the spread and evolution of the Tp resistance gene, both on R-plasmids and on resistance transposons, within clinical isolates.

1. Genetical Analysis

(i) Tp resistance within clinical isolates

Clinical strains isolated from urinary tract infections were kindly supplied by the Central Microbiology Laboratories, Edinburgh, on MacConkey agar plates. Each strain was cultured overnight in Oxoid No. 2 broth. The cultures were diluted 1 in 10^4 in DM base and spotted, as described in the Materials and Methods, onto DSTA plates containing Tp at 0, 10, 100 and 1000 mgL^{-1}. The results (table 15) show that an unprecedented 64% of the isolates were insusceptible to Tp at a concentration of 10 mgL^{-1} and of these 39.8% were resistant

to 1000 mgL^{-1} Tp. A relatively low percentage (45%) of the resistant isolates were E. coli and subsequently a much larger proportion were Klebsiella, Proteus and Pseudomonas species.

Table 15 Percentage of isolates resistant to trimethoprim

Species	Percentage of organisms with MIC of Tp (mgL^{-1})			
	10	100	1000	>1000
E. coli	18.0	6.5	7.0	13.5
Klebsiella spp.	12.5	4.5	8.5	4.5
Proteus spp.	5.5	5.0	3.0	4.0
Pseudomonas spp.	0.0	0.0	3.5	3.5
Aeromonas spp.	0.0	0.5	0.0	0.0

A representative sample of those organisms with minimum inhibitory concentrations (MIC) of Tp of 100 mgL^{-1} or more were examined in detail for the presence of Tp R-plasmids. Each strain was grown in nutrient broth and conjugated for 5 hours with the rifampicin resistant E. coli K-12 strain J62-2. Trimethoprim resistant recipients were selected on suitably supplemented DM plates containing rifampicin and trimethoprim. If no transfer occurred, the mating incubation was increased to 18 hours. A total of 15 strains were shown to transfer Tp resistance (table 16).

Table 16 Number of strains resistant to high levels of trimethoprim

Species	MIC of Tp (mgL^{-1})			
	100-1000		>1000	
	tra^-	tra^+	tra^-	tra^+
E. coli	4	0	3	4
Klebsiella spp.	5	0	3	3
Proteus spp.	1	4	2	4
Pseudomonas spp.	5	0	4	0

tra^- = non-transferable
tra^+ = self-transferable

Eleven of these strains had characteristic MICs of Tp greater than 1000 mgL^{-1}. However, the other four strains, all of which were identified as *Proteus* spp., had MICs of Tp of only 100 mgL^{-1}. Although the level of Tp resistance in these *Proteus* isolates was low, transfer of the R-plasmids to the standard *E. coli* J62-2 strain was accompanied by an increase in the level of Tp resistance, to greater than 1000 mgL^{-1} in the transconjugant strain.

In addition to those strains exhibiting self-transferable Tp resistance, a large number of organisms were found to possess high-level (MIC >1000 mgL^{-1}), non-transferable Tp resistance. These isolates were subsequently investigated for the presence of transposons in the chromosome.

(ii) Characterisation of R-plamsids

The resistance patterns were determined for each of the Tp R-plasmids isolated. The Tp resistant transconjugant strains were grown in nutrient broth and diluted 1 in 10^4 in DM base. The diluted cultures were then inoculated onto DSTA plates containing various drugs at the concentrations listed in the Materials and Methods. The plates were incubated overnight at 37°C. The results (table 17) show that five different resistance patterns were exhibited by the R-plasmids.

Table 17 Characterisation of Trimethoprim R-plasmids

No. of plasmids	Resistance pattern	Size (Mdal)
1	TpAp	25.0
1	TpAp	37.1
2	TpApSx	38.9
2	TpApSx	28.8
3	TpApSxSm/Sp	40.1
2	TpApSxSm/SpTcCe	39.8
4	TpApSxSm/SpTcCeCmGm	35.0

In addition, the molecular weights of the R-plasmids were determined. R-plasmid DNA was extracted from each of the E. coli transconjugant strains by the method of Meyers et al. (1976) as described in the

Materials and Methods. Alcohol precipitated DNA was then analysed by agarose gel electrophoresis. The results (table 17) show that there was a minimum of seven different plasmid types present within the Gram negative rods isolated from these urine specimens, because some plasmids of the same resistance pattern were of different sizes.

(iii) Extraction of Tp resistance transposons from clinical R-plasmids

The diversity of R-plasmids indicates that the spread of Tp resistance was not manifested by a single R-plasmid. Thus a representative sample of six R-plasmids were subsequently investigated for the presence of transposons carrying Tp resistance. The conjugation experiments involved in the detection of two transposons are summarised in table 18. The kanamycin (Km) resistant inc P group plasmid, RP4, was employed as a vector for the identification of Tp resistance transposons that may be present in the Tp R-plasmids.

RP4 was introduced into standard E. coli J62-2 strains, each harbouring a different R-plasmid, during a 5 hour mating as described in the Materials and Methods (table 18 steps a). Selection was made on suitably supplemented DM plates for J62-2 recipients of plasmid RP4. The transconjugants were purified on the same medium and were then subcultured twice in nutrient broth in the absence of antibiotic selection pressures, to allow any transposition events to take place. The purified J62-2 transconjugants were then used as donors in a subsequent mating with E. coli strain J53. The mating was for 5

Table 18 Extraction of Tp resistance genes from the clinical plasmids pUK179 and pUK173

STEP	DONOR	RECIPIENT	TRANSCONJUGANT	SELECTION	TRANSFER FREQUENCY/ DONOR CELL
a	J53(RP4)	J62-2(pUK179)	J62-2(pUK179)(RP4)	Km	9.6×10^{-4}
b	J62-2(pUK179)(RP4)	J53	J53(RP4::Tn4129)	TpKm	2.6×10^{-6}
				Tp	2.8×10^{-5}
				Km	1.7×10^{-4}
c	J53(RP4::Tn4129)	J62	J62(RP4::Tn4129)	TpKm	1.8×10^{-5}
				Tp	3.3×10^{-5}
				Km	1.8×10^{-5}
a	J53(RP4)	J62-2(pUK173)	J62-2(pUK173)(RP4)	Km	2.0×10^{-2}
b	J62-2(pUK173)(RP4)	J53	J53(RP4::Tn4133)	TpKm	7.1×10^{-6}
				Tp	1.1×10^{-3}
				Km	1.2×10^{-3}
c	J53(RP4::Tn4133)	J62	J62(RP4::Tn4133)	TpKm	1.7×10^{-3}
				Tp	3.1×10^{-3}
				Km	1.4×10^{-3}

hours and selection was made for the transfer of Tp and Km resistance determinants both separately and together. The transfer frequencies for the individual Tp and Km resistance genes differed from those obtained when selection was made for the two resistances together (table 18 steps b). This indicates that the Tp and Km resistance determinants are transferring independently of each other. However, a single colony, isolated from the Tp/Km plate and purified on the same medium, was subsequently used as a donor in a further one hour mating with strain J62. The transfer frequencies of the Tp and Km resistance genes were again measured, both separately and together. The results (table 18 steps c) showed that the transfer frequency of each resistance determinant was now similar to that obtained when selection was made for the two resistance genes together. This indicates that the Tp resistance determinant is now transferring with the Km resistance gene and hence suggests that the Tp resistance gene has transposed into RP4.

In order to confirm that the plasmid RP4 is indeed present in these transconjugants, purified Tp/Km resistant J62 strains were tested for their sensitivity to phage PR4 which is specific for plasmids of inc groups P, N and W. Each organism was taken from a DM plate containing Tp and Km and streaked onto a nutrient agar plate. A concentrated preparation of phage PR4 was spotted onto each plate and these were then incubated overnight at 37°C. The presence of RP4 was confirmed if a clear zone of bacterial lysis had occurred at the area of inoculation with PR4. In each case lysis was observed. Strains of J62-2 containing the original clinical plasmids were also

tested and showed no lysis with phage PR4 indicating that none of the original plasmids belong to inc groups P, N or W.

Each Tp/Km resistant transconjugant was also tested for the simultaneous acquisition of the Tc and Ap resistance determinants that are also determined by RP4. Each strain was grown in nutrient broth and diluted 1 in 10^4 in DM base. The diluted cultures were then inoculated onto DSTA plates containing the appropriate antibiotics, at a concentration of 10 mgL^{-1}, with a multiple inoculator. In every case, all three RP4 resistances were present in addition to Tp.

Finally, the Tp resistant transconjugants were examined for the physical presence of plasmid DNA. DNA was extracted from the cells by the method of Meyers et al. (1976) and analysed by electrophoresis in 0.5% agarose gels. In each case only one covalently closed supercoiled band of plasmid DNA was detected, indicating the sole presence of plasmid RP4.

Tp resistance transposons were extracted from each of the six clinical plasmids investigated. These transposons were originally given the designations TnGB4-TnGB9 but have now been re-classified as Tn4129-Tn4134 (table 19).

Table 19 Clinical plasmids and their transposons

Clinical plasmid			Transposon	
No.	Resistance	Size (Mdal)	No.	Former Designation
pUK179	TpApSm/SpSxCeTc	39.8	Tn4129	TnGB4
pUK217	TpApSx	28.8	Tn4130	TnGB5
pUK218	TpApSx	28.8	Tn4131	TnGB6
pUK163	TpApSx	38.9	Tn4132	TnGB7
pUK173	TpAp	25.0	Tn4133	TnGB8
pUK176	TpApSm/SpSx	40.1	Tn4134	TnGB9

(iv) Extraction of Tp resistance transposons from the chromosome of clinical strains

Organisms with high level (MIC >1000 mgL^{-1}), non-transferable Tp resistance were investigated for the presence of transposons located in the chromosome. Preliminary experiments revealed that 8 of the 11 strains were resistant to a wide variety of antimicrobials including those carried by plasmid RP4. It was thus impossible to use RP4, or other commonly-used standard plasmids as vectors for any transposons present. Hence, only three organisms, E. coli strains E7 and E27 and Proteus sp. E16, were further investigated.

Each of the three clinical isolates was examined initially for the presence of plasmid DNA. DNA was extracted by the method of Meyers et al. (1976) and separated by electrophoresis in 0.5% agarose

gels. However, no plasmid DNA could be detected by this procedure.

The Tp resistance gene was then extracted from the chromosome of each of these organisms employing RP4 as the plasmid vector. The conjugation experiments and results for the clinical isolate E. coli strain E27 are shown in table 20. Plasmid RP4 was introduced into the clinical strains during an 18 hour mating (table 20a) and similar conjugation experiments were performed as before. After the introduction of RP4 into the clinical strains, the transconjugants were purified and then subcultured twice in nutrient broth in the absence of antimicrobial drugs to allow transposition to occur. Plasmid RP4 was then transferred into the rifampicin resistant E. coli strain J62-2 during a 5 hour mating, and selection was made for the transfer of the Tp and Km resistance determinants. Tp resistance now appeared to transfer but at a lower frequency than the Km resistance determinant of RP4 (table 20b). This suggests that the Tp resistance gene in some of the donor cells has been mobilised by plasmid RP4.

A transconjugant taken from a Tp containing selection plate was purified on the same medium and then used as a donor in a further 5 hour mating with strain J53. Selection was again made for the transfer of Tp and Km resistance genes. The results (table 20c) showed that the transfer frequencies of both resistance determinants were now identical indicating that transposition of the Tp resistance gene into RP4 had taken place.

Table 20 Extraction of the Tp resistance gene from the chromosome of *E. coli* E27

STEP	DONOR	RECIPIENT	TRANSCONJUGANT	SELECTION	TRANSFER FREQUENCY/ DONOR CELL
a	J53(RP4)	*E. coli* E27	*E. coli* E27(RP4)	Km	1.3×10^{-2}
b	*E. coli* E27(RP4)	J62-2	J62-2(RP4::Tn4126)	Tp	1.8×10^{-6}
				Km	4.1×10^{-5}
c	J62-2(RP4::Tn4126)	J53	J53(RP4::Tn4126)	Tp	4.4×10^{-3}
				Km	3.2×10^{-3}

This transposition event was confirmed using the same criteria as before. Each purified Tp resistant transconjugant was shown to be sensitive to phage PR4, resistant to both Tc and Ap in addition to Tp and Km and gave a single plasmid band when analysed by agarose gel electrophoresis.

Tp resistance transposons were extracted from the chromosome of each of the clinical isolates examined, and were designated Tn4126, Tn4127 and Tn4128 (former designations: TnGB1, TnGB2 and TnGB3 respectively).

(v) Characterisation of the Tp resistance transposons

Characterisation of the Edenhall transposons reported here initially involved the determination of their resistance pattern. E. coli J62 strains harbouring plasmid RP4 containing the transposons were grown aerobically in nutrient broth for 5 hours. Each culture was then diluted 1 in 10^4 in DM base and inoculated onto DSTA plates containing a range of antibacterial drugs. E. coli strain J62 with and without the original plasmid vector RP4 was used as a control. The results showed that the only resistances present in addition to those carried by RP4, were Tp resistance and in some strains Sm and Sp resistance. The MICs of these three drugs were then determined for each strain. The results (table 21) suggest the presence of two different transposons within this bacterial population. The three transposons Tn4126, Tn4127 and Tn4128 extracted from the chromosome of strains E27, E7 and E16 respectively, confer resistance to both Tp

Table 21 Antibacterial resistance levels conferred by transposons

Organism	Minimum inhibitory concentration (mgL^{-1})		
	Tp	Sm	Sp
J62	1	4	10
J62(RP4)	1	4	10
J62(RP4::Tn4126)	>1000	40	200
J62(RP4::Tn4127)	>1000	40	200
J62(RP4::Tn4128)	>1000	40	200
J62(RP4::Tn4129)	>1000	40	200
J62(RP4::Tn4130)	>1000	40	200
J62(RP4::Tn4131)	>1000	40	200
J62(RP4::Tn4132)	>1000	8	20
J62(RP4::Tn4133)	>1000	8	20
J62(RP4::Tn4134)	>1000	8	20

and Sm/Sp. Similarly transposons Tn4129, Tn4130 and Tn4131 extracted from plasmids pUK179, pUK217 and pUK218 also carried resistance to Sm/Sp in addition to Tp. However, the three transposons Tn4132, Tn4133 and Tn4134, extracted from plasmids pUK163, pUK173 and pUK176 were shown to possess resistance to Tp alone, with MICs of Sm and Sp of 8 mgL^{-1} and 20 mgL^{-1} respectively.

(vi) Molecular characterisation of the transposons

In order to determine the size of the transposons, the molecular weight (MW) of plasmid RP4 was measured by agarose gel electrophoresis, both before and after transposition of Tp resistance genes into the plasmid had taken place. Plasmid DNA was prepared by the method of Meyers et al. (1976) and separated by electrophoresis in 0.5% agarose gels. The position of the plasmids was compared with those of the standard plasmids, Sa (23 Mdal), R6K (26 Mdal), R15 (40 Mdal) and R1 (62 Mdal), which were run concurrently (figures 21a, b). The MW of plasmid RP4 and RP4 transposon containing derivatives were then estimated (Table 22). The increase in MW of RP4 represents the size of the Tp resistance transposons.

The three chromosomal transposons, Tn4126, Tn4127 and Tn4128 each measured 9 Mdal in size (figure 21a, tracks 5, 6, 7). Similar sizes of 8 Mdal were calculated for Tn4129, Tn4130 and Tn4131 (figure 21b, tracks 9, 10, 11). These six transposons each carry a resistance determinant to Sm/Sp in addition to Tp and would therefore appear to be similar to the well characterised TpSm/Sp resistance

Figure 21a. Agarose gel electrophoresis of RP4 derivatives containing transposons extracted from the chromosome of clinical strains.

Track 1, Sa and R1; track 2, Sa; track 3, R15;
track 4, RP4; track 5, RP4::Tn4126; track 6, RP4::Tn4127;
track 7, RP4::Tn4128.
Electrophoresis was in 0.5% agarose at 80V for 16 hours.

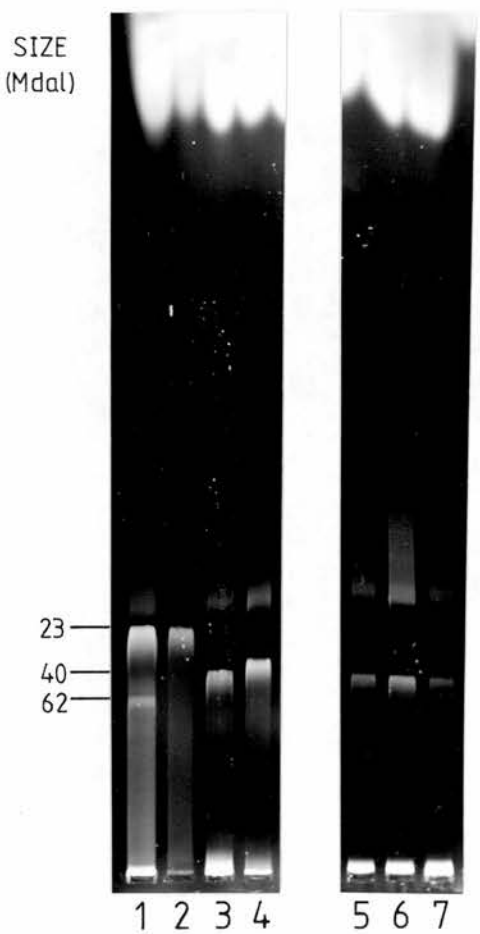
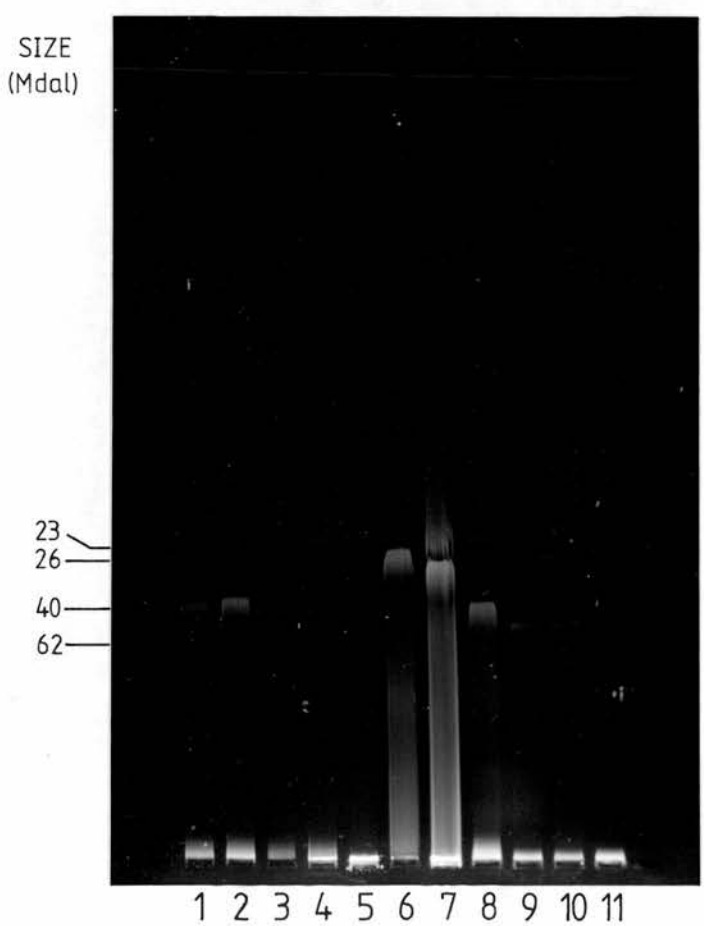


Figure 21b. Agarose gel electrophoresis of RP4 derivatives containing transposons extracted from clinical R-plasmids.

Track 1, RP4::Tn4132; track 2, RP4::Tn4133; track 3, RP4::Tn4134; track 4, R15; track 5, R1; track 6, Sa; track 7, R6K; track 8, RP4; track 9, RP4::Tn4129; track 10, RP4::Tn4130; track 11, RP4::Tn4131. Electrophoresis was in 0.5% agarose at 80V for 16 hours.



transposon Tn7 (Barth et al., 1976).

Table 22 Molecular weights of RP4 and RP4 transposon containing derivatives

Plasmid	Size (Mdal)
RP4	38
RP4::Tn4126	47
RP4::Tn4127	47
RP4::Tn4128	47
RP4::Tn4129	46
RP4::Tn4130	46
RP4::Tn4131	46
RP4::Tn4132	38
RP4::Tn4133	36
RP4::Tn4134	40

Transposons Tn4132, Tn4133 and Tn4134, however, carry the single resistance determinant to Tp and, when present in plasmid RP4, give quite different results from those obtained for the other six transposons (figure 21b, tracks 1, 2, 3). Plasmid RP4 harbouring Tn4134 showed only a small increase in the MW of RP4 (figure 21b, track 3), the size of the insertion being calculated as 2 Mdal. In contrast, RP4::Tn4133 derivatives showed a similar decrease of about 2 Mdal in the size of RP4 (figure 21b, track 2). RP4::Tn4132 derivatives, on the other hand, showed no change in the MW of RP4

(figure 21b, track 1).

Previous reports have shown that insertion of transposons into RP4 may cause small deletions of RP4 at the site of insertion (Barth et al., 1978; Datta et al., 1979; McCombie et al., 1983). This phenomenon may therefore explain the result obtained for RP4::Tn4132 derivatives. Insertion of Tn4132 into RP4 may have resulted in a small deletion of about 2 Mdal in the size of RP4. It is conceivable, therefore, that Tn4132 may be of a similar size to Tn4134. However, if the size of Tn4133 were to be explained by this deletion event, a deletion of approximately 4 Mdal would have to have occurred. This amount represents a relatively large proportion of RP4 and, therefore, the similarity of these three transposons was further investigated by restriction enzyme digestion of the RP4 transposon containing derivatives.

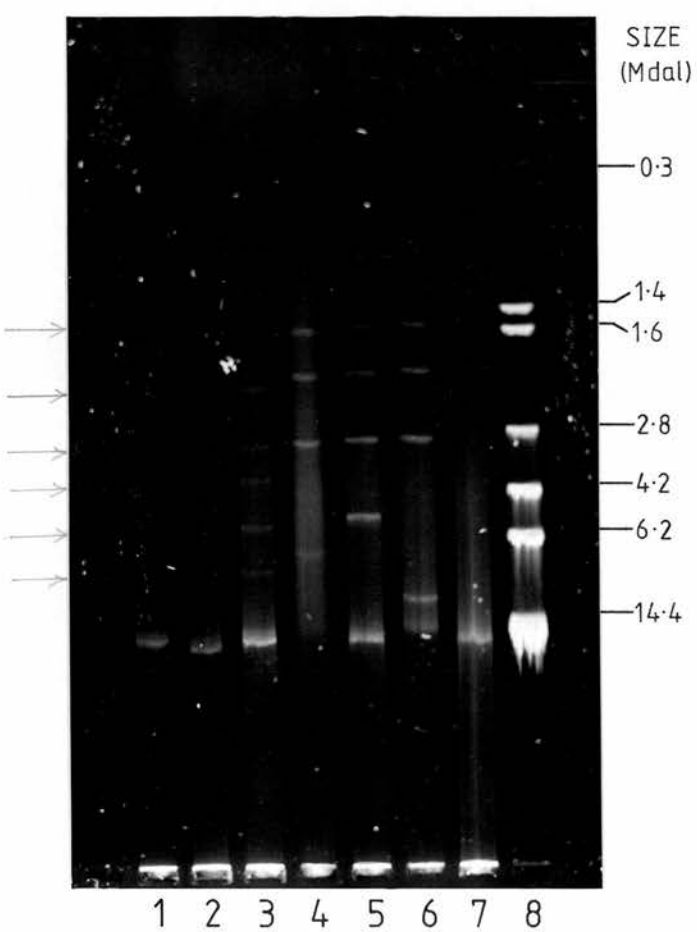
(vii) Restriction enzyme analysis of transposons

Plasmid DNA was prepared by the method of Holmes and Quigley (1981). Each preparation was digested for one hour with the restriction endonuclease Hind III, under the conditions recommended by the Boehringer Corporation Ltd. The reaction was terminated by cooling to 4°C and the simultaneous addition of the recommended 'stopping buffer'. Restricted fragments were then analysed immediately on 0.5% agarose gels run at 140 volts for three and a half hours.

The results (figure 22, tracks 1, 2, 3) show that the RP4 derivatives containing transposons Tn4132, Tn4133 and Tn4134 have

Figure 22. Agarose gel electrophoresis of RP4 and RP4 transposon containing derivatives after digestion with the restriction endonuclease Hind III.

Track 1, RP4::Tn4132; track 2, RP4::Tn4133; track 3, RP4::Tn4134; track 4, RP4::Tn4129; track 5, RP4::Tn4130; track 6, RP4::Tn4131; track 7, RP4; track 8, λ DNA after digestion with Hind III.
Electrophoresis was in 0.5% agarose at 140V for 3½ hours.



identical restriction endonuclease digest patterns. A total of seven fragments were present (arrowed) in each of these plasmid derivatives. When the original plasmid RP4 was digested with Hind III, a single band of DNA was observed (figure 22, track 7), confirming that RP4 has only one restriction site for this enzyme. The large number of fragments present in the RP4 transposon derivatives suggests, therefore, that either these transposons have several restriction sites for this enzyme, or, more probably, that the transposons have inserted into RP4 at multiple sites.

The sizes of the smaller fragments were estimated by comparison with those obtained for lambda DNA following digestion with Hind III (figure 22, track 8) (Murray and Murray, 1975). The fragment sizes were 1.65 Mdal, 2.2 Mdal, 3.3 Mdal, 4.1 Mdal, 5.7 Mdal and 8.8 Mdal. The MWs of some of the larger fragments suggest that some of them may possibly have been formed as a result of oligimerisation of smaller fragments, for example, the fragment corresponding to a MW of 3.3 million may be a result of dimerisation between two 1.65 Mdal fragments.

In order to compare the relatedness of the small transposons with Tn4129, Tn4130 and Tn4131, RP4 derivatives containing these transposons were similarly digested with Hind III. The results (figure 22, tracks 4, 5, 6) show that each derivative gives rise to five restriction fragments. Three of the fragments are present in each of the three plasmid derivatives and correspond to MWs of 1.65, 2.0 and 3.3 million. The other two fragments, which are considerably

larger, differ in size between each plasmid derivative. Digestion of RP4 containing the 9 Mdal TpSm/Sp resistance transposon, Tn7, with Hind III is known to give rise to two small fragments and two much larger fragments (Datta et al., 1979). If the 3.3 Mdal fragment present in the digests performed here is assumed to be a dimer of the 1.65 Mdal fragment, then the transposons Tn4129, Tn4130 and Tn4131 may be classified as Tn7-like Tp resistance transposons. The different sizes of the larger fragments are due to the insertion of each transposon into a different site on RP4.

