ABSTRACT OF THESIS

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Title of Thesis The DNA Modification Enzyme of Bacteriophage P1.

Many strains of E.Coli are able to degrade or 'restrict' foreign DNA that enters the cell. The enzyme responsible is a specific endonuclease which cleaves bacteriophage DNA into discrete fragments of high molecular weight. The host DNA is protected from breakage by host-induced 'modification' of the DNA. The modification enzyme methylates specific adenine residues in the sequence recognised by the restriction enzyme. This thesis is concerned with the modification enzyme coded for by phage P1 when it is resident in E.Coli.

The modification enzyme has been detected in extracts of P1-infected E.Coli by assaying for the transfer of tritium labelled methyl groups from S-adenosylmethionine to unmodified phage 82 DNA. It was not detectable in extracts of E.Coli infected with a modificationless mutant of P1. The activity has been purified approximately 500 fold by chromatography on DE-cellulose and phosphocellulose followed by zonal sedimentation through a glycerol gradient where it sedimented to a position corresponding to 6S. The glycerol gradient fraction is free of detectable P1 restriction activity, endodeoxyribonuclease and exodeoxyribonuclease activities. Methylation is specific for native unmodified DNA.

The pH optimum in potassium morpholinoethanesulphonate buffer is 6.0 - 6.25; the K_M S-adenosylmethionine is 5μM. At 30°C the purified enzyme takes 3 h to incorporate 20-24 methyl groups per phage 82 DNA molecule. The extent of methylation is not limited by inactivation of the enzyme and the sole product of methylation is 6-methylaminopurine. Methylation of unmodified phage λ DNA confers protection against a challenge by purified P1 restriction enzyme. The methylated λ DNA is, however, broken by the heterospecific E.Coli K restriction enzyme.

The enzyme has been purified approximately 1400-fold from induced lysogens of a thermoinducible mutant of P1. The most purified fraction shows two principle stained bands when analysed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. The two bands co-sediment with the modification activity at 6S in glycerol gradients. Their mobilities on sodium dodecyl sulphate polyacrylamide gel electrophoresis correspond to

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polypeptides of molecular weight 7 x 10^4 and 4.5 x 10^4 and they are present in equimolar amounts. It is concluded that the 6S species of the enzyme is a dimer of distinct subunits.

An attempt has been made to investigate the nucleotide sequences around the methylated base by 'fingerprinting' ^{14}C-methylated DNA after DNAse digestion. The resulting oligonucleotides have been analysed by partial exonucleolytic digestion with snake venom phosphodiesterase.

Various aspects of the mechanism of the modification enzyme have been discussed.

THE DNA MODIFICATION ENZYME OF BACTERIOPHAGE P1

by Jeremy Patrick Brockes

A thesis presented for the degree of

Doctor of Philosophy of the University of Edinburgh

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PREFACE

This thesis describes work done in the Department of Molecular Biology during the tenure of an M.R.C. Scholarship between October 1969 and September 1972. All of the experiments described were performed by myself. I have, in certain places, cited collaborative experiments performed with Dr P.R.Brown, Dr K. Murray and Dr R.Yuan.

ACKNOWLEDGEMENTS

I should like to thank my supervisors, Dr K.Murray and Dr N.E.Murray, for their patient advice and encouragement throughout this work. I thank Dr P.R.Brown, Dr R.Yuan and Mr R.W.Old for many helpful and enjoyable discussions. I also thank Mr T.Bruce and Mr S.G.Hughes for growing the large batches of cells that I required. I thank the Medical Research Council for a scholarship for training in research methods and Professors W.Hayes and M.R.Pollock for a place in their department. Finally, Linda, in the unlikely event that you should ever read this, I am grateful to you.

SUMMARY

Many strains of E.Coli are able to degrade or 'restrict' foreign DNA that enters the cell. The enzyme responsible is a specific endonuclease which cleaves bacteriophage DNA into discrete fragments of high molecular weight. The host DNA is protected from breakage by host-induced 'modification' of the DNA. The modification enzyme methylates specific adenine residues in the sequence recognised by the restriction enzyme. This thesis is concerned with the modification enzyme coded for by phage P1 when it is resident in E.Coli.

The modification enzyme has been detected in extracts of P1-infected E.Coli by assaying for the transfer of tritium labelled methyl groups from S-adenosylmethionine to unmodified phage 82 DNA. It was not detectable in extracts of E.Coli infected with a modificationless mutant of P1. The activity has been purified approximately 500-fold by chromatography on DE-cellulose and phosphocellulose followed by zonal sedimentation through a glycerol gradient where it sedimented to a position corresponding to 6S. The glycerol gradient fraction is free of detectable P1 restriction activity, endodeoxyribonuclease and exodeoxyribonuclease activities. Methylation is specific for native unmodified DNA.

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The enzyme has been purified approximately 1400-fold from induced lysogens of a thermoinducible mutant of P1. The most purified fraction shows two principle stained bands when analysed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate. The two bands co-sediment with the modification activity at 6S in glycerol gradients. Their mobilities on sodium dodecyl sulphate polyacryalamide gel electrophoresis correspond to polypeptides of molecular weight 7×10^4 and 4.5×10^4 and they are present in equimolar amounts. It is concluded that the 6S species of the enzyme is a dimer of distinct subunits.

An attempt has been made to investigate the nucleotide sequences around the methylated base by 'fingerprinting' ^{14}C-methylated DNA after DNAse digestion. The resulting oligonucleotides have been analysed by partial exonucleolytic digestion with snake venom phosphodiesterase.

Various aspects of the mechanism of the modification enzyme have been discussed.

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I. INTRODUCTION

Three reviews on restriction and modification have recently been published (Arber, 1971; Boyer, 1971; Meselson et al., 1972). In conjunction with the earlier reviews of Arber (1965) and Arber & Linn (1969), they provide an exhaustive collation of the available data.

(i) Early Observations

About 20 years ago a number of observations were made about a non-heritable change that could be imposed on bacteriophages by their bacterial host (Anderson & Felix,1952; Bertani & Weigle, 1952; Luria & Human, 1952). Although these observations were made with a variety of bacterial and bacteriophage systems, the example studied by Bertani & Weigle (1952) will be considered in more detail. Bacteriophage λ was grown on E.Coli strain C and the resulting lysate (denoted as λ.C) titred on E.Coli strain C and E.Coli strain K. The efficiency of plating (e.o.p.) on strain K, that is the titre on K ÷ titre on C, was only 10^{-4}. Phage from the rare plaques that formed on K (λ.K) had an e.o.p. of 1 and could, thus far, be explained as derived from mutants in the original lysate. If, however, the phage from such a plaque were grown on C, then the resulting lysate again showed an e.o.p. of 10^{-4} on K (Diagram 1). Thus the quality of the λ.K phage that allowed them to plate equally well on K or C was lost when the phage were grown on C. This quality, which therefore reflected the strain of the bacterial host, was

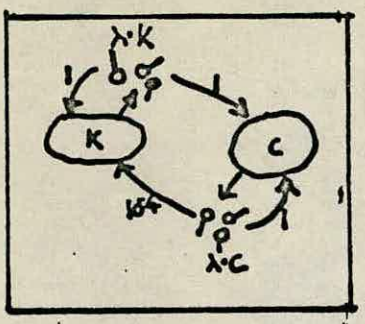

Diagram 1. Host controlled modification and restriction of phage λ in E.coli strains K12 and C. The numbers give the efficiency of plating of the λ.C and λ.K phage on the hosts indicated by the arrows.

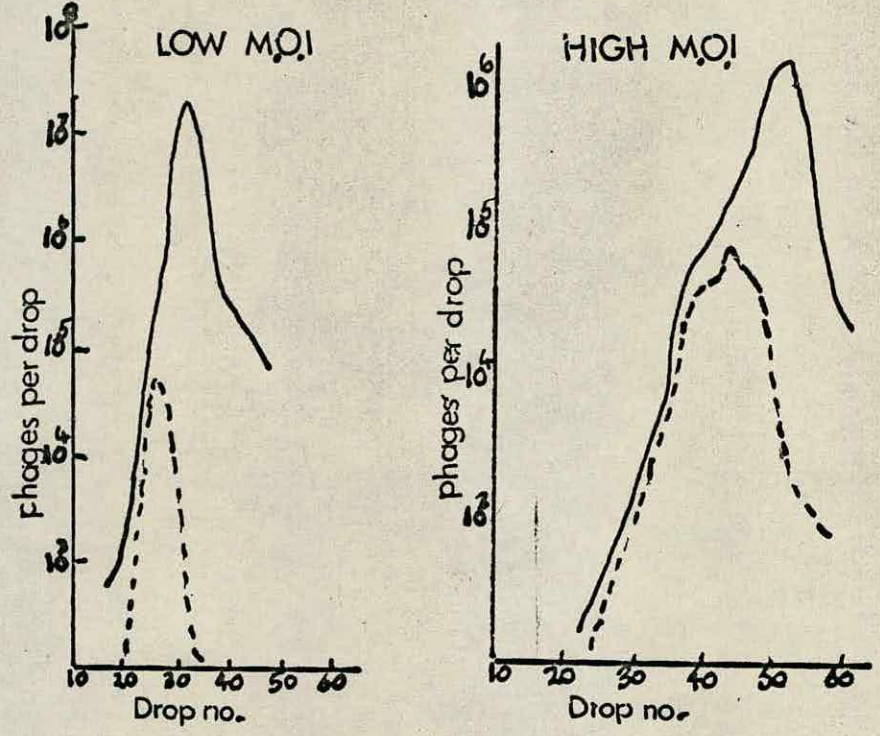

Diagram 2. The experiments of Arber & Dussoix (1962, see text). The dotted line is the titre on K(P1), the continuous line is the titre on K.

termed a host-induced 'modification'. Its distinctive feature was its non-heritable nature. The loss of infectivity observed when λ.C phage were plated on K was termed host-induced 'restriction'. The 'host-specificity' of K, that is its ability to discern the identity of the phage's previous host, is a distinctive feature of many E.Coli strains which have their own unique host-specificities. Host specificity is found in several species of bacteria (Boyer, 1971), although it should be noted that not all bacteriophages are subject to a given host-specificity system.

The molecular basis of restriction and modification remained unclear for the next decade although Lederberg (1957) made several interesting observations. He showed that certain prophages, in particular P1, specified their own restriction and modification systems which were imposed on the host bacteria. Furthermore, he provided an important clue to the molecular basis of restriction by showing that the ^{32}P-labelled DNA of phage T1 was quite extensively acid-solubilised as a consequence (direct or indirect) of restriction by prophage P1.

(ii) Restriction and modification of DNA

Arber and Dussoix (Arber & Dussoix, 1962; Dussoix and Arber, 1962) investigated the restriction and modification of λ by prophage P1 in strain K. Their biophysical investigations showed that modification was a property of the phage DNA. They investigated the loss of modification when a modified phage

replicates in a non-modifying host. λ was propagated on strain K (P1) in a medium enriched with D_2O. The "heavy" λ.K (P1) was used to infect strain K for a single cycle of growth in a "light" H_2O medium, and the resulting lysate was centrifuged to equilibrium in a CsCl density gradient. The tube was pierced and the emerging drops collected and titred on K and K (P1) to determine the distribution of unmodified and P1-modified phages. The modified phage (Diagram 2) banded at a position corresponding to phages with one heavy strand and one light strand of DNA, that is parental phage DNA molecules that had undergone one semi-conservative duplication (Meselson & Stahl 1958; Meselson & Weigle, 1961). When the multiplicity of infection was increased such that some parental phage DNA molecules escaped duplication (Meselson & Weigle, 1961), a shoulder appeared at a position corresponding to phages with two heavy strands of DNA (Diagram 2). Thus modification was associated only with those phages carrying oneor both parental strands. In addition, Dussoix and Arber (1962) confirmed Lederberg's observations by showing that ^{32}P-labelled unmodified λ DNA was degraded within a few minutes after its penetration into a restrictive host.

The concept of DNA restriction and modification was extended by showing that it is not confined to bacteriophage infection. Thus DNA transferred by transduction or conjugation (Arber and Morse 1963) or transfection (Arber & Dussoix, 1964) was also subject to the constraint of host-specificity. In the latter case, λ DNA that had been purified by phenol extraction

still retained host-induced modification, as assayed in the λ transfection system (Kaiser & Hogness, 1960).

(iii) DNA Restriction in vitro

Meselson & Yuan (1968) detected, purified and characterised the DNA restriction enzyme of strain K. The enzyme was detected in extracts of the endonuclease I^- strain 1100 (Dürwald & Hoffman-Berling, 1968) as an ATP-dependent nucleolytic activity that broke ^{32}P-labelled λ.C DNA but not ^{3}H-labelled λ.K DNA. The activity was extensively purified and shown to be absolutely dependent on Mg^{2+}, ATP and S-adenosylmethionine (SAM). The detailed role of the cofactors is unknown, but the ATP is extensively hydrolysed to ADP and orthophosphate. The most exciting property of this enzyme was that in a limit digest it broke unmodified λ DNA into discrete fragments of high molecular weight. The acid-solubilisation that is observed in vivo is presumably a consequence of secondary nucleolytic activity at the new ends that are created by the restriction enzyme.

The endonucleolytic action of the enzyme was clearly shown by its effect on the twisted circular form of λ DNA. An elegant sedimentation analysis showed that the enzyme breaks one strand and then, a few seconds later, breaks the complementary strand at a point nearly or directly opposite. The enzyme did not, however, attack both kinds of synthetic λ heteroduplex containing one modified and one unmodified strand. Thus "the enzyme somehow takes account of the structure of both

chains before cutting either" (Meselson & Yuan, 1968). The large sedimentation coefficient (12S) and the complexity of the co-factor requirements strongly suggested a subunit aggregate. In fact the homogeneous enzyme gives three bands when analysed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (quoted in Meselson et al, 1972). Similar restriction enzymes have been described for P1 and strain B (Meselson & Yuan, 1968) Linn & Arber, 1968; Roulland-Dussoix & Boyer, 1968). The K restriction enzyme was the first nuclease to have its biological role unequivocally established. Not only was it specific for unmodified DNA but it was also absent in a restrictionless mutant of strain 1100 (Meselson & Yuan,1968). Together with the repressors of the lactose operon (Gilbert & Müller-Hill, 1967) and bacteriophage λ (Ptashne, 1967), it was one of the first proteins which demonstrated recognition of specific sequences in duplex DNA.

The restriction reaction was further delineated by using the filter binding assay of Riggs & Bourgeois (1968). The nucleolytic steps can be stopped by adding excess EDTA after recognition has taken place. Under these conditions the enzyme formed a relatively stable, non-filterable complex with λ.C DNA but not with λ.K DNA. Complex formation required Mg^{2+}, SAM and ATP although the latter can be used at lower concentrations (10^{-8}M) than are required for DNA breakage (10^{-4}M) (Yuan & Meselson, 1970). Thus modification blocks the process of sequence recognition by the purified restriction enzyme. What is the molecular basis of modification?

(iv) DNA modification

Enzymatic methylation of nucleotide bases in E.Coli DNA was discovered over ten years ago (Mandel & Borek,1961) and was suggested for some time as a possible basis of non-heritable modification of DNA. The DNA methylating enzymes characterised by Gold & Hurwitz (1963) did not, however, show the requisite specificity while investigations of methylated bases following labelling with methionine (a biosynthetic precursor of the methyl-donating cofactor SAM) did not detect any change in the levels of methylation in modified and unmodified DNA (Gough & Lederberg, 1966). These latter experiments were limited by the available specific activity of the methionine and could not rule out low level changes in methylation as a consequence of modification. Arber (1965) showed that methionine starvation of a methionine auxotroph during λ DNA replication in a K strain drastically reduced modification. No other amino acid tested in the same kind of experiment gave such an inhibition. Thus methionine apparently plays an essential role in the modification process. The implication of nucleotide methylation in DNA modification has been clarified by investigation of B-specific modification of the filamentous bacteriophage fd. Base analyses of fd DNA have demonstrated a correlation between B-specific modification and the presence of 6-methylaminopurine (6 MAP) (Smith, Künlein & Arber, 1972). Phage fd was grown on isogenic, methionine auxotrophic strains of C, K and B in the presence of ^{14}C-methyl methionine. DNA was extracted from the purified phage stocks and analysed by acid hydrolysis. Phage fd.B was

found to carry about twice as much 6MAP in its DNA as fd.C or fd.K. Furthermore fd mutants that were insensitive to B restriction also contained half as much 6MAP as wild type. Modification of wild type fd DNA corresponds to formation of only two 6MAP residues per single-stranded circle of 6,000. nucleotides.

These observations have been extended by the partial purification of the B modification enzyme. This activity was detected by using a spheroplast infectivity assay for circular double-stranded fd DNA (Benzinger 1968). Modification was detected in extracts as an SAM-dependent activity that protected unmodified fd DNA against a loss of infectivity due to restriction by the appropriate spheroplasts (Linn & Arber, 1968). The activity was purified 200-fold by ion-exchange chromatography on phosphocellulose and DEAE-cellulose, and characterised by using the infectivity assay (Kuhnlein et al. 1969). This thesis describes the purification and properties of the P1 modification enzyme by using the chemical assay of methylation of unmodified DNA. The published properties of the two enzymes are fairly similar (Kühnlein and Arber, 1972; Brockes et al. 1972) and this will be considered in more detail in the discussion. These two enzymes provide the first example of DNA methylases of known function.

(v) Genetic analysis of the enzymes

Glover et al (1963) were able to obtain mutants that

were defective in P1 restriction by selecting for the rare lac^+ clones formed when a mutagenised culture of lac^-, P1-lysogenic bacteria was mated with unmodified F-lac elements. The mutation was shown to be present within the phage genome since it was transferred with the P1 phage on lysogenisation of a new host strain. Similar selection protocols have subsequently been used by other investigators in a variety of bacterial and plasmid systems. The surprising observation in the P1 system, and in the B and K systems, was that about half of the r^-_{P1} (restrictionless) mutants were m^-_{P1} (modificationless) as well. In view of their frequency, the phenotypically $r^- m^-$ mutants are unlikely to be double mutants. There are several possible explanations, but Wood (1966) suggested that the $r^- m^-$ mutants resulted from a single mutation in a gene (denoted s) whose product was common to the restriction and modification enzymes. It is also possible to start with an $r^- m^+$ mutant and derive a 'two-step' $r^- m^-$ mutant which retains s gene function as detected by complementation. The complementation data, which are discussed below, have been interpreted (Arber & Linn, 1969) as supporting a 'three-gene model' for the enzyme. The model postulates that gene s makes a product which is responsible for site-specific (sequence)recognition for both restriction and modification. Two other products, coded for by genes r and m, are responsible for carrying out the restriction and modification reactions, respectively, but act only in conjunction with the s gene product. The evidence for this model will now be discussed.

For E.Coli K and B, the host-specificity (hs) genes have been mapped close to the origin of HfrH ('12 o'clock'). The construction of F-prime elements carrying the hs genes provides partial diploids for complementation analysis of the various mutants in K and B, using λ as the tester phage. The principal observations made (Boyer & Roulland-Dussoix, 1969; Glover, 1970; Arber & Linn, 1969), and their interpretation on the three-gene model are given below.

(a) $F'r^-m^-$ (derived as a one-step mutant)/r^-m^+ has an r^+m^+ phenotype. On the model, the r^+ and m^+ subunits produced by the episome complement the s^+ subunit produced by the chromosome.

(b) $F'r^-m^-$ (derived as a two-step mutant)/r^-m^- (one-step) gives an r^+m^+ phenotype. The s^+ subunit produced by the episome complements the r^+ and m^+ subunits produced by the chromosome.

(c) In heterozygous partial diploids for the K hs genes in B (or vice versa), there is no inhibition of one type of hs function (K or B) by the presence of the other. Furthermore, the r^-m^+ phenotypes recover their restriction function in the presence of r^+m^+ or single-step r^-m^- mutants of the other strain. The single-step r^-m^- mutants do not, however, recover their particular function in the presence of various hs regions from the other strain. These observations support the hypothesis for the role of gene s, and show that between B and K the r and m gene products can act with either s gene product, but take their strain specificity from the s product. The

r and m products of P1, however, will not complement in this way with either B or K. (Glover, 1970; Boyer & Roulland-Dussoix, 1969).

These ideas of the relationship between the three gene products have been extended by a clever analysis of temperature sensitive mutations (Hubacek & Glover, 1970). A series of temperature sensitive restriction mutants was isolated by mutagenesis of K followed by a suitable selection procedure. Many of the mutants proved to be temperature sensitive for modification as well. Complementation analysis located the lesion for two of these restriction mutants within the m gene. The authors concluded that the m gene product was required for restriction, as well as for modification. Thus the most recent statement of the three gene model (Hubacek & Glover, 1970) is that the restriction enzyme is composed of the products of the r, m and s genes, while the modification enzyme is composed of at least the m and s gene products. The possibility that one complex or aggregate enzyme has both the restriction and modification activities was considered both by these authors and by Meselson & Yuan (1968).

How convincing is the evidence for the three gene model? It certainly provides a logical and consistent interpretation of the genetic data. There may be other models that explain these data but the present author is unable to think of any that present a plausible alternative. The observation that the s_K product complements with the r_B and m_B products to give efficient K specific restriction and modification, is particularly

hard to reconcile with other interpretations. Furthermore, Linn & Arber (1968) observed in vitro complementation for restriction of phage fd DNA on mixing extracts of $r^-_B m^+_B$ and $r^-_B m^-_B$ (one step) bacteria. It is, however, worth stating the formal possibility that other gene products might participate. The ultimate verification of the model depends on purifying the enzymes to homogeneity, separating the subunits in a functional state, assaying them for various relevant partial activities and reconstituting the active enzymes.

Perhaps the remaining outstanding problem in the genetics of the enzymes is the fine structure mapping of the hs gene cluster. The lack of both convenient flanking markers and selective techniques makes this a difficult problem.

(vi) Genetics of restriction and modification substrates

Just as it is possible to obtain O^c mutations in the lactose operator such that repressor is not bound (Gilbert & Müller-Hill, 1967), so it is possible to obtain mutations in the sites of restriction and modification. Unmodified phage fd has an efficiency of plating on B of 7×10^{-4}. Arber & Kuhnlein (1967) obtained a spontaneous mutant that had an e.o.p. of 3×10^{-2}, and then a second-step mutant with an e.o.p. of 1. No intermediate e.o.p. values were observed. This was interpreted to mean that the wild-type phage have 2 distinct sites of B-specific restriction. Arber & Linn (1969) have called them s_B-1 and S_B-2. Mutation in one of these sites,

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for example S_B-1, gives an S_B-1°, S_B-2 phage with an intermediate e.o.p., while mutation in both gives an S_B-1°, S_B-2° phage which is unrestricted. The two sites can be mapped on the fd chromosome by using a conventional recombination analysis (Boon & Zinder, 1970). Furthermore, closed circular DNA extracted from these mutants shows distinct patterns of cleavage by the B restriction endonuclease; the patterns are consistent with the presence of 2, 1 and 0 targets in the different mutants (Boyer et al., 1971). Finally such DNA can accept 4, 2 and 0 methyl groups in reactions with the purified B modification enzyme (Kuhnlein & Arber, 1972). The latter result is evidence for the model, implied throughout this discussion, that the adenine residues that are methylated on modification are located in the sequence(s) recognised by the restriction enzyme.

A similar analysis has been performed on the S_K sites in λ DNA. The analysis is complicated by the fact that λ is strongly restricted (e.o.p. 10^{-4}) since it has more than 2 S_K sites, and mutation of a single site does not cause a significant alteration in e.o.p. The related phage, Ø80, is, however, weakly restricted (e.o.p. = 10^{-1}) and may be crossed with λ to give various hybrid phages (Franklin et al., 1965). After selecting for the assortment of the immunity and host range characters peculiar to λ or Ø80, Franklin and Dove (1969) observed three discrete e.o.p. values in the recombinants. This provides a tool for mapping the various S_K sites and for deriving phages with isolated restriction targets. In this way two of the S_K sites have been located on the λ genome.

Phage λ lacking both S_K-1 and S_K-2 retains at least three sites for the K restriction system (Murray et al, 1972). The DNA of the various phages can be used in vitro to study the reaction of the restriction enzyme with isolated targets, and also to fragment the λ chromosome in specific locations.

One curious feature of these analyses deserves comment. If a restriction site occurs in a gene whose function is essential, then mutation of the restriction sequence might well be a lethal event. Thus, one might expect that it would not be possible to obtain S^o mutations in all sites. Nevertheless, in the eight cases investigated, namely the S_B sites on fd, the S_A site on λ and the S_K sites on λ, 82 and 80 (Arber & Kühnlein, 1967; Boyer et al, 1971; Arber et al., 1972; N.E.Murray, personal communication), S^o mutants have been easily isolated and the resulting phage showed no detectable alteration in physiology. Further genetical and biochemical analysis will be required to clarify this point.

Finally, it is possible to create a restriction site by mutation. Schnegg & Hofschneider (1969) were able to obtain a mutant of phage ϕX174 that is sensitive to B restriction, while the wild type is insensitive.

(vii) Conclusion

The investigation of restriction and modification since the analysis of Arber & Dussoix (1962) has provided quite a detailed picture of the molecular basis of these effects.

The restriction endonuclease and modification methylase probably recognise the same or overlapping sequences of nucleotides in duplex DNA. The restriction enzyme introduces a double strand break into the sequence, while the modification enzyme methylates an adenine residue. Methylation prevents the binding of the restriction enzyme. There is currently considerable interest in these enzymes, firstly as a tool to fragment DNA and secondly as a favourable opportunity to study protein-nucleic acid recognition.

The restriction enzymes are the most specific endonucleases that have been characterised. A restriction enzyme from Hemophilus influenzae (Smith & Wilcox, 1970), has been used to cleave the closed circular DNA of SV40 virus into eleven fragments (Danna & Nathans, 1972). This cleavage provides a method for 'fingerprinting' different isolates of SV40 DNA (Nathans & Danna, 1972a), and for analysing the bidirectional nature of SV40 replication in vivo (Nathans & Danna, 1972b). The availability of an increasing number of these enzymes should, in principle, allow quite small homogeneous fragments to be obtained for sequence analysis.

The mechanism by which certain proteins are able to recognise specific sequences in double-stranded DNA is a central question in molecular biology. The restriction and modification enzymes offer an excellent opportunity to investigate this problem. It has been suggested that the recognition sequences may display structural singularities such as loops

(Gierer, 1966) or unpaired regions (Crick 1971). Our current knowledge of these sequences is so meagre that we cannot critically examine these ideas. The recognition sequence, or at least a substantial part of it, has been determined only for the Hemophilus restriction enzyme (Kelly & Smith,1970). The sequence shows an intriguing two-fold axis of symmetry which the authors rationalise in terms of the enzyme's function, that is to cut both strands of a DNA duplex. Are all such sequences symmetrical, and does the symmetry necessarily encourage the formation of short looped-out regions (Messlson et al.,1972)? Why does the presence of the methyl group in modified DNA block the process of recognition by the restriction enzyme? In order to answer these questions it is essential that initially we learn more about the sequences recognisedby a series of restriction and modification enzymes.

There are also uncertainties in our understanding of the protein's behaviour during recognition. The lactose and lambda repressors react so rapidly with the operator sequence that the reaction cannot be accounted for by a normal diffusion-limited process (Riggs et al., 1970). An extreme solution to this problem is to suggest that the sequence is located by a two-dimensional walk along the DNA molecule (Gilbert & Müller-Hill, 1970), although there is little evidence to support this notion. While it has been asserted that the B restriction endonuclease shows similar kinetics (Boyer et al., 1971), the detailed quantitation and analysis remains to be done. Finally we might ask questions about the

proteins themselves. Does the active site lie in a shallow groove as it does in other enzymes (Crick, 1971), or does it form a protruding structure that could be inserted between base pairs in duplex DNA (Adler et al. 1972)? Our knowledge of the structure of the enzymes, and of the roles of the different subunits and cofactors, is inadequate to answer these questions. The technical problems involved, particularly in isolating large quantities of homogeneous enzyme, are formidable but not insoluble.