A comparison of the digest patterns obtained for each of the six RP4 transposon containing derivatives suggests that the smallest 1.65 Mdal fragment of Tn7 may be present in all six transposons, implying that the large and small transposons may be related, the small transposon comprising of only a fraction of the large transposon.

It should be noted that summation of the molecular weights of the fragments obtained for each digestion does not correspond to the MW of the RP4 transposon derivative. This may be explained by the inability to measure the size of the larger fragments, since fragments of molecular weights of greater than about 8 million are unable to be assigned an accurate molecular size by this method (Grinsted et al., 1977).

(viii) Mutation to Sm/Sp resistance of the small transposons

An examination of the resistance patterns of the original clinical R-plasmids harbouring both large and small transposons

revealed that two of the plasmids, pUK217 and pUK218, from which TpSm/Sp resistance transposons had been extracted, did not express Sm/Sp resistance in the original plasmid (table 19). In addition, plasmid pUK176, which does express Sm/Sp resistance, possessed a transposon conferring resistance to Tp alone (table 19). It, therefore appears that under certain conditions the Sm/Sp resistance genes of plasmids are not expressed. For this reason, the ability of the small Tp resistant transposons to mutate to Sm/Sp resistance was investigated in order to determine if these genes are also present within the smaller transposons.

Overnight nutrient broth cultures of the three E. coli J62 strains possessing plasmid RP4 harbouring the transposons Tn4132, Tn4133 and Tn4134 were harvested and resuspended in the same volume of DM base. 0.1 ml of the washed cultures was then spread on DSTA plates containing either Sm (20 mgL^{-1}) or Sp (100 mgL^{-1}). A viable count of each culture was also made on MacConkey agar plates. In addition, similar cultures of E. coli J62 and E. coli J62(RP4) were treated in the same way and acted as controls. The plates were incubated overnight at 37°C and then examined for the presence of resistant colonies. The ratio of the viable count on MacConkey agar to that on plates containing either Sm or Sp was taken as the mutational frequency. The results (table 23) show that strains harbouring transposons Tn4132, Tn4133 and Tn4134 mutate at a frequency of about 4×10^{-7} to Sm/Sp resistance. This frequency of mutation is at least 100 times greater than that observed with J62 and J62 harbouring RP4. The presence of these transposons within the

Table 23 Sm resistance and Sp resistance mutation frequency by strains carrying Tn4132, Tn4133 and Tn4134

The mutation frequency shown is the viable count obtained from an overnight culture on MacConkey agar divided by that obtained on DSTA plates containing $20mgL^{-1}$ Sm or $100mgL^{-1}$ Sp.

Bacterial Strain	Mutation frequency	
	Sm	Sp
J62	$<1.0 \times 10^{-9}$	$<3.0 \times 10^{-9}$
J62(RP4)	2.8×10^{-9}	$<3.0 \times 10^{-9}$
J62(RP4::Tn4132)	2.5×10^{-7}	8.6×10^{-7}
J62(RP4::Tn4133)	4.2×10^{-7}	7.4×10^{-7}
J62(RP4::Tn4134)	4.8×10^{-7}	1.1×10^{-7}

bacterial cell appears, therefore, to increase the frequency of mutation to Sm/Sp resistance.

In order to determine whether this mutation had occurred on the bacterial chromosome or on plasmid RP4, the Sm/Sp resistant mutants were purified on DSTA containing both drugs and used as donors in a one hour mating with E. coli strain J53. Selection was made for the transfer of Tp, Sm and Km resistance determinants, both individually and in combination with each other. The results (table 24b) show that Sm resistance is transferable and hence that the mutation is not chromosomally located. The transfer frequencies of the Tp and Sm resistance determinants measured both separately (table 24a, b) and together (table 24c) appear to be identical, indicating that both these resistance determinants are being transferred together.

However, the transfer frequency of the Tp and Km resistance determinants when measured together (table 24f) is significantly lower than the values obtained when selection is made for the transfer of each resistance gene separately (table 24a, d), implying that the Tp and Km resistance determinants are no longer transferring together. In addition, the transfer frequencies of the Sm and Km resistance determinants, when measured both separately (table 24b, d) and together (table 24e) differ significantly and this would suggest, therefore, that the mutation to Sm resistance has occurred within the Tp resistance transposon rather than on RP4. These mutated transposons will be referred to as Tn4132*, Tn4133* and Tn4134* throughout the remainder of this thesis.

Table 24 Transfer of Tp, Sm and Km resistance determinants from the transposon containing E. coli J62 Sm/Sp resistant mutants to E. coli J53

Step	Donor	Recipient	Selection	Transfer frequency/ donor cell
a	J62(RP4::Tn4132)*	J53	Tp	5.4×10^{-4}
b			Sm	5.0×10^{-4}
c			TpSm	3.6×10^{-4}
d			Km	3.3×10^{-3}
e			KmSm	4.6×10^{-5}
f			KmTp	3.8×10^{-5}
g			TpSmKm	3.2×10^{-5}
a	J62(RP4::Tn4133)*	J53	Tp	2.8×10^{-3}
b			Sm	3.4×10^{-3}
c			TpSm	4.4×10^{-3}
d			Km	3.8×10^{-3}
e			KmSm	5.3×10^{-5}
f			KmTp	4.3×10^{-5}
g			TpSmKm	6.5×10^{-5}
a	J62(RP4::Tn4134)*	J53	Tp	3.1×10^{-3}
b			Sm	1.0×10^{-3}
c			TpSm	1.5×10^{-3}
d			Km	2.9×10^{-3}
e			KmSm	6.7×10^{-5}
f			KmTp	4.0×10^{-5}
g			TpSmKm	1.6×10^{-4}

* represents a Sm resistant, Sp resistant derivative.

(ix) RP4-independent transfer of the mutated transposons

The results of the transfer experiments (table 24) indicate that following mutation of the J62(RP4) transposon containing derivatives to Sm/Sp resistance, the Tp resistance transposons are no longer co-transferring with the Km resistance gene on plasmid RP4. The differences in transfer frequencies of these two resistance determinants, however, may not be indicative of the independent transfer of RP4 and the transposons. That is to say, the transposons may still be transferring on RP4, but in some instances insertion of the transposon into RP4 may have resulted in the 'switching off' of some or all of the RP4 resistance determinants. This hypothesis was tested by determining the proportion of Tp resistant strains which express the RP4 resistance determinants, ie. resistance to Ap, Tc, and Km. Transconjugants selected on Tp containing plates were purified. Overnight broth cultures were diluted 1 in 10^6 and 0.1 ml amounts of the diluted cultures were spread on DSTA plates containing each of the drugs at concentrations of 10 mgL^{-1}. The results (table 25) indicate that approximately 8% of transconjugants harbouring transposon Tn4132*, 5% harbouring Tn4133* and only 3% harbouring Tn4134* express resistance to Tc, Ap and Km.

Those strains, which do not exhibit the RP4 determined resistances, were then examined physically to determine if they still possessed RP4 even though its genes are not being expressed. The DNA was extracted from the Tp resistant Km sensitive transconjugants and analysed by the method of Meyers et al. (1976).

Table 25 Percentage of Tp resistant transconjugants resistant to Km, Ap and Tc

Strain	Percentage resistant strains					
	Tp	Km	Ap	Tc	KmApTc	TpKmApTc
J53(RP4::Tn4132*)	100	10.0	10.0	7.9	7.9	8.6
J53(RP4::Tn4133*)	100	6.7	6.1	5.6	5.2	4.7
J53(RP4::Tn4134*)	100	3.2	2.4	2.6	2.6	2.5

However, no plasmid DNA could be detected by this method. In addition, each transconjugant was also tested for sensitivity to phage PR4 as described previously. All three transposon containing strains were found to be resistant to this phage. Taken together these results suggest that the Tp resistance transposons in these transconjugants are now present within the bacterial chromosome as opposed to the plasmid RP4.

From these results it would appear that the transposons have transferred on RP4, but that on entry into the recipient cell the transposon has immediately inserted into the bacterial chromosome with the simultaneous loss of RP4 from the majority of the recipients. In order to confirm the presence of the transposon within the bacterial chromosome, Tp resistant strains which had been shown to be devoid of plasmid DNA by agarose gel electrophoresis, were employed as donors in a one hour mating with E. coli strain J62. Selection was made for the transfer of Tp resistance. The results

(table 26), however, indicate that Tp resistance is still transferable, the transfer frequency of Tp for each strain being about 1 x 10^{-3} per donor cell.

Table 26 Transfer of the Tp resistance determinant from E. coli J53 strains harbouring transposons Tn4132*, Tn4133* and Tn4134

Donor	Recipient	Transconjugant	Selection	Transfer Frequency/ Donor Cell
J53(Tn4132*)	J62	J62(Tn4132*)	Tp	1.1 x 10^{-3}
J53(Tn4133*)	J62	J62(Tn4133*)	Tp	1.5.x 10^{-3}
J53(Tn4134*)	J62	J62(Tn4134*)	Tp	8.4.x 10^{-4}

These results suggest, therefore, that the transposons Tn4132*, Tn4133* and Tn4134* are able to transfer independently of a plasmid vector. However, it is possible that agarose gel electrophoresis may not have detected the presence of an extremely large plasmid within the donor cell and therefore, to exclude this possibility, a further test was made to ensure that no plasmid DNA was present within the donor strain.

A one litre stationary phase culture of the donor strain J53(Tn4133*) was harvested and the DNA extracted as before. Alcohol

precipitated DNA was then subjected to caesium chloride density gradient centrifugation in the presence of ethidium bromide (EtBr). Centrifugation was for 64 hours at 18°C and at 38,000 rpm (96,000g) in a Beckman SW50 rotor. Following centrifugation the tube was examined under the UV light for the presence of EtBr stained bands of DNA. Two controls, a J62 strain known to be devoid of plasmid DNA and a J62 strain possessing the 62 Mdal plasmid R1, were run concurrently. The results (figure 23) show that the J62 control (tube 1) possesses one band of DNA corresponding to linear fragments of chromosomal DNA. The J62(R1) control (tube 2), on the other hand, gave 3 bands, an upper band consisting of linear fragments of chromosomal DNA, a middle band representing open circular plasmid DNA and a lower band corresponding to covalently closed circular plasmid DNA. The results for J53(Tn4133*) (tube 3) show the presence of only one band of linear chromosomal DNA.

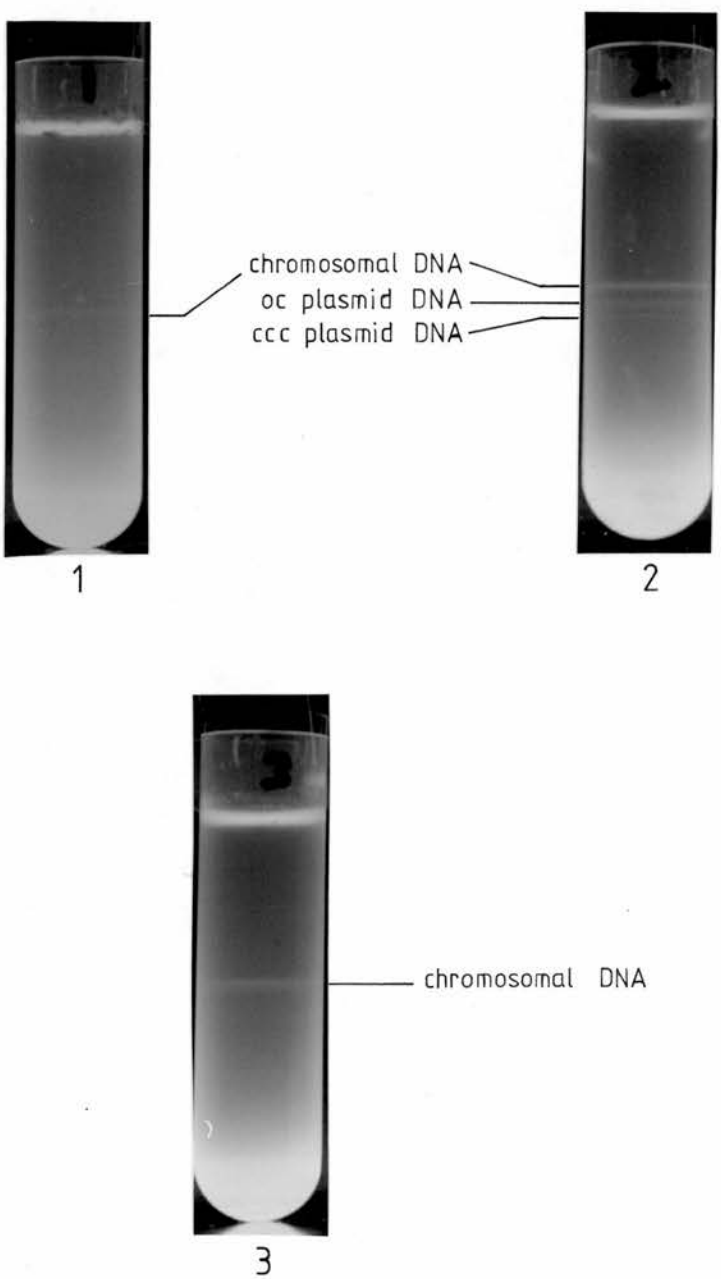
Thus it would appear that the Tp resistant donor strain did not possess any plasmid DNA and hence that the Tp resistance transposon is now somehow able to mediate its own transfer.

(x) Transposition of the mutated transposons into plamsid R1E

The presence of the mutation to Sm resistance within the Tp resistance transposon suggests that further transposition of Tp resistance to another replicon should now be accompanied by the simultaneous acquisition of resistance to Sm and Sp. In order to test this hypothesis an attempt was made to transpose the mutated Tp

Figure 23. Ethidium bromide/caesium chloride density gradient centrifugation of DNA extracted from E. coli J53(Tn4133*).

Tube 1, DNA extracted from E. coli J62 control;
tube 2, DNA extracted from E. coli J62(R1) control;
tube 3, DNA extracted from E. coli J53(Tn4133*).
oc = open circular; ccc = covalently closed circular.



resistance transposons from RP4 into the inc F_{II}, Cm resistance plasmid RlE. The conjugation experiments involved were similar to those described previously for transposition into RP4. The results for the mutated transposon, Tn4133*, are shown in table 27. Plasmid RlE was introduced into the J53(RP4::Tn4132*), J53(RP4::Tn4133*) and J53(RP4::Tn4134*) transconjugants by a five hour mating (table 27a). Following two subcultures in nutrient broth in the absence of antibiotic selection pressures, RlE was transferred into J62 during a 1 hour mating and selection was made for the transfer of Tp and Cm resistance determinants both separately and together. The results (table 27b) show that each resistance determinant is transferring independently of the other. A TpCm resistant transconjugant was then purified and used as a donor in a 1 hour mating with E. coli strain J53. Selection was again made for the transfer of Tp and Cm resistance determinants. The results (table 27c) again suggest that these two resistance determinants are transferring independently.

Following purification, the TpCm resistant transconjugants were tested for the presence of plasmid RP4 both by multiple inoculation onto DSTA plates containing Ap, Km and Tc, each at 10 mgL^{-1}, and by testing their sensitivity to phage PR4. All three transposon containing transconjugants were found to be sensitive to these drugs but resistant to phage PR4 indicating that plasmid RP4 had been lost from the cell. Similarly, each transconjugant was tested for resistance to Sm and Sp. However, none expressed resistance to these antibiotics, indicating the the Sm/Sp resistance mutation present within the Tp resistance transposons is unstable. Thus resistance to

Table 27 Transfer of Tn4133* from plasmid RP4 to plasmid RlE

STEP	DONOR	RECIPIENT	TRANSCONJUGANT	SELECTION	TRANSFER FREQUENCY/ DONOR CELL
a	J62(RlE)	J53(RP4::Tn4133*)	J53(RP4::Tn4133*)(RlE)	Cm	3.9×10^{-2}
b	J53(RP4::Tn4133*)(RlE)	J62	J62(RlE::Tn4133*)	Tp	1.2×10^{-2}
				Cm	8.1×10^{-3}
				TpCm	7.7×10^{-5}
c	J62(RlE::Tn4133*)	J53	J53(RlE::Tn4133*)	Tp	6.0×10^{-3}
				Cm	3.1×10^{-2}
				TpCm	3.1×10^{-4}
d	J53(RlE::Tn4133*)	J62	J62(RlE::Tn4133*)	Tp	7.6×10^{-3}
				Cm	1.1×10^{-2}
				TpCm	4.3×10^{-5}

* represents a Sm resistant Sp resistant derivative.

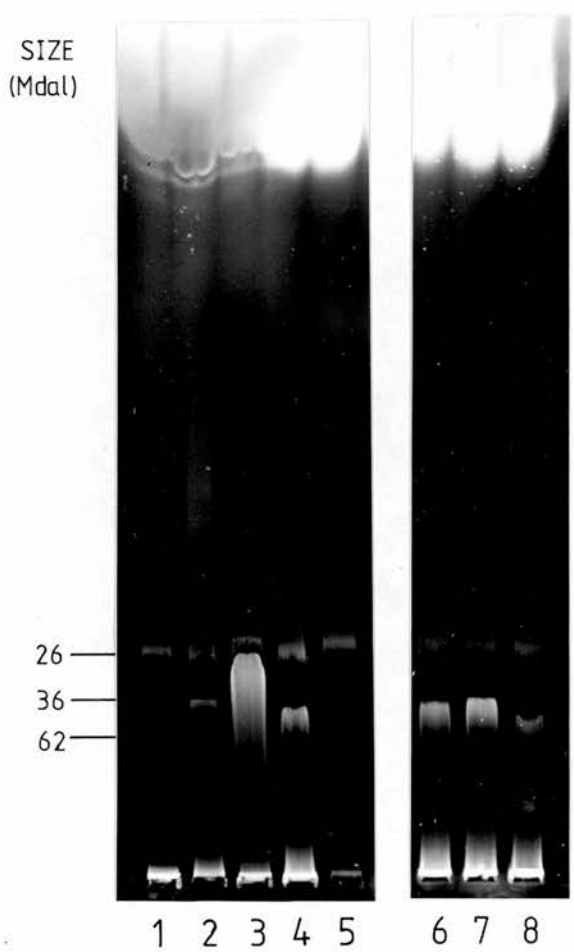
Sm/Sp is lost during subculture in the absence of selective pressures.

Despite a further transfer of Tp and Cm resistance determinants, similar transfer frequencies for both resistance genes could not be demonstrated (table 27d). Nevertheless, following purification, plasmid DNA was extracted from the final J62 TpCm resistant transconjugants and subsequently analysed by agarose gel electrophoresis as described previously. The results (figure 24) show that both strains harbouring Tn4132* (track 7) and Tn4133* (track 6) possess plasmid RlE with a MW of 41 Mdal. The relatively large size of RlE, however, renders it impossible to determine whether these small MW transposons are present on this R-plasmid. On the other hand, the results obtained with Tn4134* (figure 24, track 8) show that RlE has increased in size to 49 Mdal. This result suggests, therefore, that Tn4134* could now measure 8 Mdal as opposed to 2 Mdal when inserted into plasmid RP4.

The transfer frequencies obtained during these conjugation experiments with RlE showed a similarity to the results obtained when the mutated transposons were transferred from their J62(RP4) hosts into J53 and selection was made for the transfer of Tp and Km resistance. Similar analysis of Tp resistant transconjugants obtained from a mating between J53(RlE::Tn4133*) and J62 showed that only 5% of J62 transconjugants were Cm resistant. Examination of Tp resistant, Cm sensitive J62 transconjugants both by agarose gel electrophoresis (figure 24, track 5) and CsCl density gradient

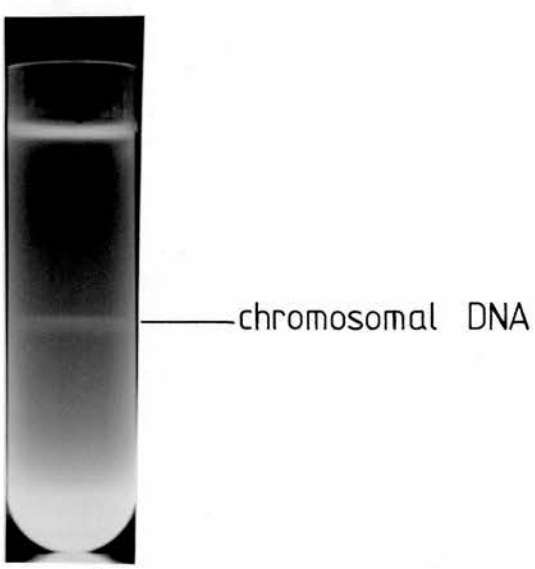
Figure 24. Agarose gel electrophoresis of R1E and R1E transposon containing derivatives and DNA extracted from E. coli J62(Tn4133*) transconjugants selected on Tp containing plates.

Track 1, R1; track 2, RP4; track 3, R6K; track 4, R1E; track 5, DNA extracted from J62(Tn4133*); track 6, R1E::Tn4133*; track 7, R1E::Tn4132*; track 8, R1E::Tn4134*.



centrifugation (figure 25) failed to reveal the presence of plasmid DNA. In addition, the J62(Tn4133*) transconjugant was shown to transfer Tp resistance to E. coli J53 at a frequency of 6.8×10^{-4} per donor cell during a 1 hour mating. Thus transfer of this small molecular weight transposon again appears to be occurring without the need for a plasmid vector. These observations, may offer an explanation for the differences in transfer frequencies obtained when selection is made for the transfer of Cm resistance, determined by RlE, and Tp resistance, determined by the transposons.

Figure 25. Ethidium bromide/caesium chloride density gradient centrifugation of DNA extracted from E. coli J62(Tn4133*).



2. Biochemical analysis

(i) Investigation of the DHFR coded by Tp resistance transposons

The degree of similarity between the six transposons extracted from Tp R-plasmids was further investigated by an examination of their gene products. E. coli J62 strains harbouring the RP4 transposon derivatives were used for the preparation of all six DHFRs. A preliminary experiment was done to determine the DHFR activities of strains containing either large or small molecular weight transposons. The specific activities were compared with the chromosomal DHFR activity of a Tp sensitive J62 strain. One litre exponential cultures of E. coli J62, E. coli J62(RP4::Tn4129) and E. coli J62(RP4::Tn4133), grown in DM minimal medium, were harvested by centrifugation and resuspended in 2.0 ml of 50 mM sodium phosphate buffer pH 7.4 containing 10 mM β-mercaptoethanol and 1 mM EDTA (buffer A). Crude enzyme preparations were then made. The cells were lysed on ice in the French Press and the lysate cleared by high speed centrifugation for 1 hour in a Sorvall RC-5B. The specific activity of DHFR in all three extracts was measured in sodium phosphate buffer pH 6.0 by the method of Osborn and Huennekens, (1958). All assay results were corrected for the DHF-independent NADPH oxidase activity. The results (Table 28) show that both the transposon containing strains exhibit high levels of DHFR activity characteristic of Type 1 DHFRs previously associated with transposon mediated Tp resistance.

Table 28 The specific activity of DHFR in crude extracts of E. coli J62, E. coli J62(RP4::Tn4129) and E. coli J62(RP4::Tn4133)

The specific activity is expressed in nmoles dihydrofolate being converted per minute per mg protein in a 1.0 ml volume assay.

Strain	Specific Activity	Relative Specific Activity
J62	2.48	1.00
J62(RP4::Tn4129)	22.06	8.89
J62(RP4::Tn4133)	13.25	5.34

These preliminary results suggested that the two transposons may be encoding similar gene products. Therefore the DHFR encoded by each of the Tp transposons was purified and characterised.

(ii) Purification of transposon encoded DHFR

Ten litre amounts of J62 strains containing the transposons, inserted into plasmid RP4, were grown to logarithmic phase in DM medium. Enzyme extracts were prepared, in buffer A, as described in the Materials and Methods. Partial purification of the enzymes was performed by a modification of the method of Amyes and Smith (1974a) as described previously. Comparison of the purification and recovery of each of the enzymes revealed little difference between them (Tables 29-34). Usually about 30% of DHFR activity was recovered following dialysis against 50% ammonium sulphate with the exception of the DHFRs from J62(RP4::Tn4129) (7.6%) and J62(RP4::Tn4131) (60%).

Table 29 Purification of the dihydrofolate reductase activity from E. coli J62(RP4::Tn4129)

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	14.0	83.85	1529.95	18.25	21419.30	100	1
STREPTOMYCIN SULPHATE	12.2	51.17	347.34	6.79	4237.55	19.8	0.37
50-80% $(NH_4)_2SO_4$ SUPERNATANT	5.6	40.64	289.45	7.12	1620.92	7.6	0.39
SEPHADEX FRACTION 45	2.0	1.09	90.97	83.46	181.94	0.8	4.55

Table 30 Purification of the dihydrofolate reductase activity from E. coli J62(RP4::Tn4130)

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	16.5	71.81	496.20	6.90	8187.30	100	1
STREPTOMYCIN SULPHATE	13.5	46.44	132.32	2.85	1786.32	21.8	0.41
50-80% $(NH_4)_2SO_4$ SUPERNATANT	6.0	31.61	330.80	10.46	1984.80	24.2	1.51
SEPHADEX FRACTION 47	2.0	1.72	78.60	45.68	157.20	1.9	6.61

Table 31 Purification of the dihydrofolate reductase activity from E. coli J62(RP4::Tn4131)

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	12.3	53.11	645.10	12.15	7934.73	100	1
STREPTOMYCIN SULPHATE	11.0	43.43	810.46	18.66	8915.06	112.4	1.53
50-80% $(NH_4)_2SO_4$ SUPERNATANT	4.5	79.71	1058.60	13.28	4763.70	60.0	1.09
SEPHADEX FRACTION 53	2.0	0.87	116.00	133.33	232.00	2.4	9.10

Table 32 Purification of the dihydrofolate reductase activity from E. coli J62(RP4::Tn4132)

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	16.5	65.36	297.70	4.56	4912.05	100	1
STREPTOMYCIN SULPHATE	15.5	54.83	148.86	2.71	2307.33	46.97	0.59
50-80% $(NH_4)_2SO_4$ SUPERNATANT	7.5	40.21	206.75	5.14	1550.62	31.57	1.13
SEPHADEX FRACTION 53	2.0	1.98	124.05	62.65	248.10	5.05	13.75

Table 33 Purification of the dihydrofolate reductase activity from E coli J62(RP4::Tn4133)

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phospate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	13.5	35.48	388.69	10.96	5247.32	100	1
STREPTOMYCIN SULPHATE	11.5	43.65	380.42	8.72	4374.83	83.4	0.79
50-80% $(NH_4)_2SO_4$ SUPERNATANT	5.0	38.06	297.72	7.82	1488.60	28.4	0.71
SEPHADEX FRACTION 53	2.0	1.50	78.56	52.38	157.12	3.0	4.78

Table 34 Purification of the dihydrofolate reductase activity from E coli J62 (RP4::Tn4134)

The units of dihydrofolate reductase activity are nM dihydrofolate reduced per minute in 40 mM sodium phosphate buffer pH 6.0 at 30°C.

STAGE	a VOLUME	b PROTEIN CONC.	c DHFR ACTIVITY	c÷b SPECIFIC ACTIVITY	a x c TOTAL DHFR ACTIVITY	RECOVERY	PURIFICATION
	ml	mg/ml	U/ml	U/mg protein	U	%	-fold
BULK	17.0	55.47	215.02	3.88	3655.34	100	1
STREPTOMYCIN SULPHATE	16.5	44.50	140.59	3.16	2319.74	63.5	0.81
50-80% $(NH_4)_2SO_4$ SUPERNATANT	8.0	57.84	165.40	2.86	1323.20	36.2	0.73
SEPHADEX FRACTION 53	2.0	2.97	181.94	61.26	363.88	9.9	15.79

The purification at this stage ranged between 0.4 and 1.5. Both the levels of recovery and purification were much lower than those reported by Amyes and Smith (1976) for the R388 Type II enzyme. However, the R388 enzyme is known to be much more stable than the Type I enzyme (Tennhammar-Ekman and Sköld, 1979) and this may account for the lower recovery found with the enzymes studied here.

The 50-80% precipitate, containing the DHFR activity, was collected and further purified by sephadex exclusion chromatography by the method of Pattishall et al. (1977). The pellet resuspended in buffer A was applied to a Sephadex G-75 superfine column ($2cm^2$ x 90cm) and DHFR activity was eluted with buffer A at a flow rate of between 6 and 8 ml per hour as described previously. Two ml fractions were collected and assayed for DHFR activity in the presence and absence of 4×10^{-6}M Tp, a concentration sufficient to abolish the activity of the chromosomal enzyme. This allowed the identification of peaks of Tp-resistant and Tp-sensitive DHFR.

Elution patterns for the DHFRs encoded by transposons Tn4129 (Figure 26a) and Tn4130 (Figure 27a) were in most respects identical and showed two clearly defined peaks of activity corresponding to the Tp-resistant transposon encoded enzyme, which eluted at about fraction 46 and the Tp-sensitive chromosomal encoded enzyme which eluted between fractions 55 and 57

The enzyme profiles for the DHFRs encoded by transposons Tn4131 (Figure 28a), Tn4132 (Figure 27b), Tn4133 (Figure 26b) and Tn4134 (Figure 28b) were also identical but did, however, differ markedly

Figure 26. Elution of DHFR activities of E. coli J62(RP4::Tn4129) and E. coli J62(RP4::Tn4133) on Sephadex G-75 superfine gel filtration.

a = J62(RP4::Tn4129); b = J62(RP4::Tn4133).
Total DHFR activity, O ; DHFR activity in the presence of 4 x 10^{-6}M Tp, ● . Enzyme activity refers to DHFR level in units/ml.

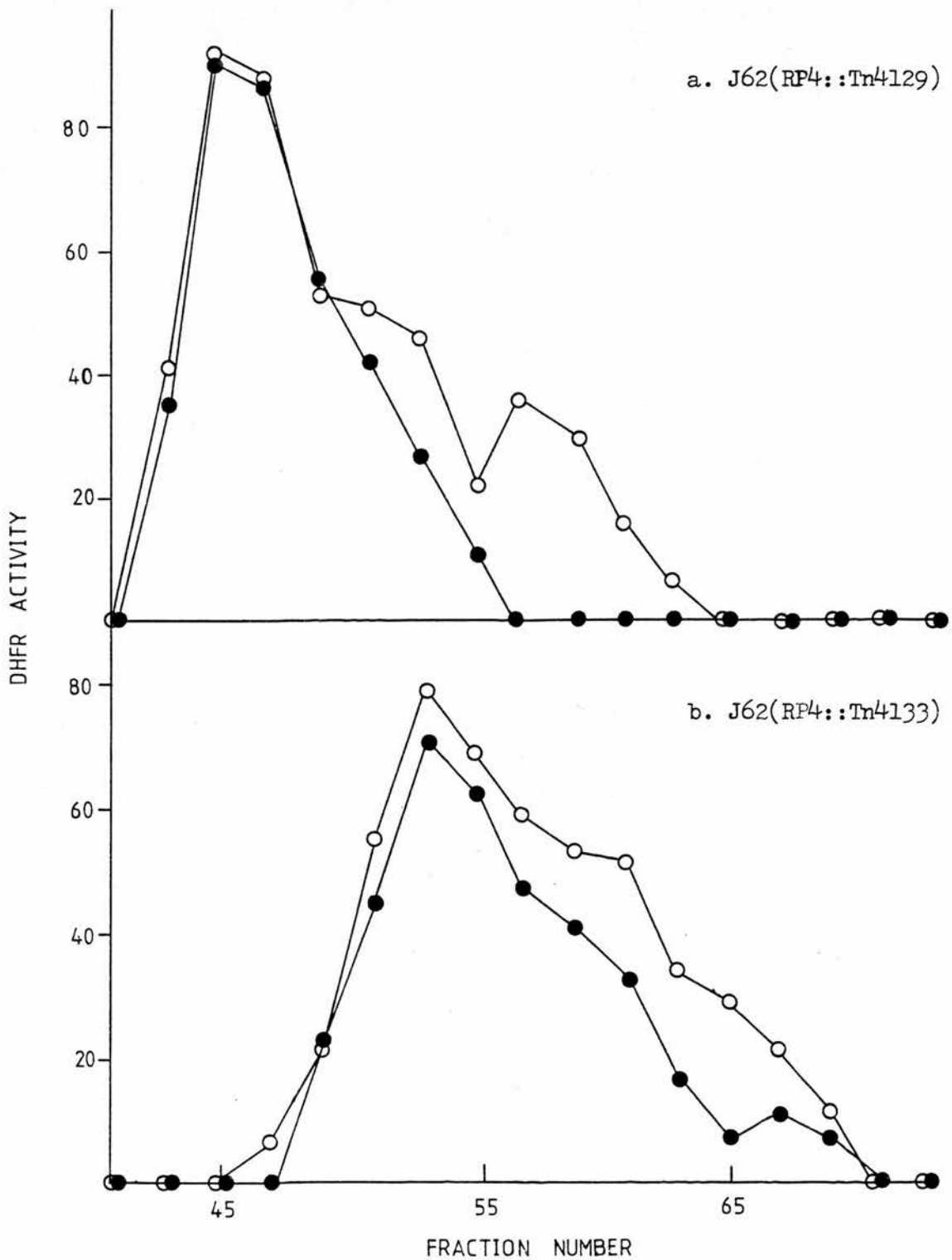


Figure 27. Elution of DHFR activities of E. coli J62(RP4::Tn4130) and E. coli J62(RP4::Tn4132) on Sephadex G-75 superfine gel filtration.

a = J62(RP4::Tn4130); b = J62(RP4::Tn4132).
Total DHFR activity, O ; DHFR activity in the presence of 4 x 10^{-6}M Tp, ● . Enzyme activity refers to DHFR level in units/ml.

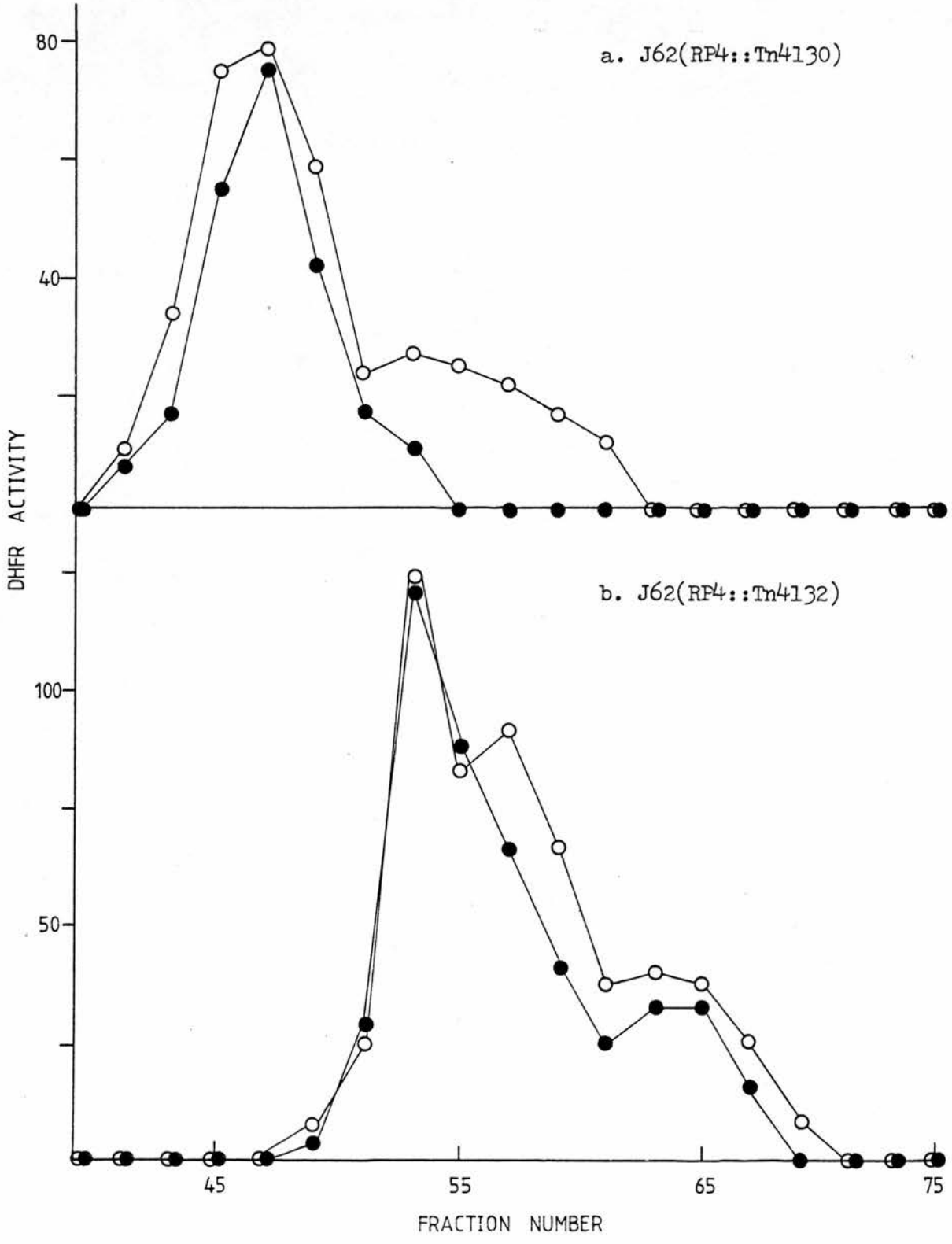
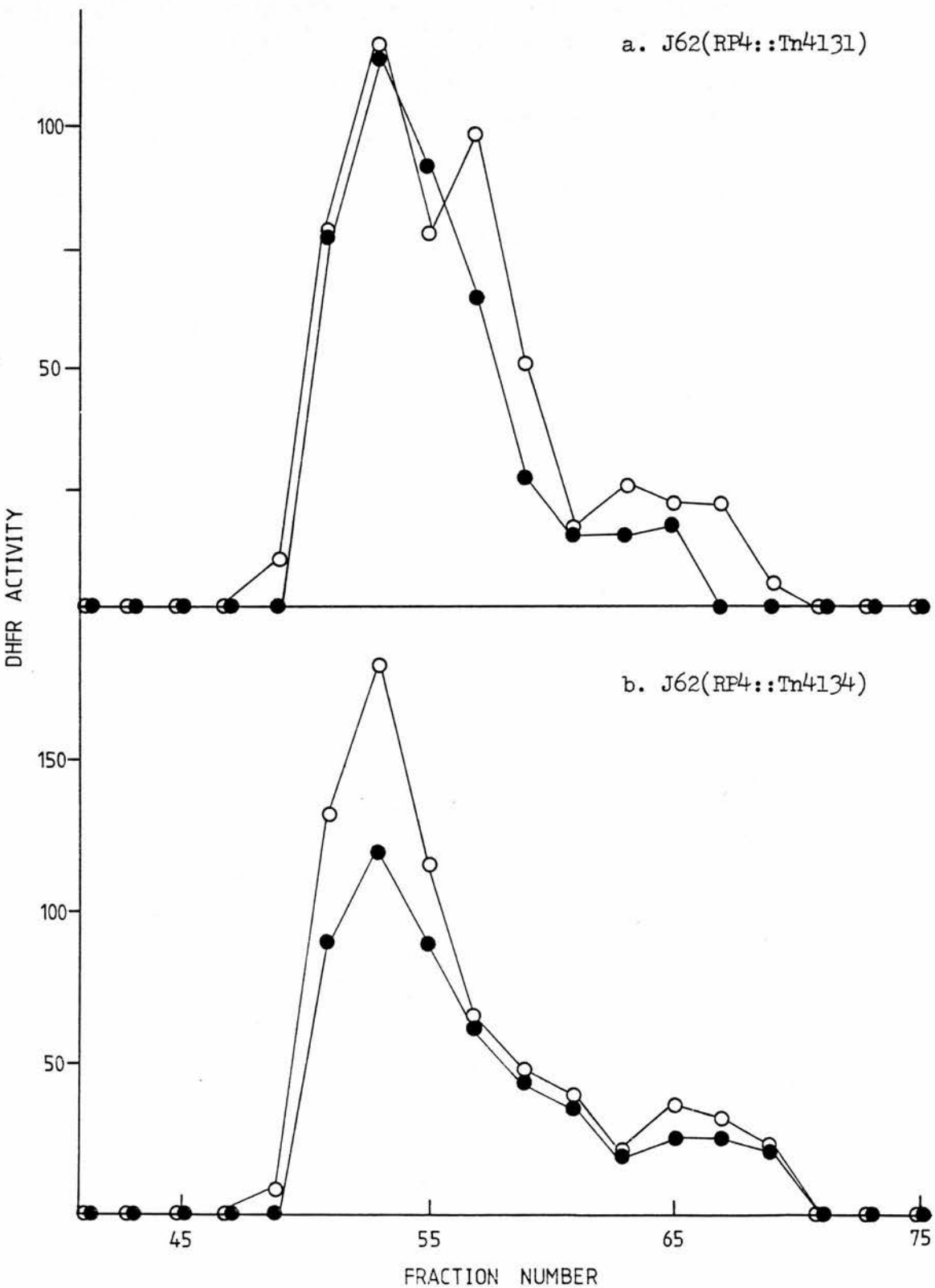


Figure 28. Elution of DHFR activities of E. coli J62(RP4::Tn4131) and E. coli J62(RP4::Tn4134) on Sephadex G-75 superfine gel filtration.

a = J62(RP4::Tn4131); b = J62(RP4::Tn4134).
Total DHFR activity, O ; DHFR activity in the presence of 4×10^{-6}M Tp, ● . Enzyme activity refers to DHFR level in units/ml.



from the Tn4129 and Tn4130 enzyme profiles. Each enzyme profile shows two less well defined peaks of activity eluting at fractions 53 and 57. These peaks often merged to form a single, slightly broader peak of activity (Figures 26b, 28b). The enzyme in fraction 53 was resistant to Tp at a concentration of 4×10^{-6}M, unlike the peak at fraction 57 and therefore represents the transposon encoded DHFR. In addition to these enzyme peaks, a small shoulder of activity was found to elute around fraction 66. This shoulder of Tp resistant enzyme activity was only present in enzymes encoded by transposons Tn4131, Tn4132, Tn4133 and Tn4134.

(iii) Molecular Weight of DHFR coded by tranposons

The different elution profiles of these enzymes suggested a difference in their molecular weight (MW). Thus sephadex exclusion chromatography was repeated in the presence of marker proteins of known molecular weight in order to determine the enzyme molecular weights. Ten litre exponential cultures of the six transposon containing strains were prepared and treated with streptomycin sulphate and ammonium sulphate as described before. The 50-80% ammonium sulphate pellet was resuspended in buffer A and mixed with the molecular weight markers described in the Materials and Methods. This sample was then applied to a Sephadex G-75 superfine column and fractions were collected and assayed for Tp resistant DHFR activity.

The Ve/Vo ratio was found for each of the three molecular weight marker proteins, as described previously, and this value was plotted

against molecular weight on a semi-logarithmic graph scale. The results showed that the MW of the Tp sensitive DHFR encoded by strains harbouring Tn4129 and Tn4130 was about 21000 which agrees well with the published value of 20500 (Amyes and Smith, 1978). Similar MWs were obtained for the Tp sensitive enzymes from the other four transposon containing strains.

The position of the Tp resistant DHFR encoded by Tn4129 was commensurate with a molecular weight of 35000 which is in full agreement with the published value of 35000 for the Tn7 encoded DHFR of plasmid R483 (Pattishall et al., 1977). The MW of the DHFR encoded by transposon Tn4130 was found to be 32000 (table 35) which is also in good agreement with the MW of Type I plasmid encoded enzymes (Pattishall et al., 1977; Amyes and Smith, 1978).

Table 35 Molecular weights of dihydrofolate reductases

Source of enzyme	Molecular Weight
Tn4129	35000
Tn4130	32000
Tn4131	24500, 12000
Tn4132	24500, 12400
Tn4133	24000, 10600
Tn4134	24500, 12400

However, when the 50-80% precipitate of strain J62(RP4::Tn4133) was eluted with marker proteins on Sephadex G-75 superfine, the Tp resistant DHFR peak was found to correspond to a MW of 24000 (table 35). This DHFR molecular weight differs from all previously characterised Tp resistant, plasmid encoded DHFRs. In addition a shoulder of Tp resistant DHFR activity was found corresponding to a MW of 10600. Similar MWs were determined for the DHFRs coded by transposons Tn4131, Tn4132 and Tn4134 (table 35). These four transposons, therefore produce a DHFR which is two thirds the MW of the Type I and Type II plasmid enzymes. The presence of a second Tp resistant shoulder of activity suggests that this enzyme may be composed of two subunits with MWs between 10600 and 12400 which possess low levels of DHFR activity.

(iv) pH profile of DHFRs

All the assays described have been performed at pH 6.0 in sodium phosphate buffer. Previously characterised R-plasmid DHFRs have all been shown to exhibit a sharp peak of activity at this pH (Amyes and Smith, 1978). Trimethoprim sensitive enzymes, on the other hand, show a broad spectrum of activity over almost the complete pH range of 5 to 9 (Amyes and Smith, 1978). The pH profiles of all six transposon mediated Tp resistant DHFRs were compared in order to determine if the large and small enzymes exhibit different pH optima. Following Sephadex G-75 superfine gel filtration, fractions containing Tp-resistant DHFR activity were pooled and assayed at pH values between 5.0 and 9.0. Controls were performed at each pH value

tested to correct for decreases in absorbance in the absence of the enzyme. The results (figure 29) show that all six enzymes exhibit a pH optimum at pH 6.0. The enzymes coded by transposons Tn4129, Tn4131, Tn4132 and Tn4134 show a slightly broader range of activity, with 95% of the maximum activity between pH 5.5 and 6.0 for enzymes from Tn4129 and Tn4132 and between pH 6.0 and 6.5 for enzymes from Tn4131 and Tn4134. This slightly broader pH optimum is characteristic of Type I DHFRs as opposed to the Type II enzymes which sharply peak at pH 6.5 (Pattishall et al., 1977).

It should be noted however, that three of the enzymes (from Tn4130, Tn4131 and Tn4134) showed an anomalously high level of activity, between 90 and 100%, at pH 5.0. This result was checked several times both by repeating the assay of each enzyme and in addition by the preparation of new enzymes which were then assayed as before. In each case a high level of activity was observed at pH 5.0.

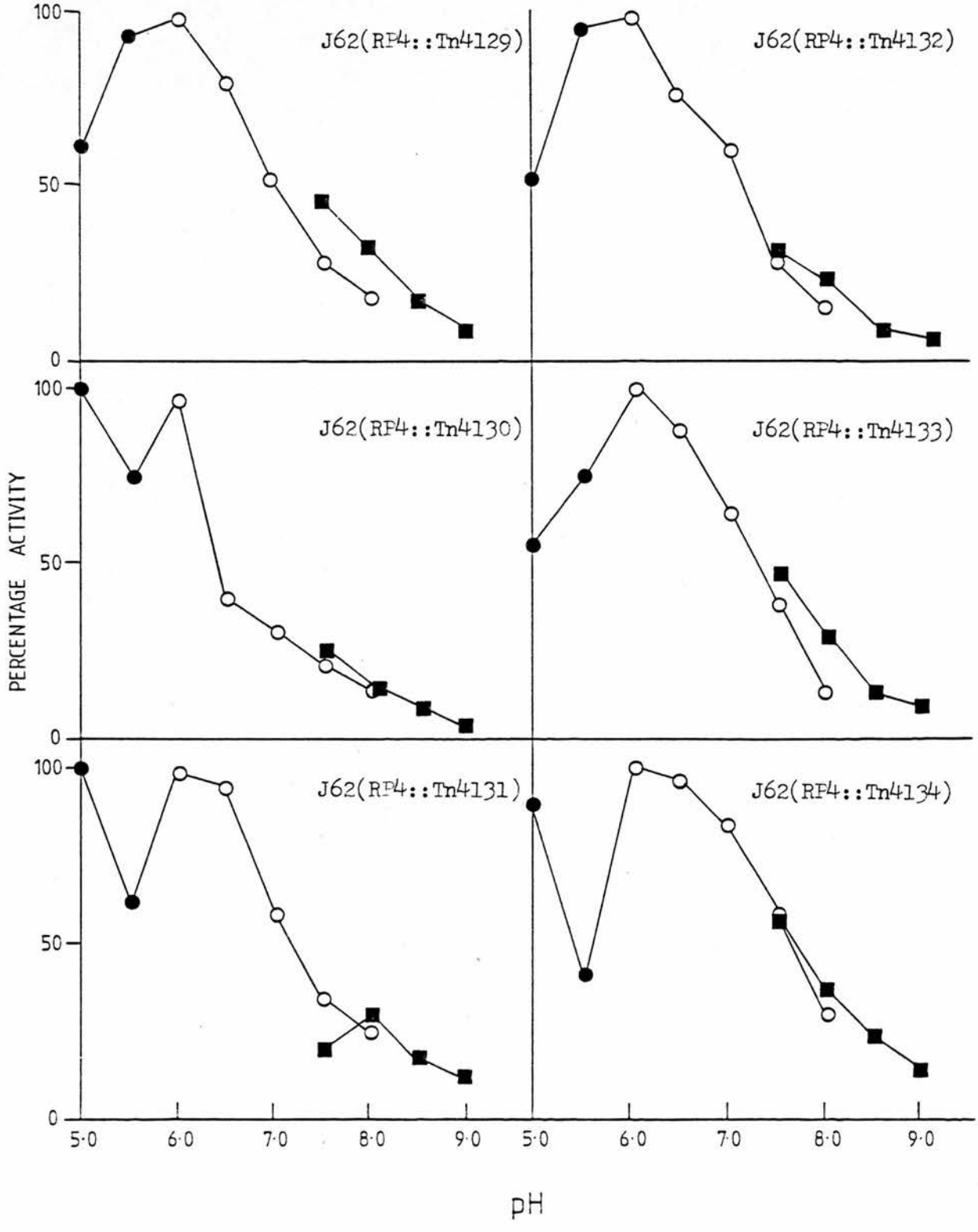
However, with the exception of this anomalous result the six enzymes show similar pH profiles 95% activity being retained over a pH range of 5.5 to 6.5 in sodium phosphate buffer. All subsequent assays were therefore performed in sodium phosphate buffer at pH 6.0.

(v) Substrate profile of DHFRs

A feature which distinguishes Tp resistant plasmid encoded enzymes from the host chromosomal enzyme of E. coli is the difference in substrate specificity profiles. With the Tp-sensitive E. coli 114

Figure 29. The pH activity curve for the Tp^R DHFRs from E. coli J62 strains harbouring Tp resistance transposons.

The activity of DHFR is expressed as a percentage of the maximum activity at pH 6.0.
The buffers employed were sodium acetate, ● ; sodium phosphate, O ; Tris-HCl, ■ .



DHFR, about 16% of the activity seen with NADPH is observed when NADH is substituted (Amyes and Smith, 1976). Similarly, the J62 enzyme exhibits 8.2% of the NADPH activity when NADH is used (Amyes and Smith, 1978). However, neither of these chromosomal enzymes show any activity when folic acid is substituted for DHF (Amyes and Smith, 1978).

Purified enzyme preparations of all six Tp-resistant DHFRs obtained from pooled gel filtration fractions were assayed in the presence of NADH instead of NADPH and/or folic acid substituting for DHF. No activity could be detected when NADH was substituted for NADPH, and none of the six enzymes exhibited any activity when folic acid was substituted for DHF. Therefore, both the large and small DHFRs differ from chromosomal DHFRs, in their response to NADH, but have similar substrate profiles to R-plasmid determined DHFRs (Amyes and Smith, 1978).

(vi) Inhibition of DHFR activity at 45°C

A major distinguishing feature of Type I DHFRs is their heat sensitivity. It is well documented that the Type I enzymes are inactivated to 50% (TD_{50}) within two or three minutes of heating to 45°C. (Amyes and Smith, 1978; Tennhammar-Ekman and Sköld, 1979; Broad and Smith, 1982). Type II DHFRs, on the other hand, are much more heat resistant with TD_{50}s of greater than 15 minutes (Tennhammar-Ekman and Sköld, 1979; Broad and Smith, 1982).

The heat sensitivities of the six partially purified (gel

filtration pooled fractions) Tp resistant enzymes were measured. The temperature inactivation curves of both large and small MW DHFRs showed similar linear relationships between the logarithm of the activity and the time that the enzyme was maintained at 45°C. All six enzymes were found to be sensitive on heating to 45°C, each losing 50% activity after about 2 minutes (table 36). These six transposon mediated enzymes therefore appear to be more closely related to the Type I DHFRs than the Type II enzymes.