II. MATERIALS

1. E.Coli Strains

The strains of E.Coli used in this work are given in table 1. All of these strains are E.Coli K12 derivatives.

2. Bacteriophage strains

The bacteriophage strains used in this work are given in table 2.

3. Media

The following media were used for growing bacteria and bacteriophages. All quantities are given in gm/Litre H_2O unless otherwise stated.

L Broth (pH 7.2); Difco Tryptone 10, yeast extract 5, NaCl 10; BBL trypticase 10, Difco agar 10, NaCl 5.

Tryptone (B) agar; Difco tryptone 10, NaCl 5, Difco agar 10, pH 7.5.

P1 agar; Difco agar 10, tryptone 10, NaCl 5, Yeast extract 5, 2.5 mM $CaCl_2$, 0.1% glucose.

Phage buffer; KH_2PO_4 3, $Na_2HPO_4$7, NaCl 5, 1mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.001% gelatin.

Low phosphate medium (J.Abelson, personal communication)

KCl 1.5, NaCl 5, NH_4Cl 1, Tris 12.1, adjusted to pH 7.4 with HCl.20% Bactopeptone (Difco) was adjusted to pH9 with NH_4OH, centrifuged to remove the precipitate and adjusted to pH 7.5. The product was diluted 1:100 in the pH 7.4 salts solution, autoclaved, and made 0.4% in glucose and 1 mM in $MgSO_4$ before use.

TABLE I. E.Coli Strains

	STRAIN	RELEVANT GENOTYPE	REFERENCE	REMARKS	SOURCE
1.	1100	Endonuclease I^- r_K^+ m_K^+	Dürwald & Hoffman-Berling (1968)		I.R.Lehman
2.	1100 r_K^-	r_K^- m_K^+ derivative of 1100	-	Derived by ethylmethane-sulphonate mutagenesis	N.E.Murray
3.	1100 r_K^-(P1)	r_{P1}^+ m_{P1}^+	-	Derived by lysogeny of 1100 r_K^- with P1KC	Author
4.	K140(P1c$_I$162)	r_{P1}^+ m_{P1}^+	Scott (1968)	Thermoinducible P1.	J.R.Scott
5.	W1485	r_K^+ m_K^+	-		N.E.Murray
6.	803	r_K^- m_K^-	Wood (1966)		N.E.Murray
7.	C600	r_K^+ m_K^+	Appleyard (1954)		N.E.Murray
8.	C600 (P1--)	r_K^+ m_K^+, r_{P1}^- m_{P1}^-	Glover et al(1963)		S.W .Glover
9.	C600(P1-+)	r_K^+ m_K^+, r_{P1}^- m_{P1}^+	Glover et al(1963)		S.W Glover
10.	C600(P1)	r_K^+ m_K^+, r_{P1}^+ m_{P1}^+	-		S.W.Glover
11.	Ymel	SU_{III}^+, r_K^+ m_K^+	-	Standard host for $\lambda C_I 857\ S_7$.	N.E.Murray
12.	W3110 SU_{III}^+(P1)	SU_{III}^+, r_K^+ m_K^+, r_{P1}^+ m_{P1}^+	-	Derived by transduction of SU_{III}^+ (with trp+) into W3110 (P1)	Author

Table 2. Bacteriophage Strains.

	STRAIN	RELEVANT GENOTYPE	REFERENCE	REMARKS	SOURCE
1.	λC_{26}		Meselson (1964)	HNO_2 induced clear plaque mutant	N.E.Murray
2.	$\lambda C_I 857\ S_7$		Goldberg & Howe (1969)	Thermoinducible, lysis defective; convenient for making λDNA. S_7 is amber mutationsuppressible by SU_{III}^+.	N.E.Murray
3.	82C		Arber(unpublished)	Clear plaque mutant	W.Arber
4.	P1KC	$r_{P1}^+\ m_{P1}^+$	Lennox (1955)	Phage P1 'adapted'for growth on strain K	W.J.Brammar
5.	P1 KC ψ2	$r_{P1}^-\ m_{P1}^-$	Hayward & Glover (unpublished)	Two step (i.e. $r_{P1}^-\ m_{P1}^-\ s_{P1}^+$) mutant derived by nitrosoguanidine mutagenesis.	R.S.Hayward

4. Enzymes and Proteins

(a) Endonuclease R.P and endonuclease R.K. both purified to the glycerol gradient stage according to Meselson and Yuan (1968) were the generous gifts of Mr R.W.Old and Dr R. Yuan respectively. Neither of these preparations gave detectable breakage of ^{32}P-labelled λ.K(P1) DNA as indicated by sedimentation in neutral sucrose gradients. Both restriction enzymes were stored at 0°C.

(b) Pancreatic deoxyribonuclease (DNAse I), electrophoretically pure, was obtained as a solid from Worthington Biochemical Corporation, Freehold, New Jersey. It was stored at -20°C as a solid and generally dissolved in buffer (either 0.1 M-sodium acetate, 5mM-$MgCl_2$ pH 5.0, or 10 mM-tris-HCl, 5mM-$MgCl_2$ pH 7.4) just before use.

(c) Snake venom phosphiodiesterase was obtained as a solid from the Worthington Biochemical Corporation and stored at -20°C in 0.02M-tris-HCl pH 8.5.

(d) Bacterial alkaline phosphatase was obtained from Worthington, or from Whatman Biochemicals Ltd., Maidstone, Kent and was stored at -20°C or 4°C.

(e) Yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase, phosphorylase a, and bovine haemoglobin were obtained from the Sigma Chemical Company and stored at -20°C or 4°C.

(f) L-amino acid oxidase (Crotalus terrificus) and pig-heart fumarase were obtained from Boehringer Mannheim.

5. Chemicals

All chemicals were reagent grade and, with the exception of SAM (see Methods 2(e)) and phenol, they were used without further purification.

The following chemicals were obtained from the Sigma Chemical Company; Coomassie brilliant blue, N7-methylguanine, 6-methylaminopurine, 5-methylcytosine, adenine, dAMP, dGMP, dCMP, dTMP, calf thymus DNA, 'Trizma base', SAM-iodide [generally grade 1 specified as 85-90% pure, although one grade II ("approximately 70% pure") batch was also used].

The following chemicals were obtained from British Drug Houses, Poole, Dorset: dithiothreitol, 2-(N-morpholino)-ethanesulphonic acid, N-tris-(hydroxymethyl)methyl-2-amino-ethanesulphonic acid, piperazine-NN'-bis-2-ethanesulphonic acid, CsCl, $(NH_4)_2$ SO_4 ('Aristar' grade), EDTA, phenol, acrylamide, methylenebisacrylamide, tetramethyl-1:2-diaminoethane, 2-mercaptoethanol.

Streptomycin sulphate was a kind gift from Glaxo Laboratories Ltd., Hemel Hempstead, Herts.

The scintillants 2,5-diphenyloxazole (PPO), and 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP) were obtained from the Packard Instrument Company, Inc. Illinois, U.S.A. The scintillants 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (Butyl PBD) and 2,5-bis-(5-tertiarybutylbenoxazol-2-yL) thiophen (BBOT) were obtained from Ciba Ltd., Duxford, Cambs, U.K.

6. Chromatographic and Ionophoretic media

The following Whatman ion exchange resins were obtained from H.Reeve Angel and Co.Ltd., London E.C.4, U.K; P11 phosphocellulose, DE23 fibrous diethylaminoethyl cellulose, DE52 microgranular diethylaminoethyl cellulose.

Diethylaminoethyl-Sephadex A25 and carboxymethyl-Sephadex C25 were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Bio-gel Agarose A0.5M was obtained from Calbiochem, 10 Wyndham Place, London W1H 1HS. Cellulose acetate ('oxoid') was obtained from Oxo Ltd., London E.C.4, U.K. Ion exchange papers were Whatman Chromedia DE81 and AE81.

Cellulose thin layers, impregnated with polyethyleneamine, were obtained from Macherey-Nagel and Co., Düren, Germany. Thin layer cellulose sheets MN300 were also obtained from Macherey-Nagel.

7. Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks.

S-adenosyl-L-methionine, tritiated in the donor methyl group (referred to as 'CT_3SAM') was purchased at a specific activity of 8.5 or 8.9 curies/mMole. This material was supplied in dilute H_2SO_4 pH3, at a radiochemical purity of at least 96%. It was stored at -20°C and used without further purification.

S-adenosyl-L-methionine, labelled in the donor methyl group with ^{14}C (referred to as '^{14}C-SAM') was purchased at a specific activity of 55 or 58m curies/mMole. This material was

supplied in dilute H_2SO_4 at a radiochemical purity of at least 90%. It was stored at -20°C and used without further purification.

^{32}P as orthophosphate in dilute HCl solution pH2-3, was purchased at a specific activity of 1 mCi/ml or 10 mCi/ml. Before use the HCl was removed in a vacuum dessicator and the residue dissolved in about 0.5 ml of sterile H_2O.

8. DNA preparations

(a) Bacteriophage 82c DNA. E.Coli, either C600 (P1--) or C600 (P1 -+), was grown in 1 litre of L broth at 37°C to E_{650} = 0.5 (approx. 5 x 10^8 cells/ml). The culture was made 1mM in $MgSO_4$ and bacteriophage 82c was added at a multiplicity of infection of 0.3 - 0.5 phage/bacterium. The culture was shaken vigorously at 37°C for 3-4h and lysed with 5 ml of chloroform. The lysate was clarified by centrifugation (10,000g for 15 min at 4°C) and the titre determined on the appropriate indicator strains to check the presence or absence of P1 modification. The bacteriophages (generally 10^{11} plaque formers/ml) were collected by centrifugation (45,000g for 2½ h), resuspended by gentle agitation overnight in 25 ml of bacteriophage buffer and twice banded by centrifugation (30 h 27,000 rev.min, MSE superspeed 65 centrifuge, 3 x 23 ml swing-out rotor) in 41.5% ($\frac{w}{w}$) CsCl. The final band was collected, dialysed against 10mM Tris-HCl, 1mM-EDTA, pH 7.4 and extracted by three times rolling with freshly distilled phenol that had been equilibrated with 0.5 M Tris-HCl pH8. The aqueous layer was dialysed exhaustively against 10mM Tris-HCl, 1mM EDTA-50mM NaCl, pH 7.4, and stored at 0°C.

(b) Unlabelled $\lambda C_I 857\ S_7$.K and $\lambda C_I 857\ S_7$.K(P1) DNA.

In collaboration with Dr P.Batten, large scale preparations of both of these phages were made by the following method. E.Coli W1485 ($\lambda C_I 857\ S_7$) or E.Coli W1485 ($\lambda C_I 857\ S_7$) (P1) were grown to 5×10^8 cells/ml1 in 30 litres of L broth in a 50 litre fermentor at 30°C. The temperature was shifted to 42°C for 10 minutes and then lowered to 37°C, for 3-4 h. The cells were sedimented in a continuous flow Alfa-Lavall centrifuge, resuspended in 1 litre of phage buffer, and lysed by the addition of 20 ml of $CHCl_3$, followed by 1 mg of pancreatic DNAse. After incubating for 1 h at 37°C the thick suspension (titer on Ymel = 5×10^{12} p.f.u./ml) was left overnight at 0°C. The suspension was clarified by centrifugation (MSE High Speed 18, 6 x 250 ml1 rotor, 10,000 r.p.m. 30'), and the suspension incubated at 37°C for 1 h while N_2 was gently blown over the surface in order to remove residual $CHCl_3$. The phage were pelleted by centrifugation (10 x 100 ml rotor, 20,000 r.p.m., 2.5 h, 20°C) and the pellets were resuspended by shaking each overnight with 10 ml of phage buffer at 4°C. The milky supernatant was decanted from the dirty looking pellet and digested at room temperature (20°C) for 3 h with 10 µg/ml (each) of pancreatic DNAse and pancreatic RNAse. The phage were pelleted as before and resuspended in 30 ml of phage buffer. The milky supernatants were pooled and combined with the product of re-extracting the pellets. The total phage were divided into 2 equal portions. Each portion was made up to about 140 ml with phage buffer, solid CsCl was added to give 41.5% ($\frac{w}{w}$) CsCl, and the phage were

banded by centrifugation (3 x 65 ml SW rotor, 40 h, 21,000 r.p.m., 20°C). The bands were collected by aspiration, pooled, and rebanded in the 3 x 65 ml SW rotor by an identical procedure. The final bands were collected, pooled and stored in sterile glass tubes at 0-4°C. The titer of the $\lambda C_I 857\ S_7.K$ on

$$\frac{\text{W3110 su III}^+\text{(P1)}}{\text{Ymel}} \quad \text{was} \quad \frac{2.7 \times 10^9}{1.4 \times 10^{14}} = 2 \times 10^{-5}.$$

The titer of the $\lambda C_I 857.K(P1)$ on

$$\frac{\text{W3110 su III}^+\text{(P1)}}{\text{Ymel}} \quad \text{was} \quad \frac{7 \times 10^{12}}{5 \times 10^{13}} = 0.14,$$

although this weak restriction may not be real because the titering strains are not precisely isogenic.

DNA was prepared from these phages by diluting them at least 10 fold with phage buffer, dialysing out the CsCl and then extracting with redistilled phenol as described above for phage 82 DNA.

Unlabelled $\lambda C_I 857\ S_7.C$ DNA was made by a similar protocol from strain 803 ($\lambda C_I 857\ S_7$). Strain 803 (see Materials 1) is an $r_K^-\ m_K^-$ derivative of strain K.

(c) ^{32}P-labelled $\lambda C_I 857\ S_7.K$ or $\lambda C_I 857\ S_7.K$ (P1) DNA. 100 ml of Abelson low phosphate medium was innoculated with 1 ml of an overnight culture of W1485 ($\lambda C_I 857\ S_7$) or W1485 ($\lambda C_I 857\ S_7$) (P1) and grown in a shake flask at 34°C to approx. 5×10^8 cells/ml. 5 mCi of neutralised ^{32}P-orthophosphate was added, and sufficient hot water was poured into the bath to reach the induction temperature of 43°C for 15-20 min. The

temperature was returned to 37°C by adding cold water and maintained at 37°C for a further 3-4 h. The bacteria were pelleted and lysed with $CHCl_3$. The phage were purified by two successive bandings in CsCl essentially as described for the unlabelled preparations. Occasionally the band was not visible and was located by counting fractions from the CsCl gradient. The DNA was extracted from the purified phage by rolling with redistilled phenol.

(d) ^{32}P-labelled phage T7 DNA was a kind gift from Mr R.W.Old.

^{3}H-labelled phage M13 twisted circular RFI DNA purified by propidium iodide-CsCl dye buoyant density centrifugation, was a kind gift from Mr G.Peters.

^{3}H-labelled λDNA preparations were kindly provided by Drs K. and N.E.Murray.

III METHODS

1. Bacterial and Bacteriophage Genetics

(a) Efficiency of plating (e.o.p.)assays. The appropriate indicator bacteria were grown in L broth to mid log phase, pelleted in a bench centrifuge and resuspended in one half the volume of 1mM-$MgSO_4$. After shaking at 37°C for 20 minutes, the bacteria (0.2 ml) were preadsorbed for 10 minutes at room temperature with 0.1 ml of an appropriate dilution of phage. BBL top layer agar (2 ml) was added and the mixture poured onto the surface of a petri plate containning BBL bottom layer agar. The plates were incubated overnight at 37°C or 30°C. Assays were always performed in duplicate and at least 100 plaques per plate were counted. The e.o.p. values are very sensitive to physiological variation and fluctuate by a factor of two or three.

(b) General methods for lysogenising with P1, transducing with P1, and propagating phages 82, λ and P1 by plate or liquid lysis are given in Clowes and Hayes (1968). For phages 82 and λ, tryptone medium was used, for phage P1 the special P1 agar (see Materials 3) was used.

2. Enzymological methods

(a) Preparation of extracts (i) Extracts of P1-infected cells; 2.5 ml of an overnight culture of E.coli 1100 r_K^- was innoculated into 1 litre of L broth in 5 litre conical flasks. The flasks were grown in a rotary shaker at 37°C to E_{650}=0.4 (approx. 4×10^8 cells/ml). 2 ml of 1M-$CaCl_2$ was added to each followed by 2×10^{12} plaque formers of the appropriate P1 strain.

After swirling once to mix the phage, the flasks were stood at 37°C for 3 min to allow adsorption and then shaken vigorously for 12 min. The infection was terminated by pouring the cells onto 500 g of crushed frozen (-20°C) 0.05M-NaCl. The cells were sedimented (HS 18 centrifuge, 6 x 250 ml rotor, 2°C, 10,000 r.p.m., 20 min), resuspended in 10 ml of 0.05M-tris-HCl pH 8.0, 5mM-2-mercaptoethanol, and disrupted with 6 x 20 sec bursts (with 30 sec cooling in ice between each) in an MSE sonicator at 8 amps. The debris were removed by centrifugation (HS18, 8 x 50 ml rotor, 10,000 r.p.m. 20 min) and 0.3 ml of 1M-$MgCl_2$ added. The resulting precipitate was removed (HS 18, 8 x 50 ml rotor, 10,000 r.p.m. 30 min), and the supernatant extracted by adding 2.25 ml of fresh 5% ($\frac{w}{v}$) streptomycin sulphate. The streptomycin precipitate was removed (HS 18, 8 x 50 ml rotor, 10,000 r.p.m., 15 min) and the supernatant was precipitated by the addition of 4.9 g solid $(NH_4)_2SO_4$. The precipitate was collected (HS 18, 8 x 50 rotor, 15,000 r.p.m., 30 min), resuspended in 3 ml breaking buffer and dialysed extensively against 0.02M-potassium phosphate pH6.7, 5mM-2-mercaptoethanol, 0.5mM-EDTA. The protein content of each extract (generally $\sim$20 mg/ml) was determined by the method of Lowry et al (1951) with bovine serum albumin as standard.

(ii) Extracts of P1 induced cells ; Strain K140 (Plc_I162) was grown to approx. 10^9 cells/ml in L broth at 30°C and induced by shifting to 42°C for 10 min. The temperature was dropped to 37°C for 10 min and the induction was terminated by pouring the cells onto frozen 0.05M NaCl as described

above. The cells were harvested (see part (i)), and 5 g resuspended in 15 ml of buffer (0.05M-tris-HCl pH7.4, 5mM-2-mercaptoethanol, 10 mM-$MgCl_2$, 0.1mM EDTA, 5% ($\frac{v}{v}$) glycerol). After sonication, 0.02 ml of a solution of pancreatic deoxyribonuclease (1 mg/ml) was added to the lysate which was left for 30 min in the cold room (0-4°C). After removing the debris (see part (i), the ribosomes were removed by centrifugation (MSE Superspeed 65, 3 x 20 SW rotor, 30,000 r.p.m., 4°C, 2.5h). The supernatant was fractionally precipitated by adding dry $(NH_4)_2$ SO_4 to 0-25% saturation, 25-55% saturation, and 55-100% saturation. The precipitates were dissolved in 0.05 M-potassium phosphate pH 6.8, 5mM-2-mercaptoethanol, 0.1 mM-EDTA, 5% glycerol, and dialysed extensively against the same buffer. This fractionation was performed in parallel on 5 g of 1100 r_K-(Pl) cells.

(b) Pretreatment of resins for column chromatography. P11 phosphocellulose was defined, pre-cycled and equilibrated as described by Burgess (1969). Special care was taken to ensure that this high capacity exchanger was exactly equilibrated just before use.

DE23 fibrous diethylaminoethyl cellulose was pre-cycled and equilibrated according to Whatman technical bulletin IE2.

DE52 microgranular diethylaminoethyl cellulose was suspended in H_2O, defined, and then equilibrated by gently stirring with several changes of buffer until the pH and conductivity of the buffer were unaffected by contact with the resin.

Diethylaminoethyl-Sephadex A25 and carboxymethyl-Sephadex C25 were allowed to swell in excess buffer for a few hours and were then defined and equilibrated by gently stirring with several changes of buffer.

(c) Sucrose or glycerol gradients for zonal sedimentation analysis. These were constructed by using a two-chambered device of the sort described by Britten & Roberts (1960). After centrifugation the gradients were fractionated by piercing the bottom of the tube and collecting drops. When labelled DNA samples were fractionated, the emerging drops were collected on 2.1 cm Whatman GFC glass fibre discs. The discs were dried and assayed for radioactivity in a liquid scintillation spectrometer after the addition of 2.5 ml of toluene based scintillant (either 4 g BBOT or 4 g Butyl-PBD/litre AnalaR toluene). Before all sedimentation assays,,preparations of λ DNA were heated at 60°C for 10 min to dissociate concatamers. Before all alkaline sucrose gradients, the centrifuge tubes were boiled in 0.01M-EDTA pH 8.0 for 1 h.

(d) Polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS). SDS gels were run essentially as described by Weber and Osborn (1969). The gels were 8% acrylamide, 0.27% methylenebisacrylamide and were polymerised with 0.17% (final concentration) tetramethylethylenediamine and 0.075% ammonium persulphate. After electrophoresis the gels were stained in Coomassie brilliant blue as described by Weber and Osborn and destained by soaking for 7-10 days in frequent changes of 7.5% (v/v) acetic acid, 5% (v/v) methanol.

The gels were scanned at 550 nm using the 2410-S linear transport attachment to the Gilford model 2000 spectrophotometer.

(e) Purification of SAM by ion exchange chromatography (communicated by Dr R.Yuan). A small column of Zeokarb 226 resin (previously washed with 1M-NaOH and 4 M-acetic acid) was set up in a pasteur pipette and equilibrated with 0.01 M-sodium phosphate pH7.5. 25 mg of crystalline SAM-iodide was dissolved in 0.25 ml of 0.01M-sodium phosphate pH7.5. The sample was applied and washed through with 5 ml of 0.01 M-sodium phosphate pH 7.5, followed by elution with 5 ml of 0.25 M-acetic acid and then 5 ml of 4 M-acetic acid. As soon as elution with 4 M-acetic acid was begun, 10 drop fractions were collected. 10µl aliquots of each fraction were diluted 100 fold and the extinction at 257 nm was measured. The peak fractions of the 4M elution were pooled and the concentration of SAM was determined spectrophotometrically (E'_{257} = 14,700 in 1 M-H_2SO_4). A 2µl aliquot was applied to a 20 cm x 2 cm strip of thin layer cellulose MN 300 and chromatographed in ethanol: glacial acetic acid: water (165 : 34: 11 $\frac{v}{v}$). On examination under UV light the chromatogram showed one strong spot with an Rf of 0.4 and one very faint spot with an Rf of 0.7. Commercial preparations (90% pure ')or aged purified preparations sometimes showed as many as six spots on this t.l.c. system.

The purified SAM was stored in 4 M-acetic acid at -20°C.

(f) Methylation assay for the DNA modification enzyme.

Two protocols have been used.

(i) The reaction mixture contained, in a total volume of 0.2 ml 10μmoles potassium phosphate pH6.5, 1μmole 2-mercaptoethanol, 0.1μmoles EDTA, 2.4nmoles of CT_3-SAM (3 Ci/n mole), 5μg phage 82 DNA and the sample to be assayed. After 1 h incubation at 30°C, 0.5% bovine serum albumin (0.1 ml), 0.2 M tetrasodium pyrophosphate (0.2 ml) and 1 M $HClO_4$-2mM sodium pyrophosphate (0.5 ml) (were added. After 5 min at 0°C, cold water (1 ml) was added and the precipitate collected by centrifugation (5,000 g, 5 min). The precipitate was dissolved in 0.2 M NaOH (0.3 ml) and re-precipitated with 0.2 M sodium pyrophosphate (0.2 ml) and 1 M $HClO_4$-2mM sodium pyrophosphate (0.5 ml). This procedure was repeated twice more and the final precipitate was collected by filtration under reduced pressure through a 2.1 cm Whatman GFC glass-fibre disc. The disc was washed with 1 M-$HClO_4$ (5 ml), water (5 ml) and ether (2 ml). After drying under an i.r. lamp the discs were counted for radioactivity in 2.5 ml of scintillant [4 g of Butyl-PBD/litre of AnalaR toluene] in a Nuclear Chicago 'Unilux' liquid-scintillation spectrometer, at approximately 20% efficiency. This procedure was generally used for assaying relatively large volumes (up to 0.15 ml) of column fractions. Assays were always done in parallel with P1 modified and unmodified phage 82 DNA. The blank value for incorporation into modified DNA was subtracted. This blank was generally 100-150 c.p.m., except for the dialysed $(NH_4)_2SO_4$ fraction where it was 300-500 c.p.m. owing

to the presence of non-specific methylating activity (Gold & Hurwitz 1966). More recently, 10 μ moles of potassium morpholinoethanesulphonate pH 6.0 have been used in place of potassium phosphate.

(ii) The reaction mixture contained, in a total volume of 0.05 ml, 2.5 μ moles potassium morpholinoethane sulphonate pH 6.0, 0.25 μ moles 2-mercaptoethanol, 25 n moles EDTA, 0.12 n moles CT_3-SAM, (8.9 μ Ci/n moles), 5 μg phage 82 DNA and the sample. The mixture was incubated and assayed as for method (i). This was used for smaller enzyme samples.

3. DNA and nucleotide methodology

(a) Ionophoresis and mapping procedures. Full details of these procedures are given by Sanger et al. (1965) and by Murray (1970). The following systems were used (i) Ionophoresis on AE paper at pH 3.5 (5% ($\frac{v}{v}$) pyridine-acetate) was used to separate the four mononucleotides, to resolve the products of partial venom phosphodiesterase digestion and, when run for longer periods, to serve as a first dimension in conjunction with DE paper. (ii) Ionophoresis on DE paper at pH 9 (5% ($\frac{w}{v}$) triethylamine carbonate) or at pH 2 (7% ($\frac{w}{v}$) formic acid) was used in two dimensional mapping procedures or for resolving partial venom digestions. (iii) Ionophoresis on cellulose acetate ('oxoid') at pH 3.5 (5% ($\frac{v}{v}$) pyridine-acetate, 7M-urea), followed by thin layer chromatography on polyethyleneimine impregnated layers of cellulose with 1.5M-pyridine-formate pH 3.7 was according to Southern and Mitchell (1971).

(b) Analysis of bases by acid hydrolysis. The DNA from a methylation reaction was purified by phenol extraction and prolonged dialysis of the aqueous layer against high salt buffer (generally 0.01 M-tris-HCl pH 7.4, 0.5M-NaCl, 1mM-EDTA) or else by successive acid precipitation in the presence of carrier calf thymus DNA (generally 0.2 mg). The sample was evaporated to dryness and hydrolysed with 1 M-HCl (0.1 or 0.2 ml) for 1 h at 100°C. Marker bases (generally 20µl of 25 mM stock solutions) were added and the mixture evaporated to dryness. The residue was dissolved in the minimum volume of 10% ($\frac{v}{v}$) acetic acid - 10% ($\frac{v}{v}$) propan-2-ol, and applied to Whatman No.1 paper. After descending chromatography in butan-1-ol-water (43:7, $\frac{v}{v}$) in an NH_3 atmosphere for 24 h the paper was dried, markers were located under U.V. light and the paper was cut into strips (2cm x 1cm); the radioactivity of each strip was determined by liquid scintillation counting. The strips containing 6 MAP were swirled in toluene to remove scintillant, dried, eluted overnight with 0.1 M HCl and chromatographed on Whatman No.1 paper in an ascending system of methanol-water-concHCl (7 : 1 : 2, by vol) for 24 h. The chromatogram was again examined under U.V. light, cut up and counted for radioactivity.

In some experiments the residue was dissolved in 10 or 20µl of water and applied to a sheet (20 cm x 20 cm) of MN 300 thin layer cellulose. The chromatogram was developed in two dimensions as described by Razin et al. (1970). Marker bases were located under U.V. light, eluted and counted for

radioactivity, together with the origin region, as described by Razin et al.(1970).

(c) Analysis of DNA and nucleotides by enzymic digestion

(i) Degradation to mononucleotides: Bacteriophage λ or 82 DNA was purified from a methylation reaction by extracting twice with an equal volume of phenol. The aqueous layer was extensively dialysed against the DNAse I buffer (either 0.1 M sodium acetate pH 5.0, 5 mM $MgCl_2$ or 10 mM tris-HCl pH 7.4-5 mM-$MgCl_2$) and a solution of DNAse I (1 mg/ml; 0.1 ml) was added and incubated at 37°C for 2 h. The reaction was adjusted to pH 8.0 with 1 M-tris-HCl pH 8.0 and incubated with snake venom phosphodiesterase (1 mg/ml; 20µl; 37°C; 4 h). The solution was evaporated to dryness, the residue dissolved in 50µl of water and applied, together with mononucleotide markers, to AE81 cellulose paper. Electrophoresis was at 50V/cm in pyridine-acetate buffer pH 3.5, until the blue marker dye (Xylene Cyanol FF) had run 25 cm. The paper was dried overnight at room temperature, examined under U.V. light, cut into strips (2 cm x 1 cm) and the radioactivity determined in a liquid-scintillation counter.

(ii) Sequence analysis of nucleotides by partial digestion with snake venom phosphodiesterase was according to Murray (1970).

(iii) Digestion of DNA with DNAse I was according to Murray (1970) except that 10 mM-Tris HCl pH 7.4-5mM $MgCl_2$ or $Mg(CH_3COO)_2$ was sometimes preferred to the acetate buffer.

(d) De-salting of nucleotides. The ionophoretic systems for resolving nucleotides are sensitive to salt in the sample. This is particularly so for the cellulose acetate and DE81 systems. Therefore it is important when fingerprinting large quantities (>100µg) of nucleotide to desalt them effectively. The following procedure is based on adsorption to and elution from a DE ion exchange resin (Rushizsky & Sober, 1962).

DE52 cellulose or DE Sephadex A25 was equilibrated with 0.02 M-NH_4HCO_3 pH8. An aliquot (generally 0.2 ml was sufficient) of the settled slurry was pipetted into a solution of nucleotide that had been diluted ten fold with water. The solution was stirred magnetically for 30 minutes at room temperature and then allowed to settle. The supernatant was assayed for radioactivity or for its extinction at 260 nm to ensure that >90% of the nucleotide had been adsorbed by the exchanger. If this had not occurred, a further aliquot of exchanger was added and the procedure repeated. The exchanger was finally collected on a 2.5 cm Whatman No.1 disc in a Millipore filtration apparatus, washed with 40 ml of 0.2 M-NH_4HCO_3 pH8, and eluted with 5 ml of 2M-NH_4HCO_3 pH8. The eluate was repeatedly evaporated to dryness in a rotary evaporator to remove the bicarbonate. The desalted nucleotides were finally evaporated to dryness in a small siliconised tube, taken up in 5-20 µl of H_2O, and applied to the ion-exchange paper.

This procedure used for smaller amounts of exchanger than column procedures and hence desalted more effectively.

The recovery of radioactive or UV-adsorbing nucleotide was always >80% and often near 100%.

IV. RESULTS

A. General Enzymology

(i) P1 restriction of bacteriophage 82.

The P1 modification enzyme was selected for study because the efficient restriction of certain bacteriophages by the phage P1 system presumably indicates a relatively large number of sites at which the restriction and modification enzymes act. This facilitates the use of a methylation assay for modification. The DNA of bacteriophage 82 was chosen as substrate rather than that of bacteriophage λ because of the relative efficiencies of plating of the two phages on a P1 lysogen (see Table 1). The assay for the P1 modification enzyme measures the extent of methylation of bacteriophage 82.C600(P1--) DNA compared with DNA from bacteriophage 82.C600(P1-+) that has been modified *in vivo*. If modification *in vivo* is efficient, then the latter substrate should be inert to the activity *in vitro*.

(ii) Detection of P1 modification activity in fractionated extracts.

Arber and Linn (1968) have described a simple fractionation procedure that produced extracts with B specific modification activity as detected by infectivity assays on bacteriophage fd DNA. This fractionation was therefore applied to various P1 infected cells and the resulting extracts were assayed for methylation of 82.C600 (P1--) DNA and 82.C600 (P1-+) DNA. The P1-infected cells were initially investigated in preference to P1 lysogens,

Table 1. Efficiencies of plating of bacteriophages 82 and λ on P1 lysogenic hosts.

The efficiencies of plating (titre on bacteriophage P1 lysogenic host ÷ titre on E.coli C600) of bacteriophage 82c and bacteriophage λc_{26} grown on various hosts were determined as described in Methods section 1a.

Bacteriophage	E.coli C600(P1)	E.coli C600(P1-+)
82.C600	$1 \times 10^{-6} - 3 \times 10^{-6}$	1
82.C600(P1-+)	1	1
82.C600(P1--)	$1 \times 10^{-6} - 3 \times 10^{-6}$	1
λ.C600(P1--)	$1 \times 10^{-5} - 3 \times 10^{-5}$	1

because the genetical observations of Arber and Dussoix (1962) indicated that early lytic infection by P1 is a period of intense P1 modification activity.

The extracts were prepared as described in Methods Section 2a(i) and assayed as shown in the legend to Table 2. The results (Table 2) showed that 82.C600 (P1--) DNA was preferentially methylated by an activity that was present in P1 infected cells but not in uninfected cells, nor in cells infected with the modificationless mutant P1KCφ2. The increase in tritium incorporation shown by all extracts was probably due to the nonspecific methylating activity described by Gold and Hurwitz (1963). The presence of this activity made it important to perform all assays in parallel with P1 modified and unmodified DNA.

In later experiments the activity in extracts of induced lysogens of the thermoinducible mutant P1 C_I162 (Scott, 1970) was investigated. The extracts were prepared as described in Methods section 2a(ii) and the data is presented in Table 3. The 25-55% $(NH_4)_2SO_4$ cut from the heat induced strain has approximately 4 x the activity of that from the normal 1100r_K-(P1) lysogen. In both cases the 25-55% cut contains the majority of the $(NH_4)_2SO_4$ precipitable protein. The heat inducible lysogen was subsequently used for the purification of homogeneous modification enzyme (see Results, section B).

Table 2. Methylation of 82.C600(P1--) DNA and 82.C600(P1-+) DNA by various extracts.

Reaction mixtures (vol 0.275 ml) containing 14 µmoles potassium phosphate pH6.5, 1.4 µmoles EDTA, 1.3 µmoles 2-mercaptoethanol, 12 nmoles CT_3-SAM (3 µCi/nmole), 15 µg of 82 DNA and 1mg of extract protein were incubated at 30°C. Aliquots (0.1 ml) were withdrawn at 0 min and 45 min and assayed for acid precipitable radioactivity as described in Methods section 2f except that at this time 0.04 mg of salmon sperm DNA was used as carrier and sodium pyrophosphate was omitted from the precipitation reaction. This latter omission is probably responsible for the rather variable 0 minute incorporation. The results are expressed as c.p.m. acid-insoluble tritium.

Source of extract	SUBSTRATE				
	82.C600(P1--) DNA			82.C600(P1-+)DNA	
	0'	45'	95'	0'	45'
1. P1KC infected $1100r_k$-	316	1243	2072	344	481
2. P1KCφ2 infected $1100r_k$-	599	654		472	606
3. $1100r_k$- (uninfected)	382	613		402	601

Table 3. Methylation of 82 DNA by extracts of heat induced K140(Plc_I162) and uninduced 1100r_K-(Pl).

Reaction mixtures (vol. 0.2 ml) containing 10 µmoles potassium morpholinethanesulphonate pH 6.0, 1 µmole 2-mercaptoethanol, 0.1 µmole EDTA, 0.45 n mole CT_3-SAM (8.9 µCi/n mole), 6 µg 82 DNA and 1 mg of extract protein, were incubated at 30°C for 100 min and assayed for acid-insoluble ^{3}H-radioactivity(c.p.m.).

Source of extract		% total $(NH_4)_2SO_4$ precipitable protein	SUBSTRATE: 82.C600 (Pl−) DNA	SUBSTRATE: 82.C600 (Pl+) DNA
K140(Plc_I162)	1. 0-25% $(NH_4)_2SO_4$	3	5390	466
	2. 25-55% "	66	6286	433
	3. 55-100% "	31	1263	279
1100r_K-(Pl)	4. 0-25% $(NH_4)_2SO_4$	5	512	414
	5. 25-55% "	55	1652	386
	6. 55-100% "	40	921	321

(iii) Purification of the P1 modification activity from 1100r$_K$-(P1).

Although the activity has successfully been purified from 27 g of P1 infected cells, the difficulty of growing large quantities of P1 has made this unattractive as a source of purified enzyme. The enzyme was therefore generally prepared from the 1100r$_K$-(P1) lysogen by the following method. All the buffers contained 5% ($\frac{v}{v}$) glycerol, 5mM-2-mercaptoethanol and 0.5mM-EDTA unless otherwise stated. All potassium phosphate buffers were pH 6.7 and phosphate concentration was determined by use of a calibrated conductivity meter.

Step 1: preparation of dialysed $(NH_4)_2SO_4$ fraction. [Linn & Arber (1968), with minor modifications.] E.coli 1100r$_K$-(P1) was grown in L broth at 37°C to 10^9 cells/ml, sedimented in a continuous-flow Alfa-Lavall centrifuge and stored at -20°C. All subsequent operations were performed at 4°C. Cells (150g) were resuspended in 225 ml of 0.05M-tris-HCl pH 8.0, then 300 g of acid-washed glass beads was added and the mixture was blended for a total of 20 min with intermittent cooling in an ice-salt bath so that the temperature did not rise above 5°C. The supernatant was decanted from the beads, which were washed with 100 ml of 0.05M-tris-HCl pH8.0. The pooled supernatant and washings were centrifuged (10,000 g, 20 min). The supernatant (volume 354 ml) was made 0.035M in $MgCl_2$ and the resulting precipitate was removed by centrifugation (10,000g 15 min). The supernatant was precipitated by adding 180g of solid $(NH_4)_2SO_4$ which was dissolved over 30 min at 0°C. The precipitate was collected by centrifugation in the MSE High-Speed 18 centrifuge (6 x 100 ml

rotor, 17,000 r.p.m. 30 min, 2°C). The pellet was dissolved in 0.02M-potassiumphosphate (30 ml) and dialysed against the same buffer.

Step 2: chromatography on DEAE-cellulose. The dialysed $(NH_4)_2SO_4$ fraction was applied to a column (17 cm x 4.5 cm diam.) of Whatman DE52 cellulose that had been equilibrated with 0.02 M-potassium phosphate. The column was washed successively with 500 ml of 0.02 M-potassium phosphate, 500 ml of 0.05M-potassium phosphate, then eluted with a linear potassium phosphate gradient (1.5 litres), running from 0.05M to 0.3M . Fractions (approx. 45 ml) were collected and the enzyme activity was found in five neighbouring fractions with a mean phosphate concentration of 0.11M (Fig.1). The pooled fractions (volume 240 ml) were precipitated with $(NH_4)_2SO_4$ (150g). The precipitate was collected by centrifugation, dissolved in 0.02M-potassium phosphate (30 ml) and dialysed extensively against 0.02M-potassium phosphate. The precipitate that formed during dialysis was removed by centrifugation; the total protein in the supernatant was 301 mg (volume 44 ml).

Step 3: chromatography on phosphocellulose. Of the concentrated DE fraction, 34 ml was applied to a column (18 cm x 1.25 cm diam) of Whatman P11 phosphocellulose that had been equilibrated with 0.02M-potassium phosphate. The column was eluted with 0.02M-potassium phosphate (75 ml), 0.1M-potassium phosphate (100 ml), 0.2M-potassium phosphate (75 ml), 0.3M-potassium phosphate (100ml) and 0.5M-potassium phosphate (100 ml). The methylation activity was found in the 0.2M step and was immediately concentrated by

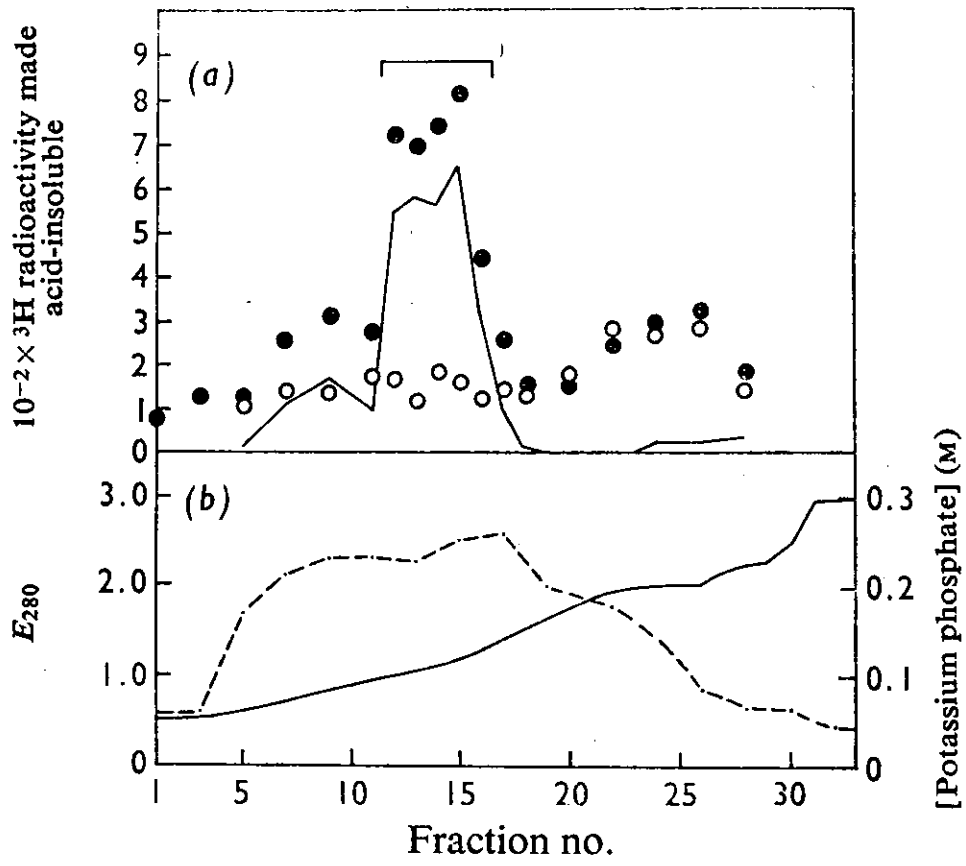


Fig.1. Chromatography of dialysed $(NH_4)_2SO_4$ fraction on DEAE-cellulose.

Adsorption and elution was as described in Results A(iii). Samples (0.15 ml) of the fractions were assayed in parallel for methylation of bacteriophage 82.C600(P1--) DNA(●) and bacteriophage 82.C600(P1-+) DNA (o). The continuous line in (a) is the difference in radioactivity incorporated into the two substrates. Fractions enclosed by the bar were pooled and concentrated as described in Results A(iii).

(b) - - - E_{280}; ———, [potassium phosphate]

the addition of $(NH_4)_2SO_4$ (40g). The precipitate was collected by centrifugation and dissolved in 0.02M-potassium phosphate (3 ml).

Step 4: glycerol-gradient sedimentation. After dialysis for 2 h against 0.02M-potassium phosphate (1 litre), the concentrated phosphocellulose fraction was layered in 1.2 ml portions on 20 ml of 10-25% ($\frac{v}{v}$) glycerol gradients made up in 0.02 M-potassium phosphate - 0.1mM EDTA - 5mM dithiothreitol, pH 6.5. After centrifugation for 36 h at 30,000 r.p.m. in the MSE Superspeed 65 centrifuge, 3 x 23 ml swing-out rotor at 2°C, the gradients were collected in 1 ml fractions. The activity was found at a position corresponding to approximately 6S. The active fractions (see Figure 2) were made 50% ($\frac{v}{v}$) in glycerol and stored at -20°C. No significant loss of activity (less than 10%) was observed over 4 months.

Comments on the purification procedure

The purification of the enzyme is summarised in Table3. Assays with a limiting amount of glycerol gradient fraction were conducted with and without the addition of extract (1 mg protein of the dialysed $(NH_4)_2SO_4$ fraction) of the $1100r_K$-strain. The extract had no significant effect on the extent of the reaction, showing that the activity of the $1100r_K$-(P1) extract can be directly compared with that of the purified enzyme in computing the degree of purification achieved. The extent of reaction was approximately linear with protein concentration over the range of activity involved in the assays. The enzyme

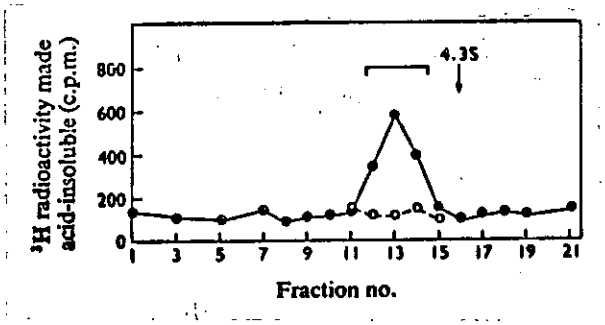


Fig.2. Glycerol-gradient sedimentation of concentrated phosphocellulose fraction.

Sedimentation of the phosphocellulose fraction was as described in the Results A(iii). Portions (0.025 ml) were assayed for methylation of bacteriophage 82.C600 (Pl−) DNA (●) or bacteriophage 82.C600 (Pl+) DNA (○). The 4.3S standard was bovine haemoglobin which was located by its extinction at 410 nm. Fractions enclosed by the bar were stored as described in A(iii). Sedimentation was from right to left.

TABLE 3. Summary of enzyme purification

1 unit of activity makes 0.16 p mol of methyl groups acid-insoluble/5 μg of unmodified phage 82 DNA per 60 min incubation at 30°C. This is equivalent to 125 c.p.m. under the conditions of assay (Methods 2f(i)). The blank value for incorporation into modified DNA is subtracted. The recovery values for the phosphocellulose and glycerol-gradient fractions are corrected for purification of total pooled DEAE-cellulose fraction.

Purification stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Crude supernatant	10,700			
Dialysed $(NH_4)_2SO_4$ fraction	4,820	7520	1.56	100
Pooled DEAE-cellulose fraction	301	4420	14.7	59
Pooled phosphocellulose fraction	9.1	2161	230.8	29
Pooled glycerol-gradient fraction	1.5	1230	820	16

was thus purified approximately 500 fold from the dialysed $(NH_4)_2SO_4$ fraction (Table 3).

The dilute column fractions were all unstable and the glycerol gradient fraction lost activity if stored at 0°C. The fractions from the phosphocellulose column were particularly unstable and it was important to concentrate them as soon as possible. In all preparations of this enzyme the steps before the glycerol gradient were completed in 2-3 days.

(iv) Properties of the purified modification activity

Although formal proof of the modification properties of this activity will not be presented until section g, it will be referred to as M·P, after the suggestion of Arber and Linn (1969).

All of the following experiments were performed with the glycerol-gradient fraction.

a. Contaminating activities

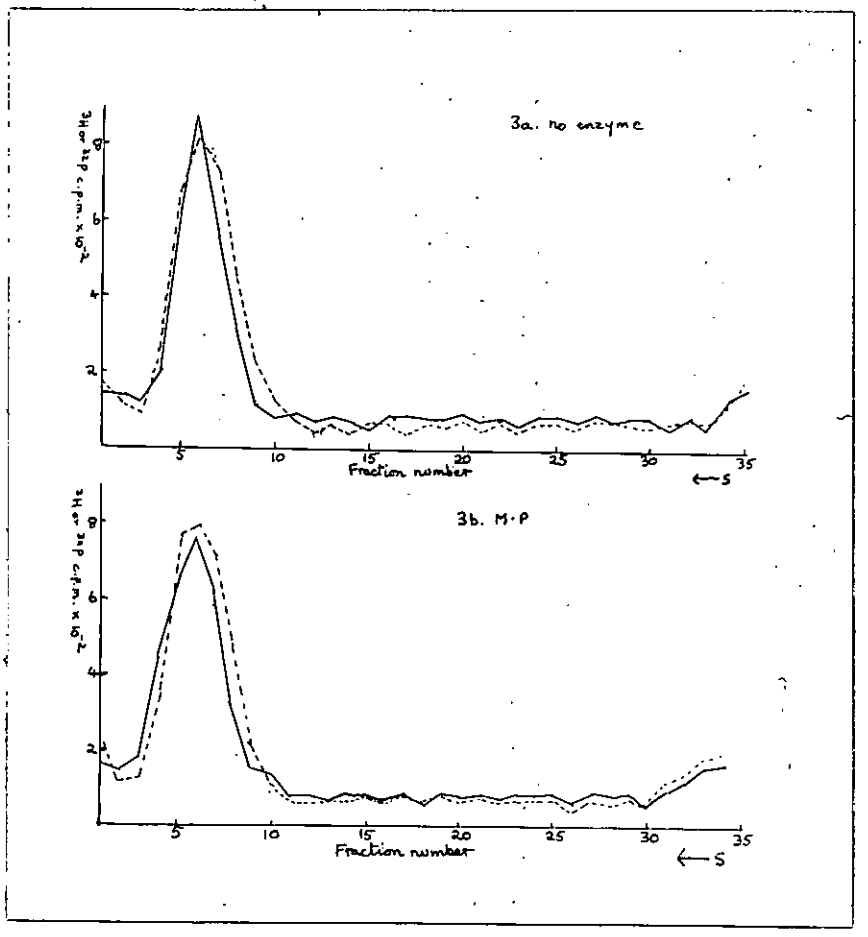
Pl restriction enzyme: Fig. 3 shows the sedimentation profiles of ^{32}P-labelled λ·K(Pl) DNA and ^{3}H-labelled λ.K DNA incubated, in the presence of appropriate cofactors, without enzyme (a), with M.P (b), with purified Pl restriction enzyme (c), with a mixture of both enzymes (d). The near superposability of the ^{32}P and ^{3}H distributions in (a) and (b) shows that neither DNA species was significantly degraded by the M.P. The λ.K DNA was, however, extensively broken both by Pl restriction enzyme and by the mixture (see (c) and (d)). Thus the M.P

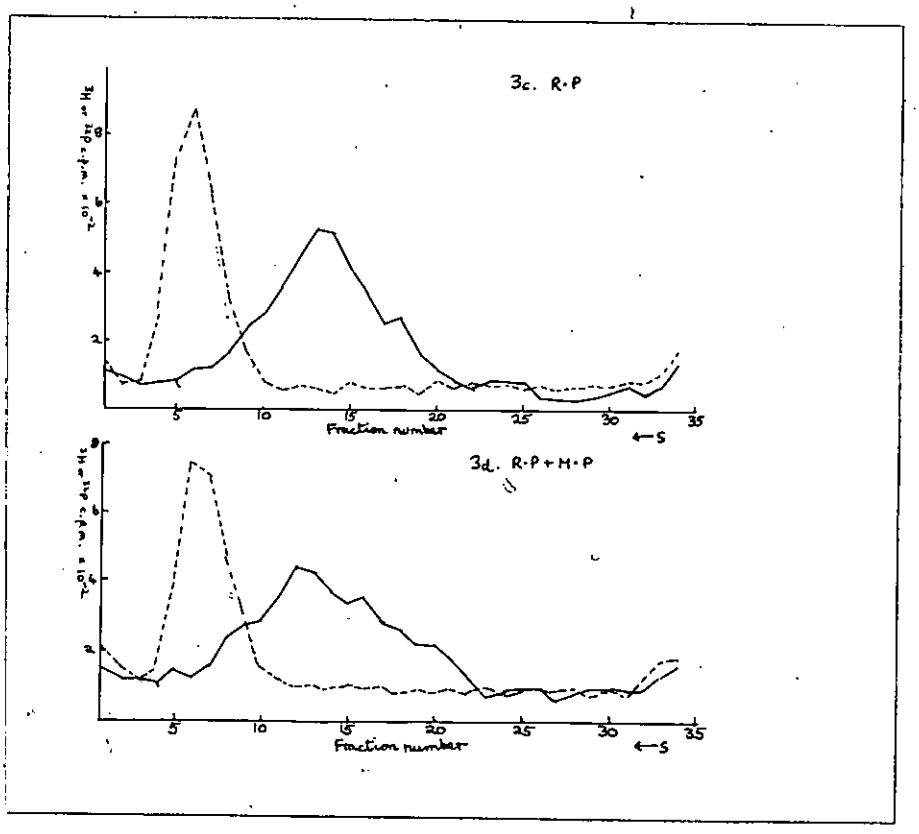
Fig.3. Sedimentation profiles in neutral sucrose gradients of ^{3}H λ.K DNA and ^{32}Pλ.K(Pl) DNA incubated with various enzyme fractions.

The reactions (vol 0.1 ml) contained 10 µmoles potassium tris-(hydroxymethyl)methyl-2-aminoethanesulphonate pH 8.0, 0.12 µmoles EDTA, 0.8 µmoles $MgCl_2$, 0.6 µmoles 2-mercaptoethanol, 0.25 µmoles ATP, 1 nmole SAM, $6x10^3$ c.p.m. of ^{3}H $\lambda c_I 857 S_7$.K DNA, $5x10^3$ c.p.m. of ^{32}P $\lambda c_I 857 S_7$.K(Pl) DNA, and either 4 µg of M.P or 10 µl (protein concentration unknown) of Endonuclease R.P. After incubation at 30°C for 20 min, the reactions were terminated by adding 5 µl of 0.5M EDTA pH8.0 and layered on 1.8 ml 6-20% (w/v) sucrose gradients in 10 mM tris-HcL(pH7.4)-1mM EDTA- 0.04% sodium dodecyl sulphate. After centrifugation (MSE superspeed 50) in a 3x3 ml swing-out rotor at 50,000 r.p.m. for 2 h at 20°C, the gradient was collected in 3 drop fractions and counted, (see Methods section 2(c)).

--- ^{32}P, λ.K(Pl) DNA

—— ^{3}H, λ.K DNA


3a. no enzyme
3b. M·P
Fraction number
S


3c. R·P
3d. R·P + M·P
Fraction number
S

preparation contained no detectable P1 restriction activity and this absence was not due to the presence of an inhibitor, unless such an inhibitor was effectively titrated. The absence of P1 restriction activity is consistent with the fact that it sediments at 10S on glycerol-gradients and is thus clearly resolved from the M.P in the final step of the purification procedure (Murray, Brown & Brookes, unpublished results).

Exodeoxyribonuclease activity: The M.P preparation was incubated with ^{32}P-labelled phage T7 DNA at pH 7.4, 5mM-Mg^{2+}. The production of mononucleotides by exonucleolytic degradation was measured by the method of Kelly and Smith (1970). The sample was applied together with mononucleotide markers, to one end of a strip (1 cm x 20 cm) of polyethyleneimine impregnated thin layer cellulose. The chromatogram was developed with 2 M-HCl until the solvent front was approximately 0.5 cm from the top. The strip was dried and the solvent front region, which contained the UV absorbing mononucleotide markers, was cut out and counted in a Nuclear Chicago gas-flow counter. The origin region, containing the higher molecular weight nucleotide, was also cut out and counted. As shown in Table 4, no detectable mononucleotides were produced after 1 h of incubation at 37°C. When the T7 DNA was incubated with the dialysed $(NH_4)_2SO_4$ fraction, however, labelled mononucleotides were detected (Table 4). If it is assumed that 100 c.p.m. above background could have been detected, then since the input counts are approximately 20,000 (Table 4), less than 0.5% of the DNA has been reduced to mononucleotides. Since the molecular weight of T7 DNA is

Table 4. Assays for contaminating exonuclease in purified M.P and in the dialysed $(NH_4)_2SO_4$ fraction.