Table 36 Time taken to lose 50% dihydrofolate reductase activity at 45°C

Source of enzyme	Time (mins)
Tn4129	2.9
Tn4130	1.4
Tn4131	1.2
Tn4132	1.2
Tn4133	2.2
Tn4134	2.1

(vii) Inhibition of DHFR activity by antifolate compounds

In order to compare the transposon determined enzymes further the extent of their resistance to trimethoprim was determined. The enzyme preparations used were the partially purified, pooled, gel

filtration fractions. In each case the DHFR activity was assayed in increasing concentrations of Tp at pH 6.0, and the concentration required to give 50% inhibition (ID_{50}) of each enzyme was determined. Inhibitor profiles of both the large and small MW enzymes were very similar and in each case the activity was directly proportional to the logarithm of the Tp concentration. The ID_{50}s for all six enzymes were similar, with values between 30 and 60 μM (table 37).

Table 37 Trimethoprim and methotrexate concentrations which cause 50% inhibition of dihydrofolate reductase activities

Source of enzyme	Trimethoprim (μM)	Methotrexate (μM)
Tn4129	60	7.1
Tn4130	63	6.3
Tn4131	50	6.3
Tn4132	32	2.8
Tn4133	30	2.5
Tn4134	45	2.5

These values are in good agreement with those found by Pattishall et al. (1977) for Type I DHFRs. The results of Amyes and Smith (1978), however, estimate the ID_{50} of the Type I enzyme to be between two to three times greater at about 150 μM. These discrepancies may be explained by the use of pure Tp by Amyes and Smith (1978), as opposed to Tp lactate which was employed here and by Pattishall et al.

(1977), which may result in a greater value for the ID_{50}.

The activity of the six enzymes was also assayed in the presence of increasing concentrations of methotrexate, to compare their relative sensitivities. Inhibitor profiles of both large and small MW enzymes were very similar, the activity again being directly proportional to the logarithm of methotrexate concentration. The ID_{50}s for methotrexate of the six enzymes were also similar, ranging from 2.5 to 7.1 μM (table 37). These values are again in good agreement with those for Type I DHFRs (Pattishall et al., 1977; Amyes and Smith, 1978).

The large and small MW DHFRs, therefore, exhibit inhibition profiles similar both to each other and to Type I DHFRs.

(viii) Michaelis-Menten kinetics of DHFRs

In order to determine whether the resistant enzymes have similar affinities for the primary substrate (dihydrofolate), the activities of the partially purified enzymes were investigated under conditions of partial saturation with DHF and the results analysed by the method of Lineweaver and Burk (1934) to determine the Km values.

The results for both large and small MW enzymes showed that in the absence of Tp straight lines were obtained by plotting the reciprocal of the substrate concentration (1/s) against the reciprocal of the rate of the enzyme reaction (1/v) and hence the enzymes obey Michaelis-Menten kinetics at pH 6.0 and 30°C. The Km

value for each of the enzymes was estimated (table 38) and found to be about 20 μM with DHF. This value is similar to that obtained for the Tp sensitive chromosomal determined DHFR (Amyes and Smith, 1978). The Km value for Tp resistant plasmid mediated DHFRs has been reported to vary between 4 μM and 10 μM with DHF (Pattishall et al., 1977; Amyes and Smith, 1978; Tennhammar-Ekman and Sköld, 1979). Since Km values are subject to several errors this variation is probably not significant. Thus all six enzymes show similar affinities for DHF to those previously reported for both chromosomal and plasmid determined enzymes.

The results of the ID_{50} experiments with Tp suggested that the inhibitor constant (Ki) of the enzymes should also be similar. In order to confirm this the activity of the enzymes was assayed as before at various limiting DHF concentrations in the presence of two relatively high concentrations of Tp, to determine the kinetic effects of the drug. In each case the double reciprocal plots of 1/v against 1/s, in the presence of Tp both intercepted the ordinate at the same position. This intercept was also that obtained in the absence of Tp, indicating that the enzymes, although resistant to Tp, are inhibited competitively by the drug with respect to DHF, as the maximum rate is unaffected. However, the plots in the presence of Tp intercepted the abscissa nearer the origin than the uninhibited slope.

The Ki values for the two Tp concentrations used were calculated as described previously and the mean Ki value determined for each enzyme. The mean Ki values for the six enzymes ranged between 2.1 μM

Table 38 Michaelis-Menten kinetics of dihydrofolate reductases with dihydrofolate as substrate and trimethoprim as inhibitor

Source of Enzyme	Km for dihydrofolate (μM)	Ki for trimethoprim (μM)
Tn4129	20	33.0
Tn4130	21	4.8
Tn4131	14	5.9
Tn4132	11	41.0
Tn4133	11	45.0
Tn4134	57	2.1

and 45 μM (table 38). The small variations between the inhibitor constants of these enzymes are probably due to experimental error arising as a result of the low levels of activity being tested and are therefore probably not significant. The results indicate, however, that each enzyme has a very low affinity for Tp, the mean Ki value being approximately 10,000 times greater than that reported for the chromosomal DHFR (Amyes and Smith, 1978). These Ki values are also in good agreement with the published results for Type I DHFRs (Pattishall et al., 1977; Amyes and Smith, 1978; Tennhammar-Ekman and Sköld, 1979). Therefore, whether the transposon mediated DHFRs are assayed for their ID_{50} or their Ki value a similar decrease in affinity for Tp is obtained which agrees well with the increase in resistance level observed with intact bacteria.

(ix) Polyacrylamide gel electrophoresis of DHFRs

From the previous sections it would appear that all six enzymes are biochemically very similar. However, the results of the gel filtration experiments suggest that the enzymes may be divided into two groups, differing significantly in their molecular weight. Since size appears to be the only significant difference between the enzymes it was important to confirm that the two DHFRs were different enzymic entities by employing another method of protein separation by molecular size. Therefore, two of the enzymes, coded by Tn4129 and Tn4133, were examined by polyacrylamide gel electrophoresis (PAGE), which separates proteins both by their molecular weight and their charge.

Initially the method of Tennhammar-Ekman and Sköld (1979) was employed. Crude enzyme extracts from 10 litres of exponential phase cultures of E. coli J62(RP4::Tn4129) and E. coli J62(RP4::Tn4133) were prepared by the modified method of Amyes and Smith (1974a). Following sephadex exclusion chromatography, fractions containing DHFR activity were pooled and the enzymes re-precipitated by dialysis against an 80% saturated solution of ammonium sulphate. The pellet was collected by centrifugation resuspended in buffer A and then dialysed overnight against 100 volumes of the same buffer. Between 100-200 µg of each enzyme preparation was mixed with sucrose solution and applied directly to the separation gels. The gels were set up as described in the Materials and Methods and the samples were run from the cathode to the anode. Electrophoresis was conducted at 4°C and pH 8.6 under the conditions described by Davis (1964) and until the marker of bromophenol blue migrated to the bottom of the gel. Bands of DHFR were located on the gels using the zymographic staining procedure as described in the Materials and Methods. Following incubation of the gels with NADPH and dihydrofolate, enzymatically formed tetrahydrofolate was detected by the addition of the tetrazolium salt MTT which is reduced by tetrahydrofolate to give a blue, insoluble formazan deposit. Unspecific bands of MTT reduction, not related to tetrahydrofolate were identified by control incubations without added dihydrofolate. To distinguish between bands corresponding to the Tp resistant transposon encoded enzymes and those corresponding to the host chromosomal enzyme, further incubations were carried out in the presence of 4×10^{-6}M Tp, a

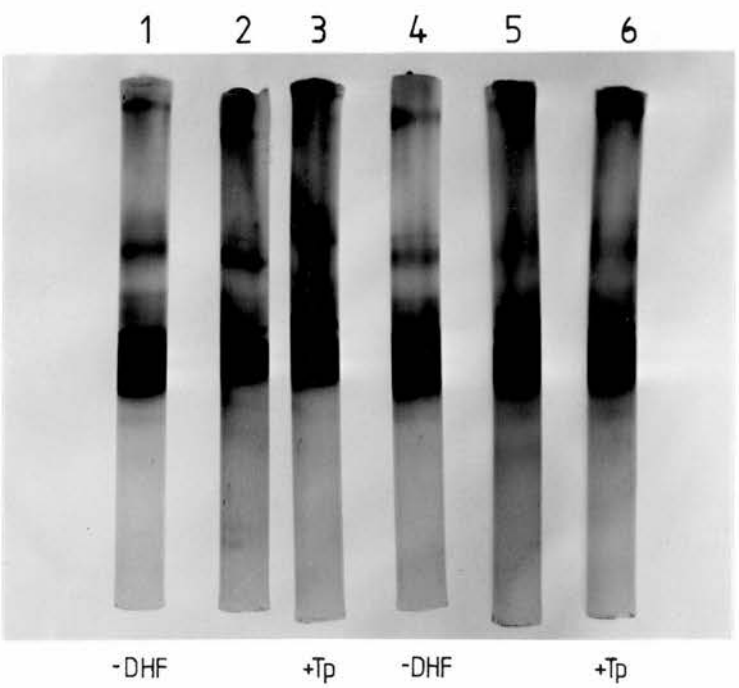
concentration sufficient to abolish chromosomal DHFR activity. The gels were then examined for specific bands of Tp resistant DHFR activity.

The results (figure 30) show, however, the presence of several bands of MTT reduction, each band being present in both control and test gels, making it impossible to distinguish those bands corresponding to DHFR activity. The large number of non-specific bands is most probably due to the low level of purification of the DHFRs prior to electrophoresis. Fling and Elwell (1980), studying the subunit structure of Type I and Type II DHFRs by PAGE performed extensive purification of the enzymes through several affinity columns before subjecting them to electrophoresis. The inability here to distinguish between the DHFRs using zymography, together with the fact other authors have found it necessary to use extensive purification procedures, casts some doubt on the results of Tennhammar-Ekman and Skőld (1979) who claim to have shown differences in the electrophoretic mobility and size of the R388, R751 and R483 encoded DHFRs by this method.

In order to overcome the problems of identification of the enzymes in the polyacrylamide gels, the experiment was repeated employing an alternative method for the detection of the DHFRs following electrophoresis. Gel-chromatographed enzyme samples were again used and applied to the separation gels as before. Following electrophoresis the gels were sliced into 3 mm segments using a scalpel. Each segment was placed in 0.5 ml of buffer A and DHFR

Figure 30. Polyacrylamide gel electrophoresis and zymographic staining of DHFRs produced by E. coli J62(RP4::Tn4129) and E. coli J62(RP4::Tn4133).

Columns 1-3 contained partially purified enzyme from E. coli J62(RP4::Tn4129) and columns 4-6 enzyme from E. coli J62(RP4::Tn4133).
In columns 1 and 4 the zymographic incubation took place in the absence of dihydrofolate (-DHF) and in columns 3 and 6 in the presence of 4×10^{-6} M Tp (+Tp).

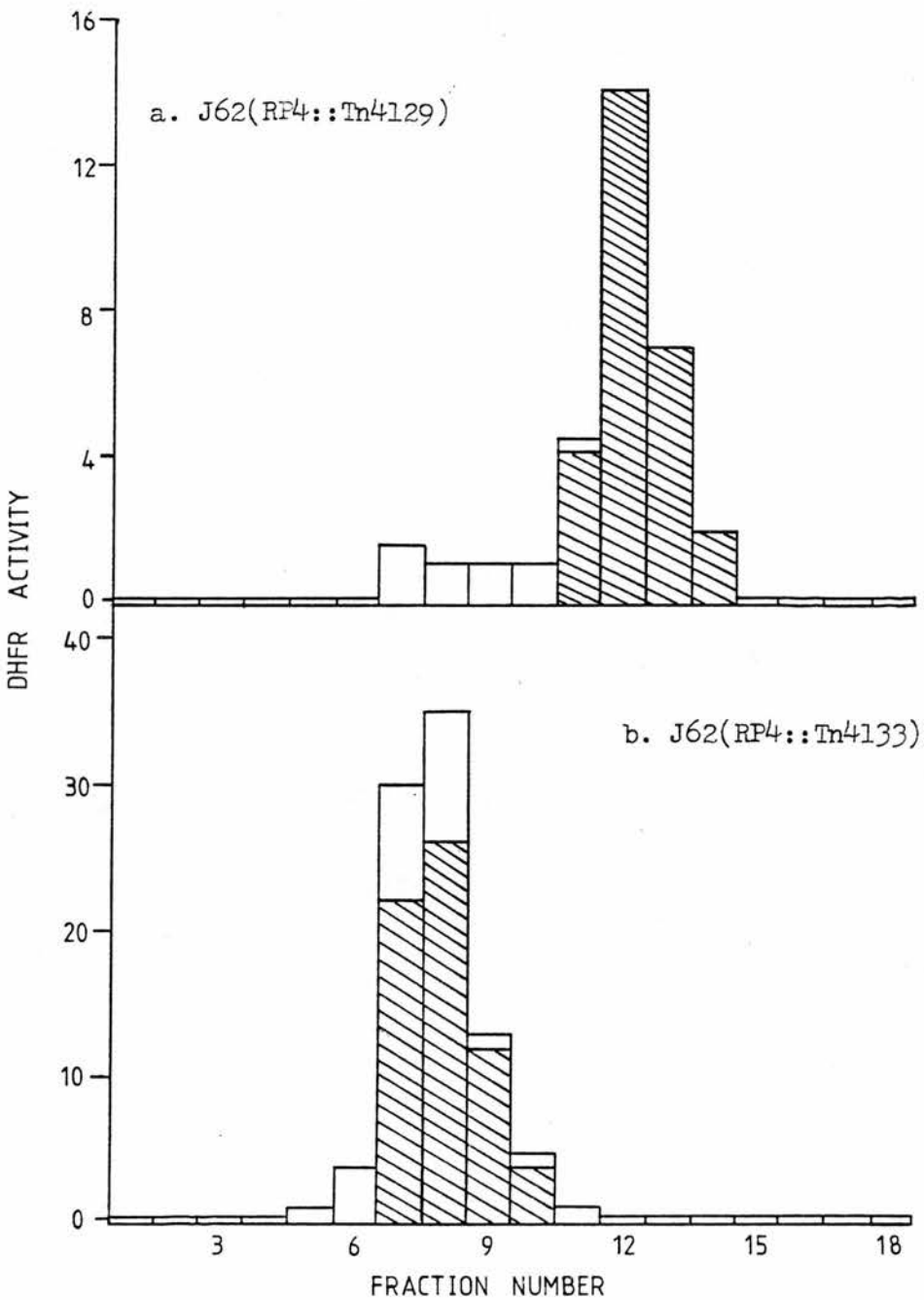


activity was allowed to elute overnight in the buffer at 4°C. The following day the eluate from each slice was assayed for DHFR activity, both in the presence and absence of 4 x 10^{-6}M Tp. The fractions were numbered from the bromophenol blue end of the gel so that fraction 1 contains this marker.

The results (figure 31) show that the sample from the Tn4129 containing strain exhibited two separate peaks of DHFR activity (figure 31a). A small peak of activity was found in fraction 7, which was completely abolished when assayed in the presence of 4 x 10^{-6}M Tp. A second, much larger peak of DHFR activity eluted from fraction 12. This enzyme continued to display maximum activity when assayed in the presence of 4 x 10^{-6}M Tp. The sample from J62(RP4::Tn4133), on the other hand, shows a single peak of DHFR activity (figure 31b). The maximum activity of DHFR was at fraction 8. Seventy-five percent of the DHFR activity in fraction 8 was resistant to 4 x 10^{-6}M Tp indicating that three quarters of the acitivity in this peak represents the Tn4133 encoded enzyme, the remaining 25% corresponding to the host chromosomal enzyme. The absence of a second peak of DHFR activity at fraction 12 confirms that the two transposons, Tn4129 and Tn4133, do indeed encode DHFRs of different molecular weight, the enzyme produced by Tn4133 being closer in size to the host chromosomal enzyme than the enzyme encoded by Tn4129.

Figure 31. Polyacrylamide gel electrophoresis of partially purified DHFRs from E. coli J62(RP4::Tn4129) and E. coli J62(RP4::Tn4133).

The histograms show the activity of DHFR (units/ml) in each fraction. The shaded areas show the level of DHFR activity in the presence of 4×10^{-6} M Tp.
a = J62(RP4::Tn4129); b = J62(RP4::Tn4133).



4. DISCUSSION

A. Origins of the Tp resistance genes

1. Klebsiella species

Recent research into the origins of plasmid borne antibiotic resistance genes has indicated that mobilization of chromosomal genes has probably played an important role in the evolution of certain R-plasmids (Ambler and Scott, 1978; Sutcliffe, 1978; Nugent and Hedges, 1979; Thomson and Grey, 1983). The initial aim of this thesis was to investigate possible sources of the Tp resistance genes present on R-plasmids and transposons. The ubiquitous nature of the enzyme dihydrofolate reductase suggests that the highly resistant enzymes encoded by Tp R-plasmids may also have arisen from the mobilization of chromosomal genes, possibly following their mutation to Tp resistance.

In the search for the precursor of the plasmid encoded enzymes, the DHFR encoded by spontaneous Tp resistant mutants of *Klebsiella sp.* D770 was isolated and partially characterised. This strain was one of the first Tp R-plamsid containing organisms isolated (Fleming *et al.*, 1972) and was of particular interest because of its unusual biochemical characteristics (Datta and Hedges, 1972). The failure of the original workers to isolate *Klebsiella* strains similar to strain D770 but devoid of Tp R-plasmids, rendered it necessary to perform preliminary experiments aimed at removing the Tp R-plasmid, R389, from strain D770 before the strain itself could be studied.

Initial investigations revelaed that in addition to the R-plasmid R389 (plasmid 2) which carried resistance determinants to Tp, Sx and Sm there was also a second R-plasmid (plasmid 3) of molecular weight 42 million present within this strain, which conferred resistance to Tc and Cm. This plasmid, however, was unstable and lost spontaneously at high frequency from the cell. On the other hand subsequent efforts to remove R389 by selection for Tp sensitive derivatives resulted, not in the loss of the whole plasmid, but rather surprisingly, in the loss of a 5 Mdal piece of DNA from R389. The high frequency of loss of this resistance determinant, without the concommitant loss of the R-plasmid, suggests that the Tp resistance determinant of R389 resides on a transposable element.

Previous reports of Tp resistance transposons have usually been associated with the gene responsible for the production of Type I DHFRs (Fling et al., 1982). The only exception was the discovery of the 5 Mdal transposon, Tn402, present on plasmid R751 (Shapiro and Sporn, 1977). This transposon, which has only been shown to transpose to bacteriophage lambda, encodes an unusual DHFR which has recently been classified as a sub-group of the Type II DHFRs (Broad and Smith, 1982). It has also been suggested, however, that the Tp resistance gene of the inc W R-plasmid, R388, may also be on a transposon (Amyes and Smith, 1977). This R-plasmid was isolated from a patient in the St. Pancras Branch, University College Hospital, London shortly before the isolation of R389 (Fleming et al., 1972). However, no detailed molecular analysis of this strain has yet been performed, so the exact nature of the 'transposon' has not been

confirmed.

The isolation and partial characterisation of the DHFR encoded by R389 in this thesis, indicates that it should also be classified as a Type II DHFR. However, it is interesting to note that the R389 enzyme is synthesised in relatively large amounts; a characteristic usually associated with the Type I plasmid mediated enzymes but not the Type II enzymes. Since the R389 enzyme is both heat stable and highly insensitive to Tp (both characteristics of Type II DHFRs) it is most likely that the levels of production are under the control of the host species. Nevertheless, although no direct evidence exists to confirm that the high levels of synthesis of Type I DHFRs is a result of their location within a transposon, it is conceivable that the elevated levels of the enzyme may be related to the location of the gene within a transposon, the transposon providing a regulatory function controlling the level of enzyme production. The increased synthesis of the R389 Type II enzyme may, therefore, be similarly related to its presence within a transposon.

After production of a Tp sensitive derivative of Klebsiella sp. D770 initial attempts to mutate this strain to high level Tp resistance were unsuccessful. Since the original Tp R-plamsid, R389, present within strain D770 belonged to incompatibility group W, it was thought that perhaps the introduction of an inc W group plasmid might provide an additional factor required for the mutational process. Indeed Tp sensitive derivatives of Klebsiella sp. D770, possessing the inc W R-plasmid Sa, were shown to mutate with relative

ease to high level Tp resistance. However, an examination of the resistance mechanism revealed that the mutation to Tp resistance did not involve the production of either an additional large molecular weight enzyme or a mutant chromosomal enzyme with similar resistance levels to Tp as the plasmid mediated DHFRS. Interestingly, however, the resistant mutant did appear to be coding for a chromosomal enzyme of a slightly greater molecular weight. This was distinguishable from the original chromosomal enzyme which appeared as a shoulder of activity corresponding to a molecular weight of 21300.

The resistance mechanism employed by this mutant strain resembles those previously reported for Tp resistant mutants of E. coli (Breeze et al., 1975; Sheldon and Brenner, 1976; Sheldon, 1977) and Klebsiella (Grey et al., 1979). In each case, resistance was shown to result from either a single or successive mutations within the DHFR gene altering both the levels of production of the enzyme and its affinity for Tp. Similar elevated levels of synthesis of the DHFR coupled with a decrease in the enzyme affinity for Tp were observed for the DHFR encoded by this Klebsiella derivative. However, it is clear that this organism is not the source of the plasmid enzymes. The molecular weight of the mutant Klebsiella DHFR is only 24300 as opposed to 35000 for the plasmid mediated enzymes. In addition, the Klebsiella enzyme is at least a thousand times more sensitive to Tp than the plasmid enzymes.

Nevertheless, the ease with which Klebsiella strains harbouring R-plasmids were shown to mutate to Tp resistance, coupled with the

intrinsic low levels of Tp resistance often observed with this species (Fruensgaard and Komer, 1974; Jobanputra and Datta, 1974; Hamilton-Miller and Grey, 1975) may be indicative of their possible role in providing a suitable environment for the continuing evolution of Tp R-plasmids. Indeed the inherent resistance to a variety of antimicrobials of many pathogenic strains of Klebsiella spp. harbouring Tp R-plasmids, has been shown to result in their gradual predominance over E. coli strains, during treatment of urinary tract infections with drugs other than Tp (Grüneberg and Bendall, 1979). Similarly strains of Proteus and Pseudomonas species, which are also inherently resistant to many antibacterial agents, may also provide a 'protective' background for evolving R-plasmids and resistance genes. It is hence significant that only 45% of the highly resistant bacterial population studied during this thesis belonged to the species E. coli and over 54% were Klebsiella, Pseudomonas and Proteus species.

2. Incompatibility W group plasmids

It seemed feasible that Tp-sensitive plasmids might also be a potential source of DHFRs that could be precursors to the Tp resistant plasmid enzymes. It was for this reason the inc W plasmid Sa was investigated. This plasmid was isolated in 1962 (Watanabe et al., 1968) and has a very extensive sequence homology with the first Tp R-plasmid, R388 (Gorai et al., 1979; Ward and Grinsted, 1982). It seemed possible, therefore, that Sa could have been the precursor of R388. However, no plasmid determined enzyme could be detected, as

only one DHFR was ever found in Sa containing cells. Therefore, it would appear that if Sa is indeed the immediate precursor of R388, the Tp resistance gene present on R388 would have to have been acquired by Sa, either by transposition, or some other recombinational mechanism, from elsewhere, and was not inherent to Sa.

The essential role of the inc W plasmid Sa in the mutation to Tp resistance of Klebsiella strains implied that this plasmid may be providing an additional factor resulting in the increased mutation frequency of certain genes. This hypothesis was extended to investigate the possible role of plasmid Sa in the mutation to Tp resistance of Escherichia coli K-12 strains. Two auxotrophic mutant strains of E. coli K-12 were examined for their ability to mutate to high levels of Tp resistance. Strain J62, which is auxotrophic for the amino acids histidine, proline and tryptophan (Clowes and Rowley, 1954) showed no significant mutation to resistance to even moderate levels of Tp. In addition, the introduction of plasmid Sa into this strain did not alter the mutation frequency. On the other hand, when the experiments were repeated with the auxotrophic mutant strain J53 (Bachmann, 1972), the strain mutated with relative ease to high levels of Tp resistance. The presence of Sa within strain J53 did not significantly increase the mutation frequency. Hence plasmid Sa is not performing the same role when present within E. coli K-12 strains as it did in the Klebsiella sp.

3. Escherichia coli

Mutation to high-level Tp resistance of E. coli J53 was achieved in three sequential subcultures. The resultant mutant strain, J53TplK^R, had an MIC of Tp greater than 1000 mgL^{-1}. Breeze et al. (1976) also isolated mutant E. coli K-12 strains, with MICs greater than 1000 mgL^{-1}, by serial subculture in progressively higher concentrations of Tp. However, a minimum of six subcultures was necessary to achieve this level of resistance. Therefore, strain J53 has an increased capacity to mutate to Tp resistance when selection is made for such mutants.

The mutant J53 strains were found to mediate the production of elevated levels of chromosomal encoded DHFRs. Interestingly the molecular weights of the DHFRs from both the original J53 parent strains and the J53 mutant derivatives were slightly higher than those previoulsy reported for the E. coli J53 chromosomal DHFR (Pattishall et al., 1977). This is most probably due to the slightly better separation achieved here by the use of Sephadex G-75 superfine. The first publication on the purification of plasmid and chromosomal encoded DHFRs failed to achieve separation of the two enzymes by Sephadex exclusion chromatography (Amyes and Smith, 1974a). However, Pattishall et al. (1977) found that by reducing the flow rate of the column to less than 5 ml/h, they obtained clearly defined peaks of Tp-resistant and Tp-sensitive DHFR activity, from cell extracts of E. coli strains harbouring Tp R-plasmids. The results of this thesis show that, by employing a Sephadex G-75

superfine matrix, enzymes which differ in size by as little as 2000 daltons may be resolved. Hence it is conceivable that some chromosomal DHFRs, previously estimated to be of molecular weight 21000 may, by the use of Sephadex G-75 superfine, be nearer 23000 daltons in size. In addition to the increased levels of production, the enzyme synthesised by the E. coli J53 mutant was also highly resistant to Tp with an inhibition constant (Ki) for Tp of 8.81 x 10^{-6}M. This value is similar to those of R-plasmid specified DHFRs (Pattishall et al., 1977; Amyes and Smith, 1978). No other mutant chromosomal DHFRs have ever been shown to be as insensitive to Tp as the enzyme isolated here. Sheldon and Brenner (1976), Baccanari et al. (1977) and Poe et al. (1979) have all isolated mutant E. coli DHFRs with slightly decreased affinities for Tp. However the Ki value for Tp of each of the enzymes was only between 1.0 x 10^{-9}M and 1.0 x 10^{-7}M. Therefore, strain J53Tp1K^{R} appears to be producing a new enzyme which is possibly more closely related to the plasmid-mediated DHFRs than those chromosomal mutants previously described and may be a precursor of the plasmid enzymes.