The reactions (vol 0.03 ml) containing 1.5 µmoles tris-HCl pH 7.4, 0.12 µmoles $Mg(CH_3COO)_2$, 0.09 µmoles 2-mercaptoethanol, 38,760 c.p.m. of ^{32}P-labelled T7 DNA and either 4 µg of purified M.P or 4 µg of the dialysed $(NH_4)_2SO_4$ fraction, were incubated at 37°C. At the indicated times, 20 µl aliquots were spotted onto PEI strips and analysed as described in the text. The results are expressed as ^{32}P c.p.m.

		Front	Origin
60' incubation with no enzyme		68	22,708
M.P	0' incubation	41	23,742
	5' "	53	21,683
	15' "	72	22,471
	30' "	61	21,050
	60' "	78	23,216
$(NH_4)_2SO_4$ fraction			
	0' incubation	46	22,418
	5' "	1,482	20,612
	15' "	4,057	18,114
	30' "	5,342	17,672
	60' "	7,161	14,814

2.6×10^7 (Studier 1965), or 8×10^4 nucleotides, this means that less than 200 nucleotides were removed from each double stranded end of the intact molecule.

Endodeoxyribonuclease activity: The M.P preparation was incubated with ^{3}H-labelled λ.C DNA in 5mM-Mg^{2+} pH 7.4. The λ DNA was analysed by sedimentation in neutral and alkaline sucrose gradients and compared with λ DNA that had been incubated without enzyme. As shown in Fig.4 (a-d), the sedimentation profile of the λ DNA in both neutral and alkaline media was not detectably altered by the incubation. Therefore the M.P preparation was free of detectable endodeoxyribonuclease activity as scored by this test.

The most sensitive method of scoring endodeoxyribo nuclease activity is by its effect on twisted circular DNA. Only one single-stranded break is sufficient to convert the twisted circle to the less rapidly sedimenting non-twisted circular form. The M.P preparation was incubated with a mixture of ^{32}P-labelled λ.K (P1) linear DNA and ^{3}H-labelled bacteriophage M13 twisted circular DNA, containing a small amount of the non-twisted circular form, under the conditions of methylation reactions (0.05M - potassium morpholinoethane-sulphonate pH6.0, 0.25mM-EDTA). As shown in Fig.4 (e and f) the sedimentation profile of the M13 DNA was not detectably altered by the incubation.

Fig. 4. Analysis of contaminating endodeoxyribonuclease activity by zonal sedimentation in sucrose gradients.

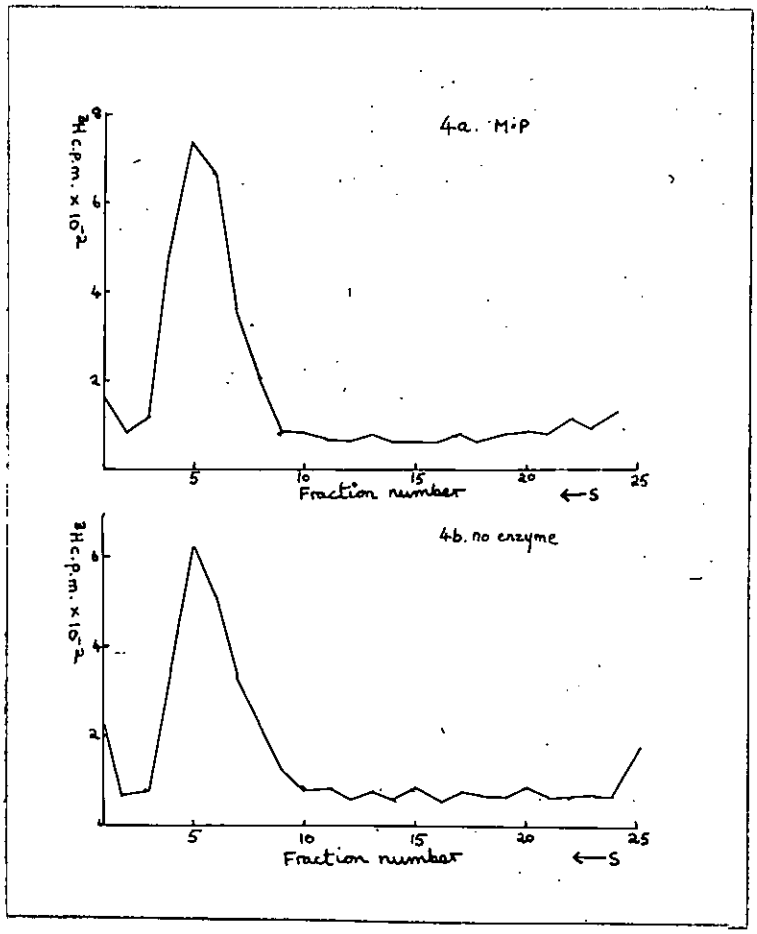
1. λDNA as substrate. Reactions (vol 0.1 ml) contained 5 μmoles tris-HCl pH 7.4, 0.5 μmoles $Mg(CH_3COO)_2$, 0.5 μmoles 2-mercaptoethanol, 3×10^3 c.p.m. of $\lambda c_I 857 S_7$.C DNA and either 4 μg of M.P (Fig.4a and 4c) or no enzyme (Fig. 4b and 4d). After incubating at 37°C for 30 min, the reaction mixtures were layered on 1.8 ml 6-20% (w/v) sucrose gradients in neutral medium (0.01M tris-HClpH7.4, 0.9M-NaCl, 1mM-EDTA) or alkaline medium (0.3M-NaOH, 0.9M-NaCl, 1mM-EDTA), centrifuged at 20°C, 50,000 r.p.m., (MSE superspeed 50, 3 x 3 ml swing-out rotor) for 2 h (neutral) or 2.75 h (alkaline) and collected in two drop fractions. Fig. 4a and 4b, neutral. Fig. 4c and 4d alkaline.

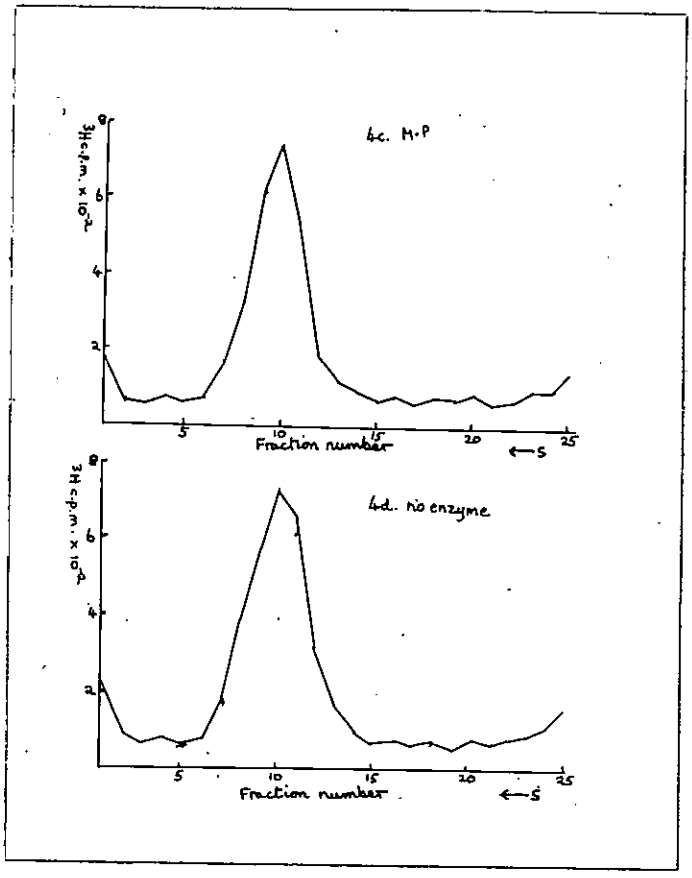
—— ^3H, λ.C DNA

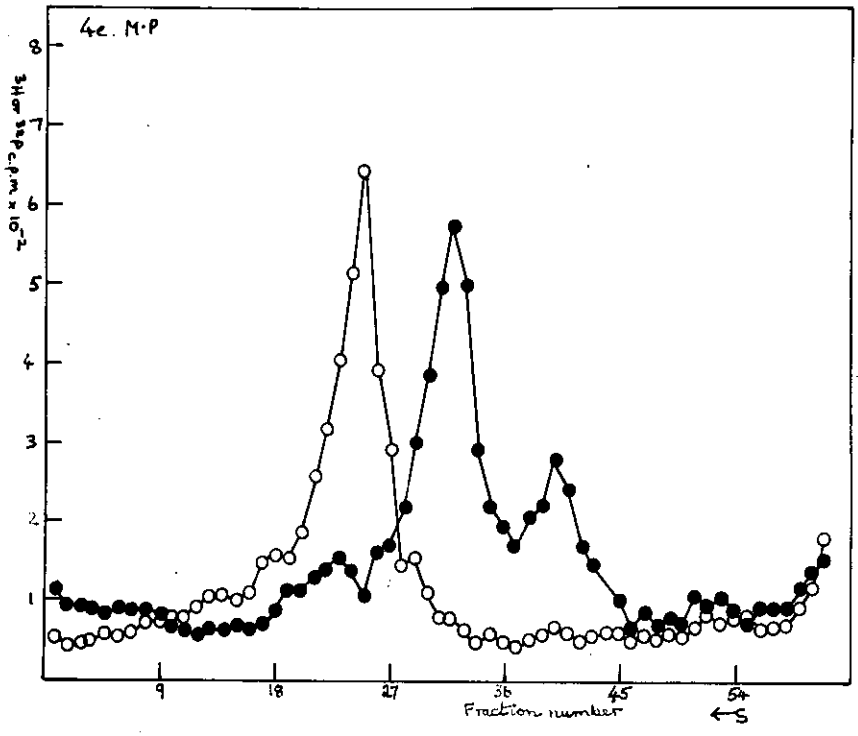
2. M13 twisted circular DNA as substrate. Reactions (vol 0.1ml) contained 5 μmoles potassium morpholinoethanesulphonate pH 6.0, 0.025 μmoles EDTA, 0.5 moles 2-mercaptoethanol, 3.5×10^3 c.p.m. of ^3H-labelled M13 RFI DNA (720 c.p.m./μg), 3.5×10^3 c.p.m. of ^{32}P-labelled $\lambda c_I 857 S_7$.K(P1) DNA and 4 μg of M.P(Fig.4e) or no enzyme (Fig. 4f). After incubating for 3 h at 30°C, the reaction mixtures were layered on 4 ml of 6-20% (w/v) sucrose gradients in 0.01M-tris-HCl pH 7.4, 1mM-EDTA, 0.04% (w/v) sodium dodecyl sulphate and centrifuged (MSE Superspeed 75) at 40,000 r.p.m. 20°C, 2.5 h in a 3 x 5 ml swing-out rotor. The gradients were collected in 4 drop fractions

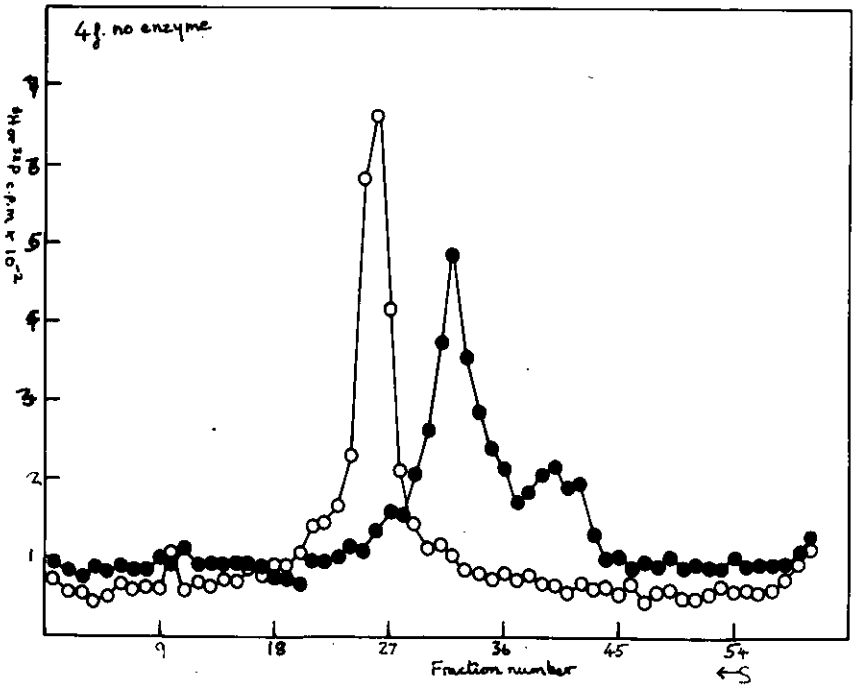
○—○ ^{32}P, λ.K(P1) DNA

●—● ^3H, M13 DNA


4a. M·P
^{3}H c.p.m. x 10^{-2}
2
4
6
8
5
10
15
20
25
Fraction number
←S
4b. no enzyme
^{3}H c.p.m. x 10^{-2}
2
4
6
5
10
15
20
25
Fraction number
←S


4c. M·P
^{3}H c.p.m. x 10^{-2}
2
4
6
8
5
10
15
20
25
Fraction number
←S
4d. no enzyme
^{3}H c.p.m. x 10^{-2}
2
4
6
8
5
10
15
20
25
Fraction number
←S


4e. M·P
^{3}H acid ppt. c.p.m. x 10^{-2}
8
7
6
5
4
3
2
1
9
18
27
36
45
54
Fraction number
←S


4f. no enzyme
^{3}H acid ppt. c.p.m. x 10^{-2}
8
7
6
5
4
3
2
1
9
18
27
36
45
54
Fraction number
←S

(b) Requirements for the activity

The requirements were investigated as shown in Table 5. The enzyme is specific for native, unmodified DNA and is stimulated 1.5 fold by 5mM-Mg^{2+}, a property also noted by Kühnlein et al.(1969)with the E.coli strain B modification activity. A threefold inhibition by 0.1mM-ATP was also observed.

(c) pH-dependence of methylation

The pH-dependence of the activity in potassium morpholinoethanesulphonate and potassium piperazine-NN'-bis-2 ethanesulphonate buffers is shown in Fig.5. In 0.05M-potassium morpholinoethanesulphonate the pH optimum is between 6.0 and 6.25, although significant methylation was observed from pH 5.5 to pH 8.0. No significant methylation of P1 modified DNA was observed over this pH range.

(d) Product of methylation

A sample of methylated DNA was analysed by enzymic digestion to mononucleotides and high-voltage electrophoresis on aminoethylcellulose paper at pH 3.5 (see Methods section 3c(i)). A single peak of radioactivity (Fig. 6a) migrated with dAMP, indicating that a single mononucleotide had been methylated. Another sample of methylated DNA was analysed by acid hydrolysis and paper chromatography (see Methods section 3b). The radioactivity migrated on two solvent systems with 6-methylaminopurine (Fig. 6b and 6c). 6-methylaminopurine is thus the sole detectable product of methylation.

Table 5 Requirements for methylation

The complete system (vol 0.1 ml) contained 5 μ moles potassium morpholinoethanesulphonate pH 6.0, 0.025 μ moles EDTA, 0.5 μ moles 2-mercaptoethanol, 0.4 n moles CT_3-SAM (8.9 μ Ci/n mole), 6 μ g of bacteriophage 82.C600 (Pl--) DNA and 2 μ g of M.P. After incubation for 45 min at 30°C the mixtures were assayed for acid precipitable ^3H-radioactivity. Bacteriophage 82DNA was denatured by heating at 100°C for 7 min and cooling in ice. Enzyme was heat-inactivated at 100°C for 5 min in a stoppered tube.

	Acid-insoluble ^3H radioactivity (c.p.m.)
Complete system	2333
Minus DNA	134
Substitute bacteriophage 82.C600 (Pl-+)DNA	147
Substitute heat-denatured DNA	185
Minus enzyme	78
Substitute heat-inactivated enzyme	113
Plus 5mM-$MgCl_2$	3392
Plus 0.1mM-ATP	713
Plus 5mM-$MgCl_2$ and 0.1mM-ATP	2984

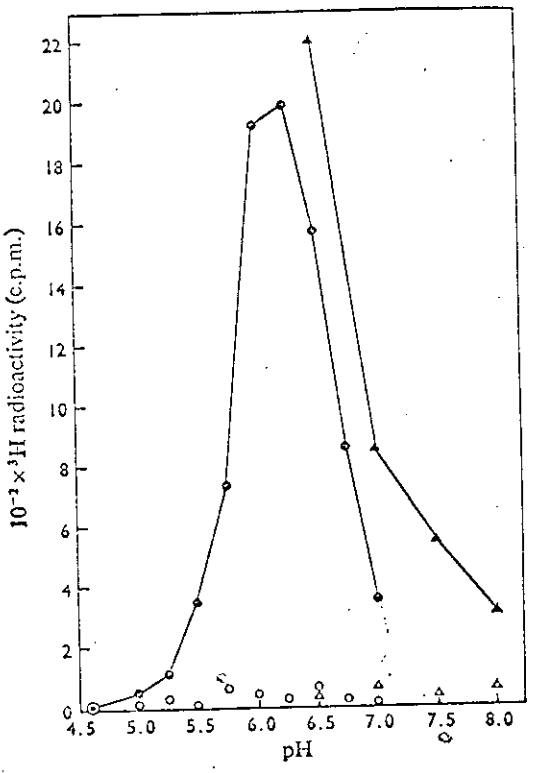


Fig. 5 pH-dependence of methylation in potassium morpholinoethanesulphonate (pKa = 6.15) and potassium piperazine-NN'-bis-2-ethanesulphonate (pKa = 6.8) buffers.

The complete system was made up as for Table 5 in 0.05M-potassium morpholinoethanesulphonate or 0.05M-potassium piperazine-NN'-bis-2-ethanesulphonate buffers of the appropriate pH value, which was measured at 27°C on a Vibron model 39a pH-meter with manual temperature compensation and standardized against air-free phthalate buffer, pH7.0. Control experiments with larger volumes of the reaction components indicated that the initial pH of the buffer was altered less than 0.01 pH units by the addition of the other reactants. The reaction mixtures were incubated for 40 min at 30°C and assayed for acid insoluble ^3H-radioactivity. Bacteriophage 82.C600(Pl--) DNA: ●, potassium morpholinoethanesulphonate; ▲,potassium piperazine-NN'-bis-2-ethanesulphonate. Bacteriophage 82.C600 (Pl-+) DNA: o, potassium morpholinoethanesulphonate; Δ, potassium piperazine-NN'-bis-2-ethanesulphonate.

Fig.6. Analysis of the product of DNA methylation

(a) Mononucleotide analysis of methylated bacteriophage 82 DNA on aminoethylcellulose paper (see Methods section 3c(i)). 'Blue' = Xylene Cyanol FF marker dye.

(b) and (c) Base analysis of bacteriophage 82 DNA by paper chromatography (see Methods section 3b) on butan-1-ol-water (NH_3 atmosphere) then methanol-water-HCl. The material remaining at the origin (indicated by an arrow) after the butan-1-ol-water system was eluted with 0.1M-HCl and hydrolysed with 1M-HCl at 100°C for 2 h. On re-chromatography more than 90% of the radioactivity remained at the origin. This material was also found in the hydrolysate of a reaction from which the DNA was omitted and hence is not a product of DNA methylation. Methylated DNA for both analyses was obtained by incubating 4 µg of bacteriophage 82 DNA for 4 h at 30°C in the complete system described in Table 5. 7 MeG, N-7-methylguanine; Ad, adenine; 5 MeC, 5-methylcytosine; 6 MAP, 6-methylaminopurine.

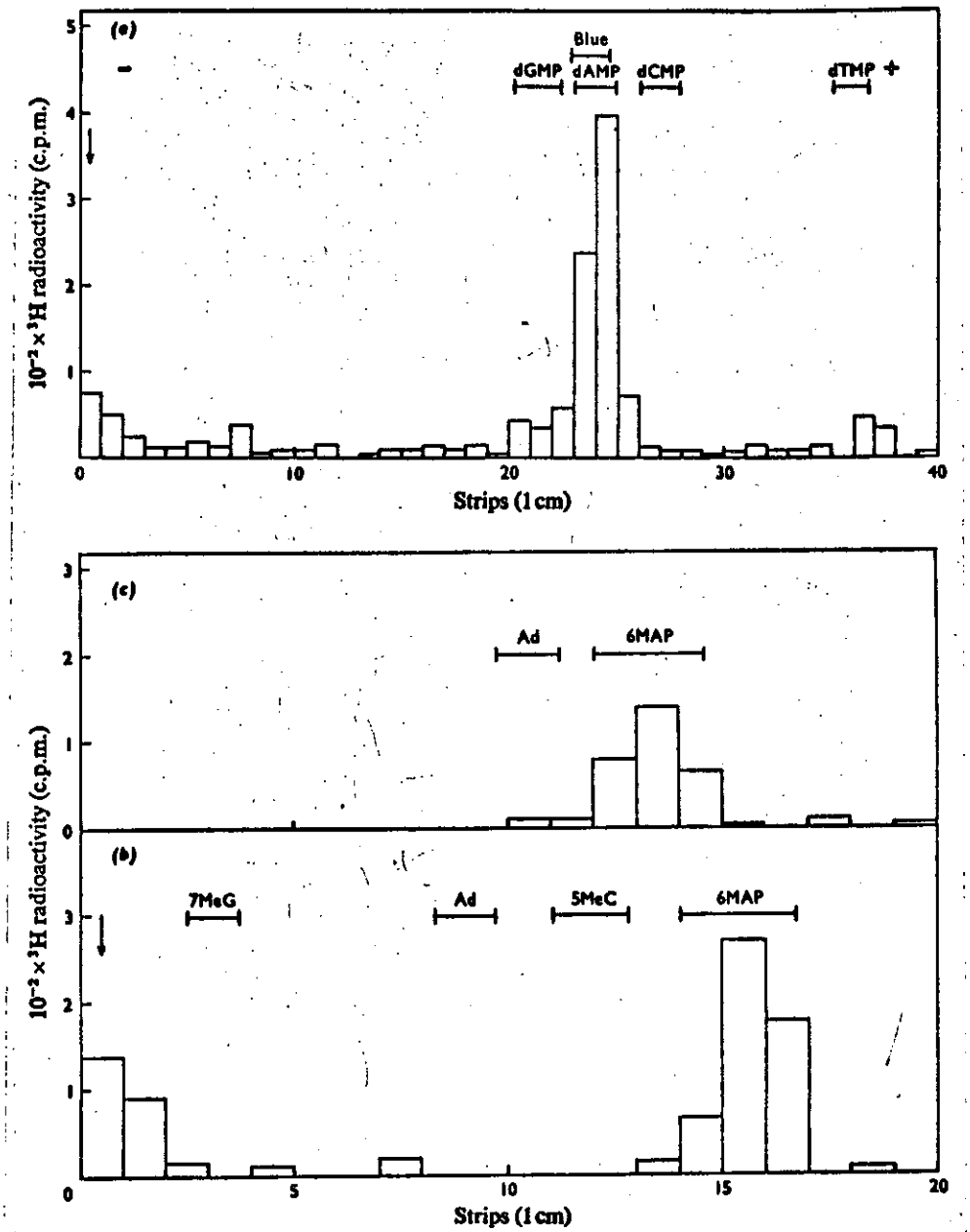


Fig. 6. *Analysis of the product of DNA methylation*

(e) Time-course of methylation

The time-course of methylation of unmodified bacteriophage 82 DNA was investigated by incubating a series of duplicate reaction mixtures for various times and then assaying for acid-insoluble ^{3}H-radioactivity. As shown in Fig.7, incorporation of methyl groups was complete after 3 h of incubation at 30°C. The extent of methylation was not limited by inactivation of the enzyme, since the addition of more enzyme after 5 h was without effect, whereas the addition of more DNA produced a detectable stimulation. The number of methyl groups incorporated at the plateau was about 20-24/DNA molecule (containing 10^5 bases), although this number is difficult to estimate accurately because of differences in recovery and quenching during the acid precipitation procedure.

(f) Dependence of methylation on SAM.

The SAM dependence was investigated as described in the legend to Fig.8. Analysis of the results by a double-reciprocal plot (Lineweaver & Burk, 1934), gave an apparent K_M for SAM of 5 µM (Fig.8). The value obtained by Kuhnlein and Arber (1972) for the E.coli B modification enzyme was 4 µM.

(g) Modification and restriction of bacteriophage λ DNA in vitro.

The modifying action of the purified M.P can be demonstrated by incubating methylated DNA with purified P1 restriction enzyme. Unlabelled λ.C DNA was methylated with the enzyme and CT_3-SAM in the presence of ^{32}P-labelled λ.K (P1) DNA. After the

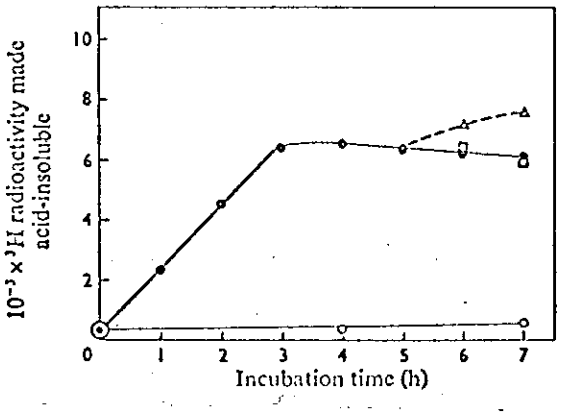


Fig.7. Time-course of methylation

The reaction mixtures had the same composition as the complete system in Table 5, except that 4 µg of bacteriophage 82.C600(Pl--) DNA (●) or bacteriophage 82.C600 (Pl-+) DNA (o) was used. Reaction mixtures were incubated at 30°C for the times indicated and then assayed for acid-insoluble ^3H-radioactivity. To one set of reaction mixtures (Δ) 3 µg of bacteriophage 82.C600(Pl--) DNA was added after 5 h; to another set (□) a further 25 µl of enzyme was added. All tubes had a blank value of 129 c.p.m. Subtracted for an incubation without enzyme assayed at 0 min. No attempt has been made to correct for any fluctuation of this blank with time of incubation.

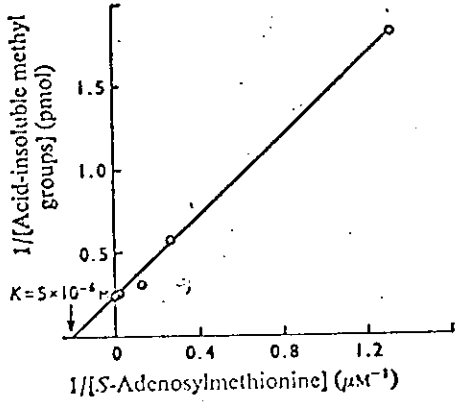


Fig.8. SAM dependence of methylation

Reaction mixtures (vol 0.15 ml) contained 7.5 µmoles potassium morpholinoethanesulphonate pH 6.0, 0.03 µmoles EDTA, 0.75 µmoles 2-mercaptoethanol, 7.5 µg of bacteriophage 82.C600 (Pl--) DNA, 4 µg of M.P and CT_3-SAM adjusted with the required amount of unlabelled SAM to give the concentrations shown in the figure. After 30 min incubation at 30°c the reaction mixtures were assayed for acid-insoluble methyl groups. Each result was corrected for a blank incorporation performed in the absence of enzyme.

incubation the DNA species were purified by phenol extraction. After removal of the phenol and SAM by dialysis, a sample of DNA was analysed by acid hydrolysis followed by thin layer chromatography according to Razin et al.(1970). As shown in Table 6, 85% of the ^{3}H was found in 6-methylaminopurine. The remainder of the ^{3}H-methylated DNA was divided into three equal portions, which were incubated with endonuclease R.P (Fig.9a), or endonuclease R.K (Fig 9b) or without enzyme (Fig.9c). The activity of the endonuclease R.P was checked by incubating it with an equivalent amount of ^{3}H-labelled λ.C DNA and ^{32}P-labelled λ.K(P1) DNA that had been subjected to the same protocol of incubation and phenol extraction, except that SAM was omitted (Fig.9d). All reaction mixtures were analysed by neutral sucrose-gradient centrifugation in buckets on the same rotor (see Fig.9 and legend).

The near-superposability of the ^{3}H and ^{32}P distributions in Fig.9 (a) and 9 (c) shows that the ^{3}H-methylated DNA was not significantly degraded by incubation with endonuclease R.P. The ^{3}H-methylated DNA was, however, broken by the heterospecific endonuclease R.K (Fig. 9b), and, in the control incubation mixture (Fig. 9d) the ^{3}H-labelled λ.C DNA was also broken, showing that the endonuclease R.P was active. The nearly identical distribution of ^{32}P in all four gradients shows that all breakage was specific for unmodified DNA. Upon repetition of the entire experiment, these features of the various distributions were found to recur.

This experiment demonstrates the role of DNA

Table 6. Base analysis of ^{3}H-methylated λ DNA.

10 µl of reaction mixture 1 (see Fig.9) were analysed by acid hydrolysis followed by t.l.c. (see Methods 3b). The ^{3}H-radioactivity in various areas of the chromatogram was determined by the elution and counting procedure of Razin et al (1970). The radioactivity applied to the chromatogram was derived by applying 10 µl of reaction mixture 1 DNA together with 10 µl of 25 mM each 5-methylcytosine and 6-methylaminopurine to a sheet of the thin layer cellulose. The radioactivity in this spot was also determined by the above procedure. A background of 124 c.p.4m. has been subtracted from all results. This was derived by eluting and counting a blank area of the chromatogram.

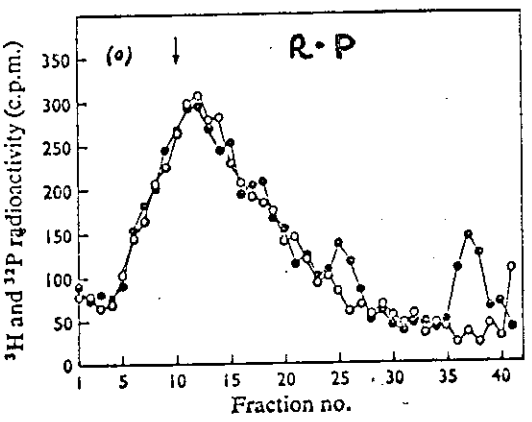
	c.p.4m.
Total radioactivity applied	1561
origin region	288
5-methylcytosine region	12
6-methylaminopurine region	1356

% Recovery of radioactivity = 105

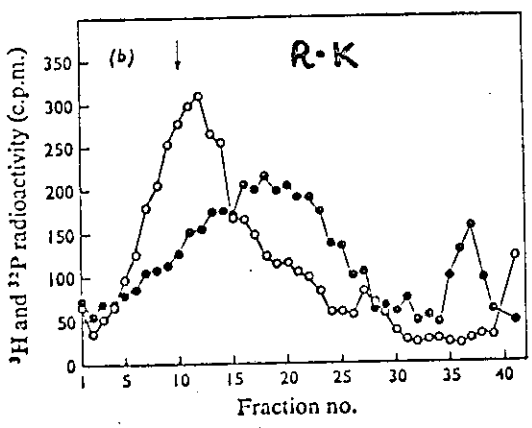
% radioactivity in 6-methylaminopurine = 85

Fig.9 Zone sedimentation in neutral sucrose gradients of the products of restriction endonuclease reactions with modified λ DNA

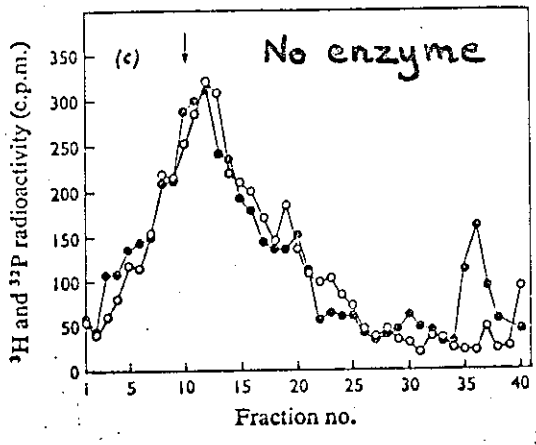
Methylation reactions were carried out in small 'Dunham' tubes to facilitate phenol extraction. Reaction mixture 1 (vol 0.35 ml) contained 17.5 μmoles potassium morpholinoethanesulphonate pH 6.0, 0.087 μmoles EDTA, 1.75 μmoles 2-mercaptoethanol, 1.4 nmoles CT_3-SAM (8.9 μCi/nmole), 20 μg of $\lambda c_I 857 S_7$.C DNA, 2 x 10^4 c.p.m. of ^{32}P-labelled $\lambda c_I 857 S_7$.K (P1) DNA and 18 μg of M.P. Reaction mixture 2 (vol 0.115 ml) contained 5.5 μmoles potassium morpholinoethanesulphonate pH 6.0, 0.029 μmoles EDTA, 0.55 μmoles 2-mercaptoethanol, 6 μg of $\lambda c_I 857 S_7$.C DNA, 5.5 x 10^3 c.p.m. of ^{3}H-labelled $\lambda c_I 857 S_7$.C DNA (1 μg), 6 x 10^3 c.p.m. of ^{32}P-labelled $\lambda c_I 857 S_7$.K (P1) DNA and 6 μg of M.P. After incubation at 30°C for 3.0 h, both reactions were extracted three times with an equal volume of freshly distilled phenol. The aqueous layers were dialysed twice against 1 litre of 10mM-tris-HCl-0.1mM-EDTA-0.4M-NaCl pH7.4, then twice against 1 litre of the same buffer without NaCl. Both samples were heated at 60°C for 10 min to dissociate concatenates.



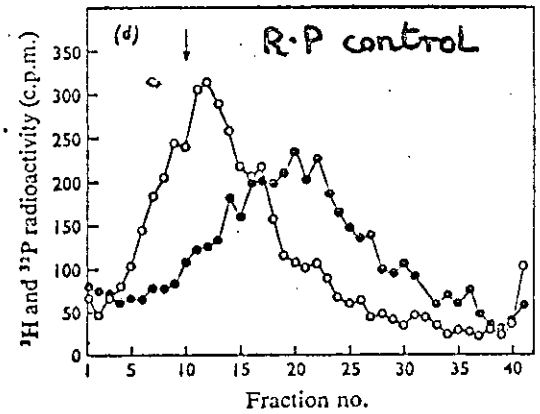
Restriction reactions (a) Reaction mixture (vol 0.36 ml) contained 36 μmoles potassium-N-tris(-hydroxymethyl)methyl-2-aminoethanesulphonate pH 8.0, 0.43 μmoles EDTA, 2.88 μmoles $MgCl_2$, 2.16 μmoles 2-mercaptoethanol, 0.9μmoles ATP, 3.6 nmoles SAM, 0.15 ml of DNA from reaction mixture 1 (5.5 x 10^3 c.p.m. of ^{3}H and 6.3 x 10^3 c.p.m. of ^{32}P), and 0.15 ml of endonuclease R.P.



(b) Reaction mixture (vol. 0.36 ml) contained DNA, potassium-N-tris(hydroxymethyl)-methyl-2-aminoethanesulphonate, EDTA, $MgCl_2$, 2-mercaptoethanol and ATP as for (a) but 7.2 n moles SAM, and 0.1 ml of endonuclease R.K instead of endonuclease R.P.



(c) As (a) except that water (0.15 ml) replaced endonuclease R.P.



(d) as (a) except that 0.15 ml of DNA reaction mixture 2 (5.2×10^3 c.p.m. of ^{3}H and 6×10^3 c.p.m. of ^{32}P) replaced DNA from reaction mixture 1.

The restriction reactions were incubated at 30°C for 20 min, terminated with 10 µl of 0.5 M EDTA pH 8.0, and layered on 6-20% ($\frac{W}{V}$) sucrose gradients in 0.01 M-tris-HCl-1mM-EDTA-0.04% ($\frac{W}{V}$) sodium dodecylsulphate pH 7.4 (vol 4 ml). After centrifugation (MSE Superspeed 65) in a 6 x 5 ml swing-out rotor at 50,000 r.p.m. for 130 min at 20°C,5 drop fractions were collected into vials and counted in a Beckman liquid scintillation spectrometer after the addition of 1.5 ml of scintillant (30 g of napthalene, 2 g of 2,5-diphenyloxazole, 0.1g of dimethyl 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 50 ml of methanol and 500 ml of p-dioxan). The arrows in (a) - (d) indicate the position of sedimentation of whole molecule ^{32}P-labelled λ.K (P1) DNA and ^{3}H-labelled λ.C DNA that were run in the fifth bucket of the rotor. The small peak of ^{3}H at the top of the gradient in (a), (b) and (c) is CT_3-SAM remaining after dialysis. Sedimentation was from right to left.

●, ^{3}H; o, ^{32}P.

methylation in protection against the P1 restriction enzyme. The extensive phenol extraction (legend to Fig.9) rules out the possibility that this protection derives from binding of the modification enzyme, and thus shows that it is a property of the DNA. It proved critical to exhaustively methylate the DNA by incubating for 3 h at 30°C (Fig.7). A series of previous attempts to demonstrate the modifying activity were only partially successful because the period of incubation was shorter (data not shown).

B. The sub-unit structure of DNA methylase M.P.

(i) Purification of the M.P

In order to purify the enzyme it is very helpful to try and augment the level in the cell. Scott (1970) has described a thermoinducible mutant of P1. This phage seemed an attractive source of the enzyme since the induction of the prophage should lead to a considerable increase in the number of gene copies. As described in Table 3, the specific activity of the modification enzyme in extracts of the induced lysogen is at least four-fold higher than that in the lysogenic strain 1100 r_K-(P1), which was previously used as a source of enzyme. The following procedure was used to prepare pure enzyme. It differs in the following salient characteristics from the method described in Results A (iii).

(a) The crude lysate was treated with DNAse I and then centrifuged to remove ribosomes. This replaced the $MgCl_2$ and streptomycin precipitation steps.

(b) The $(NH_4)_2SO_4$ precipitation was 'cut' between 35% and 60% saturation.

(c) Two successive DEAE cellulose fractionations were used.

(d) The phosphocellulose column was eluted with a phosphate gradient.

(e) An additional fractionation on CM-sephadex was used.

Growth of cells: Strain K140 (P1 C_I162) was grown in 50 litres of L broth at 30°C to 10^9 cells/ml. The temperature was shifted to 42°C for 15 minutes and then

lowered to 0-5°C by adding frozen 0.05 M NaCl. The cells were harvested by sedimentation in an Alfa-Laval centrifuge and stored at -20°C.

Step 1: Preparation of crude extract. 850g of cells were resuspended in 900 ml of breaking buffer (0.05 M-tris-HCl pH8.0, 0.1 mM-EDTA, 1mM-2-mercaptoethanol, 10 mM-$MgCl_2$, 5% ($\frac{v}{v}$) glycerol). Glass beads (1.5 kg) were added and the mixture was blended (with intermittent cooling) for 5 min at low speed and 7.5 min at high speed. The supernatant was decanted and the beads re-extracted with 200 ml of breaking buffer. Pancreatic DNAse (5 mg) was added to the pooled extract which was incubated at 5-8°C for 40 min, and then centrifuged at low speed (10,000g, 20 min, 0°C), and at high speed (Superspeed 65, 6 x 250 rotor, 18,000 r.p.m., 2°C, 4 h). The supernatant (vol 1.2 litres) had 22 mgs protein/ml.

Step 2: $(NH_4)_2SO_4$ fractionation. $(NH_4)_2SO_4$ (233g) was added slowly to the supernatant which was left to precipitate at 0°C for 30 min. The precipitate was collected by centrifugation (12,000 g, 20 min, 2°C) and the supernatant precipitated again by adding $(NH_4)_2SO_4$ (183 g). The precipitate was collected by centrifugation, dissolved in 300 ml of 0.02 M-PM(potassium phosphate pH 6.7, 5 mM-2-mercaptoethanol, 5% ($\frac{v}{v}$) glycerol), and dialysed against 6 changes of 2 litres of 0.02 M-PM.

Step 3: DE23 fractionation. The 400 ml of dialysed $(NH_4)_2SO_4$ fraction was diluted to 2 litres with 0.02 M-PM and applied to a column (17 cm x 9.5 cm diameter) of DE23 cellulose

that had been equilibrated with 0.02 M-PM. The column was washed with 800 ml of 0.02 M-PM, 800 ml of 0.05 M-PM and then eluted with 0.3 M-PM. The conductivity of the effluent was equivalent to 0.3 M-PM after the passage of 940 ml. The 0.05 M-0.3 M-PM batch was concentrated by adding $(NH_4)_2SO_4$ (950 g), the precipitate was collected by centrifugation, dissolved in 90 ml of 0.02 M-PM and dialysed against 4 changes of 3 litres of 0.05 M-PM.

Step 4. DE52 chromatography. The DE23 fraction was applied to a column (14.5 cm x 6.25 cm diameter) of DE52 cellulose that had been equilibrated with 0.05M-PM. The column was washed with 600 ml of 0.05M-PM and eluted with a linear 2 litre gradient of 0.05 M-PM to 0.3 M-PM which was collected in 30 ml fractions. The activity emerged in six neighbouring fractions (mean [phosphate] = 0.12 M, total volume 190 ml, 2.1 mg protein/ml) which were concentrated by adding $(NH_4)_2SO_4$ (155g). The precipitate was collected by centrifugation, dissolved in 30 ml of 0.05 M-PEM (PM containing 0.5mM-EDTA) and dialysed against 3 changes of 1.5 litres of 0.05 M-PEM.

Step 5: P11 chromatography. The concentrated DE52 fraction was diluted to 80 ml with 0.05 M-PEM and applied at approximately 40 ml/hr to a column (7.2 cm x 3.75 cm diameter) of P11 phosphocellulose equilibrated with 0.05 M-PEM. The column was washed with 75 ml of 0.05 M-PEM and then eluted with a linear 500 ml gradient of 0.05 M-PEM to 0.3 M-PEM which was collected in 10 ml fractions. The activity emerged after passage of approximately 0.6 of the gradient. Three neighbouring fractions (31, 32 & 33) were pooled, concentrated by

ultrafiltration in a Diaflo Amicon pressure cell (PM10 membrane, 20 lb/in^2 of N_2) and 'dialysed' by concentrating on four occasions after addition of a ten fold excess of 0.02 M-PED (potassium phosphate pH 6.5 1mM-dithiothreitol, 0.5 mM-EDTA, 5% glycerol). The volume of the final concentrate was 5 ml (1.3 mg protein/ml).

Step 6: CM-Sephadex chromatography. 4 ml of the P11 fraction was applied to a column (5 cm x 1.25 cm diameter) of CM-Sephadex C25 equilibrated with 0.02 M-PED. The column was washed with 20 ml of 0.02 M-PED and eluted with 0.1 M-PED (30 ml), 0.2 M-PED (20 ml) and 0.4 M-PED (30 ml). Fractions of 5 ml were collected. The activity eluted sharply in the first two fractions of the 0.4 M-PED step. These were pooled, concentrated in the Amicon cell and 'dialysed' into 0.01 M-potassium morpholinoethanesulphonate pH 6.5, 1 mM-dithiothreitol, 0.1 mM-EDTA, 5% glycerol by successive concentration as employed for the P11 fraction. The volume of the final concentrate was 1.5 ml (0.6 mg protein/ml).

The purity of the CM-Sephadex fraction was investigated by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulphate (see Methods 2d). The anionic detergent dissociates oligomeric proteins into their component subunits which then run on the gel with a mobility that is inversely proportional to the logarithm of their molecular weight (Shapiro et al. 1967, Weber & Osborn, 1969). As shown in Figure 10, the CM-Sephadex fraction shows two prominent bands and a number of minor components. It is known from genetic studies that

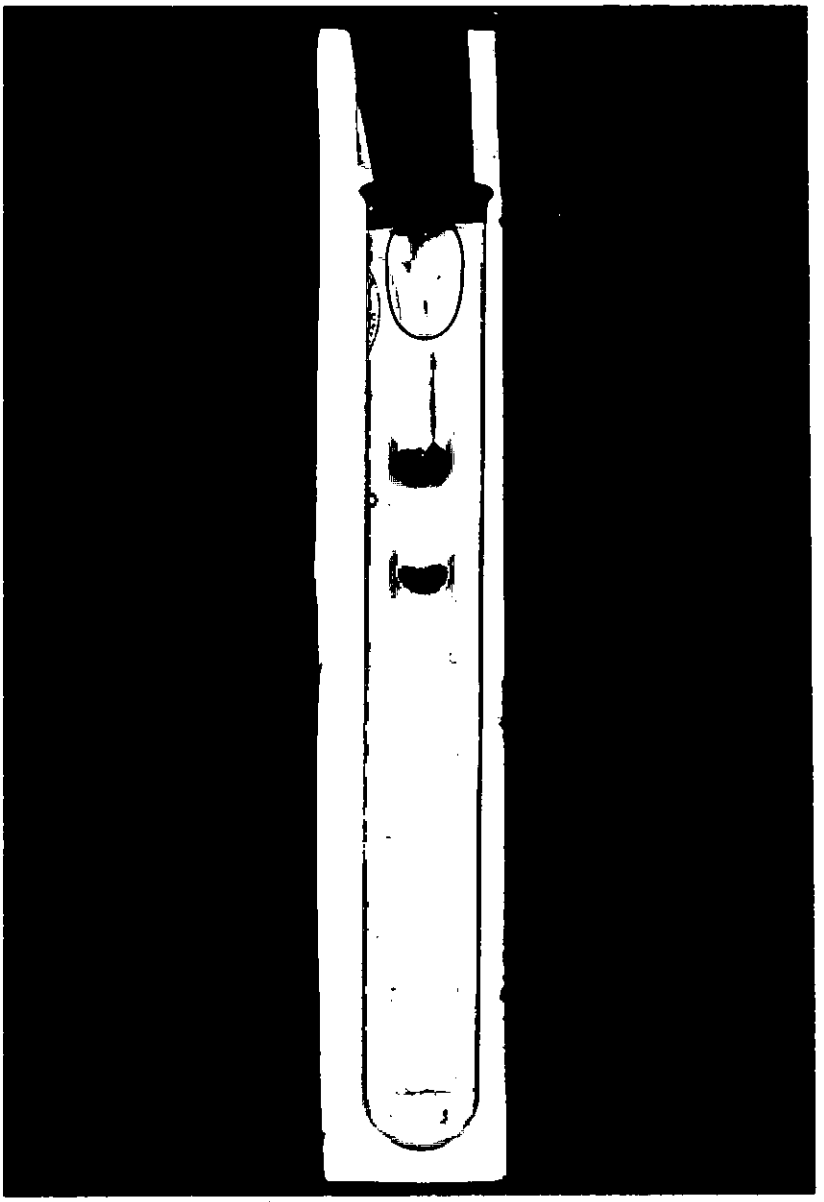


Fig. 10 Analysis of the CM-sephadex fraction by polyacrylamide gel electrophoresis in SDS.

24 µg of protein (CM-sephadex fraction) was analysed by SDS gel electrophoresis as described in Methods 2(d).

the m_{p1}^- phenotype can result from mutations in two distinct genes (Glover et al. 1963). Do these two bands represent the subunits of the M.P? The following experiment was performed to answer this question. An aliquot of the CM-Sephadex fraction was sedimented through a long glycerol gradient that was calibrated by reference to marker proteins run in a sister bucket of the some rotor. The fractions of the gradient were assayed for M.P activity. A single peak of activity (Fig.11) was found at a position corresponding to 6S. The active fractions were concentrated by TCA precipitation and analysed by acrylamide gel electrophoresis in SDS. As shown in Fig.11, the intensity of the two bands increased and decreased co-ordinately across the peak of activity.

Although close inspection of the stained gels revealed trace contaminating bands in some of them, none of the trace bands exhibited such a change in intensity across the peak of enzyme activity.

On the basis of the association of these two bands through the purification procedure and in the sedimentation analysis, it was concluded that they are the components of the 6S M.P enzyme. As with all such purifications, however, minor contaminants that might not be detectable could concievably be responsible for the activity.

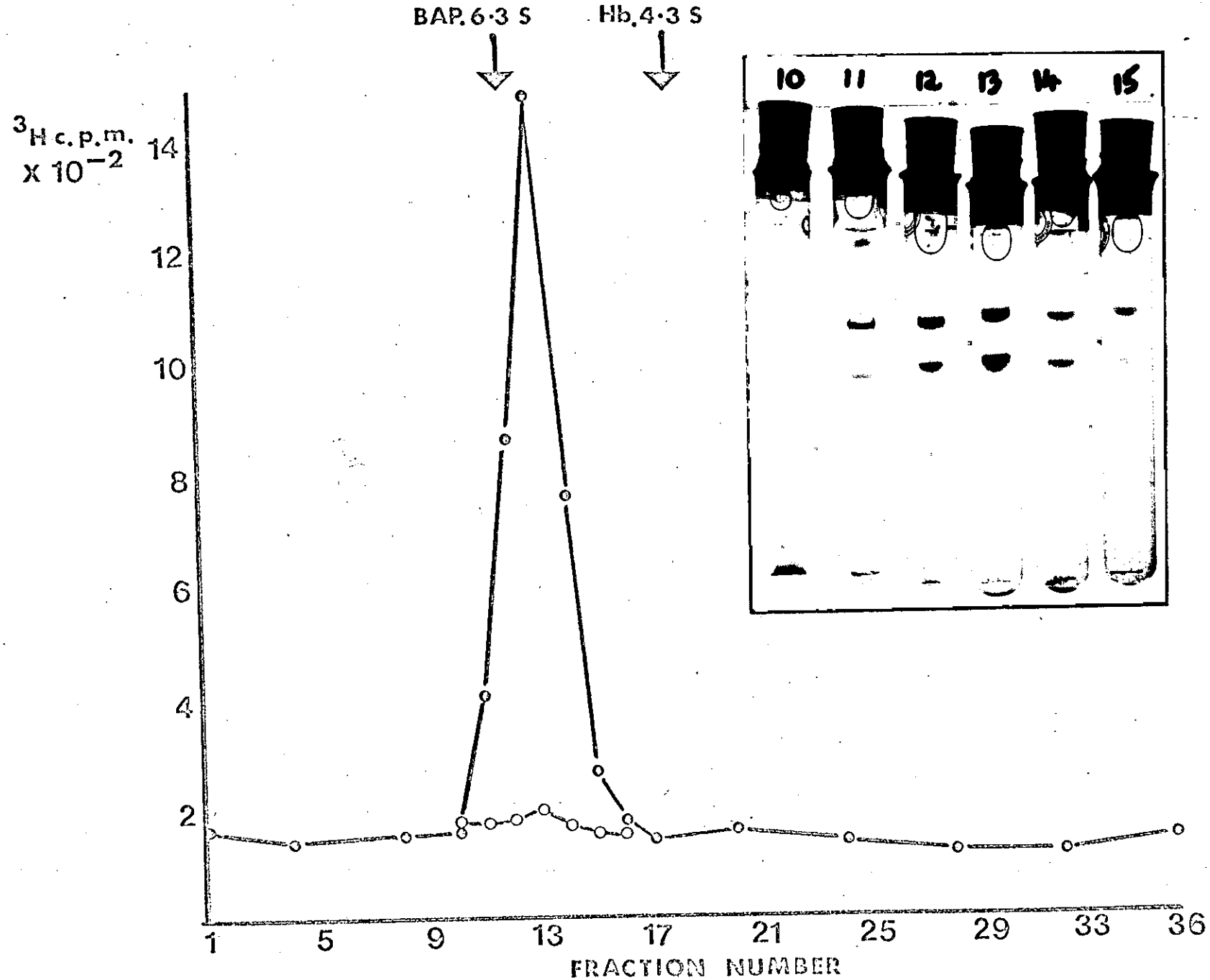
(ii) Molecular weights and mole fractions of the two components

The molecular weights of the two components were estimated by determining their mobilities in SDS gels, relative

Fig. 11. Analysis of the M.P by glycerol-gradient sedimentation and SDS gel electrophoresis.

A 0.5 ml portion (0.3 mg protein) of the CM-sephadex fraction was layered on a 12-25% ($\frac{v}{v}$) glycerol gradient (vol 13.5 ml) in 0.01 M-potassium morpholineethanesulphonate pH 6.5, 1mM-dithiothreitol, 0.5 mM-EDTA. After centrifugation (S875, 6 x 15 ml SW rotor, 70 h, 24,000 r.p.m. at approx. 2°C), the gradient was collected in 15 drop fractions. Aliquots (0.02 ml) were assayed for methylation of $\lambda C_I 857.K$ DNA (●) and $\lambda C_I 857\ S_7.K(P1)$ DNA (○). The remainder of fractions 10-15 were precipitated by adding 10% ($\frac{w}{v}$) TCA (0.4 ml). After 15 min at 0°C, the precipitate was collected by centrifugation ('Quickfit' microcentrifuge, 20,000 g, 5 min) and dissolved in 0.05 ml of 0.01 M-sodium phosphate pH 7.0, 1%($\frac{w}{v}$)-SDS, 1%($\frac{w}{v}$)-2-mercaptoethanol, prior to SDS gel electrophoresis (Methods 2(d)).

A parallel gradient was layered with 0.5 ml of a solution containing bovine haemoglobin (5 mg) and bacterial alkaline phosphatase (0.4 mg). After sedimentation and fractionation, the haemoglobin was located by its extinction at 410 nm, and the phosphatase by following (at 410 nm) the hydrolysis of 1 mM p-nitrophenylphosphate in 1M-tris-Hcl pH 8.0.


BAP. 6·3 S
Hb. 4·3 S
^{3}H c.p.m. X 10^{-2}
14
12
10
8
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25
29
33
36
FRACTION NUMBER
10
11
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13
14
15

to eight authentic protein standards. The accuracy of this method is said to be within 10% (Weber & Osborn, 1969). In the experiment described in Fig.12, the mobilities of the two components indicated molecular weights of 68,000 and 43,000. Four independent determinations gave the following values for the larger component - 68,000, 71,000, 69,000, 73,000 and gave the following values for the smaller component - 43,000, 44,000, 48,000, 45,000. In each of these determinations, the marker proteins clustered tightly around a straight line in the log plot of Fig.12. It is concluded that the molecular weight of the larger component is 70,000 $\pm$ 5,000, and of the smaller component is 45,000 $\pm$ 5,000.