This hypothesis is reinforced by the discovery in this thesis of a new class of transposon-mediated enzymes, which share many of the biochemical characteristics of the Type I DHFRs but differ markedly in their molecular weight. The size of these new transposon encoded enzymes shows considerable resemblance to the molecular weight of the J53 mutant enzymes. Although these mutant strains have been obtained in vitro, the possibility that the transposon enzymes may have arisen by the incorporation of a spontaneously derived mutant chromosomal

enzyme *in vivo* into a transposable element cannot be dismissed.

In addition to the 22400 dalton enzyme encoded by J53TplK^R, a second enzyme of molecular weight 16000 was distinguished as a shoulder of Tp resistant DHFR activity. Although this 16000 dalton enzyme was not characterised it is evident from the DHFR elution profile of strain J53TplK^R that the shoulder of activity corresponding to this molecular weight is resistant to at least 4 x 10^{-6}M Tp. Fling *et al.* (1982) recently detected a 'new' plasmid-encoded enzyme of similar molecular weight to the shoulder observed here. Preliminary characterisation of their 'new' enzyme showed it to have an ID_{50} for Tp of about 10^{-6}M. The plasmid p699, encoding the 'new' enzyme was identified as a small 8kb non-self transmissible plasmid, thought to be mobilized by a second R-plasmid present in the original host (Fling *et al.*, 1982). In view of the identification of a chromosomal determined enzyme of a similar size and resistant to similar levels of Tp in this thesis, it is possible that Fling *et al.* (1982) may in fact have been observing the mobilization of part of the bacterial chromosome carrying a spontaneously derived mutant DHFR gene encoding an enzyme similar to the one isolated from strain J53TplK^R. However, more detailed characterisation of both these small molecular weight enzymes is required before a definite relationship between them can be established.

Detailed biochemical characterisation of the J53TplK^R DHFR showed that, not only is the enzyme resistant to Tp, but also that it is slightly less sensitive to the 2,4-diaminopteridine methotrexate

than the J53 parent enzyme. However, the resistance to methotrexate expressed by the J53Tp1K^R enzyme is not as great as that exhibited by the plasmid-mediated enzymes, the J53Tp1K^R DHFR being about one hundred times more susceptible to this drug than the Type I and Type II DHFRs. Nevertheless, the slightly decreased susceptibility to methotrexate may contribute to the theory that this mutant enzyme is indeed a stage in the process to produce an enzyme similar to those encoded by Tp R-plasmids.

One of the most striking differences between the J53 parent Tp-sensitive DHFR and its Tp resistant derivative is the pH profile of the enzymes. The parent enzyme displayed a characteristic pH profile previously associated with Tp sensitive chromosomal enzymes, maintaining over 60% of its activity throughout the pH range 5.0 to 9.0 (Pattishall et al., 1977; Amyes and Smith, 1978). However, the Tp resistant derivative of strain J53 showed two very distinct peaks of activity at pH 5.0 and 6.0. Poe et al. (1979) studying the DHFRs from two mutant E. coli K-12 strains have shown that differences observed in their pH profiles are probably related to slight differences in the local environment of histidine residues at the active site of the enzymes. A similar modification in the local environment may also account for the altered pH profile observed with the J53Tp1K^R DHFR. The results of this thesis indicate that DHFRs encoded by Tn7-like transposons can also exhibit two pH optima at pH 5.0 and 6.0 as can the new transposon mediated enzyme identified here. The differences in pH profile of the transposon mediated enzymes do not, however, appear to reflect alterations in their

susceptibility to antifolate inhibitors. Thus the different pH profiles observed for the J53 and J53Tp1K^R DHFRs are unlikely to have resulted from the change in their respective susceptibilities to Tp.

B. The continuing evolution of the Tp resistance gene

Since the first report of the 9 Mdal transposon, Tn7, encoding Tp and Sm/Sp resistances (Barth et al., 1976) the incidence of Tp resistance within the Enterobacteriaceae has risen markedly (Amyes et al., 1978; Datta et al., 1980; Amyes et al., 1981; Fling et al., 1982; Towner et al., 1982). Examination of many of these isolates has revealed the presence of Tn7 within both the bacterial chromosome and R-plasmids of many different incompatibility groups. This transposon appears therefore to be playing a major role in the spread of Tp resistance.

Investigation of Tp R-plasmids isolated during an epidemic of Tp resistance within the Enterobacteria isolated from patients in Edenhall Hospital revealed that the spread of the resistance gene was again being manifested by transposons within this bacterial population. Transposons were extracted from six of the plasmids. Three of the transposons, Tn4129, Tn4130 and Tn4131 were found to carry the determinants for Sm/Sp resistance in addition to Tp resistance. When these transposons were measured in the vector plasmid RP4 they were estimated to be about 9 Mdal in size which suggests a similarity to Tn7. Each of these transposons possess three restriction sites for the endonuclease Hind III, showing the

presence of two small DNA fragments characteristic of Hind III cleavage of Tn7 (Datta et al., 1979; Lichtenstein and Brenner, 1982; Taylor, 1983). The three other transposons Tn4132, Tn4133 and Tn4134 also carried the gene for Tp resistance but not for Sm resistance. They were estimated to be about 2 Mdal in size when inserted into plasmid RP4. However, the size of the inserted DNA was not easily determined, since transposition of these small transposons was sometimes accompanied by a decrease in the molecular weight of the RP4 containing derivative. This phenomenon has been observed previously with other transposons following their insertion into plasmid RP4 (Barth et al., 1978; Datta et al., 1979; McCombie et al., 1983). Hence, in common with other workers, transposon sizes were determined when an increase in the RP4 transposon containing derivative was observed (McCombie et al., 1983). One of the transposons, Tn4134, when inserted in the plasmid RlE showed an increase in the molecular weight of this plasmid, of 9 million. This suggests that Tn4134 has a molecular size of 9 Mdal. However, if this was the case, insertion of the same transposon into plasmid RP4 would have to have been accompanied by a deletion of at least 7 Mdal, since an increase of only 2 Mdal was calculated for RP4::Tn4134 derviatives. Datta et al. (1979) identified a 9 Mdal transposon, Tn78, in an inc M R-plasmid isolated form a Tp resistant strain of Klebsiella aerogenes K-16. This transposon, like Tn4134, also carried the single resistance determinant for Tp but caused only a very small reduction (1 Mdal) in the molecular weight of RP4 derivatives harbouring Tn78. In addition, unlike Tn4134, no

reversion to Sm/Sp resistance could be demonstrated for Tn78 demonstrating that Tn4134 is quite distinct from Tn78.

The deletions frequently observed following insertion of transposons are usually thought to be a result of the spontaneous loss of small fragments of DNA during or immediately after the insertion process (Weisberg and Adhya, 1977). However, an alternative explanation for the deletions occurring here is that multiple insertion of the transposon into the vector plasmid may have occurred. This event would then provide two or more regions of homologous DNA, between which recombination can occur, with the ultimate loss of the intervening DNA. Transposon mediated deletions of this nature have previously been proposed by Kleckner et al. (1977). This process of multiple insertion also offers an explanation for the observed increase in size of 9 Mdal occurring after acquisition of Tn4134 by plasmid RlE, if recombination between homologous DNA failed to take place.

Evidence for the multiple insertion of the transposons described in this thesis, within plasmid RP4, is provided by the restriction endonuclease digest patterns of the RP4 transposon containing derivatives. Each of the RP4 small transposon derivatives showed a similar restriction pattern. The 2.0 Mdal fragment, obtained following restriction of Tn7 with Hind III, was absent in these digests, confirming that these small transposons are distinct from both Tn7 and Tn78 which in contrast give rise to such fragments (Datta et al., 1979). However, the estimated size of the transposons

at 2 Mdal, indicates that only the smallest fragment of molecular weight 1.65 million could have resulted from two restriction sites in the transposon itself. Plasmid RP4 is known to have a single restriction site for the endonuclease Hind III (Grinsted et al., 1977) and therefore the other fragments obtained following digestion of RP4::Tn4132, RP4::Tn4133 and RP4::Tn4134 must be a result of the insertion of these transposons into several sites of the RP4 genome. Therefore this 'new' transposon, like Tn7, does not appear to exhibit the phenomenon of cis-acting transposition immunity (Hassan and Brevet, 1983) which has so far only been reported for Tn3-like transposons (Robinson et al., 1977).

The presence of the smallest Hind III fragment, of molecular weight 1.65 million, in the digests of each of the six transposons suggests that, despite their different molecular weights, the transposons might be related. Indeed, isolation and detailed characterisation of the DHFR gene product of each transposon revealed that each element encodes the synthesis of a Tp-resistant DHFR with biochemical properties almost indistinguishable from each other and from the Type I Tn7-mediated enzyme of plasmid R483 (Pattishall et al., 1977; Amyes and Smith, 1978). Each of the enzymes is produced in large quantity, about ten times that of the normal E. coli host DHFR. All are heat labile, losing their activity rapidly when raised to 45 °C, and each enzyme is inhibited by 50% by similar levels of Tp and methotrexate with ID_{50}s of about 50 μM for Tp and 5 μM for methotrexate. In addition, the pH and substrate profiles of the enzymes are similar.

The enzymes, however, are not identical as they differ strikingly in their molecular weight. The size of the enzymes produced by Tn4129 and Tn4130 at 35000 and 32000 daltons respectively, is more similar to those found for Type I DHFRs (Pattishall et al., 1977; Amyes and Smith, 1978), whereas the DHFR encoded by each of the small transposons, Tn4132, Tn4133 and Tn4134 has a molecular weight of about 24000. Enzymes of this novel molecular weight, ie. 24000, have never previously been associated with plasmid or transposon mediated resistance. However, Purohit et al. (1981) reported that the DHFR encoded by the T4 bacteriophage comprised of two subunits of about 23000 daltons each. Although, as dimers, the T-even DHFRs are moderately resistant to Tp and temperature sensitive, both characteristics of the new enzymes described in this thesis, it is unclear whether the 23000 dalton monomer is similarly active. In spite of these similarities, the phage enzyme is however distinct from the new transposon encoded enzyme both by its sensitivity to 2,4-diaminopteridines (Mathews, 1967) and its precipitation by ammonium sulphate at 0-40% saturation (Purohit et al., 1981).

The plasmid p699-determined enzyme reported by Fling et al. (1982) is also of a lower molecular weight (between 16000 and 18000) than the Type I and Type II enzymes. Preliminary characterisation of this p699-mediated enzyme, however, shows it to be quite distinct from the enzyme reported here. The level of resistance conferred by plasmids encoding the small molecular weight DHFR isolated by Fling et al. (1982) is only moderate, the MIC of Tp being 64 mgL^{-1} in E.

coli, compared with the high level (MIC >1000 mgL^{-1}) of resistance achieved in strains producing the enzyme reported here. In addition, the Tp 50% inhibitory concentration for the p669 enzyme is approximately 1.5 μM compared with about 50 μM for the enzyme described here. However, more properties of the p669 enzyme require to be studied before a true comparison between it and the new enzyme detected here can be made.

The similarity between the two enzymes described here suggests a common evolutionary origin. In favour of a common origin of the transposons and the DHFR genes themselves is the observation that the 9 Mdal Tn7-like transposon Tn4131, carrying the resistance determinants for Sm/Sp in addition to Tp, encodes a DHFR of 24500 daltons. It would therefore appear that a Tn7-like transposon is coding for a small molecular weight enzyme.

It is known that the Tn7 determined enzyme is made up of two identical subunits about 19000 daltons (Fling and Elwell, 1980; Novak et al., 1983). However, these subunits are not active on their own and therefore may not be directly related to the small molecular weight enzyme reported here. The small transposon DHFR appears also to be made up of subunits, as the enzyme profile shows a small peak at between 10000 and 12000 daltons which has activity. Although the Type II enzyme coded by the R-plasmid R388 has also been shown to consist of subunits of about 10500 (Fling and Elwell, 1980) it is more likely that the new enzyme is made up of part of the two subunits of the Tn7 DHFR, the redundant regions of which are missing.

A point of particular interest was the presence of TpSm/Sp resistance transposons within clinical plasmids which did not express resistance to either Sm or Sp. Several possible explanations for this observation exist. It is conceivable that insertion of the TpSm/Sp transposon within the original clinical plasmid may have occurred adjacent to a resident IS sequence, the IS element exerting a polar effect on the transposon and "switching off" the expression of the Sm/Sp resistance gene (Jordan et al., 1968). Alternatively, an IS element may have inserted into the actual transposon between the promoter and the Sm/Sp resistance gene itself. This again could result in the "switching off" of Sm/Sp resistance gene expression. The role of IS sequence as mobile promoters resulting in the switching "on" or "off" of bacterial genes has been suggested previously by Glansdorff et al. (1980). Following transposition of the transposon to another replicon (in this case RP4) the IS element may be spontaneously lost restoring the expression of the Sm/Sp resistance gene. However, it is also important to consider that the host species may be causing the observed changes in MIC of Sm/Sp. Indeed, the Sm resistance transposon, Tn904, has been shown to express a gene dosage effect when present in E. coli which is not mirrored when the transposon is present within P. aeruginosa (McCombie et al., 1983). A similar gene dosage effect has been suggested for the β-lactamase specified by the plasmid R-1818 when present in Proteus mirabilis (Smith, 1969).

The observed differences in Sm/Sp resistance gene expression of the Tn7-like transposons Tn4130 and Tn4131 suggests that the small

transposons may also be carrying the Sm/Sp resistance gene in a "dormant switched off" state. Selection for spontaneous Sm/Sp resistant mutants of E. coli J62 strains harbouring the small transposons revealed them to mutate at a greater frequency than strains which do not carry the transposons. The location of the mutation was subsequently identified within the Tp resistance transposon. However, this mutation was shown to be unstable and the strains rapidly became sensitive to Sm/Sp again following removal of selective pressures. This would imply that the selective pressure of subculturing in the presence of concentrations of Sm and Sp greater than the MIC of the organism has resulted in the selective amplification of a Sm/Sp resistance gene, which normally exerts a very low level of resistance on its host, with a correlated amplification in the organisms MIC to the drugs. This selective amplification and induced resistance mechanism has previously been reported for several resistance genes including the Tc resistance gene of Tn10 (Spies and Laufs, 1983; Spies et al., 1983) and the kanamycin/neomycin resistance gene of plasmid pRR983 (Peterson and Rownd, 1983). Following removal of the selective pressure, the MIC decreases and the gene copy number returns to normal.

After mutation of RP4 transposon containing derivatives to Sm/Sp resistance it was found that the Km resistance determinant of RP4, and the Tp resistance gene of each of the transposons, Tn4132, Tn4133 and Tn4134, no longer transferred together. Further experiments in which Tp resistant transconjugants were tested for the presence of RP4, suggested that the transposons were probably

transferring on this plasmid vector but that following entry into the recipient cell the transposon was excised and inserted into the bacterial chromosome with the subsequent loss of RP4 from the majority of transconjugants. Gawton-Burke and Clewell (1982) have observed that the Tc resistance transposon Tn916, when present on a conjugative plasmid is excised at fairly high frequency when transferred to recipient strains with between 43 and 77% of transconjugant strains losing the transposon. It is possible that a similar excision event is occurring here but that in this case integration into the chromosome, of the excised transposon, subsequently takes place. Philips and Novick (1979) have suggested that the erythromycin transposon, Tn554, upon entry into recipient cells, undergoes a "zygotic induction" of an integrase or transposase which facilitates its insertion into the chromosome.

However, contrary to the expected results, the Tp resistance gene in transconjugants which had been shown to be devoid of plasmid DNA, was found to be transferable, suggesting that somehow the mutated transposons were now able to mediate their own transfer. A number of recent reports have suggested similar transfer of resistance genes in the apparent absence of extrachromosomal DNA. Franke and Clewell (1980, 1981) and Gawton-Burke and Clewell (1982) have reported the transfer of Tn916 between strains of Streptococcus faecalis devoid of plasmid DNA. These authors suggest that this transposon, having an approximate size of 10 Mdal, may possess a fertility potential capable of mobilizing determinants that reside on it and as such Tn916 may represent a new class of genetic elements

that could be referred to as "conjugative transposons". Smith et al. (1981) have reported similar findings concerning transferable Tc resistance in Clostridium difficile, and transfer of clindamycin and Tc resistance in Bacteroides fragilis is also believed to occur by a conjugal transfer system in the absence of plasmid DNA (Mays et al., 1982; Smith et al., 1982).

However, in view of the size of transposons Tn4132, Tn4133 and Tn4134 it seems unlikely that these transposons would possess the genes required, to encode the enzymes necessary, to mediate their own transfer. A more plausible explanation would be the integration of RP4 into the bacterial chromosome. In favour of this hypothesis are reports of certain R-plasmids in E. coli (Nugent, 1981) and Haemophilus (Stuy, 1980) which prefer to be integrated in the chromosome. However, although Watson and Scaife (1980) have shown that insertion of RP4 derivatives containing the att region of lambda, into the bacterial chromosome of E. coli, results in loss of expression of their incompatibility function, it is generally believed that integration of R-plasmids into the chromosome of E. coli is not usually accompanied by the complete loss of expression of plasmid function, as would appear to be the case here (Datta and Barth, 1976; Martin et al., 1981; Nugent, 1981). Nevertheless, several authors have indicated that formation of R' plasmids by recombination of inc P plasmids, notably R68.45, with the bacterial chromosome, can result in structural instability of the R-plasmid while the chromosomal markers remain stable (Haas and Holloway, 1976, 1978). This instability is not of the type in which the whole

plasmid is lost as a unit but instead results in a progressive loss of plasmid markers during bacterial replication, until either the remnant stabilizes as a more or less permanent function of the original plasmid or the whole plasmid is lost (Godfrey et al., 1980). It is thought that the deletions which occur are generated by intramolecular recombination between regions of homology, possibly generated following mobilization of the bacterial chromosome (Godfrey et al., 1980). It is conceivable, therefore, that a similar situation may have occurred here. Following recombination between RP4 and the transposons, the added selective pressure of subculturing in high concentrations of Sm/Sp may have enhanced the selective replication of the transposon, providing additional regions of homology and subsequently, through intramolecular recombination, causing extensive structural instability of RP4, the transposon itself remaining stable. This could then lead to the loss to the RP4 resistance genes and result in the ultimate formation of a stable RP4 transposon containing plasmid which may preferentially become integrated into the bacterial chromosome, from where it could subsequently mediate the transfer of the Tp resistance gene.

In order to determine whether transposition of the mutated Tp resistance transposon resulted in the simultaneous transposition of Sm/Sp resistance, plasmid RlE was introduced into strains harbouring RP4 containing the mutated transposons and employed as a second vector for the transposons. However, a similar phenomenon of resistance gene transfer in the apparent absence of plasmid DNA was

again found to occur. It seems likely, therefore, that complete loss of RP4 was not being observed, but rather that an RP4 transposon-containing remnant derivative had again integrated into the bacterial chromosome. The spontaneous loss of plasmid RlE from the cells could then be a result of successive subculture of the strains in the absence of favourable selective pressures for this plasmid, ie. subcultures in the absence of Cm.

Although plasmid integration into the chromosome is an attractive hypothesis, it is important to consider that the observed genetic exchange may possibly be occurring by some specialised transduction, by a difficult-to-detect (perhaps defective) prophage as a means of transfer.

C. Conclusions

Although the source of the Tp resistance genes remains unsolved, it is clear from the results of this thesis that the resistance genes are continuing to evolve. It is difficult to assess accurately the order in which the evolutionary processes have occurred. Nevertheless, it would appear that since their origin the Tp resistance genes have gradually become more efficient and promiscuous genetic elements.

The properties of the mutant enzyme produced by E. coli J53TplK^R suggest that the plasmid genes may possibly have originated as chromosomal genes. Alternatively, further studies of Tp-sensitive R-plasmids may reveal the presence of DHFR genes capable of similar

mutations to Tp resistance. The mutated gene, encoding a Tp resistant DHFR, might then have become incorporated, through legitimate recombination, into a wide host range plasmid, allowing its diversification into many bacterial species. During recombination between the resistance genes and the chromosome or plasmid genomes, insertion of the Type I DHFR gene may conceivably have occurred within, or close to, resident IS sequences. Such an event may possibly contribute to the formation of a transposable element.

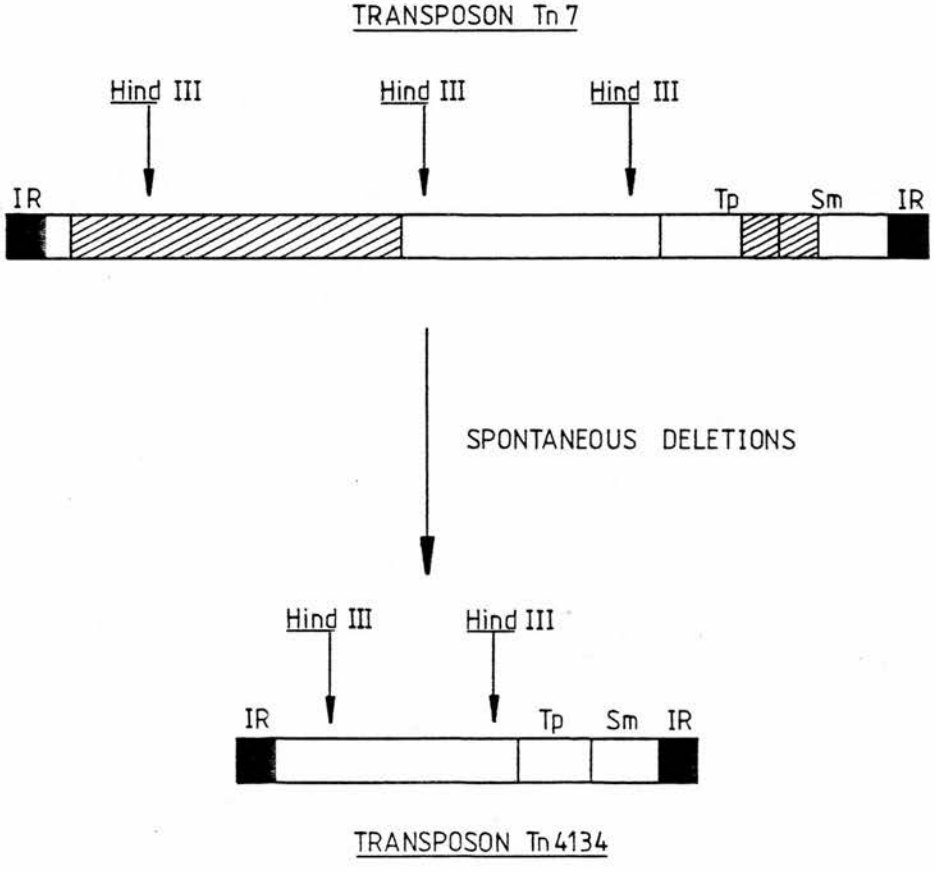
During the last four years the spread of the TpSm/Sp resistance transposon Tn7 has become more prevalent, with increased reports of its presence within both the chromosome and plasmids of pathogenic bacteria. The bacterial population studied in this thesis also owed much of its resistance to Tp resistance transposons. However, in addition to Tn7-like transposons, a second much smaller transposon, epitomised by Tn4134 was also identified which confers resistance to Tp alone. Biochemical, molecular and genetic analysis of both the Tn7-like and new transposons suggests that they are related.

The absence of expression of the Sm/Sp resistance genes, the lower molecular weight of the Tp resistant DHFR and the presence of only one fragment of DNA following digestion of the small transposon with Hind III, all suggest that the small transposon has probably evolved from Tn7 by the loss of less important regions of Tn7 DNA (figure 32). The ability of the small transposons to mutate back to Sm/Sp resistance implies that these genes are still present. How-

Figure 32. Proposed model for the evolution of transposon Tn4134 from transposon Tn7.

IR = inverted repeat sequence; Tp = trimethoprim resistance gene; Sm = streptomycin/spectinomycin resistance gene; Hind III = Hind III restriction site. Shaded areas represent spontaneously deleted fragments of DNA.

Note: Diagrams are not to scale.



ever, if formation of the small transposon is a result of the loss of Tn7 DNA it is possible that the excised fragment of DNA contains a gene encoding an inducer enzyme required for Sm/Sp resistance gene expression. The decreased DHFR molecular weight could also be accounted for by the loss of redundant regions of DNA within the DHFR gene. Finally, the presence of only one Hind III restriction fragment could be a result of the excision of an internal fragment of Tn7 containing one of the three Hind III restriction sites.

The production of this small molecular weight Tp resistance transposon may hence be a further stage in the evolution of Tp resistance genes.

REFERENCES

ABRAHAM, E.P. and CHAIN, E. (1940). An enzyme from bacteria able to destroy penicillin. Nature (London), 146, 837.

ACAR, J.F., GOLDSTEIN, F.W., GERBAUD, G.R. and CHABBERT, Y.A. (1977). Plasmides de résistance au triméthoprim: transférabilité et groupes d'incompatibilité. Ann. Inst. Pasteur, 128, 41.

AKIBA, T., KOYAME, K., ISHIKI, Y., KUMIRA, S. (1960). On the mechanism of the development of multiple drug-resistant clones of Shigella. Jap. J. Microbiol., 4, 219.

ALIET, B. (1979). Mu insertion duplicates a 5 base pair sequence at the host inserted site. Cell, 16, 123.

AMBLER, R.P. and SCOTT, G.K. (1978). Partial amino acid sequence of penicillinase coded by Escherichia coli plasmid R6K. Proc. Nat. Acad. Sci. USA., 75, 3732.

AMYES, S.G.B. (1974). The susceptibility of gram-negative bacteria to antifolate chemotherapeutic agents. Ph.D. thesis, London.

AMYES, S.G.B. (1982) Bactericidal activity of trimethoprim alone and in combination with sulphamethoxazole on susceptible and resistant Escherichia coli K-12. Antimicrob. Ag. Chemother., 21, 288.