The following method was used to estimate the mole fractions of the two components. The stained SDS gels of the CM-sephadex and various glycerol-gradient fractions were scanned in a densitometer. The areas under the two peaks were determined by xeroxing the densitometer traces, cutting out the peaks with a razor blade and weighing the paper to the nearest 0.1 mg on aMettler balance. The investigations of Fazekas de St. Groth et al (1963) indicated that the amount of Coomassie brilliant blue bound to various proteins differs by less than 10% on a weight for weight basis. Thus if the two components are present in equimolar amounts, the amount of Coomassie blue that is bound, and hence the areas under the densitometer peaks, should be in the ratio 7 : 4.5 that is 1.55. As shown in Table 7, the ratios observed are in excellent agreement with this and in distinct disagreement with other possibilities. The

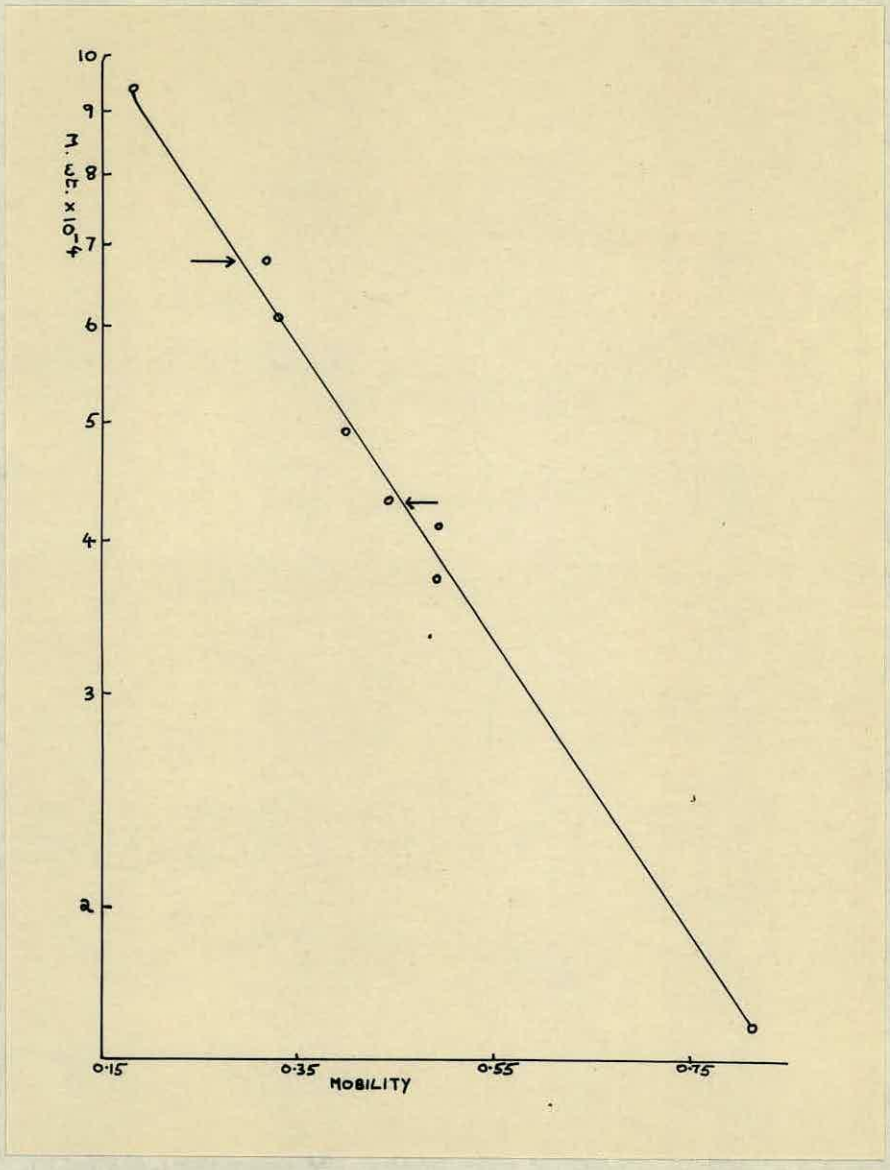

Fig. 12 Plot of the mobility of various proteins on 8% polyacrylamide gels (run in 0.1% SDS) against the logarithm of their molecular weight.

The mobilities of the two components, and of various marker proteins, with respect to bromophenol blue ~~was~~ were determined as described by Weber & Osborn (1969) (see also Methods 2(d)). The two components (applied as 12 µg of CM-sephadex fraction) were run on one gel and the markers (approx 10 µg of each) were run in two groups of four on parallel gels. The markers were phosphorylase a (94,000), bovine serum albumin (68,000), L-amino acid oxidase (61,000), fumarase (49,000), ovalbumin (43,000), liver alcohol dehydrogenase (41,000), yeast alcohol dehydrogenase (37,000) and myoglobin (17,200).

TABLE 7 Estimation of mole fraction of the two components in various fractions

The mole fraction of the two components in various fractions was estimated by densitometry and weighing (see text).

Fraction		Weight of peak 1 (g)	Weight of peak 2 (g)	Weight ratio $\frac{\text{peak 1}}{\text{peak 2}}$
CM-sephadex		0.0613	0.0414	1.48
Glycerol-gradient	No.11 (see Fig.11)	0.0238	0.0142	1.68
	No.12	0.0453	0.0286	1.58
	No.13	0.0592	0.0417	1.42
	No.14	0.0317	0.0219	1.45
	No.15	0.0107	0.0072	1.49

Average weight ratio for 6 gels = 1.52

Expected weight ratio for 1 heavy:1 light molar ratio* = 1.55
Expected weight ratio for 1 heavy:2 light molar ratio* = 1.29
Expected weight ratio for 2 heavy:1 light molar ratio* = 3.1

*Assuming molecular weights 70,000 and 45,000 (see Text)

TABLE 8 Summary of enzyme purification

The unit of activity was defined in Table 3.

Purification Stage	Protein (mgs)	Activity (units)	Specific activity (units/mg)	Recovery (%)
High speed supernatant	26,400			
$(NH_4)_2 SO_4$ cut	17,800	106,800	6.0	100
DE23 batch	5,640	98,352	17.4	92
Pooled DE52 fraction	400	48,481	121.2	45
Pooled P11 fraction	6.5	17,563	2702	16
Pooled CM-Sephadex fraction	0.9	7,831	8702	7

association of the two components in a molecule that sediments at 6S (Fig.11) precludes the possibility of anything other than a 1 : 1 dimer, assuming that the conformation is not grossly abnormal.

Thus it is concluded from this analysis that the 6S species of the M.P is a dimer of ( ) distinct subunits.

C. Analysis of the nucleotide sequences around the methylated base.

What is the nucleotide sequence or sequences that the M.P recognises? In order to answer this question, an attempt has been made to analyse the sequences around the methylated base.

Initially, a double labelling method was investigated. Phage λ or 82 DNA was methylated with M.P and CT_3-SAM. After purification by phenol extraction and dialysis, the DNA was digested with pancreatic deoxyribonuclease, treated with phosphatase to remove the 5' terminal phosphate groups, and re-phosphorylated with polynucleotide kinase (Richardson, 1965) and γ^{32}P-labelled ATP. The oligonucleotides were then fractionated by two-dimensional ionophoresis and located by autoradiography. The spots were cut out and counted for ^{32}P and ^{3}H. Pancreatic DNAse lacks base specificity (Murray, 1970) and should give a population of ^{3}H-labelled nucleotides around the methylated base. Several experiments by Dr P.R.Brown, and latterly by the author have failed to detect significant ^{3}H in the ^{32}P-labelled oligonucleotides. In two experiments by the author, the amount of ^{3}H initially present in the digest was enough to have been detected even if it had entered every ^{32}P-labelled oligonucleotide. The two dimensional systems used in these experiments were (i) AE paper pH 3.5, DE paper pH2 (ii) DE pH9, DE pH2 (iii) cellulose acetate pH 3.5, polyethyleneimine impregnated thin layer cellulose t.l.c. (Southern & Mitchell,1971).

Two explanations have been considered for the failure of these experiments.

(i) After digestion, the abundance of the methylated oligonucleotides is very low relative to their unmethylated counterparts. Therefore the success of the method depends on the ionophoretic mobility of the ^{32}P-labelled methylated and unmethylated oligonucleotides being identical. If the presence of the methyl group alters the mobility then the ^{3}H will not be associated with the detectable ^{32}P-labelled unmethylated sequences.

(ii) The ^{3}H is lost by an exchange process occurring on the ionophoretic systems at the pH values employed.

The author has been reluctant to spend more time investigating these possibilities. It is clear, however, that the method is not promising.

An alternative approach was to methylate unlabelled λ DNA with ^{14}C-SAM and M.P. The ^{14}C-labelled product was digested with DNAse and 'fingerprinted' by two dimensional ionophoresis followed by autoradiography (Fig.13). As shown in Fig.13 this procedure was successful in producing a number of oligonucleotides. An attempt has been made to sequence these nucleotides by Holley's method of partial exonucleolytic digestion. For example, a partial digest of the 5'-tetranucleotide AGCT with snake venom phosphodiesterase would contain AGCT, AGC, AG and the four mononucleotides. If the overlapping nucleotides are resolved by ionophoresis on AE paper (pH 3.5) or DE paper (pH2) then the mobility difference between successive nucleotides depends on the mononucleotide that has been removed. This method is discussed in more detail by Murray (1970).

Fig.13. Fingerprint of ^{14}C-modified DNA

The reaction (vol 3.05 ml) containing 50 µmoles potassium morpholinoethanesulphonate pH 6.0, 0.25 µmoles EDTA, 5 µmoles 2-mercaptoethanol, 0.042 µmoles ^{14}C-SAM (55 µCi/µmole), 0.5 mg $\lambda C_I 857 S_7$.K DNA and 20 µg of M.P (glycerol gradient fraction, see Results A(iii)), was incubated at 30°C for 4 h. The reaction was extracted twice by rolling with distilled phenol (equilibrated with 0.5 M-Tris-HCl pH 8.0) and the aqueous layer was dialysed three times versus 2L of 0.01 M-Tris-HCl pH 7.4, 0.5M-NaCl, 0.001M-EDTA, then versus 2L of this buffer without NaCl, and finally versus two changes of 2L of 0.01M-sodium acetate pH 5.0, 0.5mM-$MgCl_2$. The dialysate was evaporated to 0.5 ml and pancreatic DNAse (1 mg) was added. After incubation (37°C, 18 h) the DNA was desalted by adsorption to DE-cellulose (see Methods 3(d)) and applied to AE 81 paper. After electrophoresis (pH 3.5, 50 v/cm) until the xylene cyanol FF blue marker dye had reached the bottom layer of buffer (35 cms), the AE paper was dried and stitched onto a sheet of DE 81 paper which was electrophoresed (pH2, 15 v/cm) until the blue dye had moved 25 cm. The fingerprint was exposed to X-ray film for 4 weeks. Total incorporation of radioactivity was 9.84×10^3 c.p.m., approximately 15 methyl groups per input λ DNA molecule.

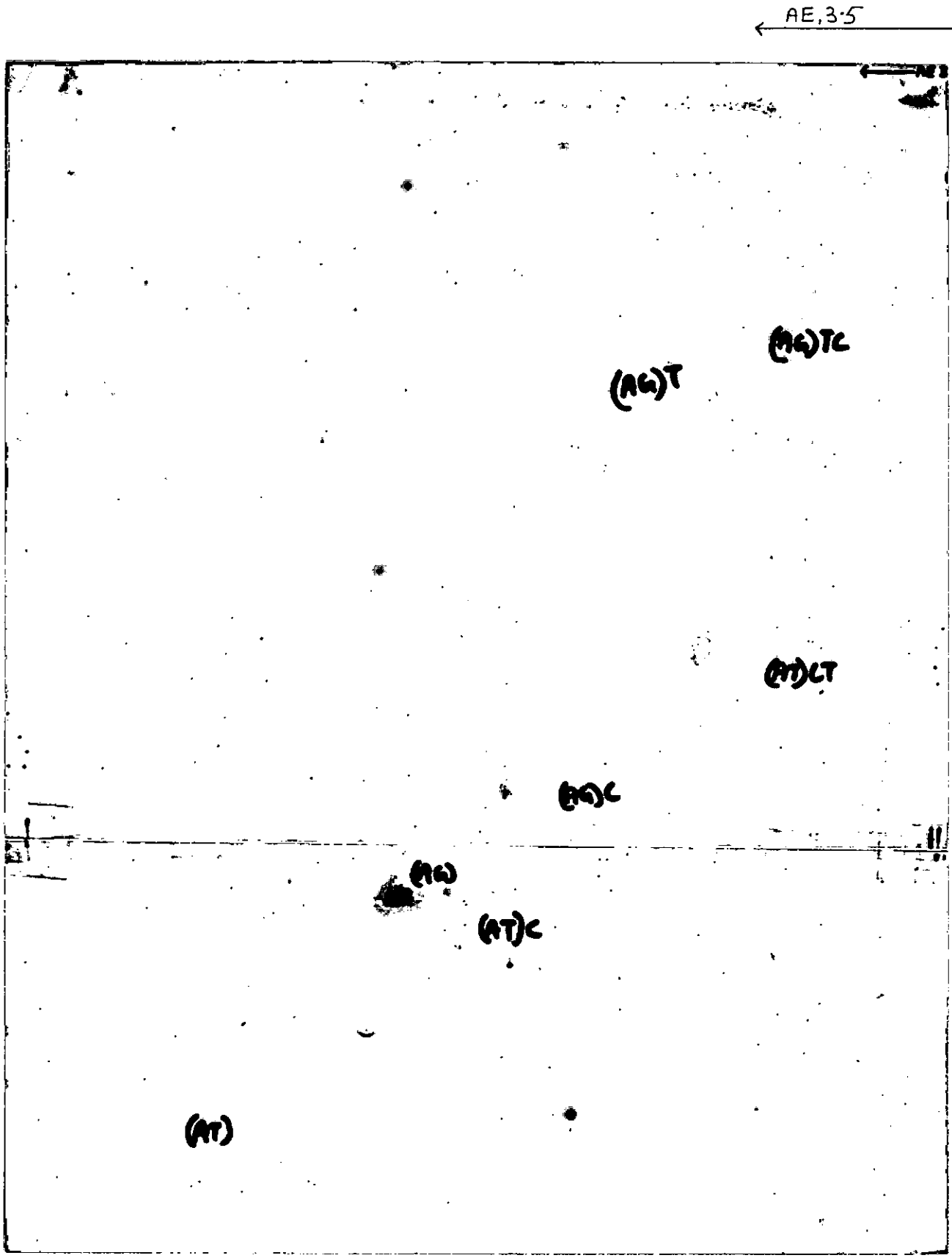

FIG. 13

The analysis of the ^{14}C-methylated nucleotides by this method is difficult for three reasons.

(i) The ^{14}C-radioactivity in the original nucleotide is already low and this is split up into several components by the analysis. Thus long exposures are required to visualise the products of partial digestion.

(ii) Some of the spots from the fingerprint are mixtures of nucleotides and thus after partial digestion they produce a series of products that are difficult to interpret.

(iii) The only label in the nucleotide is the ^{14}C-methylated A residue. Thus when the venom exonuclease has removed this residue, no further products will be seen. For example an oligonucleotide with 3'-methyl A will only give the original compound and methyl A.

These three difficulties have hampered the sequence analysis of the ^{14}C nucleotides. It has been possible, however, to obtain some sequence information and this is shown in Table 9. The sequences are derivatives of (AG) and (AT). The (AG) and (AT) sequences are currently being determined by eluting the dinucleotides, removing the 5' phosphate with phosphatase, and treating half with venom exonuclease (a 3' exonuclease) and half with spleen exonuclease (a 5' exonuclease). If the dinucleotide is pApG then only the spleen enzyme will give methyl AMP; if it is pGpA then only the venom enzyme will do so.

It is important to try to confirm and extend these sequences and this is currently being attempted.

Table 9 Sequences of some ^{14}C-methylated nucleotides by partial venom analysis

Nucleotide Number	Blue[1]	Original[2] & Products	Mononucleotide[3]	Sequence
2	23	14.2	A*	(A*T)
3	30	12.4 —C→ 19.7	A*	(A*T)C
4	30	14	A*	(A*G)
6	28.5	6.6 —C→ 10.9	A*	(A*G)C
7	22.9	3.1 —A/T→ 8.9 —C→ 14.4	A*	(A*T)CT[5]
13	29	6.4 —A/T→ 13.5 (A*G)	n.d.	(A*G)T[5]
Identical 15	29.7	3.7 —C→ 6.1 —A/T→ 13.4	A*	(A*G)T[5]C
Identical 28				
23	29.5	5.5	A*	(AG)A*
A[4]	29.8	2.2? —— 5.0 —A/T→ 14.6	A*	(A*G) A[5]
B[4]	29.8	6.0 —A/T→ 14.5	A*	(A*G)A[5]

Notes All of these partial digests were resolved on AE paper pH 3.5.

* = ^{14}C-methyl group
The sequence of nucleotides enclosed in brackets is not known.

n.d = not detectable

1 = position (cms) from origin) of xylene cyanol FF marker

2 = position of original oligonucleotide followed by successive products of partial digestion and nucleotides inferred from the mobility differences (Murray 1970).

3 = ^{14}C-mononucleotide product of digestion

4 = nucleotide obtained from a fingerprint on cellulose acetate/ PEI t.l.c. (Southern & Mitchell, 1971)

5 = A/T ambiguity resolved on the basis of position of original nucleotide on the fingerprint.

V. DISCUSSION

(i) Identity of the enzyme

Three lines of evidence identify the enzyme studied in this thesis as the DNA modification enzyme of phage P1 (M.P).

(a) Phage λ or 82 DNA that has been modified *in vivo* is not a substrate for the enzyme whereas unmodified phage DNA is methylated.

(b) The activity is present in extracts of cells infected with wild type P1, but is absent in cells infected with a modificationless mutant of P1.

(c) Methylation of phage λ DNA confers protection against a challenge by purified P1 restriction enzyme.

An additional criterion, which has not been tested, would be to demonstrate that methylation of phage λ DNA confers specific protection against a loss of infectivity due to restriction by recipient bacteria (Kaiser & Hogness,1960) that are lysogenic for P1.

Meselson & Yuan (1968) have suggested that the K restriction enzyme might also be the K modification enzyme. The purified M.P is free of detectable R.P activity, however, and sediments at 6S on glycerol gradients, whereas the R.P sediments at 10S (Murray, Brown & Brockes, unpublished work). Thus the purified activities are distinct.

If the purified R.P is incubated with SAM and unmodified DNA for 24h at 37°C then modification activity is detected [unpublished results quoted in Meselson et al. (1972)]. The molecular species that is responsible for modification has not

yet been identified. It is likely to be the result of dissociation or partial denaturation of the 10S R.P into the 6S species studied by the author.

(ii) General properties of the purified enzyme.

The sole product of the M.P, 6MAP, is also the product of B specific modification both *in vivo* (Smith et al., 1972) and *in vitro* (Kühnlein & Arber, 1972), and of K specific modification *in vitro* (unpublished results of the author). Prophage P1 also restricts the unglucosylated (Ugt) mutants of phages T2 and T4 (reviewed in Revel & Luria, 1970), but, for reasons that are obscure, P1 modification does not operate on these phages. It is possible, however, to obtain a mutant of T4 ugt that is unglucosylated but not restricted. This mutant has hypermethylated DNA and the product of methylation is 6MAP (Hattman, 1970). The extent of methylation is in excess of the number of sites of P1 restriction (Hattman, 1970). The simplest explanation is that a phage-induced enzyme has mutated to an activity that confers protection against P1 restriction. The interpretation of T-even phage restriction phenomena is complicated, however, by the failure of purified R.P to break unglucosylated DNA *in vitro* (quoted in Revel & Luria, 1970). Revel & Luria speculate that the restriction enzyme may need to act on T-even DNA in conjunction with the bacterial membrane. Further investigation is required to clarify these phenomena.

The characteristic properties of the M.P, in particular its pH optimum, SAM dependence and kinetics are similar to

those of the M.B (Kühnlein & Arber, 1972). The slow kinetics of methylation pose a particular problem. Figure 7 shows that at the K_M for SAM, saturating levels of enzyme takes 3h to incorporate the full quota of methyl groups into phage 82 DNA Kühnlein & Arber (1972) have presented similar data for B specific modification of phage fd DNA, although they did not comment on the slowness. In collaboration with Dr. R. Yuan, the author has investigated the binding of the M.P to phage λ DNA by using the filter binding method (Riggs & Bourgeois, 1968; Yuan & Meselson, 1970). In the presence of SAM, the glycerol gradient fraction (see Methods A(iii)) binds within 2 minutes (at 30°C) to both λ.K and λ.K(P1) DNA. Since these DNA species are the substrate and product of the M.P, the enzyme should be retained on the DNA after methylation. This is found to be the case (unpublished experiments of R.Yuan & the author). The presence of the enzyme, bound at a site of potential methylation (possibly on the opposite strand), could 'repress' subsequent methylation until the enzyme comes off the DNA. Although the details of the reaction remain unclear, this effect may well provide a basis for the slow kinetics. It is important, however, to determine whether the M.P is binding at a site of methylation. This question could be answered by binding the M.P to ^{3}H-methylated DNA and digesting the complex with DNAse. If the enzyme is binding at the site of methylation, then the ^{3}H-radioactivity should be protected from digestion.

It is unclear why unmodified DNA is restricted and not modified on entering the cell. The observation that the M.P

binds to both modified and unmodified DNA suggests that the molecules of modification enzyme spend most of their time on the many modified or partly modified sites, on the bacterial chromosome. The restriction enzyme does not bind to unmodified DNA (Yuan & Meselson, 1970) and is thus free to attack incoming unmodified DNA. These considerations suggested the use of DNAse to remove DNA in the purification procedure (Results B(i)), rather than streptomycin sulphate which might precipitate enzyme-DNA complexes. No systematic study has been made, however, to determine if this modified procedure increases yield.

(iii) Subunit structure of the enzyme

The dimeric structure of the enzyme is consistent with the genetic data of Glover et al (1963). The three gene model (see Introduction) indicates that one subunit is the product of gene m_{Pl} and one is the product of gene s_{Pl}. If the subunits could be successfully renatured from SDS gels (Weber & Kuter, 1971), then their identity could be tested by determining if one subunit (the m_{Pl} product) bound SAM, or the other (the S_{Pl}product) bound to DNA. If, however, these properties were dependent on the dimeric structure then such an approach would be unsuccessful.

(iv) Nucleotide sequences around the methylated base

It is clearly premature to draw any firm conclusions on the basis of the sequence data presented in the Results (section C). The failure of the $^{3}H/^{32}P$ double labelling methods has made the sequence analysis rather difficult.

Attempts are being made, however, to try to confirm and extend these sequences. The sequences around the break points made by the R.P are also being determined in this laboratory. It is hoped that consideration of these data will contribute some insight into the general problem of how proteins are able to recognise specific nucleotide sequences in duplex DNA.

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APPENDIX I. Construction of an Endonuclease I⁻ Pol A1 strain

The mutant Pol A1 (De Lucia & Cairns, 1969), which lacks detectable DNA polymerase I, has proved valuable in studies of DNA synthesis. The combination of Pol A1 with the endonuclease I⁻ mutation (Dörwald & Hoffman-Berling, 1968) provides a favourable strain for investigating various aspects of DNA enzymology *in vitro*. Extracts of endonuclease I⁻ strains lack at least 95% of the total endonuclease activity of wild-type extracts (Dörwald & Hoffman-Berling, 1968). The doubly mutant strain was constructed by isolating a rhamnose⁻ (rha⁻) mutant of strain 1100 (endo I⁻) and transducing to rha⁺ with a lysate grown on Pol A1. The rha+ pol A1 transductants were detected by their sensitivity to methylmethanesulphonate (MMS).

Step I. Isolation of 1100 rha⁻. Strain 1100 su⁻ was grown overnight in L broth, pelleted in a bench centrifuge, and resuspended in an equal volume of phage buffer. After irradiation with ultra-violet light (1,000 ergs, 26 sec), the survivors were plated on rhamnose tetrazolium agar and incubated overnight at 37°C. Six deep red colonies (rha⁺ colonies are white on this indicator agar) were picked and purified by streaking on rhamnose tetrazolium agar. The isolates were tested for growth on minimal agar supplemented with rhamnose, glucose or lactose. Of the six isolates, two gave leaky growth on rhamnose, two had a pleiotropic defect in sugar catabolism since they grew on none of the carbon sources, and two were non-reverting rhamnose negatives. One of this latter pair (strain 7) was retained for the P1 transduction.

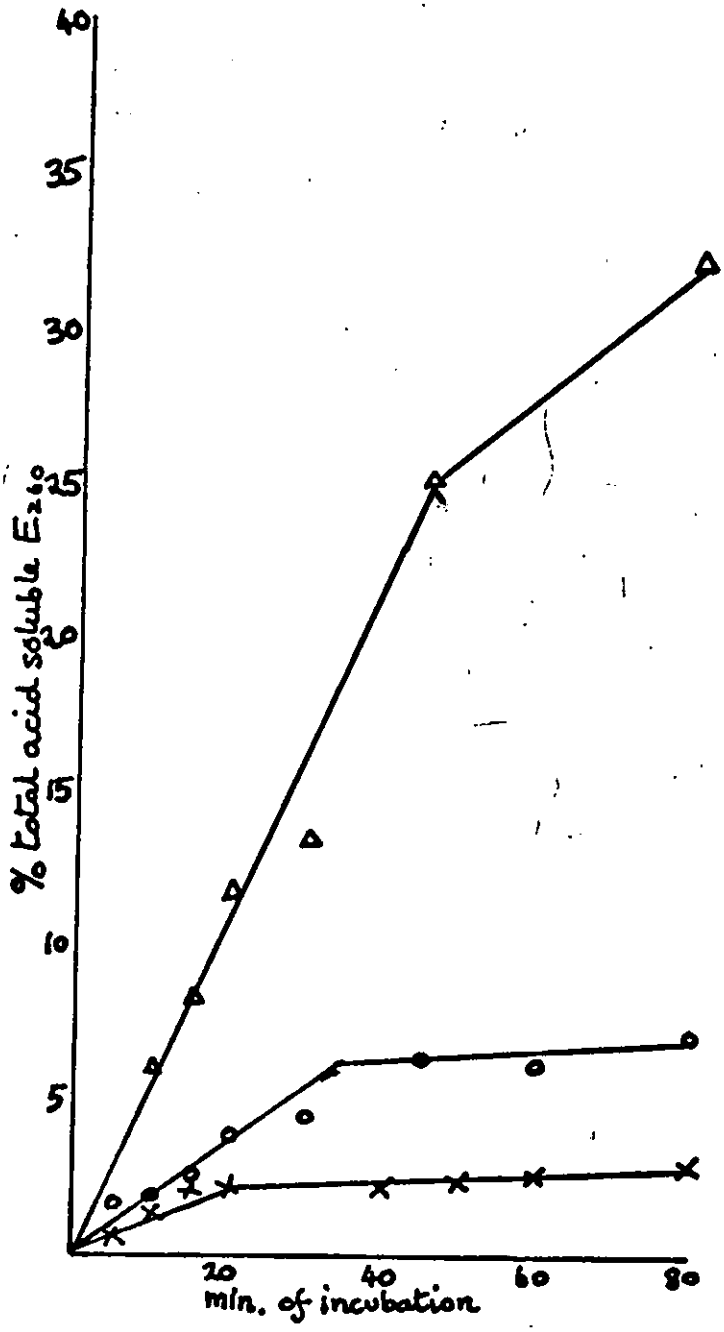
Step II Isolation of 1100 Pol A1. Strain 7 was transduced with a P1 lysate (a gift from Dr M.Monk) grown on Pol A1. Rha^+ transductants were selected on rhamnose minimal agar and 300 were patched onto the same medium. The patches were replicated onto rhamnose minimal plates with and without MMS. Twelve of the patches showed negligible growth on MMS plates. One of the isolates (strain 8) was verified as follows.

(a) Endo I^- character. Parallel extracts of W1485 endo I^+, 1100 su^- and strain 8 were made as described by Dürwald and Hoffman-Berling (1968). The extracts were assayed for endonuclease activity by following the appearance of acid soluble E_{260} units (Dürwald & Hoffman-Berling,1968). This presumably reflects the combined action of endonuclease and exonuclease activity. As shown in Figure A1, strain 8 had a lower level of nuclease than the endo I^+ strain W1485 and had a comparable level to its parent strain 1100. It is interesting that the level is lower than that of 1100. This might be a consequence of the absence of the exonuclease activity of DNA polymerase I (Kornberg, 1969).