AMYES, S.G.B. (1983). Trimethoprim resistance in bacteria isolated from farm animals. J. Pharm. Pharmacol., 35, 65p.

AMYES, S.G.B. and SMITH, J.T. (1974a). R-factor trimethoprim resistance mechanism: an insusceptible target site. Biochem. Biophys. Res. Commun., 58, 412.

AMYES, S.G.B. and SMITH, J.T. (1974b). Trimethoprim action and its analogy with thymine starvation. Antimicrob. Ag. Chemother., 5, 169.

AMYES, S.G.B. and SMITH, J.T. (1975). Thymineless mutants and their resistance to trimethoprim. J. Antimicrob. Chemother., 1, 85.

AMYES, S.G.B. and SMITH, J.T. (1976). The purification and properties of the trimethoprim-resistant dihydrofolate reductase mediated by the R-factor, R388. Eur. J. Biochem., 61, 597.

AMYES, S.G.B. and SMITH, J.T. (1977). Trimethoprim resistance controlled by a combination of plasmid and chromosomal genes. Genet. Res., 29, 35.

AMYES, S.G.B. and SMITH, J.T. (1978). R-factor mediated dihydrofolate reductases which confer trimethoprim resistance. J. Gen. Microbiol., 107, 263.

AMYES, S.G.B., EMMERSON, A.M. and SMITH, J.T. (1978). R-factor mediated trimethoprim resistance: result of two three-month clinical surveys. J. Clin. Pathol., 31, 850.

AMYES, S.G.B., McMILLAN, C.J. and DRYSDALE,J.L. (1981). Transferable trimethoprim resistance amongst hospital isolates. In New trends in antibiotics: research and therapy. Grassi and Sabath (eds.). Elsevier/North Holland Biomedical Press, Amsterdam, p325.

AMYES, S.G.B., DRYSDALE, J.L., McMILLAN, C.J. and YOUNG, H.K. (1982). The diversity of trimethoprim resistance within hospital isolates. J. Pharm. Pharmacol., 34, 60p.

ANDERSON, E.S. (1968). The ecology of transferable drug resistance in the Enterobacteria. Annu. Rev. Microbiol., 22, 131.

ANDERSON, E.S. and DATTA, N. (1965). Resistance to penicillins and its transfer in Enterobacteriaceae. Lancet, i, 407.

ANDERSON, E.S., HUMPHREYS, G.O. and WILLSHAW, G.A. (1975). The molecular relatedness of R-factors in enterobacteria of human and animal origin. J. Gen. Microbiol., 91, 376.

ANDERSON, J.D., LACEY, R.W., LEWIS, E.L. and SELLIN, M.A. (1974). Failure to demonstrate an advantage in combining sulphamethoxazole with trimethoprim in an experimental model of urinary infection. J. Clin. Pathol., 27, 619.

ANDREWS, P. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem. J., 91, 222.

ANGIER, R.B., BOOTHE, J.H., HUTCHINGS, B.L., MOWAT, J.H., SEMB, J., STOKSLAD, E.L.R., SUBBAROW, Y., WALLER, C.W., COSULICH, D.B., FAHRENBACH, M.J., HULTQUIST, M.E., KUH, E., NORTHEY, E.H., SEEGER, D.R., SICKELS, J.P., SMITH, J.M.Jr. (1946). Synthesis of a compound identical with the L. casei factor isolated from liver. Science, 103, 667.

ARTHUR, A. and SHERRATT, D.J. (1979). Dissection of the transposition process: A transposon-encoded site-specific recombination system. Mol. Gen. Genet., 175, 267.

ASHFORD, W.A., GOLASH, R.G., HEMMING, V.G. (1976). Penicillinase-producing Neisseria gonorrhoeae. Lancet, ii, 657.

AVERY, O.T., MacLEOD, C.M. and McCARTY, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a deoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med., 79, 137.

BACCANARI, D.P., DALUGE, S. and KING, R.W. (1982). Inhibition of dihydrofolate reductase: Effect of reduced nicotinamide adenine dinucleotide phosphate on the selectivity and affinity of diaminobenzylpyrimidines. Biochemistry, 21, 5068.

BACCANARI, D.P., PHILLIPS, A., SMITH, S., SINSKI, D. and BURCHALL, J. (1975). Purification and properties of Escherichia coli dihydrofolate reductase. Biochemistry, 14, 5267.

BACCANARI, D.P., AVERETT, D., BRIGGS, C. and BURCHALL, J. (1977). Escherichia coli dihydrofolate reductase: Isolation and characterization of two isoenzymes. Biochemistry, 16, 3566.

BACHMANN, B.J. (1972). Pedigrees of some mutant strains of Escherichia coli K-12. Bacteriol. Rev., 36, 525.

BALL, P.R., SHALES, S.W. and CHOPRA, I. (1980). Plasmid mediated tetracycline resistance in Eshcerichia coli involves increased efflux of the antibiotic. Biochem. Biophys. Res. Commun., 93, 74.

BARNER, H.D. and COHEN, S.S. (1959). Virus-induced acquisition of metabolic function. IV. Thymidylate synthetase in thymine-requiring Escherichia coli infected by T_2 and T_5 bacteriophages. J. Biol. Chem., 234, 2987.

BARTH, P.T., GRINTER, N.J. and BRADLEY, D.E. (1978). Conjugal transfer system of plasmid RP4: Analysis by transposon 7 insertion. J. Bacteriol., 133, 43.

BARTH, P.T., DATTA, N., HEDGES, R.W. and GRINTER, N.J. (1976). Transposition of a deoxyribonucleic acid sequence encoding trimethoprim and streptomycin resistance from R483 to other replicons. J. Bacteriol., 125, 800.

BATLEY, K.E. and MORRIS, H.R. (1977). Dihydrofolate reductase from Lactobacillus casei: N-terminal sequence and comparison with the substrate binding region of other reductases. Biochem. Biophys. Res. Commun., 75, 1010.

BEADLE, G.W. and TATUM, E.L. (1941). Genetic control of biochemical reactions in Neurospora. Proc. Nat. Acad. Sci. USA., 27, 499.

BENNETT, C.D., RODKEY, J.A., SONDEY, J.M. and HIRSCHMANN, R. (1978). Dihydrofolate reductase: the amino acid sequence of the enzyme from a methotrexate-resistant mutant of Escherichia coli. Biochemistry, 17, 1328.

BENNETT, P.M., de la CRUZ, F. and GRINSTED, J. (1983). Cointegrates are not obligatory intermediates in transposition of Tn3 and Tn21. Nature (London), 305, 743.

BENVENISTE, R. and DAVIES, J. (1971). R-factor mediated gentamicin resistance: a new enzyme which modifies aminoglycoside antibiotics. FEBS Letts., 14, 293.

BENVENISTE, R., DAVIES, J. (1973). Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Nat. Acad.Sci. USA., 70, 2276.

BERG, D.E. and DRUMMOND, M. (1978). Absence of DNA sequences homologous to transposable element Tn5(Kan) in the chromosome of Escherichia coli K-12. J. Bacteriol., 136, 419.

BIRDSALL, B., BURGEN, A.S.V., ROBERTS, G.C.K. (1980). Binding of coenzyme analogues to Lactobacillus casei dihydrofolate reductase: Binary and ternary complexes. Biochemistry, 19, 3723.

BITAR, K.G., BLANKENSHIP, D.T., WALSH, K.A., DUNLAP, R.B., REDDY, A.V. and FREISHEIM, J.H. (1977). Amino acid sequence of dihydrofolate reductase from an amethopterin-resistant strain of Lactobacillus casei. FEBS. Letts., 80, 119.

BLAKELY, R.L. (1969). The biochemistry of folic acid and related pteridines. North Holland, Amsterdam.

BLAKELY, R.L. and McDOUGAL, B.M. (1961). Dihydrofolic reductase from Streptococcus faecalis R. J. Biol. Chem., 236, 1163.

BREEZE, A.S., SIMS, P. and STACEY,K.A. (1975). Trimethoprim-resistant mutants of Escherichia coli K-12: preliminary genetic mapping. Genet. Res., 25, 207.

BROAD, D.F. and SMITH, J.T. (1982). Classification of trimethoprim-resistant dihydrofolate reductases mediated by R-plasmids using isoelectric focussing. Eur. J. Biochem., 125, 617.

BROWN, G.M. (1962). The biosynthesis of folic acid. Inhibition by sulphonamides. J. Biol. Chem., 237, 536.

BROWN, G.M. (1971). The biosynthesis of pteridines. Advan. Enzymol., 35, 35.

BROWN, G.M., WEISMAN, R.A. and MOLINAR, D. (1961). The biosynthesis of folic acid. I. Substrate and cofactor requirements for enzymatic synthesis by cell free extracts of Escherichia coli. J. Biol. Chem., 236, 2534.

BRUMFITT, W. and PURSELL, R. (1972). Double blind trial to compare ampicillin, cephalexin, cotrimoxazole and trimethoprim in treatment of urinary infection. Brit. Med. J., ii, 673.

BRUMFITT, W. and PURSELL, R. (1973). Trimethoprim/sulphamethoxazole in the treatment of urinary infection. Med. J. Aust. (Special Suppl.), 1, 44.

BREZEZINSKA, M., BENVENISTE, R., DAVIES, J., DANIELS, P.J.L. and WEINSTEIN, J. (1972). Gentamicin resistance in strains of Pseudomonas aeruginosa mediated by enzymatic N-acetylation of the deoxystreptamine moiety. Biochemistry, 11, 761.

BUKHARI, A.I. and ZIPSER, D. (1972). Random insertion of Mu-1 DNA within a single gene. Nature (London), 236, 240.

BUKHARI, A.I., SHAPIRO, J.A. and ADHYA, S. (1977). DNA insertion elements, episomes and plasmids. Cold Spring Harbor.

BURCHALL, J.J. (1969). Comparative studies of dihydrofolate reductase. Postgrad. Med. J., 45, (Suppl.), 29.

BURCHALL, J.J. (1971). Comparative biochemistry of dihydrofolate reductase. Ann. N.Y. Acad. Sci., 186, 143.

BURCHALL, J.J. (1979). The development of the diaminopyrimidines. J. Antimicrob. Chemother., 5, (Suppl. B), 3.

BURCHALL, J.J. and HITCHINGS, G.H. (1965). Inhibitor binding analysis of dihydrofolate reductases from various species. Mol. Pharmacol., 1, 126.

BURCHALL, J.J. and HITCHINGS, G.H. (1968). The role of metabolites and antimetabolites in the control of folate coenzyme synthesis. Advan. Enzyme Regul., 6, 323.

BUSHBY, S.R.M. (1969). Combined antibacterial action in vitro of trimethoprim and sulphonamides. Postgrad. Med. J., 45, (Suppl.), 10.

BUSHBY, S.R.M. (1973). Trimethoprim-sulphamethoxazole: in vitro microbiological aspects. J. Infec. Dis., 128, (Suppl.), S442.

BUSHBY, S.R.M. and HITCHINGS, G.H. (1968). Trimethoprim, a sulphonamide potentiator. Brit. J. Pharmacol. Chemother., 33, 72.

CALOS, M.P., JOHNSRUD, L. and MILLER, J.H. (1978). DNA sequences at the integration sites of the insertion element IS1. Cell. 13, 411.

CAUDRY, S.D. and STANISICH, V.A. (1979). Incidence of antibiotic-resistant Escherichia coli associated with frozen chicken carcasses and characterization of conjugative R-plasmids derived from such strains. Antimicrob. Ag. Chemother., 16, 701.

CAVALLI-SFORZA, L.L., LEDERBERG, J. and LEDERBERG, E.M. (1953). An infective factor controlling sex compatibiltiy in Bacterium coli. J. Gen. Microbiol., 8, 89.

CHAIN, E., FLOREY, H.W., GARDNER, A.D., HEATLEY, N.G., JENNINGS, M.A., ORR-EWING, J., SANDER, A.G. (1940). Penicillin as a chemotherapeutic agent. Lancet, ii, 226.

CHANG, F.N. and FLAKS, J.G. (1972). Binding of dihydrostreptomycin to Escherichia coli ribosomes: Characteristics and equilibrium of the reaction. Antimicrob. Ag. Chemother., 2, 294.

CHOPRA, I. (1976). Mechanisms of resistance to fusidic acid in Staphylococcus aureus. J. Gen. Microbiol., 96, 229.

CHOPRA, I. and BALL, P.R. (1982). Transport of antibiotics into bacteria. Adv. Microb. Physiol., 23, 183.

CLOWES, R.C. and ROWLEY, D. (1954). Some observations on linkage effects in genetic recombination in Escherichia coli K-12. J. Gen. Microbiol., 11, 250.

COETZEE, J.N., DATTA, N. and Hedges, R.W. (1972). R-factors from Proteus rettgeri. J. Gen. Microbiol., 72, 543.

COHEN, S.N., CASADABAN, M., CHOU, J. and Tu, C. (1979). Studies of the specificity and control of transposition of the Tn3 element. Cold Spring Harbor Symp. Quant. Biol., 43, 1247.

COLEMAN, D.C., CHOPRA, I., SHALES, S.W., HOWE, T.G.B., FOSTER, T.J. (1983). Analysis of tetracycline resistance encoded by transposon Tn10: deletion mapping of tetracycline sensitive point mutations and identification of two structural genes. J. Bacteriol., 153, 921.

COMERE, B. and MENZIES, R. (1974). Haemophilus influenzae apparently resistant to trimethoprim. Brit. Med. J., i, 77.

COOKE, M.D. (1976). Antibiotic resistance among coliform and fecal coliform bacteria isolated from the freshwater mussel Hydridella menziessii. Antimicrob. Ag. Chemother., 9, 885.

DALE, J.W. and SMITH, J.T. (1971). Some relationships between R-factor and chromosomal β-lactamase in Gram-negative bacteria. Biochem J., 123, 507.

DANIEL, L.J. and NORRIS, L.C. (1947). Growth inhibition of bacteria by synthetic pterins. II. Studies with Escherichia coli, Staphylococcus aureus and Lactobacillus arabynosus showing synergism between pterin and sulphonamides. J. Biol. Chem., 170, 747.

DANIEL, L.J., NORRIS, L.C., SCOTT, M.L. and HEUSER, G.F. (1947). Growth inhibition of bacteria by synthetic pterins. I. Studies with Streptococcus faecalis, Lactobacillus casei and Lactobacillus arabynosus. J. Biol. Chem., 169, 689.

DARRELL, J.H., GARROD, L.P.and WATERWORTH, P.M. (1968). Trimethoprim: laboratory and clinical studies. J. Clin. Pathol., 21, 202.

DARWIN, C.R. (1859). On the Origin of Species. London.

DATTA, N. (1977). Classification of plasmids as an aid to understanding their epidemiology and evolution. J. Antimicrob. Chemother., 3, (Suppl C), 19.

DATTA, N. (1969). Drug resistance and R-factors in the bowel bacteria of London patients before and after admission to hospital. Brit. Med. J., ii, 407.

DATTA, N. and HEDGES, R.W. (1971). Compatibility groups among fi⁻ R-factors. Nature (London), 234, 222.

DATTA, N. and HEDGES, R.W. (1972). Trimethoprim resistance conferred by W plasmids in Enterobacteriaceae. J. Gen. Microbiol., 72, 349.

DATTA, N. and BARTH, P.T. (1976). Hfr formation by I-pilus-determining plasmids in Escherichia coli K-12. J. Bacteriol., 125, 811.

DATTA, N., NUGENT, M. and RICHARDS, H. (1980). Transposons encoding trimethoprim or gentamicin resistance in medically important bacteria. Cold Spring Harbor Symp. Quant. Biol., 45, 45.

DATTA, N., HEDGES, R.W., SHAW, E.J., SYKES, R.B. and RICHMOND, M.H. (1971). Properties of an R-factor from Pseudomonas aeruginosa. J. Bacteriol., 108, 1244.

DATTA, N., HUGHES, V.M., NUGENT, M.E. and RICHARDS, H. (1979). Plasmids and transposons and their stability and mutability in bacteria isolated during an outbreak of hospital infection. Plasmid, 2, 182.

DAVIS, B.D. and MINGIOLI, E.S. (1950). Mutants of Escherichia coli requiring methionine or vitamin B_{12}. J. Bacteriol., 60, 17.

DAVIS, B.J. (1964). Disc electrophoresis - II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci., 121, 404.

DIXON, M. and WEBB, E.C. (1958). Enzymes, London. Longmans, Green and Co. Ltd. p21.

DATTA, N. (1962) Transmissible drug resistance in an epidemic strain of Salmonella typhimurium. J. Hyg. 60, 301.

DOMAGK, G. (1935). Ein Beitrag zur Chemotherapie der bakteriellen Infektionen. Deut. Med. Wochenschr., 61, 250.

DOOLITTLE, R.F. (1981). Similar amino acid sequences: chance or common ancestry? Science, 214, 149.

DUBNAU, E. and STOCKER, B.A.D. (1964). Genetics of plasmids in Salmonella typhimurium. Nature (London), 204, 1112.

EHRLICH, P. (1913). Address in Pathology on Chemotherapeutics: Scientific Principles, methods and results. Lancet, ii, 445.

ELWELL, L.P., de GRAAF, J., SEIBERT, D. and FALKOW, S. (1975). Plasmid mediated ampicillin resistance in Haemophilus influenza type b. Infect. Immun., 12, 404.

ERICKSON, J.S. and MATHEWS, C.K. (1971). T4 bacteriophage-specific dihydrofolate reductase: purification to homogeneity by affinity chromatography. Biochem. Biophys. Res. Commun., 43, 1164.

ERICKSON, J.S. and MATHEWS, C.K. (1973). Dihydrofolate reductases of Escherichia coli and Bacteriophage T4. A spectrofluorometric study. Biochemistry, 12, 372.

FALCO, E.A., GOODWIN, L.E., HITCHINGS, G.H., ROLLO, I.M. and RUSSELL, P.B. (1951). 2,4-Diamino-pyrimidines - A new series of antimalarials. Brit. J. Pharmacol. Chemother., 6, 185.

FALKOW, S., GUERRY, P., HEDGES, R.W. and DATTA, N. (1974). Polynucleotide sequence relationships among plasmids of the I compatibility complex. J. Gen. Microbiol., 85, 65.

FIANDT, M., SZYBALSKI, W. and MALAMY, M.H. (1972). Polar mutations in lac, gal and phage λ consist of a few IS-DNA sequences inserted with either orientation. Mol. Gen. Genet., 119, 223.

FLEMING, A. (1929). On antibacterial action of cultures of a penicillium with special reference to their use in isolation of B. influenzae. Brit. J. Exp. Pathol., 10, 226.

FLEMING, M.P. (1973). Trimethoprim resistance and its transferability in Escherichia coli isolated from calves treated with trimethoprim-sulphadiazine: a two year study. J. Hyg., 71, 669.

FLEMING, M.P., DATTA, N. and GRÜNEBERG, R.N. (1972). Trimethoprim resistance determined by R-factors. Brit. Med. J., i, 726.

FLING, M.E. and ELWELL, L.P. (1980). Protein expression in Escherichia coli minicells containing recombinant plasmids specifying trimethoprim-resistant dihydrofolate reductase. J. Bacteriol., 141, 779.

FLING, M.E. and RICHARDS, C. (1983). The nucleotide sequence of the trimethoprim-resistant dihydrofolate reductase gene harboured by Tn7. Nucleic Acids Res., 11, 5147.

FLING, M.E., WALTON, L. and ELWELL, L.P. (1982). Monitoring of plasmid-encoded, trimethoprim-resistant dihydrofolate reductase genes: detection of a new resistant enzyme. Antimicrob. Ag. Chemother., 22, 882.

FRANKE, A.E. and CLEWELL, D.B. (1980). Evidence for conjugal transfer of a Streptococcus faecalis transposon (Tn916) from a chromosomal site in the absence of plasmid DNA. Cold Spring Harbor Symp. Quant. Biol., 45, 77.

FRANKE, A. and CLEWELL, D.B. (1981). Evidence for a chromosome-borne resistance transposon (Tn916) om Streptococcus faecalis that is capable of 'conjugal' transfer in the absence of a conjugative plasmid. J. Bacteriol., 145, 494.

FREISHEIM, J.H., SMITH, C.C. and GUZY, P.M. (1972). Dihydrofolate recuctase and thymidylate synthetase in strains of Streptococcus faecium resistant to pyrimethamine (PM), chlorguanide triazine (CGT), trimethoprim (TMP) and amethopterin (MTX). Arch. Biochem. Biophys., 148, 1.

FRUENSGAARD, K. and KOMER, B. (1974). Alterations in the sensitivity pattern after use of trimethoprim/sulphamethoxazole for two years in the treatment of urinary tract infections. Chemother., 20, 97.

FUTTERMAN, S. (1957). Enzymatic reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. J. Biol. Chem., 228, 1031.

FUTTERMAN, S. and SILVERMAN, M. (1957). The "inactivation" of folic acid by liver. J. Biol. Chem., 224, 31.

GAFFNEY, D.F., CUNDLIFFE, E. and FOSTER, T.J. (1981). Chloramphenicol resistance that does not involve chloramphenicol acetyltransferase encoded by plasmids from gram negative bacteria. J. Gen Microbiol., 125, 113.

GALAS, D. J. and CHANDLER, M. (1981). On the molecular mechanisms of transposition. Proc. Nat. Acad. Sci. USA., 78, 4858.

GAWTON-BURKE, C. and CLEWELL, D.B. (1982). A transposon in Streptococcus faecalis with fertility properties. Nature (London), 300, 281.

GILL, R., HEFFRON, F. and FALKOW, S. (1979). Identification of the protein encoded by the transposable element Tn3 which is required for its transposition. Nature (London), 282, 797.

GILL, R., HEFFRON, F., DOUGAN, G. and FALKOW, S. (1978). Analysis of sequences transposed by complementation of two classes of transposition-deficient mutants of Tn3. J. Bacteriol., 136, 742.

GLANSDORFF, N., CHARLIER, D. and ZAFARULLAH, M. (1980). Activation of gene expression by IS2 and IS3. Cold Spring Harbor Symp. Quant. Biol., 45, 153.

GLEISNER, J.M., PETERSON, D.L. and BLAKLEY, R.L. (1974). Amino acid sequence of dihydrofolate reductase from a methotrexate-resistant mutant of Streptococcus faecuim and identification of methionine residues at the inhibitor binding site. Proc. Nat. Acad. Sci. USA., 71, 3001.

GODFREY, A.J., MORGAN, A.F. and HOLLOWAY, B.W. (1980). Structural instability of inc P-1 plasmids in Pseudomonas aeruginosa PAT involves interaction with plasmid PVS1. J. Bacteriol., 144, 622.

GOLDSTEIN, A., ARANOW, L. and KALMAN, S.M. (1968). Principles of Drug Action: the basis of Pharmacology. Hoeber, New York.

GORAI, A.P., HEFFRON, F., FALKOW, S., HEDGES, R.W. and DATTA, N. (1979). Electron microscope heterduplex studies of sequence relationships among plasmids of the W incompatibility group. Plasmid, 2, 485.

GREENBERG, F.R. (1954). Role of folic acid derivatives in purine biosynthesis. Fed. Proc., 13, 745.

GREENWOOD, D. (1979). Relevance of in vitro synergy to therapy; does synergy between diaminopyrimidines and sulphonamides operate at concentrations achievable in urine? J. Antimicrob. Chemother., 5, (Suppl. B), 85.

GREENWOOD, D. and O'GRADY, F. (1976). Activity and interaction of trimethoprim and sulphamethoxazole against Escherichia coli. J. Clin. Pathol., 29, 162.

GREY, D. and HAMILTON-MILLER, J.M.T. (1977). Sensitivity of Pseudomonas aeruginosa to sulphonamides and trimethoprim and the activity of the combination trimethoprim:sulphamethoxazole. J. Med. Microbiol., 10, 273.

GREY, D., HAMILTON-MILLER, J.M.T., BRUMFITT, W. (1979). Incidence and mechanisms of resistance to trimethoprim in clinically isolated gram negative bacteria. Chemother., 25, 147.

GRIFFIN, M.J. and BROWN, M.J. (1964). The biosynthesis of folic acid. III. Enzymatic formation of dihydrofolic acid from dihydropteroic acid and of tetrahydropteroylpolyglutamic acid compound from tetrahydrofolic acid. J. Biol. Chem., 239, 310.

GRINDLEY, N.D.F. (1978). IS1 insertion generates duplication of a nine base pair sequence at its target site. Cell, 13, 419.

GRINDLEY, N.D.F. and SHERRATT, D.J. (1978). Sequence analysis at IS1 insertion sites: Models for transposition. Cold Spring Harbor Symp. Quant. Biol., 43, 1257.

GRINDLEY, N.D.F., HUMPHREYS, G.O. and ANDERSON, E.S. (1973). Molecular studies of R-factor compatibility groups. J. Bacteriol., 115, 387.

GRINSTED, J., BENNETT, P.M and RICHMOND, M.H. (1977). A restriction map of R-plasmid RP1. Plasmid, 1, 34.

GRUNBERG, E. and BESKID, G. (1977). Studies on the in vitro development of drug resistance of Proteae to sulphonamides, trimethoprim and combinations of a sulphonamide and trimethoprim. Chemother., 23, 309.

GRUNEBERG, R.N. (1976). Susceptibility of urinary pathogens to various antimicrobial substances: a four year study. J. Clin. Pathol., 29, 292.

GUYER, M.S. (1978). The $\gamma\delta$ sequence of F is an insertion sequence. J. Mol. Biol., 126, 347.

HAAS, D. and HOLLOWAY, B.W. (1976). R-factor variants with enhanced sex factor activity in Pseudomonas aeruginosa. Mol. Gen. Genet., 144, 243.

HAAS, D. and HOLLOWAY, B.W. (1978). Chromosome mobilization by the R-plasmid R68.45: A tool in Pseudomonas genetics. Mol. Gen. Genet., 158, 229.

HAMILTON-MILLER, J.M.T. (1979). Mechanisms and distribution of bacterial resistance to diaminopyrimidines and sulphonamides. J. Antimicrob. Chemother., 5, (Suppl.B), 61.

HAMILTON-MILLER, J.M.T. and GREY, D. (1975). Resistance to trimethoprim in Klebsiella isolated before its introduction. J. Antimicrob. Chemother., 1, 213.

HANSEN, I., NEILSEN, M.L., HEERFORDT, L., HENRIKSEN, B.I. and BERTELSEN, S. (1973). Trimethoprim in normal and pathological human lung tissue. Chemother., 19, 221.

HARADA, K., KAMEDA, M., SUZUKI, M,, SHIGEHARA, S. and MITSUHASHI, S. (1967). Drug resistance of enteric bacteria. VIII. Chromosomal location of non-transferable R-factor in Escherichia coli. J. Bacteriol., 93, 1236.

HARSHEY, R.M. and BUKHARI, A.I. (1981). A mechanism of DNA transposition. Proc. Nat. Acad. Sci. USA., 78, 1090.

HASSAN, D.M. and BREVET, J. (1983). Absence of cis-acting transposition immunity with Tn7. Plasmid, 10, 31.

HAYES, W. (1953a). Observations on a transmissible agent determining sexual differentiation in Bacterium coli. J. Gen. Microbiol. 8, 72.

HAYES, W. (1953b). The mechanism of genetic recombination in Escherichia coli. Cold Spring Harbor Symp. Quant. Biol., 18, 75.

HEDGES, R.W. and JACOB, A.E. (1974). Transposition of ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. 132, 31.

HEDSTROM, R.C., CRIDER, B.P. and EAGON, R.G. (1982). Comparison of kinetics of active tetracycline uptake and active tetracycline efflux in sensitive and plasmid RP4-containing Pseudomonas putida. J. Bacteriol., 152, 255.

HEFFRON, F., RUBENS, C. and FALKOW, S. (1975). Translocation of a plasmid DNA sequence which mediates ampicillin resistance: molecular nature and specificity of insertion. Proc. Nat. Acad. Sci. USA., 72, 3623.

HEFFRON, F., BEDINGER, P., CHAMPOUX, J.J. and FALKOW, S. (1977). Deletions affecting the transposition of an antibiotic resistance gene. Proc. Nat. Acad. Sci. USA., 74, 702.

HEFFRON, F., McCARTHY, J., OHTSUBO, H. and OHTSUBO, E. (1979). DNA sequence analysis of the transposon Tn3: three genes and three sites involved in transposition of Tn3. Cell, 18, 1153.

HEFFRON, F., KOSTRIKEN, R., MORITA, C. and PARKER, R. (1980). Tn3 encodes a site-specific recombination system: identification of essential sequences, genes and the actual site of recombination. Cold Spring Harbor Symp. Quant. Biol., 45, 259.

HERSHEY, A.D. and CHASE, M. (1952). Independent functions of viral protein and nucleic acid in growth of bacteriophage. J. Gen. Physiol., 36, 39.

HESSLEWOOD, S.R. (1973). Host cell regulation of enzyme production in bacteria harbouring R-factors. PhD thesis, London.

HIRSCH, H.J., STARLINGER, P. and BRACHET, P. (1972). Two kinds of insertions in bacterial genes. Mol. Gen. Genet., 119, 191.

HITCHINGS, G.H. (1969). Species differences among dihydrofolate reductases as a basis for chemotherapy. Postgrad. Med. J., 45, (Suppl.), 7.

HITCHINGS, G.H. (1970). Trimethoprim: biochemical basis for sulphonamide potentiation. South African Med. J., 44, (Suppl.), 1.

HITCHINGS, G.H. (1973). Biochemical background of trimethoprim-sulphamethoxazole. Med. J. Aust. (Suppl.), 1, 5.

HITCHINGS, G.H.and ROTH, B. (1959). Trialkoxybenzylpyrimidines and method. US Patent, 2,909,522.

HITCHINGS, G.H. and BUSHBY, S.R.M. (1961). 5-Benzyl-2,4-diaminopyrimidines, a new class of systemic antibacterial agents. In Vth International Congress of Biochemistry Moscow. Sissakian (ed), p165.

HITCHINGS, G.H. and BURCHALL, J.J. (1965). Inhibition of folate biosynthesis and function as a basis for chemotherapy. Advan. Enzymol., 27, 417.

HITCHINGS, G.H. and SMITH, S.L. (1980). Dihydrofolate reductases as targets for inhibitors. In Advances in Enzyme regulation vol 18. Weber (ed). Pergamon, Oxford, New York.

HITCHINGS, G.H., FALCO, E.A. and SHERWOOD, M.B. (1945). The effect of pyrimidines on the growth of Lactobacillus casei. Science, 102, 251.

HITCHINGS, G.H., ELION, G.B., VANDERWERFF, H. and FALCO, E.A. (1948). Pyrimidine derivatives as antagonists of pteroylglutamic acid. J. Biol. Chem., 174, 765.

HOLMES, D.S. and QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem., 114, 193.

HOWARD, A.J., HINCE, C.J. and WILLIAMS, J.D. (1978). Antibiotic resistance in Streptococcus pneumoniae and Haemophilus influenzae. Report of Study group on bacterial resistance. Brit. Med. J., i, 1657.

HU, S., OHTSUBO, E. and DAVIDSON, N. (1975). Electron microscope heteroduplex studies of sequence relations among bacterial plasmids of Escherichia coli: structure of F13 and related F-primes. J. Bacteriol., 122, 749.

JACOB, F. and MONOD, J. (1961). On the regulation of gene activity. Cold Spring Harbor Symp. Quant. Biol., 26, 193.

JOBANPUTRA, R.A. and DATTA, N. (1974). Trimethoprim R-factors in Enterobacteria from clinical specimens. J. Med. Microbiol., 7, 169.

JOHNSRUD, L., CALOS, M.P. and MILLER, J.H. (1978). The transposon Tn9 generates a 9bp repeated sequence during integration. Cell, 15, 1209.

JORDAN, E., SAEDLER, H. and STARLINGER, P. (1968). O^C and strong polar mutations in the gal operon are insertions. Mol. Gen. Genet., 102, 353.

KAHMAN, R. and KAMP, D. (1979). Nucleotide sequences of the attachment sites of bacteriophage Mu DNA. Nature (London), 280, 247.

KASANEN, A., ANTTILA, R., ELFVING, R., KAHELA, P., SAARIMAA, H., SUNDQUIST, H., TIKKANEN, R. and TOIVANEN, P. (1978). Trimethoprim. Pharmacology, antimicrobial activity and clinical use in urinary tract infections. Ann. Clin. Res., 10, (Suppl. 22), 1.

KAUFMAN, B.T. and GARDINER, R.C. (1966). Studies of dihydrofolate reductase. I. Purification and properties of dihydrofolic reductase from chicken liver. J. Biol. Chem., 241, 1319.

KIRVEN, L.A. and THORNSBERRY, C. (1978). Minimum bactericidal concentration of sulphamethoxazole-trimethoprim for Haemophilus influenzae: correlation with prophylaxis. Antimicrob. Ag. Chemother., 14, 731.

KITAMOTO, O., KASAI, N., FUKAYA, K. and KAWASHIMA, A. (1956). Drug sensitivity of the Shigella strains isolated in 1955 [in Japanese]. J. Jap. Ass. Infect. Dis., 30, 403. (Cited by Watanabe, 1963).

KLECKNER, N. (1977). Translocatable elements in prokaryotes. Cell, 11, 11.

KLECKNER, N. (1979). DNA sequence analysis of Tn10 insertions: Origin and role of 9bp flanking repetitions during Tn10 translocation. Cell, 16, 711.

KLECKNER, N., ROTH, J. and BOTSTEIN, D. (1977). Genetic engineering in vivo using translocatable drug-resistance elements. J. Mol. Biol., 116, 125.

KLECKNER, N., STEELE, D., REICHARDT, K. and BOTSTEIN, D. (1979). Specificity of insertion by the translocatable tetracycline-resistance element Tn10. Genetics, 92, 1023.

KOCH, U.J., SCHUMANN, K.P., KÜCHLER, R. and KEWITZ, H. (1973). Efficacy of trimethoprim, sulphamethoxazole and the combination of both in acute urinary tract infection. Clinical and pharmacokinetical studies. Chemother., 19, 314.

KONDO, E. and MITSUHASHI, S. (1964). Drug resistance of enteric bacteria. IV. Active transducing bacteriophage P1 CM produced by the combination of R-factor with bacteriophage P1. J. Bacteriol., 88, 1266.

KONTAMICHALOU, P., MITANI, M. and CLOWES, R.C. (1970). Circular R-factor molecules controlling penicillinase synthesis, replicating in Escherichia coli under relaxed and stringent control. J. Bacteriol., 104, 34.

KOPECKO, D.J. and COHEN, S.N. (1975). Site specific recA-independent recombination between bacterial plasmids: involvement of palindromes at the recombinational loci. Proc. Nat. Acad. Sci. USA., 72, 1373.

KUMAR, A.A., BLANKENSHIP, D.T., KAUFMAN, B.T. and FREISHEIM, J.H. (1980). Primary structure of chicken liver dihydrofolate reductase. Biochemistry, 19, 667.

LACEY, R.W. (1982). Occasional Review. Do sulphonamide-trimethoprim combinations select less resistance to trimethoprim than the use of trimethoprim alone? J. Med. Microbiol., 15, 403.

LACEY, R.W., GILLESPIE, W.A., BRUTEN, D.M. and LEWIS, E.L. (1972). Trimethoprim resistant coliforms. Lancet, i, 409.

LACEY, R.W., LORD, V.L., GUNASEKERA, H.K.W., LEIBERMANN, P.J. and LUXTON, D.E.A. (1980a). Comparison of trimethoprim alone with trimethoprim sulphamethoxazole in the treatment of respiratory and urinary infections with particular reference to selection of trimethoprim resistance. Lancet, i, 1270.

LACEY, R.W., ROGERSON, J.G. and STOKES, A. (1980b). Antibacterial effects of trimethoprim and sulphadiazine in human urine and blood. J. Med. Microbiol., 13, 121.

LEBEK, G. (1963). Übertragung der Mehrfachresistenz gegen Antibiotika und Chemotherapeutika von E. coli auf andere Species gramnegativer Bakterien. Zentralbl. Bakteriol. Hyg. I. Abt. Orig. A., 189, 213.

LEDERBERG, J. (1952). Cell genetics and hereditary symbiosis. Physiol. Rev., 32, 403.

LEDERBERG, J. and LEDERBERG, E.M. (1952). Replica plating and indirect selection of bacterial mutants. J. Bacteriol., 63, 399.

LEDERBERG, J., CAVALLI, L.L. and LEDERBERG, E.M. (1952). Sex compatibility in Escherichia coli. Genetics, 37, 720.

LEE, C-H., BHAGWAT, A. and HEFFRON, F. (1983). Identification of a transposon Tn3 sequence required for transposition immunity. Proc. Nat. Acad. Sci. USA., 80, 6765.

LEVY, S.B., McMURRAY, L. (1978). Plasmid determined tetracycline resistance involves new transport systems for tetracycline. Nature (London), 276, 90.

LICHTENSTEIN, C. and BRENNER, S. (1981). Site-specific properties of Tn7 transposition into the Escherichia coli chromosome. Mol. Gen. Genet., 183, 380.

LICHTENSTEIN, C. and BRENNER, S. (1982). Unique insertion site of Tn7 in the Escherichia coli chromosome. Nature (London), 297, 601.

LINEWEAVER, H. and BURK, D. (1934). The determination of enzyme dissociation constants. J. Amer. Chem. Soc., 56, 658.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 265.

MABECK, C.E. and VEJLSGAARD, R. (1980). Treatment of urinary tract infections in general practice with sulphamethizole, trimethoprim or co-trimazine (sulphadiazine-trimethoprim). J. Antimicrob. Chemother., 6, 701.

MARTIN, R.R., THORLTON, C.L. and UNGER, L. (1981). Formation of Eshcerichia coli Hfr strains by integrative suppression with the P group plasmid RP1. J. Bacteriol., 145, 713.

MASTERS, J.N. and ATTARDI, G. (1983). The nucleotide sequence of the cDNA coding for the human dihydrofolic acid reductase. Gene, 21, 59.

MATHEWS, C.K. (1967). Evidence that bacteriophage-induced dihydrofolate reductase is a viral gene product. J. Biol. Chem., 242, 4083.

MATHEWS, C.K. and SUTHERLAND, K.E. (1965). Comparative Biochemistry of Bacterial and Phage - induced dihydrofolate reductases. J. Biol. Chem., 240, 2142.

MATHEWS, C.K., SCRIMGEOUR, K.G. and HUENNEKENS, F.M. (1963). Dihydrofolate reductase. In Methods in Enzymology VI. Colwick and Kaplan, (eds.) p364.

MATHEWS, D.A., ALDEN, R.A., BOLIN, J.T., FILMAN, D.J., FREER, S.T., HAMLIN, R., HOL, W.G.J., KISLIUK, R.L., PASTORE, E.J., PLANTE, L.T., XUONG, N., KRAUT, J. (1978). Dihydrofolate reductase from Lactobacillus casei. J. Biol. Chem., 253, 6946.

MAYS, T.D., SMITH, C.J., WELCH, R.A., DELFINI, C. and MACRINA, F.L. (1982). Novel antibiotic resistance transfer in Bacteroides. Antimicrob. Ag. Chemother., 21, 110.

MENDEL, G.J. (1865). Versuch über Pflanzen-Hybriden. In Proceedings of the Brünn Natural History Society.

MEYERS, J.A., SANCHEZ, D., ELWELL, L.P. and FALKOW, S. (1976). Simple agarose gel electrophoretic method for the identification and characterisation of plasmid DNA. J. Bacteriol., 127, 1529.

MEYNELL, E. and DATTA, N. (1966). The relation of resistance transfer factors to the F-factor (sex-factor) of Escherichia coli K-12. Genet. Res., 7, 134.

MILES, A.A. and MISRA, S.S. (1938). The estimation of the bactericidal power of the blood. J. Hyg., 38, 732.

MILLER, J.H., CALOS, M.P., GALAS, D., HOFER, M., BUCHEL, D.E. and MULLER-HILL, B. (1980). Genetic analysis of transposition in the lac region of Escherichia coli. J. Mol. Biol., 144, 1.

MIOVIC, M. and PIZER, L.I. (1971). Effect of trimethoprim on macromolecular synthesis in Escherichia coli. J. Bacteriol., 106, 856.

MITSUHASHI, S., HARADA, K., KAMEDA, M., SUZUKI, M. and EGAWA, R. (1960). On the drug-resistance of enteric bacteria. 3. Transmission of the drug resistance from Shigella to F⁻ or Hfr strains of Escherichia coli K-12. Jap. J. Exp. Med., 30, 301.

MOORHOUSE, E.C. and FARRELL, W. (1972). Effect of co-trimoxazole on faecal enterobacteria: No emergence of resistant strains. J. Med. Microbiol., 6, 249.

MOSHER, R.A. and MATHEWS, C.K. (1979). Bacteriophage T4-coded dihydrofolate reductase: synthesis, turnover, and location of the virion protein. J. Virol., 31, 94.

MOSHER, R.A., DiRENZO, A.B., MATHEWS, C.K. (1977). Bacteriophage T4 virion dihydrofolate reductase: Approaches to quantitation and assessment of function. J. Virol., 23, 645.

MURRAY, K. and MURRAY, N.E. (1975). Phage lambda receptor chromosomes for DNA fragments made with restriction endonuclease III of Haemophilus influenzae and restriction endonuclease I of Escherichia coli. J. Mol. Biol., 98, 551.

McCLINTOCK, B. (1951). Chromosome organisation and genic expression. Cold Spring Harbor Symp. Quant. Biol., 16, 13.

McCOMBIE, W.R., HANSEN, J.B., ZYLSTRA, G.J., MAURER, B. and OLSEN, R.H. (1983). Pseudomonas streptomycin resistance transposon associated with R-plasmid mobilization. J. Bacteriol., 155, 40.

McCUEN, R.W., SIROTNAK, F.M. (1974). Hyperproduction of dihydrofolate reductase in Diplococcus pneumonia by mutation in the structure gene. Biochim. Biophys. Acta., 338, 540.

MacHATTIE, L. and JACKOWSKI, J.B. (1977). Physical structure and deletion effects of the chloramphenicol resistance element Tn9 in phage lambda. In DNA insertion elements, episomes and plasmids. Bukhari, Shapiro and Adhya (eds.). Cold Spring Harbor.

McMURRAY, L.M., PETRUCCI, R.E., LEVY, S.B. (1980). Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in Escherichia coli. Proc. Nat. Acad. Sci. USA., 77, 3974.

McMURRAY, L.M., CULLINANE, J.C., LEVY, S.B. (1982). Transport of the lipophilic analog minocycline differs from that of tetracycline in susceptible and resistant Escherichia coli strains. Antimicrob. Ag. Chemother., 22, 791.

NEVERS, P. and SAEDLER, H. (1978). Mapping and characterisation of an Escherichia coli mutant defective in ISl-mediated deletion formation. Mol. Gen. Genet., 160, 209.

NICHOL, C.A. and WELCH, A.D. (1950). On the mechanism of action of aminopterin. Proc. Soc. Exp. Biol. Med., 74, 403.

NOMURA, M. (1970). Bacterial Ribosome. Bacteriol. Rev., 34, 228.

NOVAK, P., STONE, D. and BURCHALL, J.J. (1983). R-plasmid DHFR with a dimeric subunit structure. J. Biol. Chem., 258, 10956.

NOVICK, R.P. (1963). Analysis by transduction of mutations affecting penicillinase formation in Staphylococcus aureus. J. Gen. Microbiol., 33, 121.

NOVICK, R.P., CLOWES, R.C., COHEN, S.W., CURTISS, R., DATTA, N. and FALKOW, S. (1976). Uniform nomenclature for bacterial plasmids a proposal. Bacteriol. Rev., 40, 168.

NUGENT, M.E. (1981). A conjugative 'plasmid' lacking autonomous replication. J. Gen. Microbiol., 126, 305.

NUGENT, M.E. and HEDGES, R.W. (1979). The nature of the genetic determinant for the SHV-1 beta-lactamase. Mol. Gen. Genet., 175, 239.

OCHIAI, K., YAMANAKA, T., KIMURA, K. and SAWADA, O. (1959). Studies on inheritance of drug resistance between Shigella strains and Escherichia coli strains. [in Japanese]. Nippon Iji Shimpo, 1861, 34. (Cited by Watanabe, 1963).

OHTSUBO, E., ZENILMAN, M., OHTSUBO, H., McCORMICK, M., MACHIDA, C. and MACHIDA, Y. (1980). Mechanism of insertion and cointegration mediated by ISl and Tn3. Cold Spring Harbor Symp. Quant. Biol., 45, 283.

OHTSUBO, H., OHMORI, H. and OHTSUBO, E. (1979). Nucleotide-sequence analysis of Tn3 (Ap): Implications for insertion and deletion. Cold Spring Harbor Symp. Quant. Biol., 43, 1269.

OKA, A., NOMURA, N., SUGIMOTO, K., SUGISAKI, M. and TAKANAMI, M. (1978). Nucleotide sequence at the insertion sites of a kanamycin transposon. Nature (London), 276, 845.

OSBORN, M.J. and HUENNEKENS, F.M. (1958). Enzymatic reduction of dihydrofolic acid. J. Biol. Chem., 233, 969.

PATTISHALL, K.H., ACAR, J., BURCHALL, J.J., GOLDSTEIN, F.W. and HARVEY, R.J. (1977). Two distinct types of trimethoprim-resistant dihydrofolate reductases specified by R-plasmids of different compatibility groups. J. Biol. Chem., 252, 2319.

PERCIVAL, A., ROWLANDS, J., CORKHILL, J.E. (1976). Penicillinase-producing gonococci in Liverpool. Lancet, ii, 1379.

PERINE, P.L., THORNSBERRY, C., SCHALLA, W., BIDDLE, J., SIEGEL, M.S., WONG, K-H., THOMSON, S.E. (1977). Evidence for two distinct types of penicillinase-producing Neisseria gonorrhoeae. Lancet, ii, 993.

PERKINS, J.B. and BERTINO, J.R. (1966). Dihydrofolate reductase from the L1210R murine lymphoma. Fluorometric measurements of the interaction of the enzyme with coenzymes, substrates and inhibitors. Biochemistry, 5, 1005.

PETERSON, B.C. and ROWND, R.H. (1983). Homologous sequences other than insertion elements can serve as recombination sites in plasmid drug resistance gene amplification. J. Bacteriol., 156, 177.

PFIFFNER, J.J., BINKLEY, S.B., BLOOM, E.S., BROWN, R.A., BIRD, O.D., EMMETT, A.D., HOGAN, A.G. and O'DELL, B.L. (1943). Isolation of the antianemia factor (vit Bc) in crystalline form from liver. Science, 97, 404.

PHILIPS, S. and NOVICK, R. (1979). Tn554 - A site-specific repressor-controlled transposon in S. aureus. Nature (London), 278, 476.

PHILPOTT-HOWARD, J., SEYMOUR, A. and WILLIAMS, J.D. (1983). Accuracy of methods used for susceptibility testing of Haemophilus influenzae in United Kingdom laboratories. J. Clin. Pathol., 36, 1105.

POE, M. (1973). Dihydrofolate reductase from a methotrexate-resistant strain of Eshcerichia coli: Dihydrofolate monooxygenase activity. Biochem. Biophys. Res. Commun., 54, 1008.

POE, M. (1977). Antibacterial synergism: a proposal for chemotherapeutic potentiation between trimethoprim and sulphamethoxazole. Science, 194, 533.

POE, M., GREENFIELD, N.J., HIRSHFIELD, J.M., WILLIAMS, M.N. and HOOGSTEEN, K. (1972). Dihydrofolate reductase purification and characterisation of the enzyme from an amethopterin resistant mutant of Escherichia coli. Biochemistry, 11, 1023.

POE, M., BREEZE, A.S., WU, J.K., SHORT, C.R. and HOOGSTEEN, K. (1979). Dihydrofolate reductase from trimethoprim-resistant Escherichia coli MB3746 and MB3747. J. Biol. Chem., 254, 1799.

PUROHIT, S., BESTWICK, R.K., LASSER, G.W., ROGERS, C.M. and MATHEWS, C.K. (1981). T4 Phage-coded dihydrofolate reductase: subunit structure and cloning of its structural gene. J. Biol. Chem., 256, 9121.

RABINOWITZ, J.C. and HIMES, R.H. (1960). Folic acid coenzymes. Fed. Proc., 19, 963.

RICHARDS, H. and DATTA, N. (1981). Transposons and trimethoprim resistance. Brit. Med. J., 282, 1118.

RICHARDS, H., HUGHES, V. and DATTA, N. (1981). Diversity of plasmids responsible for multiple resistance in Klebsiella serotype K2. J. Hyg., 86, 189.

RICHARDS, H., DATTA, N., SOJKA, W.J. and WRAY, C. (1978). Trimethoprim-resistant plasmids and transposons in Salmonella. Lancet, ii, 1194.

ROBINSON, M.K., BENNETT, P.M. and RICHMOND, M.H. (1977). Inhibition of TnA translocation by TnA. J. Bacteriol., 129, 407.

ROMERO, E. and PERDUCA, M. (1977). Compatibility groups of R-factors for trimethoprim resistance isolated in Italy. J. Antimicrob. Chemother., 3, (Suppl. C), 35.

ROSENBERG, M., COURT, D., SHIMATAKE, H., BRADY, C. and WULFF, D. (1978). The relationship between function and DNA sequence in an intercistronic regulatory region in phage . Nature (London), 272, 414.

ROSS, D.G., GRISAFI, P., KLECKNER, N. and BOTSTEIN, D. (1979). The ends of Tn10 are not IS3. J. Bacteriol., 139, 1097.

ROTH, B., FALCO, E.A., HITCHINGS, G.H. and BUSHBY, S.R.M. (1962). 5-Benzyl-2,4-diaminopyrimidines as antibacterial agents. I. Synthesis and antibacterial activity in vitro. J. Med. Pharm. Chem., 5, 1103.

ROTHSTEIN, S.J., JORGENSEN, R.A., POSTLE, K. and REZNIKOFF, W.S. (1980a). The inverted repeats of Tn5 are functionally different. Cell, 19, 795.

ROTHSTEIN, S.J., JORGENSEN, R.A., YIN, J.C-P., YONG-DI, Z. (1980b). Genetic organisation of Tn5. Cold Spring Harbor Symp. Quant. Biol., 45, 99.

SAEDLER, H. and HEISS, B. (1973). Multiple copies of the insertion-DNA sequences IS1 and IS2 in the chromosome of Escherichia coli K-12. Mol. Gen. Genet., 122, 267.

SAUNDERS, J.R. (1984). Genetics and evolution of antibiotic resistance. Brit. Med. Bull., 40, 54.

SCHALLER, H. (1979). The intergenic region and the origins for filamentous phage DNA replication. Cold Spring Harbor Symp. Quant. Biol., 43, 401.

SHAPIRO, J.A. (1969). Mutations caused by the insertion of genetic material into the galactose operon of Escherichia coli. J. Mol. Biol., 40, 93.

SHAPIRO, J.A. (1979). Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. Proc. Nat. Acad. Sci. USA., 76, 1933.

SHAPIRO, J.A. and SPORN, P. (1977). Tn402: a new transposable element determining trimethoprim resistance that inserts in bacteriophage lambda. J. Bacteriol., 129, 1632.

SHELDON, R. (1977). Altered dihydrofolate reductase in fol regulatory mutants of Escherichia coli K-12. Mol. Gen. Genet., 151, 215.

SHELDON, R. and BRENNER, S. (1976). Regulatory mutants of dihydrofolate reductase in Escherichia coli K-12. Mol. Gen. Genet., 147, 91.

SIMONSEN, C.C., CHEN, E.Y. and LEVINSON, A.D. (1983). Identification of the Type I trimethoprim resistant dihydrofolate reductase specified by the Escherichia coli R-plasmid R483: Comparison with prokaryotic and eukaryotic DHFRs. J. Bacteriol., 155, 1001.

SIROTNAK, F.M. (1971). High dihydrofolate reductase levels in Diplococcus pneumoniae after mutation in the structural gene. J. Bacteriol., 106, 318.

SIROTNAK, F.M. (1973). Hyperproduction mutation in the dihydrofolate reductase gene of Diplococcus pneumoniae: some effects on synthesis correlate with the presence of a modified enzyme form. Biochim. Biophys. Acta., 312, 426.

SKÖLD, O. and WIDH, A. (1974). A new dihydrofolate reductase with low trimethoprim sensitivity induced by an R-factor mediating high resistance to trimethoprim. J. Biol. Chem., 249, 4324.

SMITH, C.J., MARKOWITZ, S.M. and MACRINA, F.L. (1981). Transferable tetracycline resistance in Clostridium difficile. Antimicrob. Ag. Chemother., 19, 997.

SMITH, C.J., WELCH, R.A. and MACRINA, F.L. (1982). Two independent conjugal transfer systems operating in Bacteroides fragilis V479-1. J. Bacteriol., 151, 281.