(b) Pol A1 character. Parallel cultures of 1100, strain 8 and Pol A1 were grown in L broth at 37°C to 3×10^8 cells/ml. The cells were resuspended in 7.5 ml buffer (0.01M-Tris-HCl pH 7.0, 2mM-2 mercaptoethanol), disrupted by sonication, and centrifuged at low speed (15,000g, 15 min) and at high speed (38,000g 1.5h). The DNA polymerase activity in the extracts was assayed according to Lehman et al (1958). As shown in Table A1, the DNA polymerase activity in strain 8 and pol A1 extracts was less than 0.5% of that in strain 1100.

While this endo I^- pol A1 double mutant has not been used by the author, it has proved useful in studies of DNA replication (J.Gross, personal communication).

Figure A1. Release of acid soluble nucleotides from salmon sperm DNA by various extracts.



Reaction mixtures in 0.02M Tris-HCl pH 7.8, 0.1M-NaCl, 6mM-Mg $(CH_3COO)_2$ were set up as described by Dürwald and Hoffman-Berling (1968, Fig.2). At the times indicated 1 ml portions were withdrawn and assayed for acid soluble E_{260} units.

Δ—Δ W1485 extract
o—o 1100 extract
X—X strain 8 extract

Table A1: Reaction mixtures (vol 0.2 ml) contained 80μ moles potassium phosphate pH 6.8, 12μ moles 2-mercaptoethanol, 9μ moles $MgCl_2$, 0.075 mg salmon sperm DNA, 5n moles dGTP, 10n moles dTTP, 5.6n moles dCTP, 40n moles ^{14}C-dATP (7×10^2 c.p.m./n moles) and, when appropriate, 0.1 mg of extract protein. After incubation at 37°C for 30 min, the acid precipitable radioactivity was prepared for counting as described by Lehman et al (1958). Glass fibre discs were counted for 30 min on a nuclear Chicago low background gas flow counter.

		^{14}C, c.p.30m.	
a.	1100 endo I^- pol A^+ extract + cocktail	2071 1754	Duplicate
	extract added at 30 min to cocktail	267	
	cocktail alone	117	
b.	Pol A1 extract + cocktail	116	
	extract added at 30 min to cocktail	100	
	cocktail alone	63	
c.	Strain 8 extract + cocktail	171 124	Duplicate
	extract added at 30 min to cocktail	169	
	Cocktail alone	110	

Net promotion of incorporation by 1100 endo I^- pol A^+ extract

$$= \frac{1912 - 267}{30} = 55 \text{ c.p.m.} \equiv 60p \text{ moles dATP/30 min at 37°C/}$$

0.1 mg protein/0.075mg DNA primer.

[illegible]

[illegible]

[illegible]

APPENDIX II. A hypothesis for the role of SAM in phosphodiester bond cleavage by endonuclease R.K.

The nucleolytic activity of endonuclease R.K is absolutely dependent on Mg^{2+}, ATP and SAM (Meselson & Yuan, 1968). What is the role of SAM in this reaction? The experiments on enzyme-DNA complex formation show that SAM is required for the initial binding of enzyme to unmodified DNA (Yuan & Meselson, 1970). Another role is suggested by the close relation between the modification methylase and the restriction endonuclease. This table summarises various data discussed in the introduction.

	M.K	R.K.
1. Gene products	h_K, s_K	r_K, m_K, s_K
2. Cofactors	SAM	Mg^{2+}, ATP, SAM
3. Action	methylation of 6-amino of adenine	DNA breakage

Why is the apparatus of methylation implicated in DNA breakage? It is known from work with monofunctional methylating agents such as methyl methanesulphonate (MMS) that DNA can be broken by methylation (Lawley, 1963). MMS reacts readily with the purine nucleophilic centres such as the N7 of guanine and the N3 of adenine. This methylation labilises the N-deoxyriboside bond such that 7-methylguanine or 3 methyladenine are released from the DNA; this is a particular variety of depurination reaction. The resulting structure, while being quite stable at neutral pH, is labile to general acid-base catalysis so that the phosphodiester bonds on either side of the deoxyribose residue may break under these conditions.

It is possible, therefore, that restriction proceeds through such an unstable methylated intermediate. It is not intended to imply that the depurination and breakage steps take place without enzymic catalysis. The extensive ATPase activity of the enzyme may be important in mediating breakage (possibly through production of protons), as well as other features of the active centre.

Two lines of experimental evidence support this hypothesis,

(i) If λ DNA is restricted by endonuclease R.K, then the resulting ends are not phosphorylated by γ^{32}P-ATP and polynucleotide kinase, either before or after phosphatase treatment to remove a 5' terminal phosphate (K.Murray, unpublished observations). This result is neatly accommodated by the above mechanism if it is assumed that breakage of the phosphodiester bond occurs so as to leave a 5' terminal deoxyribose. This would not be an acceptor in the polynucleotide kinase reaction (Novogrodsky & Hurwitz, 1966).

(ii) If a restriction reaction is run with CT_3 SAM and stopped after two minutes then ^{3}H-radioactivity is associated with the DNA after gel filtration in high salt (Fig.A2). After hydrolysis with HCl, the ^{3}H-radioactivity chromatographs with authentic N3 methyladenine (from Cyclo Chemicals) on two successive solvent systems (Fig A3).

Attempts are being made to confirm and extend these findings.

Fig. A2 Agarose elution profile of λ DNA recovered from a two minute restriction reaction.

The reaction mixture (vol 0.28 ml) contained 7.5 µmoles potassium tris(hydroxymethyl)methyl-2-aminoethanesulphonate pH 8.0, 0.038 µmoles EDTA, 1.05 µmoles $Mg\ Cl_2$, 1.8 µmoles 2-mercaptoethanol, 0.1 µmoles ATP, 6.6 nmoles CT_3 SAM (8.9µCi/nmole, preneutralised with 0.01 ml of 1M potassium tris(hydroxymethyl)methyl-2-aminoethanesulphonate pH8), 5.5 µg $\lambda C_I 857 S_7$.C DNA, and 0.125 ml of endonuclease R.K (final addition). After incubation (2 min, 30°C) the reaction was stopped by adding 10% ($\frac{w}{v}$) SDS (0.01 ml) and 0.5M-EDTA pH 8.0 (0.05 ml); after 1 min at 30°C, 5M-NaCl (0.01 ml) and ^{32}P-labelled λ DNA (0.025 ml, 3,000 c.p.m.) were added and the reaction mixture was applied to a column (50 cms x 1 cm diameter) of A-50M Agarose equilibrated with 0.15M-ammonium bicarbonate pH8. The sample was washed in with small aliquots of ammonium bicarbonate and then eluted with the same solution. Fractions (approx. 0.5 ml) were collected every 6 min and aliquots (0.075 ml) were counted for ^{3}H & ^{32}P.

The following variants of the above protocol gave essentially no peak of ^{3}H that was excluded with the ^{32}P-labelled λ DNA.

(i) reaction mixture - ATP
(ii) reaction mixture with 4.8µg λ.K DNA in place of λ.C DNA
(iii) reaction mixture incubated for 30 min.

o—o ^{32}P
•—• ^{3}H

Fig A2

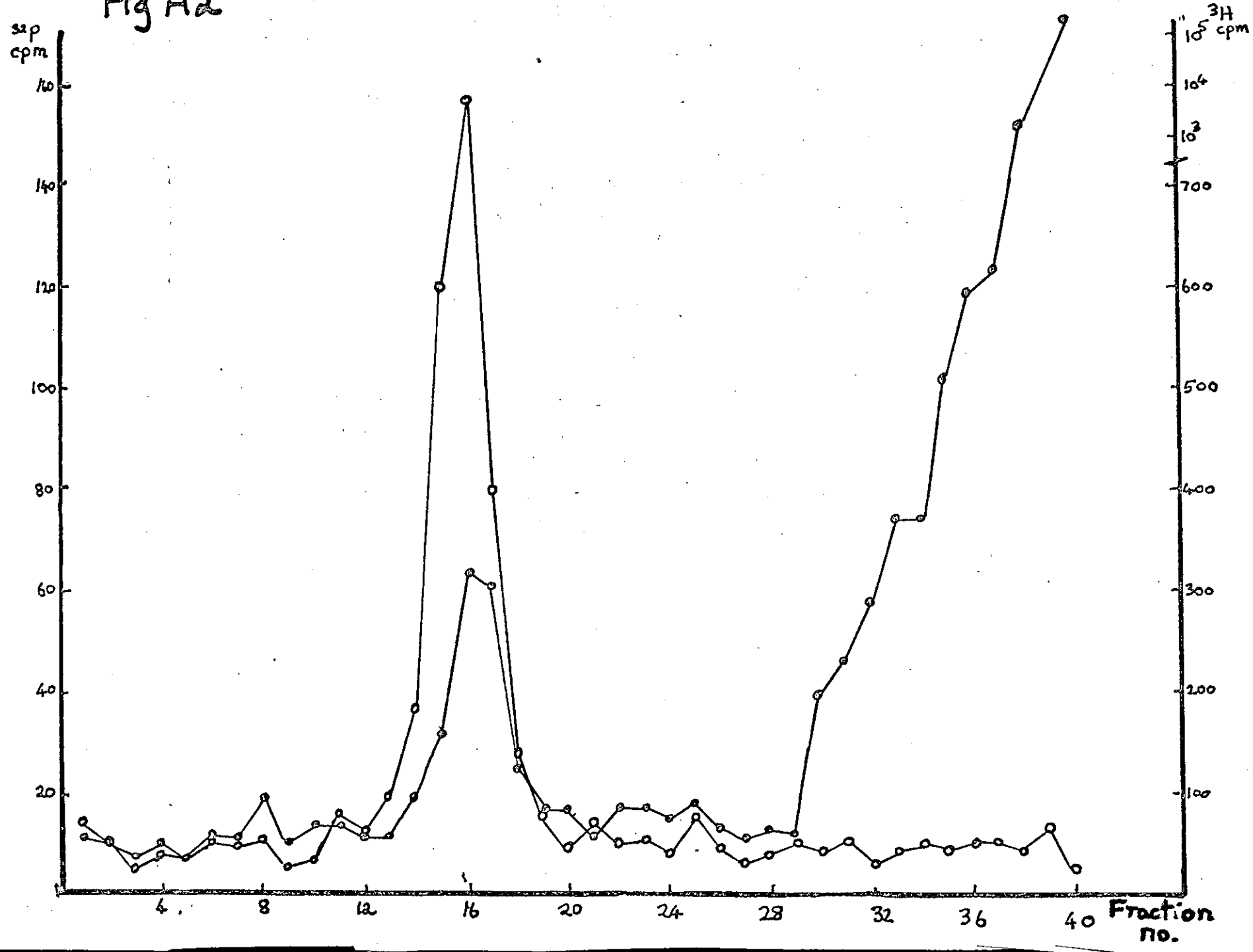
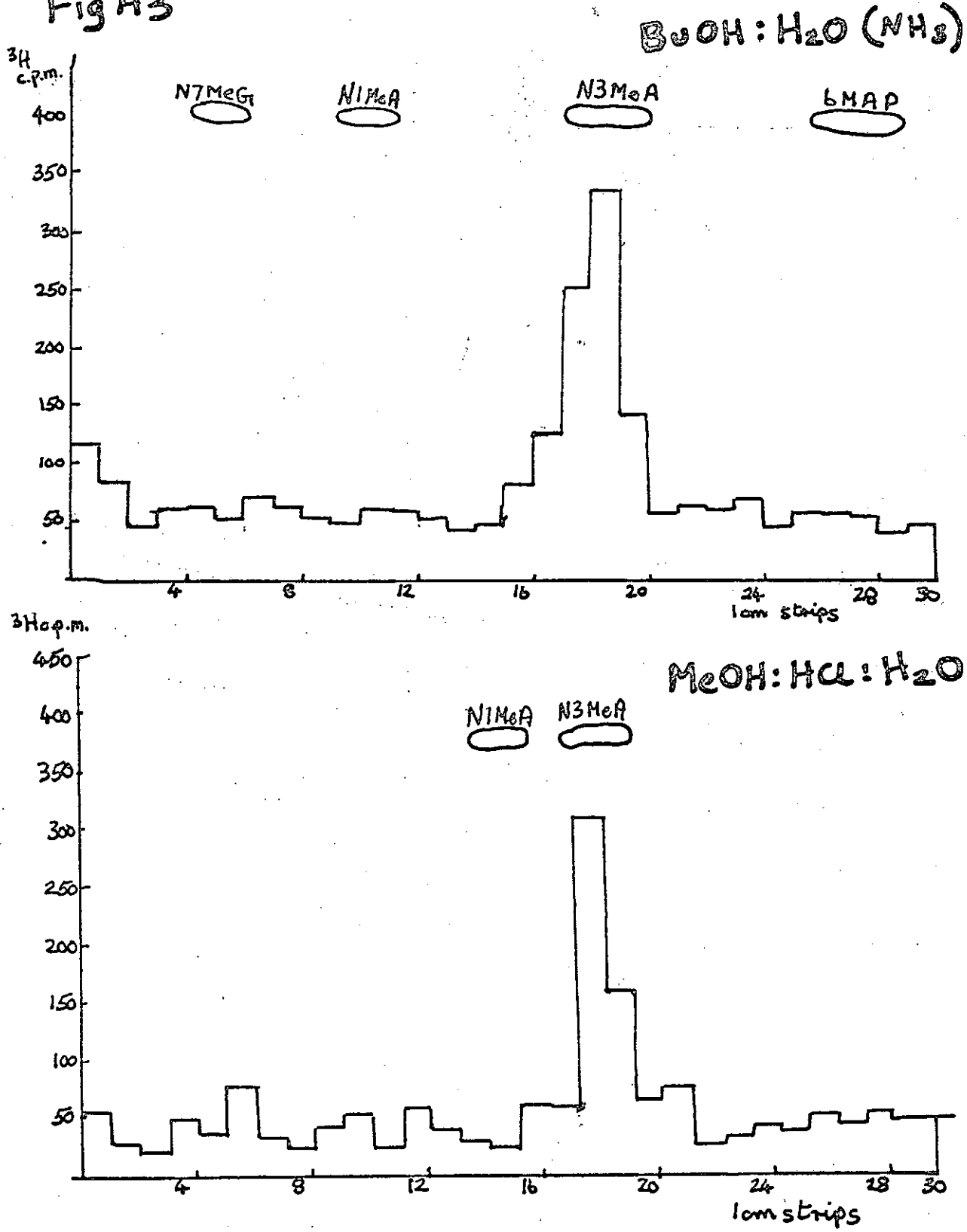

32P cpm
160
140
120
100
80
60
40
20
3H cpm
10^5
10^4
10^3
700
600
500
400
300
200
100
4
8
12
16
20
24
28
32
36
40
Fraction no.

Fig. A3. Base analysis of λ DNA recovered from a two minute restriction reaction.

Fractions 15-18 inclusive from the Agarose column (see Fig.A3) were pooled, evaporated to dryness twice, and hydrolysed with 1M-HCl (0.05 ml) for 30 min at 100°C. Marker bases were added (0.01 ml of 25 mM stock solutions) and the hydrolysate was evaporated to dryness, taken up in 0.02 ml 10% (v/v) propan-2-ol, 10% (v/v) glacial acetic acid and applied to Whatman No.1 paper. After descending chromatography for 27 hrs in 86 n-Butanol: 14 H_2O (NH_3 atmosphere) the paper was examined as described in Methods 3(b). Strips 17-20 inclusive were eluted with 1M-HCl and chromatographed on No.1 paper with 7 Methanol:2 conc HCl:1 H_2O for 10 hrs. The paper was again examined and counted.

Fig A3


BuOH : H2O (NH3)
3H c.p.m.
N7MeG
N1MeA
N3MeA
6MAP
400
350
300
250
200
150
100
50
4
8
12
16
20
24
28
30
1cm strips
3H c.p.m.
MeOH : HCl : H2O
N1MeA
N3MeA
450
400
350
300
250
200
150
100
50
4
8
12
16
20
24
28
30
1cm strips

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The Deoxyribonucleic Acid Modification Enzyme of Bacteriophage P1

PURIFICATION AND PROPERTIES

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The bacteriophage P1 modification enzyme, assayed by the specific methylation of unmodified bacteriophage 82 DNA, has been purified 500-fold from a bacteriophage P1 lysogen of *Escherichia coli*. The enzyme catalyses the incorporation of approximately 20–24 methyl groups per bacteriophage 82 DNA molecule. The sole product of methylation is 6-methylaminopurine. Methylation of unmodified bacteriophage DNA confers protection against a challenge by purified bacteriophage P1 restriction enzyme. The pH optimum is 6.0–6.25: the apparent K_m for *S*-adenosyl-L-methionine is 5×10^{-6} M.

The mechanism by which certain proteins are able to bind to or recognize particular nucleotide sequences is a central problem in molecular biology (Yarus, 1969). The enzymes of DNA restriction and modification offer an excellent opportunity for studying this problem (Arber & Linn, 1969). Many strains of *Escherichia coli* are able to degrade or 'restrict' foreign DNA that enters the cell. The enzyme responsible is a product either of the bacterial genome or of plasmids such as bacteriophage P1 (Lederberg, 1957) that are harboured by the bacteria. Several restriction enzymes have been purified and shown to be specific endonucleases which cleave bacteriophage DNA into discrete fragments of high molecular weight (Meselson & Yuan, 1968; Roulland-Dussoix & Boyer, 1969; Linn & Arber, 1968; Smith & Wilcox, 1970). This highly specific cleavage is believed to be a consequence of recognition of particular nucleotide sequences (Kelly & Smith, 1970).

The bacterial DNA, and any bacteriophage DNA that successfully infects the cell, is protected from the restriction endonuclease by host-induced 'modification' of the DNA (Arber & Dussoix, 1962). Yuan & Meselson (1970) have shown that purified *E. coli* K restriction enzyme will bind to unmodified bacteriophage λ DNA, but not to bacteriophage λ DNA carrying the strain K modification. With *E. coli* B, the modification *in vivo* of bacteriophage fd DNA involves methylation of four adenine residues at the 6-amino position (Smith *et al.*, 1972). Genetic analysis (reviewed by Arber & Linn, 1969) of the *E. coli* B, *E. coli* K and bacteriophage P1 restriction and modification systems indicates that the modification enzymes have at least one and probably two (Hubacek & Glover, 1970) polypeptide chains in common with the corresponding restriction enzymes. This presumably reflects the common specificities of the enzymes, since the adenine residue that is methylated on modification is believed to be located in the sequence recognized by the restriction enzyme (Kühnlein & Arber, 1972). The *E. coli* B modification enzyme has been partially purified (Kühnlein *et al.*, 1969), this being based on an infectivity assay for bacteriophage fd DNA (Benzinger, 1968).

The present paper describes a 500-fold purification of the bacteriophage P1 modification activity and some of the properties of this activity. The activity has been assayed by measuring the transfer of tritium-labelled methyl groups from *S*-adenosyl-L-methionine to unmodified DNA.

In this paper the general nomenclature of bacteriophages and bacteria given by Arber & Linn (1969) is followed. Thus bacteriophage P1 that has lost its restriction and modification properties is written $P1r_{P1}^{-}m_{P1}^{-}$. Bacteriophage 'pedigree' is indicated after a point by the strain of the bacterial host, e.g. $82.C600(Plr_{P1}^{-}m_{P1}^{+})$ is bacteriophage 82 grown on *E. coli* C600 lysogenized with the appropriate bacteriophage P1, and hence carrying bacteriophage P1 modification. Endonuclease R.P and endonuclease R.K are the bacteriophage P1 and *E. coli* K restriction enzymes respectively.

Materials and Methods

Bacterial and bacteriophage strains

The endonuclease I^- strain *E. coli* 1100 of Dürwald & Hofman-Berling (1968) (obtained from Dr. I. R.

Lehman, Department of Biochemistry, Stanford University, California, U.S.A.) was made $r_K^- m_K^\pm$ by Dr. N. E. Murray of this department by using ethylmethanesulphonate mutagenesis (Hubacek & Glover, 1970), and was lysogenized with bacteriophage P1kc (Lennox, 1955). *E. coli* C600 and its derivatives C600($P1r_{P1}^- m_{P1}^-$), C600($P1r_{P1}^- m_{P1}^+$) and C600 (P1) were obtained from Dr. S. W. Glover of this department. Bacteriophage 82c, a clear-plaque mutant of bacteriophage 82, was obtained from Dr. W. Arber, Biozentrum der Universität, Basel, Switzerland, and bacteriophage P1kc from Dr. W. J. Brammar of this department.

Enzymes and chemicals

Pancreatic deoxyribonuclease and snake-venom phosphodiesterase were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Endonuclease R.P and endonuclease R.K, both purified to the glycerol-gradient stage as described by Meselson & Yuan (1968), were the generous gifts of Mr. R. W. Old and Dr. R. Yuan, both of this department. Dithiothreitol, bovine liver haemoglobin, bovine serum albumin, adenine, *S*-adenosyl-L-methionine, 6-methylaminopurine, 5-methylcytosine and *N*-7-methylguanine were obtained from Sigma (London) Chemical Co., London S.W.6, U.K., Whatman DE52 cellulose (microgranular, pre-swollen), Whatman P11 phosphocellulose and AE81 aminoethyl-cellulose paper were obtained from H. Reeve Angel and Co. Ltd., London E.C.4, U.K. Glass beads (ballotini no. 11) were obtained from Jencons (Scientific) Ltd., Hemel Hempstead, Herts., U.K. Streptomycin sulphate was a kind gift from Glaxo Laboratories Ltd., Ulverston, Lancs., U.K. *S*-Adenosyl-L-methionine was purified by elution from Zeo-Karb 226 with 4M-acetic acid (Meselson & Yuan, 1968), and stored in acetic acid at −10°C. Thin-layer cellulose sheets (MN300) were obtained from Macherey Nagel and Co., Düren, Germany. [*methyl*-^{3}H]-*S*-Adenosyl-L-methionine (8.9Ci/mmol, 80μM) in H_2SO_4 (pH2.5–3.5) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and used without further purification. L broth (pH7.2) contained, per litre of water, Difco Tryptone (10g), yeast extract (5g) and NaCl (10g).

DNA preparations

^{3}H-labelled bacteriophage λ $C_{1857}S_7$.C DNA and unlabelled bacteriophage λ $C_{1857}S_7$.C DNA. These were kindly provided by Dr. N. E. Murray of this department. ^{32}P-labelled bacteriophage λ $C_{1857}S_7$.K (P1) DNA was kindly provided by Mr. R. W. Old.

Bacteriophage 82c DNA. E. coli, either C600 ($P1r_{P1}^- m_{P1}^-$) or C600 ($P1r_{P1}^- m_{P1}^+$), was grown in 1 litre of L broth at 37°C to $E_{650} = 0.5$ (approx. 5×10^8 cells/ml). The culture was made 1mM in $MgSO_4$ and bacteriophage 82c was added at a multiplicity of infection of 0.3–0.5 phage/bacterium. The culture was shaken vigorously at 37°C for 3–4h and lysed with 5ml of chloroform. The lysate was clarified by centrifugation (10000*g* for 15min at 4°C) and the titre determined on the appropriate indicator strains to check the presence or absence of bacteriophage P1 modification. The bacteriophages (generally 10^{11} plaque-formers/ml) were collected by centrifugation (45000*g* for 2½h), resuspended by gentle agitation overnight in 25ml of bacteriophage λ dil medium (Kaiser & Hogness, 1960) and twice banded by centrifugation (30h, 27000rev./min, MSE Superspeed 65 centrifuge, 3×23ml swing-out rotor) in 41.5% (w/w) CsCl. The final band was collected, dialysed against 10mM-tris–HCl–1mM-EDTA, pH7.4, and extracted three times by rolling with freshly distilled phenol that had been equilibrated with 0.5M-tris–HCl, pH8. The aqueous layer was dialysed exhaustively against 10mM-tris–HCl–1mM-EDTA–50mM-NaCl, pH7.4, and stored at 0°C.

Methylation assay

The reaction mixture contained, in a total volume of 200μl, 0.05M-potassium phosphate, pH6.5, 5mM-2-mercaptoethanol, 0.5mM-EDTA, 12μM-[*methyl*-^{3}H]-*S*-adenosyl-L-methionine (3Ci/mmol), bacteriophage 82 DNA (5μg) and the sample to be assayed. After 60min incubation at 30°C, 0.5% bovine serum albumin (0.1ml), 0.2M-tetrasodium pyrophosphate (0.2ml) and 1M-$HClO_4$–2mM-sodium pyrophosphate (0.5ml) were added. After 5min at 0°C cold water (1ml) was added and the precipitate collected by centrifugation (5000*g*, 5min). The precipitate was dissolved in 0.2M-NaOH (0.3ml) and re-precipitated with 0.2M-sodium pyrophosphate (0.2ml) and 1M-$HClO_4$–2mM-sodium pyrophosphate (0.5ml). This washing procedure was repeated twice more and the precipitate was finally collected by filtration under reduced pressure through a 2.1cm Whatman GFC glass-fibre disc. The disc was washed with 1M-$HClO_4$ (5ml), water (5ml) and ether (2ml). After drying under an i.r. lamp the discs were counted for radioactivity in 2.5ml of scintillant [4g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/l of AnalaR toluene] in a Nuclear–Chicago Unilux liquid-scintillation spectrometer. Assays were always done in parallel with P1 modified and unmodified bacteriophage 82 DNA. The blank value for incorporation into modified DNA was subtracted. This blank was always 100–150c.p.m., except for the dialysed $(NH_4)_2SO_4$ fraction, where it was 300–500c.p.m. owing to the presence of non-specific methylating activity.

Degradation of DNA

Acid hydrolysis. Bacteriophage 82 DNA from a methylation reaction was purified by acid precipitation as described in the methylation assay, except that calf thymus DNA (0.2ml, 1.2mg/ml) was used as carrier in place of serum albumin. The final precipitate was hydrolysed with 1M-HCl (0.2ml) at 100°C for 1h. The hydrolysate was evaporated to dryness (together with 20μl of 5mM solutions of various marker bases), the residue dissolved in 10% (v/v) acetic acid–10% (v/v) propan-2-ol (60μl) and applied to Whatman no. 1 paper. After descending chromatography in butan-1-ol–water (43:7, v/v) in an NH_3 atmosphere for 24h the paper was dried, markers were located under u.v. light and the paper was cut into strips (2cm × 1cm); the radioactivity of each strip was determined by liquid-scintillation counting. The strips containing 6-methylaminopurine were swirled in toluene to remove scintillant, dried, eluted overnight with 0.1M-HCl and chromatographed on Whatman no. 1 paper in an ascending system of methanol–water–conc. HCl (7:1:2, by vol.) for 24h. The chromatogram was again examined under u.v. light, cut up and counted for radioactivity.

Methylation of bacteriophage λ DNA was investigated by hydrolysing 10μl of the product with 1M-HCl (50μl) for 45min at 100°C in the presence of 6-methylaminopurine and 5-methylcytosine. The hydrolysate was evaporated to dryness, the residue dissolved in 20μl of water and applied to a sheet (20cm × 20cm) of MN300 thin-layer cellulose. The chromatogram was developed in two dimensions as described by Razin *et al.* (1970). Marker bases were located under u.v. light, eluted and counted for radioactivity, together with the origin region, as described by Razin *et al.* (1970).