SMITH, D.H. and ARMOUR, S.E. (1966). Transferable R factors in enteric bacteria causing infection of the genitourinary tract. Lancet, ii, 15.

SMITH, J.T. (1969). R-factor gene expression in Gram-negative bacteria. J. Gen. Microbiol., 55, 109.

SMITH, S.L. and BURCHALL, J.J. (1980). Studies of Escherichia coli R-plasmid dihydrofolate reductase. Fed. Proc., 39, 1771.

SMITH, S.L., STONE, D., NOVAK, P., BACCANARI, D.P. and BURCHALL, J.J. (1979). R-plasmid dihydrofolate reductase with subunit structure. J. Biol. Chem., 254, 6222.

SMITH, W. (1980). Antibiotic-resistant Escherichia coli in market pigs in 1956-1979: the emergence of organisms with plasmid-borne trimethoprim-resistance. J. Hyg., 84, 467.

SO, M., HEFFRON, F. and McCARTHY, B.J. (1979). The Escherichia coli gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of IS1. Nature (London), 277, 453.

SPIES, T. and LAUFS, R. (1983). Circularized copies of amplifiable resistance genes from Haemophilus influenzae plasmids. J. Bacteriol., 156, 1263.

SPIES, T., LAUFS, R. and RIESS, F-C. (1983). Amplification of resistance genes in Haemophilus influenzae plasmids. J. Bacteriol., 155, 839.

STACEY, K.A. and SIMSON, E. (1965). Improved method for the isolation of thymine requiring mutants of Escherichia coli. J. Bacteriol., 90, 554.

STANISICH, V.A. (1974). Properties and host range of male-specific bacteriophages of Pseudomonas aeruginosa. J. Gen. Microbiol., 84, 332.

STONE, D. and SMITH, S.L. (1979). The amino acid sequence of the trimethoprim-resistant dihydrofolate reductase specified in Escherichia coli by R-plasmid R67. J. Biol. Chem., 254, 10857.

STONE, D., PATERSON, S.J., RAPER, J.H. and PHILLIPS, A.W. (1979). The amino acid sequence of dihydrofolate reductase from the mouse lymphoma L1210. J. Biol. Chem., 254, 480.

STUART, A., PATERSON, T., ROTH, B. and AIG, E. (1983). 2,4-Diamino-5-benzylpyrimidines and analogues as antibacterial agents. 6. A one-step synthesis of new trimethoprim derivatives and activity analysis by molecular modeling. J. Med. Chem., 26, 667.

STUY, J.H. (1980). Chromosomally integrated conjugative plasmids are common in antibiotic-resistant Haemophilus influenzae. J. Bacteriol., 142, 925.

SUTCLIFFE, J.G. (1978). Nucleotide sequence of the ampicillin resistance gene of Escherichia coli plasmid pBR322. Proc. Nat. Acad. Sci. USA., 75, 3737.

SUTTON, W.S. (1903). The chromosomes in heredity. Biol. Bull., 4, 213.

SWIFT, G., McCARTHY, B.J. and HEFFRON, F. (1981). DNA sequence of a plasmid-encoded dihydrofolate reductase. Mol. Gen. Genet., 181, 441.

TAIT, R.C. and BOYER, H.W. (1978). On the nature of tetracycline resistance controlled by the plasmid pSC101. Cell, 13, 73.

TAYLOR, D.E. (1983). Transfer-defective and tetracycline-sensitive mutants of the incompatibility group H1 plasmid R27 generated by insertion of transposon 7. Plasmid, 9, 227.

TENNHAMMAR-EKMAN, B. and SKÖLD, O. (1979). Trimethoprim resistance plasmids of different origin encode different drug-resistant dihydrofolate reductases. Plasmid, 2, 334.

THEN, R.L. (1979). Neisseriaceae, a group of bacteria with dihydrofolate reductases, moderately susceptible to trimethoprim. Zentralbl. Bakteriol. Hyg. I. Abt. Orig. A., 245, 450.

THEN, R. and ANGEHERN, P. (1973). Sulphonamide induced "thymineless death" in Escherichia coli. J. Gen. Microbiol., 76, 255.

THEN, R. and ANGEHRN, P. (1979). Low trimethoprim susceptibility of anaerobic bacteria due to insensitive dihydrofolate reductases. Antimicrob. Ag. Chemother., 15, 1.

THEN, R.L. and RIGGENBACH, H. (1978). Dihydrofolate reductases in some folate-requiring bacteria with low trimethoprim susceptibility. Antimicrob. Ag. Chemother., 14, 112.

THOMAS, I.W.J., McREYNOLDS, J.W., MOCK, C.R. and BAILEY, D.W. (1974). Ampicillin resistant Haemophilus influenzae meningitis. Lancet, i, 313.

THOMSON, C.J. and GRAY, G.S. (1983). Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferases encoded by resistance plasmids. Proc. Nat. Acad. Sci USA., 80, 5190.

THRELFALL, E.J., WARD, L.R., ASHLEY, A.S. and ROWE, B. (1980). Plasmid encoded trimethoprim resistance in multiresistant epidemic Salmonella typhimurium phage types 204 and 193. Brit. Med. J., 5, 1210.

TIETZE, E. and TSCHÄPE, H. (1983). Plasmid pattern analysis of natural bacterial isolates and its epidemiological implication. J. Hyg., 90, 475.

TOWNER, K.J. (1981). A clinical isolate of Escherichia coli owing its trimethoprim resistance to a chromosomally-located trimethoprim transposon. J. Antimicrob. Chemother., 7, 157.

TOWNER, K.J. and PINN, P.A. (1981). A transferable plasmid conferring only a moderate level of resistance to trimethoprim. FEMS Microbiol. Lett., 10, 271.

TOWNER, K.J., VENNING, B.M. and PINN, P.A. (1982). Occurrence of transposable trimethoprim resistance in clinical isolates of Escherichia coli devoid of self-transmissible resistance plasmids. Antimicrob. Ag. Chemother., 21, 336.

TRÉFOUEL, J., TRÉFOUEL, J., NITTE, F. and BOVET, D. (1935). Activité du p-aminophenysulfamide sur les infections streptococciques experimentales de la souris et du lapin. Compt. Rend. Soc. Biol. Paris, 120, 756.

UMEZAWA, H., OKANISHI, M. and KONDO, S. (1967). Phosphorylative inactivation of aminoglycosidic antibiotics by Escherichia coli carrying R-factor. Science, 157, 1559.

VILLARROEL, R., HEDGES, R.W., MAENHAUT, R., LEEMANS, J., ENGLER, G., VAN MONTAGU, M. and SCHELL, J. (1983). Heteroduplex analysis of P-plasmid evolution: The role of insertion and deletion of transposable elements. Mol. Gen. Genet., 189, 390.

WADDELL, W.J. (1956). A simple ultraviolet spectrophotometric method for the determination of protein. J. Lab. Clin. Med., 48, 311.

WARD, J.M. and GRINSTED, J. (1982). Physical and genetic analysis of the inc-W group plasmids R338, Sa, and R7K. Plasmid, 7, 239.

WATANABE, T. (1963). Infective heredity of multiple drug resistance in bacteria. Bacteriol. Rev., 27, 87.

WATANABE, T. (1967). Infectious drug resistance. Scientific American, 217, 19.

WATANABE, T. and FUKASAWA, T. (1962). Episome mediated transfer of drug resistance in Enterobacteriaceae. IV. Interactions between Resistance transfer factor and F-factor in Escherichia coli K-12. J. Bacteriol., 83, 727.

WATANABE, T. and LYANG, K.W. (1962). Episome mediated transfer of drug resistance in Enterobacteriaceae. V. Spontaneous segregaton and recombination of resistance factors in Salmonella typhimurium. J. Bacteriol., 84, 422.

WATANABE, T., OGATA, C. and SATO, S. (1964a). Episome mediated transfer of drug resistance in Enterobacteria. VIII. Six-drug-resistance R-factor. J. Bacteriol., 88, 922.

WATANABE, T., NISHIDA, H., OGATA, C., ARAI, T. and SATO, S. (1964b). Episome mediated transfer of drug resistance in Enterobacteriaceae. VII. Two types of naturally occurring R-factors. J. Bacteriol., 88, 716.

WATANABE, T., FURUSE, C. and SAKAIZUMI, S. (1968). Transduction of various R-factors by phage P1 in Escherichia coli and by phage P22 in Salmonella typhimurium. J. Bacteriol., 96, 1791.

WATSON, J.D. and CRICK, F.C. (1953). Molecular structure of nucleic acids. A structure for deoxyribose nucleic acids. Nature (London), 171, 737.

WATSON, M.D. and SCAIFE, J.G. (1980). Integrative compatibility: Stable coexistence of chromosomally integrated and autonomous derivatives of plasmid RP4. J. Bacteriol., 142, 462.

WEINSTOCK, G.M., SUSSKIND, M.M. and BOTSTEIN, D. (1979). Regional specificity of illegitimate recombination by the translocatable ampicillin-resistance element Tn1 in the genome of phage P22. Genetics, 92, 685.

WEISBERG, R.A. and ADHYA, S. (1977). Illegitimate recombination in bacteria and bacteriophage. Annu. Rev. Genet., 11, 451.

WEISMAN, R.A. and BROWN, G.M. (1964). The biosynthesis of folic acid. V. Characteristics of the enzyme system that catalyses the synthesis of dihydropteroic acid. J. Biol. Chem., 239, 326.

WILKENSON, P.J. and REEVES, D.S. (1979). Tissue penetration of trimethoprim and sulphonamides. J. Antimicrob. Chemother., 5, (Suppl. B), 159.

WILLIAMS, J.D. and ANDREWS, J. (1974). Sensitivity of Haemophilus influenzae to antibiotics. Brit. Med. J., i, 134.

WILLIAMS, J.W., MORRISON, J.F. and DUGGLEBY, R.G. (1979). Methotrexate, a high-affinity pseudosubstrate of dihydrofolate reductase. Biochemistry, 18, 2567.

WISE, E.M. and ABOU-DONIA, M.M. (1975). Sulphonamide resistance mechanisms in Escherichia coli: R-plasmids can determine sulphonamide-resistant dihydropteroate synthetases. Proc. Nat. Acad. Sci. USA., 72, 2621.

WOOD, R.C. and HITCHINGS, G.H. (1959a). A study of the uptake and degradation of folic acid, citrovorum factor, aminopterin and pyrimethamine by bacteria. J. Biol. Chem., 234, 2381.

WOOD, R.C. and HITCHINGS, G.H. (1959b). Effect of pyrimethamine on folic acid metabolism in Streptococcus faecalis and Escherichia coli. J. Biol. Chem., 234, 2377.

WOOD, R.C., GELTZER, A. and HITCHINGS, G.H. (1960). Comparative effects of various antimetabolites on growth and synthesis of citrovorum factor by Streptococcus faecalis. J. Bacteriol., 79, 306.

ZOLG, J.W. and HÄNGGI, U.J. (1981). Characterisation of an R-plasmid associated, trimethoprim resistant dihydrofolate reductase and determination of the nucleotide sequence of the reductase gene. Nucleic Acids Res., 9, 697.

ZOLG, J.W., HÄNGGI, U.J. and ZACHAU, H.G. (1978). Isolation of a small DNA fragment carrying the gene for a dihydrofolate reductase from a trimethoprim resistance factor. Mol. Gen. Genet., 164, 15.

13th ICC

13th International Congress of Chemotherapy

Vienna 28th August to 2nd September 1983

SEPARATUM

TRIMETHOPRIM RESISTANCE:
AN EPIDEMIC CAUSED BY TWO RELATED TRANSPOSONS

Hilary-Kay Young and Sebastian G.B. Amyes

Epidemics of trimethoprim (Tp) resistance within Enterobacteria have been caused by the spread of Tp resistance plasmids (R-plasmids) within the bacterial population (3,5). More recently, transposable genes known as transposons have been implicated in the carriage of Tp resistance. These elements have now been found to reside not only on R-plasmids but also on the chromosome of clinical strains (4,9) and this has given the potential for a greater spread of resistance. Routine isolation of strains from the orthopaedic unit of Edenhall Hospital, near Edinburgh, has revealed that there was a surge of Tp resistance within the pathogenic Gram negative rods isolated from urine specimens. Of these strains, an unprecedented 64% were insusceptible to 10 mgL^{-1} Tp and of these 39.8% were resistant to 1 gL^{-1} Tp (4).

In a representative sample of 100 strains, the highly resistant organisms were examined initially for their ability to transfer Tp resistance and 11 strains were shown to harbour Tp R-plasmids. A further 12 strains possessing high level Tp resistance were unable to transfer their resistance and R-plasmids were not detectable by analysis of the cellular DNA.

Several of the organisms with high level, non-transferable Tp resistance were examined for the presence of transposons located in the chromosome. The method followed was similar to that described by Towner *et al* (8). The R-plasmid RP4 was introduced into the clinical strains during an 18 hour mating (7). After suitable subculture, RP4 was then transferred into the rifampicin-resistant *Escherichia coli* strain J6-2_{-2} during a five hour mating, and selection was made for the transfer of the Tp and Kanamycin (Km) resistance determinants. A further five hour mating was performed with this strain using *E. coli* strain J5-3 as recipient. Selection was again made for the transfer of Tp and Km resistance determinants. The two resistance determinants now transferred at the same frequencies indicating that transposition of the Tp resistance gene into RP4 had taken place. This was confirmed by phage sensitivities and the physical presence of only one plasmid in the recipient strain. The molecular weight of the transposons was determined by measuring the increase in size of plasmid RP4 by agarose gel electrophoresis following the method of Meyers *et al* (6). DNA was extracted from the bacterial cells by gentle lysis with Triton X-100. The protein was removed by phenol extraction and the DNA was concentrated by precipitation with absolute ethanol. The plasmid DNA was separated by electrophoresis in 0.3% agarose gels run horizontally. The position of the plasmids was compared with

those of standard plasmids of known molecular weight (MW) which were run concurrently. Three strains harboured Tp transposons in the bacterial chromosome and each transposon was 8 megadaltons (Mdal) and carried resistance determinants to Streptomycin (Sm), Spectinomycin (Sp) as well as Tp.

The presence of transposons in the autotransferable R-plasmids was detected by a modification of the above method. Each R-plasmid was transferred into strain J6-2_{-2}. R-plasmid RP4 was then introduced into this strain during a five hour mating. The plasmids were subsequently transferred into strain J5-3 and selection was made for Tp and Km resistant transconjugants. The recipient plasmid resistance determinants were then transferred back into strain J6-2 and the transfer frequencies measured. Unlike the transfer from strain J6-2_{-2} to J5-3, the transfer frequencies of Km and Tp resistance were similar in this mating. This suggested that the Tp resistance determinant had transposed into RP4. This was confirmed employing the same criteria as before. Six plasmids were tested in this manner and, in each case, a transposon carrying Tp resistance was detected.

Table 1 Properties of Tp R-plasmids and transposons

Plasmid			Transposon	
No.	Resistance	Size (Mdal)	Resistance	Size (Mdal)
pSA 163	Tp Ap Sx	39.8	Tp	2.1
pSA 173	Tp Ap	25.0	Tp	2.1
pSA 179	Tp Ap Sm/Sp Sx Ce Tc	39.8	Tp Sm/Sp	7.6
pSA 176	Tp Ap Sm/Sp Sx	40.1	Tp	2.0
pSA 217	Tp Ap Sx	28.8	Tp Sm/Sp	7.2
pSA 218	Tp Ap Sx	28.8	Tp Sm/Sp	8.6

Ap = Ampicillin
Sx = Sulphamethoxazole
Ce = Cephradine
Tc = Tetracycline

Two different Tp resistance transposons were identified. One of the transposons closely resembled those found in the chromosome with a molecular weight of about 8 million and carrying resistance determinants to Tp, Sm and Sp. Interestingly, the Sm/Sp resistance of this transposon was sometimes not expressed in the original plasmid. The second transposon carried the single resistance determinant to Tp and its molecular weight is less than half that of the other transposon. These two transposons originated from R-plasmids which seemed closely related to one another and, to examine the degree of similarity, the transposon gene products were investigated.

The Tp resistant dihydrofolate reductases from each transposon were isolated and purified by the method of Amyes and Smith (1),

modified by the use of a sephadex G-75 superfine column (2 cm^2 x 90 cm) to allow better separation and a more accurate determination of the molecular weights of the plasmid and chromosomal enzymes. The assay of the enzyme and the measurement of the biochemical properties were performed by the methods of Amyes and Smith (2). The specific activities (enzyme units/mg protein), the ID_{50} (concentration of Tp in μmoles required to inhibit the enzyme by 50%) and the TD_{50} (time taken in minutes for enzyme to lose half its activity at 45°) were each determined and the two transposon encoded enzymes were found to be very similar. However, the two enzymes differed markedly in their molecular weights (Table 2).

Table 2 Properties of Tp resistant dihydrofolate reductases

Plasmid	Transposon	Dihydrofolate Reductase			
	Size (Mdal)	Size (Mdal)	Sp. Act.	ID_{50}	TD_{50}
pSA 173	2.1	24000	13.25	30	2.2
pSA 179	7.6	35000	22.06	60	2.9

The results suggest the presence of two transposons encoding Tp resistance in this clinical population. One transposon encodes resistances for both Tp and Sm/Sp. It has a molecular weight of about 8 million and encodes the production of a 35000 dalton DHFR which has similar properties to the type I plasmid DHFR. The second transposon is smaller and only encodes Tp resistance. This transposon encodes the production of a Tp resistant DHFR with similar properties to the type I enzyme but with a much smaller molecular weight. These results suggest that within one hospital the spread of resistance to Tp was being manifested by two transposons which, by virtue of the similar but not identical properties of their gene products, were related to one another.

We should like to acknowledge the award of the Davidson Research Studentship to Hilary-Kay Young and we should like to thank E.R. Squibb & Sons Ltd for their generous financial support of this project.

REFERENCES

1. Amyes, S. G. B. and Smith, J. T.: R-factor trimethoprim resistance mechanism: an insusceptible target site. Biochemical and Biophysical Research Communications. 58: 412-418, 1974.

2. Amyes, S. G. B. and Smith, J. T.: The purification and properties of the trimethoprim-resistant dihydrofolate reductase mediated by the R-factor, R388. European Journal of Biochemistry. 61:597-603, 1976.

3. Amyes, S. G. B., McMillan, C. J. and Drysdale, J. L.: Transferable trimethoprim resistance amongst hospital isolates. New Trends in Antibiotics: Research and Therapy. Eds. G.G. Grassi and L.D. Sabath. Elsevier/North Holland Biomedical Press, Amsterdam. 325-327, 1981.

4. Amyes, S. G. B., Drysdale, J. L., McMillan, C. J. and Young, H-K.: The diversity of trimethoprim resistance within hospital isolates. Journal of Pharmacy and Pharmacology. 34:60p, 1982.

5. Grüneberg, R. N. and Bendall, M. J.: Hospital outbreak of trimethoprim resistance in pathogenic coliform bacteria. British Medical Journal. 2:7-9, 1979.

6. Meyers, J. A., Sanchez, D., Elwell, L. P. and Falkow, S.: Simple agarose gel electrophoretic method for the identification and characterisation of plasmid DNA. Journal of Bacteriology. 127:1529-1537, 1976.

7. Smith, J. T.: R-factor gene expression in Gram negative bacteria. Journal of General Microbiology. 55: 109-120, 1969.

8. Towner, K. J.: A clinical isolate of Escherichia coli owing its trimethoprim resistance to a chromosomally located trimethoprim transposon. Journal of Antimicrobial Chemotherapy. 7:157-162, 1981.

9. Towner, K. J., Venning, B. M. and Pinn, P. A.: Occurrence of transposable trimethoprim resistance in clinical isolates of Escherichia coli devoid of self-transmissible resistance plasmids. Antimicrobial Agents and Chemotherapy. 21: 336-338, 1982.

Address:
Department of Bacteriology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, United Kingdom.

THE DIVERSITY OF TRIMETHOPRIM RESISTANCE WITHIN HOSPITAL ISOLATES

S.G.B. Amyes, Judyth L. Drysdale, Catherine J. McMillan and Hilary-Kay Young, Department of Bacteriology, The Medical School, University of Edinburgh, Teviot Place, Edinburgh, EH8 9AG, United Kingdom.

Trimethoprim resistance within Enterobacteria can be determined either by the chromosome or by Resistance plasmids (R-plasmids). Epidemics of trimethoprim resistance have been caused by the spread of trimethoprim R-plasmids within the population (Grüneberg and Bendall 1979; Amyes et al 1980). Recently, however, transposons carrying trimethoprim resistance have been found to reside not only on R-plasmids but also on the chromosome of clinical strains (Towner et al 1982).

Routine isolation of strains from Edenhall Hospital in Musselburgh has revealed that a surge of trimethoprim resistance was occurring within the Gram-negative rods isolated from urine specimens. Of these strains, an unprecedented 64% were resistant to 10 mgL^{-1} trimethoprim and 25.5% to 1 gL^{-1} trimethoprim. One hundred isolates were examined in detail to determine which type of trimethoprim resistance was being observed. Fifteen strains were shown to possess auto-transferable trimethoprim resistance and examination of the plasmids by their drug resistance pattern and DNA size revealed that there was a minimum of six different types. Nine strains were Pseudomonas aeruginosa and although some of these showed a high degree of insusceptibility, no auto-transferable trimethoprim R-plasmids were shown. Four Proteus species were shown to possess low insusceptibility to trimethoprim but possessed auto-transferable R-plasmids. Within the Proteus strains these plasmids conferred a minimum inhibitory concentration (MIC) of trimethoprim of 100 mgL^{-1}. However, when these plasmids were transferred into the standard recipient strain, Escherichia coli $J6\text{-}2\text{-}_2$, they nevertheless elevated its MIC of trimethoprim to above 1 gL^{-1}.

The R-plasmids isolated from the Musselburgh clinical isolates were examined for the presence of transposons conferring trimethoprim resistance. It was found that there was a predominance of transposon 7, the 8.7 Mdal plasmid that determines trimethoprim and streptomycin resistance. The four strains that possessed high level resistance, but were unable to transfer trimethoprim resistance, were examined for the presence of transposons within the bacterial chromosome. Transposon 7 was found within the chromosome of some Enterobacteria strains but could not be detected in the chromosome of any Pseudomonas strains.

The incidence of trimethoprim resistance was higher amongst the Gram-negative rods isolated in this hospital than in other hospitals in the district. This seems to reflect the nature of the patients from whom the strains were isolated. There were many types of trimethoprim-resistant organisms within this small population and the spread of one type of strain or R-plasmid was not being observed. The high incidence of trimethoprim resistance amongst these hospital isolates seems to be linked to the clinical use of ampicillin rather than trimethoprim and this supports the results we have found in other hospitals (Amyes et al 1980). The use of ampicillin in this hospital has now been restricted.

We wish to thank E.R. Squibb and Sons Ltd for their generous financial support of this project.

Amyes,S.G.B. et al (1980) J.Pharm.Pharmacol. 32:80P
Grüneberg,R.N., Bendall,M.J. (1979) B.M.J. 2:7
Towner,K.J. et al (1982) Antimocrob.Ag.Chemother. 21:336

0022-3573/82/120060P-01$02.50/0

TRIMETHOPRIM RESISTANCE IN PLASMID-BEARING KLEBSIELLA

Hilary-Kay Young and S.G.B. Amyes, Department of Bacteriology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, United Kingdom.

Resistance plasmids (R-plasmids) conferring high level resistance to trimethoprim (Tp) were first identified in Enterobacteria in Britain in 1971 (Fleming et al 1972). All early plasmids were members of the W incompatibility group and a high proportion of them resided in an unusual Klebsiella species. It therefore seemed possible that the plasmid resistance gene could have evolved from this Klebsiella. In order to investigate this the dihydrofolate reductase (DHFR) from the original Klebsiella was studied.

To remove the Tp R-plasmid, the plasmid-containing Klebsiella (strain A) was grown on nutrient agar and replica-plated onto agar plates containing Tp. The loss of Tp resistance occurred at a frequency of $3x10^{-1}$ per cell. However, when the cellular DNA was analysed by agarose gel electrophoresis a plasmid was still found to be present, although reduced in size by 4.7 Mdaltons, suggesting the spontaneous loss of a Tp resistance transposon. The DHFR of this Tp sensitive derivative (strain B) was isolated and purified (Young & Amyes 1983). The specific activity (enzyme units/mg protein), the ID_{50} (concentration of Tp in μmoles required to inhibit the enzyme by 50%) and the TD_{50} (half life of DHFR at 45°C) were determined for the enzyme as well as the MIC (minimum inhibitory concentration in mgL^{-1}) of Tp for the strain (Amyes & Smith 1976).

Strain	MIC	DHFR MW	Sp. Activity	ID_{50}	TD_{50}
A	1000	35000	2.32	50000	>4
B	1	22000	1.94	0.02	>4
C	1000	22000	5.64	0.50	Activated

In order to determine if the enzyme from strain B could be the precursor of the plasmid DHFR, mutation of strain B to high level Tp resistance was attempted but without success. To promote this mutational event, the inc W plasmid Sa, which does not confer Tp resistance, was mated into strain B and mutants resistant to 100 mgL^{-1} Tp were again selected for. This time resistant colonies were detected. When these were purified, another selection was made for Tp resistance to 1000 mgL^{-1}. These mutants (strain C) were then tested for their ability to transfer Tp resistance but this was found to be impossible, indicating that plasmid Sa had promoted a chromosomal mutation to high level Tp resistance. The DHFR of strain C was produced in higher levels than the strain B enzyme. This enzyme property, coupled with its decreased susceptibility for Tp, is likely to be the mechanism of Tp resistance.

The results of this investigation indicate that Klebsiella spp do not provide the source for plasmid Tp resistance genes. However, the presence of the plasmid Sa, in a Tp sensitive Klebsiella, promotes a chromosomal mutation resulting in Tp resistance due to the production of higher levels of a DHFR with decreased susceptibility for Tp. Furthermore, this type of mutation may account for the inherent Tp resistance often observed within this genus.

We wish to thank E.R. Squibb & Sons Ltd for their generous financial support.

Amyes, S.G.B., Smith, J.T. (1976) Eur. J. Biochem. 61: 597
Fleming, M.P. et al. (1972) Brit. Med. J. i: 726
Young, H-K., Amyes, S.G.B. (1983) 13th Int. Congr. Chemother., Vienna

Reprinted from J. Pharm. Pharmacol. 35.