Enzymic digestion. Bacteriophage 82 DNA was purified from a methylation reaction by extracting twice with an equal volume of phenol. The aqueous layer was extensively dialysed against 0.1M-sodium acetate (pH5.0)–0.005M-$MgCl_2$ and a solution of pancreatic deoxyribonuclease (1mg/ml; 0.1ml) was added and incubated at 37°C for 2h. The reaction was adjusted to pH8.0 with 1M-tris–HCl, pH8.0 (10μl), and incubated with snake-venom phosphodiesterase (1mg/ml; 20μl; 37°C; 4h). The solution was evaporated to dryness, the residue dissolved in 50μl of water and applied, together with mononucleotide markers, to AE 81 cellulose paper. Electrophoresis was at 50 V/cm in pyridine acetate buffer, pH3.5, until the blue marker dye (Xylene Cyanol FF) had run 25cm. The paper was dried overnight at room temperature, examined under u.v. light, cut into strips (2cm × 1cm) and the radioactivity determined in a liquid-scintillation spectrometer.

Results

P1 restriction of bacteriophage 82

The bacteriophage P1 modification enzyme was selected for study because the efficient restriction of certain bacteriophages by the bacteriophage P1 system presumably indicates a relatively large number of sites at which the restriction and modification enzymes act. This facilitates the use of a methylation assay for modification. The DNA of bacteriophage 82 was chosen as substrate rather than that of bacteriophage λ because of the relative efficiencies of plating of the two phages on a P1 lysogen (see Table 1). The assay for the P1 modification enzyme measures the extent of methylation of bacteriophage 82.C600 ($P1r_{P1}^{-}m_{P1}^{-}$) DNA compared with DNA from bacteriophage 82.C600 ($P1r_{P1}^{-}m_{P1}^{+}$) modified *in vivo*. If modification *in vivo* is efficient, then the latter substrate should be inert to the activity *in vitro*.

Purification of the modification enzyme

All buffers contained 5% (v/v) glycerol, 5mM-2-mercaptoethanol and 0.5mM-EDTA unless otherwise stated. All potassium phosphate buffers were pH6.7. Protein in subcellular fractions was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Step 1: *preparation of dialysed* $(NH_4)_2SO_4$ *fraction.* [Linn & Arber (1968), with minor modifications.] *E. coli* $1100r_K^{-}$ (P1) was grown in L broth at 37°C

Table 1. *Efficiencies of plating of bacteriophages* 82 *and* λ *on* P1 *lysogenic hosts*

The efficiencies of plating (titre on bacteriophage P1 lysogenic host/titre on *E. coli* C600) of bacteriophage 82c and bacteriophage λ grown on various hosts were determined by standard bacteriophage and bacterial techniques.

Bacteriophage	*E. coli* C600 (P1)	*E. coli* C600 ($P1r_{P1}^{-}m_{P1}^{+}$)
82.C600	1×10^{-6}–3×10^{-6}	1
82.C600($P1r_{P1}^{-}m_{P1}^{+}$)	1	1
82.C600($P1r_{P1}^{-}m_{P1}^{-}$)	1×10^{-6}–3×10^{-6}	1
λ.C600($P1r_{P1}^{-}m_{P1}^{-}$)	1×10^{-5}–3×10^{-5}	1

to 10^9 cells/ml, sedimented in a continuous-flow Alfa-Lavall centrifuge and stored at −20°C. All subsequent operations were performed at 4°C. Cells (150g) were resuspended in 225ml of 0.05M-tris–HCl, pH8.0, then 300g of acid-washed glass beads was added and the mixture was blended for a total of 20min with intermittent cooling in an ice–salt bath so that the temperature did not rise above 5°C. The supernatant was decanted from the beads, which were washed with 100ml of 0.05M-tris–HCl, pH8.0. The pooled supernatant and washings were centrifuged (10000*g*, 20min). The supernatant (volume 354ml) was made 0.035M in $MgCl_2$ and the resulting precipitate was removed by centrifugation (10000*g*, 15min). Fresh 5% (w/v) streptomycin sulphate solution (125ml) was added to the supernatant and, after 20min at 0°C, the precipitate was removed by centrifugation (10000*g*, 15min). The supernatant was precipitated by adding 180g of solid $(NH_4)_2SO_4$, which was dissolved over 30min at 0°C. The precipitate was collected by centrifugation in the MSE High-Speed 18 centrifuge (6×100ml rotor, 17000rev./min, 30min, 2°C). The pellet was dissolved in 0.02M-potassium phosphate (30ml) and dialysed against the same buffer.

Step 2: *chromatography on DEAE-cellulose.* The dialysed ammonium sulphate fraction was applied to a column (17cm×4.5cm diam.) of Whatman DE52 cellulose that had been equilibrated with 0.02M-potassium phosphate. The column was washed successively with 500ml of 0.02M-potassium phosphate, 500ml of 0.05M-potassium phosphate, then eluted with a linear potassium phosphate gradient (1.5 litres) running from 0.05M to 0.3M. Fractions (approx. 45ml) were collected and the enzyme activity was found in five neighbouring fractions with a mean phosphate concentration of 0.11M (Fig. 1). The pooled fractions (volume 240ml) were precipitated with $(NH_4)_2SO_4$ (150g). The precipitate was collected by centrifugation, dissolved in 0.02M-potassium phosphate (30ml) and dialysed extensively against 0.02M-potassium phosphate. The precipitate that formed during dialysis was removed by centrifugation; the total protein in the supernatant was 301mg (volume 44ml).

Step 3: *chromatography on phosphocellulose.* Of the concentrated DE fraction 34ml was applied to a column (18cm×1.25cm diam.) of Whatman P11 phosphocellulose that had been equilibrated with 0.02M-potassium phosphate. The column was eluted with 0.02M-potassium phosphate (75ml), 0.1M-phosphate (100ml), 0.2M-potassium phosphate (75ml), 0.3M-potassium phosphate (100ml) and 0.5M-potassium phosphate (100ml). The methylation activity was found in the 0.2M step and was immediately concentrated by the addition of $(NH_4)_2SO_4$ (40g). The precipitate was collected by centrifugation and dissolved in 0.02M-potassium phosphate (3ml).

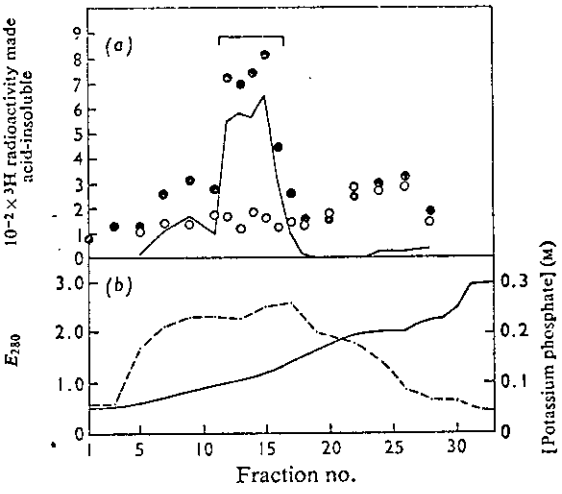

Fig. 1. *Chromatography of dialysed ammonium sulphate fraction on DEAE-cellulose*

Adsorption and elution was as described in the Results section under 'Purification of the modification enzyme'. Samples (0.15ml) of the fractions were assayed in parallel for methylation of bacteriophage 82.C600 ($P1r_{P1}^-m_{P1}^-$) DNA (●) and bacteriophage 82.C600 ($P1\ r_{P1}^-m_{P1}^+$) DNA (○). The continuous line in (*a*) is the difference in radioactivity incorporated into the two substrates. Fractions enclosed by the bar were pooled and concentrated as described under 'Purification of the modification enzyme'. (*b*) —·—, E_{280}; ——, [potassium phosphate].

Step 4: *glycerol-gradient sedimentation.* After dialysis for 2h against 0.02M-potassium phosphate (1 litre) the concentrated phosphocellulose fraction was layered in 1.2ml portions on 20ml of 10–25% (v/v) glycerol gradients made up in 0.02M-potassium phosphate – 0.1mM-EDTA – 5mM-dithiothreitol, pH6.5. After centrifugation for 36h at 30000rev./min in the MSE Superspeed 65 centrifuge, 3×23ml swing-out rotor at 2°C, the gradients were collected in 1ml fractions. The active fractions (see Fig. 2) were made 50% (v/v) in glycerol and stored at −20°C. No significant loss of activity (less than 10%) was observed over 4 months.

The purification of the enzyme is summarized in Table 2. The dilute column fractions were all unstable and the glycerol gradient fraction lost activity if stored at 0°C. It was found necessary to complete all steps before the glycerol gradient in 2–3 days. The glycerol-gradient fraction had no detectable bacteriophage P1 restriction activity, and no detectable endodeoxyribonuclease activity at pH7.5 in 5mM-$MgCl_2$, as assayed by neutral sucrose-gradient sedimentation of bacteriophage λ DNA. Under the conditions of

methylation reactions [0.05M-2-(*N*-morpholino)-ethanesulphonic acid (pH 6.0)–0.25mM-EDTA] it had no detectable effect on the sedimentation profile in neutral sucrose gradients of supercoiled bacteriophage M13 RFI DNA (kindly provided by Mr. G. G. Peters of this department).

The enzyme has also been purified from 27g of bacteriophage P1-infected cells by an identical procedure, and is currently prepared from induced lysogens of the thermoinducible mutant P1.C_i162 (Scott, 1970). This increases the specific activity of enzyme five- to ten-fold.

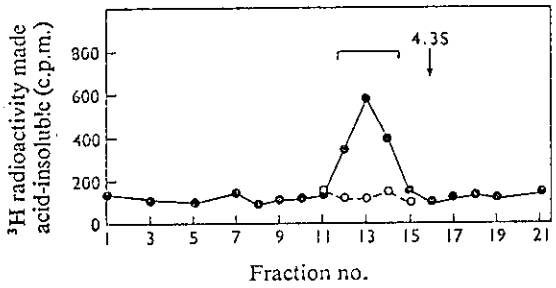

Fig. 2. *Glycerol-gradient sedimentation of concentrated phosphocellulose fraction*

Sedimentation of the phosphocellulose fraction was as described in the Results section under 'Purification of the modification enzyme'. Portions (25μl) were assayed for methylation of bacteriophage 82.C600 (P1 r_{P1}^- m_{P1}^-) DNA (●) or bacteriophage 82.C600 (P1 r_{P1}^- m_{P1}^+) DNA (○). The 4.3S standard was bovine haemoglobin, which was located by its extinction at 410nm. Fractions enclosed by the bar were stored as described under 'Purification of the modification enzyme'. Sedimentation was from right to left.

Properties of the purified modification activity

All these experiments were performed with the glycerol-gradient fraction.

Requirements for the activity. The requirements were investigated as shown in Table 3. The enzyme is specific for native, unmodified DNA and is stimulated 1.5-fold by 5mM-Mg^{2+}, a property also noted by Kühnlein *et al.* (1969) with the *E. coli* B modification activity. A threefold inhibition by 0.1mM-ATP was also observed.

pH-dependence. The pH-dependence of the activity in 2-(*N*-morpholino)ethanesulphonic acid and piperazine-*NN'*-bis-2-ethanesulphonic acid buffers is shown in Fig. 3. In 2-(*N*-morpholino)ethanesulphonic acid buffer the pH optimum is between 6.0 and 6.25, although significant methylation was observed from pH5.5 to pH8.0. No significant methylation of bacteriophage P1-modified DNA was observed over this pH range.

Time-course of methylation. The time-course of methylation of unmodified bacteriophage 82 DNA was investigated by incubating a series of duplicate reaction mixtures for various times and then assaying for acid-precipitable radioactivity. As shown in Fig. 4, incorporation of methyl groups was complete after 3h of incubation at 30°C. The extent of methylation was not limited by inactivation of the enzyme, since the addition of more enzyme after 5h was without effect, whereas the addition of more DNA produced a detectable stimulation. The number of methyl groups incorporated at the plateau was about 20–24/DNA molecule (containing 10^5 bases), although this number is difficult to estimate accurately because of differences in recovery and quenching during the acid-precipitation procedure.

Dependence of methylation on S-adenosyl-L-methionine. The *S*-adenosyl-L-methionine dependence was investigated as described in the legend to Fig. 5. Analysis of the results by a double-reciprocal plot

Table 2. *Summary of enzyme purification*

1 unit of activity makes 0.16pmol of methyl groups acid-insoluble/5μg of unmodified bacteriophage 82 DNA per 60min incubation at 30°C. This is equivalent to 125c.p.m. under the conditions of assay. The blank value for incorporation into modified DNA is subtracted, as noted in the Materials and Methods section. The recovery values for the phosphocellulose and glycerol-gradient fractions are corrected for purification of total pooled DEAE-cellulose fraction.

Purification stage	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Crude supernatant	10700			
Dialysed $(NH_4)_2SO_4$ fraction	4820	7520	1.56	100
Pooled DEAE-cellulose fraction	301	4420	14.7	59
Pooled phosphocellulose fraction	9.1	2161	230.8	29
Pooled glycerol-gradient fraction	1.5	1230	820	16

Table 3. *Requirements for methylation*

The complete system contained, in a volume of 100μl: 0.05M-2-(*N*-morpholino)ethanesulphonic acid, pH6.0, 0.25mM-EDTA, 5mM-2-mercaptoethanol, 4μM-[*methyl*-^{3}H]-*S*-adenosyl-L-methionine (8.9Ci/mmol), 6μg of bacteriophage 82.C600 ($P1r_{P1}^-m_{P1}^-$) DNA and 25μl of glycerol-gradient fraction modification enzyme. After incubation for 45min at 30°C the mixtures were assayed for acid-precipitable radioactivity as described in the Materials and Methods section. Bacteriophage 82 DNA was denatured by heating at 100°C for 7min and cooling in ice. Enzyme was heat-inactivated at 100°C for 5min in a stoppered tube.

	Acid-insoluble ^{3}H radioactivity (c.p.m.)
Complete system	2333
Minus DNA	134
Substitute bacteriophage 82.C600 ($P1r_{P1}^-m_{P1}^+$) DNA	147
Substitute heat-denatured DNA	185
Minus enzyme	78
Substitute heat-inactivated enzyme	113
Plus 5mM-$MgCl_2$	3392
Plus 0.1mM-ATP	713
Plus 5mM-$MgCl_2$ and 0.1mM-ATP	2984

(Lineweaver & Burk, 1934) gave an apparent K_m for *S*-adenosyl-L-methionine of 5×10^{-6}M. The value obtained by Kühnlein & Arber (1972) for the *E. coli* B modification enzyme was 4×10^{-6}M.

Product of methylation. A sample of methylated DNA was analysed by enzymic digestion to mononucleotides and high-voltage electrophoresis on aminoethylcellulose paper at pH3.5 (see the Materials and Methods section). A single peak of radioactivity (Fig. 6*a*) migrated with dAMP, indicating that a single mononucleotide had been methylated. Another sample of methylated DNA was analysed by acid hydrolysis and paper chromatography (see the Materials and Methods section). The radioactivity migrated on two solvent systems with 6-methylaminopurine (see Figs. 6*b* and 6*c*). 6-Methylaminopurine is thus the sole detectable product of methylation.

Modification and restriction of bacteriophage λ DNA in vitro. The effect of this methylase can be demonstrated by incubating DNA that had been methylated *in vitro* with purified bacteriophage P1 restriction enzyme. Unlabelled bacteriophage λ.C DNA was methylated with the enzyme and [*methyl*-^{3}H]-*S*-adenosyl-L-methionine in the presence of

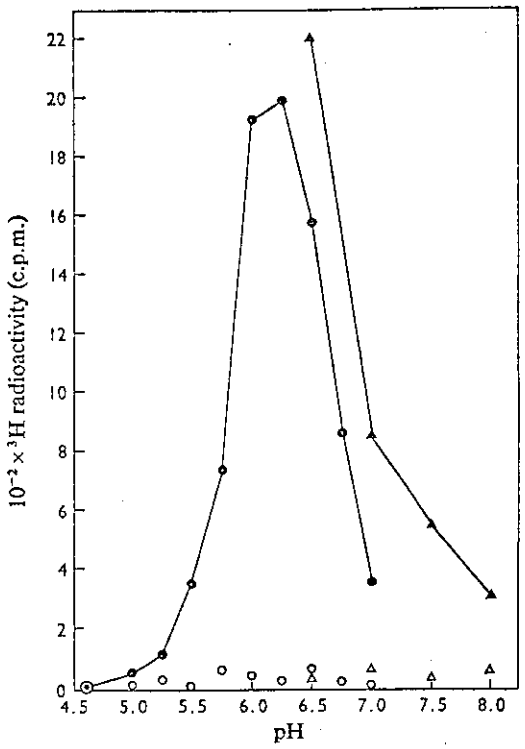

Fig. 3. *pH-dependence of methylation in 2-(N-morpholino)ethanesulphonic acid ($pK_a = 6.15$) and piperazine-NN′-bis-2-ethanesulphonic acid ($pK_a = 6.8$) buffers*

The complete system was made up as for Table 3 in 0.05M-2-(*N*-morpholino)ethanesulphonic acid or 0.05M-piperazine-*NN*′-bis-2-ethanesulphonic acid buffers of the appropriate pH value, which was measured at 27°C on a Vibron model 39a pH-meter with manual temperature compensation and standardized against air-free phthalate buffer, pH7.0. The reaction mixtures were incubated for 40min at 30°C and assayed as described in the text. Bacteriophage 82.C600 ($P1\ r_{P1}^-m_{P1}^-$) DNA: ●, 2-(*N*-morpholino)ethanesulphonic acid; ▲, piperazine-*NN*′-bis-2-ethanesulphonic acid. Bacteriophage 82.C600 ($P1\ r_{P1}^-m_{P1}^+$) DNA: ○, 2-(*N*-morpholino)ethanesulphonic acid; △, piperazine-*NN*′-bis-2-ethanesulphonic acid.

^{32}P-labelled bacteriophage λ.K (P1) DNA. After the incubation the DNA species were purified by phenol extraction. After removal of the phenol and *S*-adenosyl-L-methionine by dialysis, a sample of DNA was analysed by acid hydrolysis, followed by t.l.c.; 85% of the ^{3}H was found in 6-methylaminopurine. The remainder of the ^{3}H-methylated DNA was

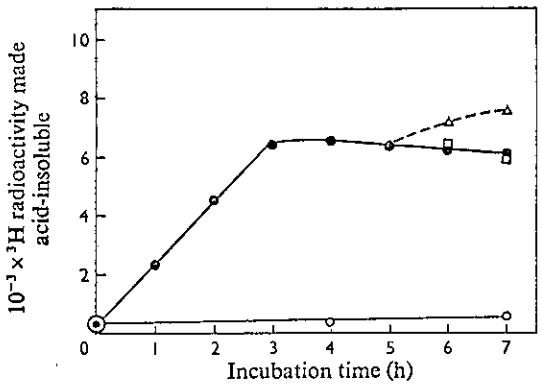

Fig. 4. *Time-course of methylation*

The reaction mixtures had the same composition as the complete system in Table 3, except that 4 μg of bacteriophage 82.C600 (P1 $r_{P1}^-m_{P1}^-$) DNA (o) or bacteriophage 82.C600 (P1 $r_{P1}^-m_{P1}^+$) DNA (○) was used. Reaction mixtures were incubated at 30°C for the times indicated and then assayed for acid-insoluble methyl groups as described in the Materials and Methods section. To one set of reaction mixtures (△) 3 μg of bacteriophage 82.C600 (P1 $r_{P1}^-m_{P1}^-$) DNA was added after 5h; to another set (□) a further 25 μl of enzyme was added. All tubes had a blank value of 129c.p.m. subtracted for an incubation without enzyme assayed at 0min. No attempt has been made to correct for any fluctuation of this blank with time of incubation.

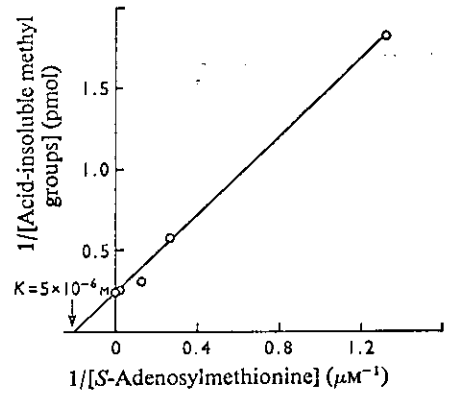

Fig. 5. *S-Adenosyl-L-methionine dependence of methylation*

The reaction mixtures contained, in a volume of 150 μl, 0.05M-2-(*N*-morpholino)ethanesulphonic acid, pH6.0, 0.2mM-EDTA, 5mM-2-mercaptoethanol, 7.5 μg of bacteriophage 82.C600 (P1 $r_{P1}^-m_{P1}^-$) DNA, 50 μl of enzyme and [*methyl*-^{3}H]-*S*-adenosyl-L-methionine adjusted with the required amount of unlabelled *S*-adenosyl-L-methionine to give the concentrations shown in the figure. After 30min incubation at 30°C the reaction mixtures were assayed for acid-insoluble methyl groups as described in the Materials and Methods section. Each result was corrected for a blank incorporation performed in the absence of enzyme.

divided into three equal portions, which were incubated with endonuclease R.P (Fig. 7*a*), or endonuclease R.K (Fig. 7*b*) or without enzyme (Fig. 7*c*). The activity of the endonuclease R.P was checked by incubating it with an equivalent amount of ^{3}H-labelled bacteriophage λ.C DNA and ^{32}P-labelled bacteriophage λ.K(P1) DNA that had been subjected to the same protocol of incubation and phenol extraction, except that *S*-adenosyl-L-methionine was omitted (Fig. 7*d*). All reaction mixtures were analysed by neutral sucrose-gradient centrifugation in buckets on the same rotor (see Fig. 7 and legend). The near-superimposability of the ^{3}H and ^{32}P distributions in Fig. 7(*a*) and 7(*c*) shows that the ^{3}H-methylated DNA had not been significantly degraded by incubation with endonuclease R.P. The ^{3}H-methylated DNA was, however, broken by the heterospecific endonuclease R.K (Fig. 7*b*), and in the control incubation mixture (Fig. 7*d*) the ^{3}H-labelled λ.C DNA was also broken, showing that the endonuclease R.P was active. The nearly identical distribution of ^{32}P in all four gradients shows that all breakage was specific for unmodified DNA. This experiment demonstrates the role of DNA methylation in protection against the P1 restriction enzyme.

Discussion

Three lines of evidence identify the methylase as the bacteriophage P1 modification enzyme: (a) bacteriophage 82 DNA that has been modified by bacteriophage P1 *in vivo* is not methylated, whereas unmodified DNA is a substrate for methylation; (b) methylation of bacteriophage λ DNA protects against breakage by purified restriction enzyme; (c) the activity is found in the dialysed $(NH_4)_2SO_4$ fraction prepared from *E. coli* 1100 infected with bacteriophage P1, but is absent (<10%) on infection with bacteriophage P1 m_{P1}^- (P. R. Brown & J. P. Brockes, unpublished work).

The product of the activity, 6-methylaminopurine, is also the product of *E. coli* B modification both *in vivo* (Smith *et al.*, 1972) and *in vitro* (Kühnlein & Arber, 1972), and of *E. coli* K modification *in vitro* (J. P. Brockes, unpublished work). 6-Methylaminopurine has been reported as the product of the bacteriophage T_4-induced methylase that protects

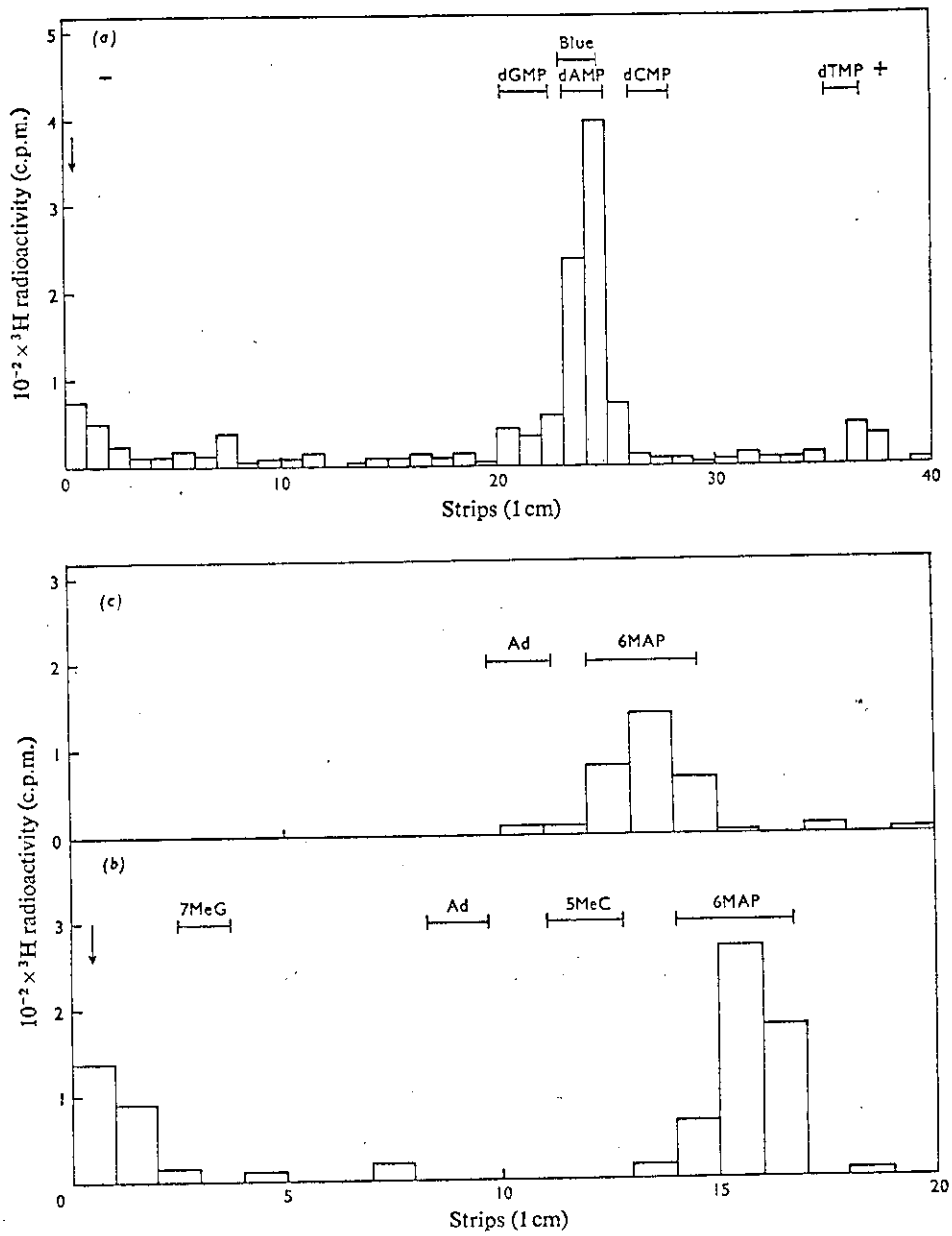

Fig. 6. *Analysis of the product of DNA methylation*

(*a*) Mononucleotide analysis of methylated bacteriophage 82 DNA on aminoethylcellulose paper (see the Materials and Methods section). 'Blue' = Xylene Cyanol FF marker dye. (*b*) and (*c*) Base analysis of methylated bacteriophage 82 DNA by paper chromatography (see the Materials and Methods section) on butan-1-ol–water (NH_3 atmosphere) then methanol–water–HCl. The material remaining at the origin (indicated by an arrow) after the butan-1-ol–water system was eluted with 0.1 M-HCl and hydrolysed with 1 M-HCl at 100°C for 2h. On re-chromatography more than 90% of the radioactivity remained at the origin. This material was also found in the hydrolysate of a reaction from which the DNA was omitted and hence is not a product of DNA methylation. Methylated DNA for both analyses was obtained by incubating 4μg of bacteriophage 82 DNA for 4h at 30°C in the complete system described in Table 2. 7MeG, *N*-7-methylguanine; Ad, adenine; 5MeC, 5-methylcytosine; 6MAP, 6-methylaminopurine.

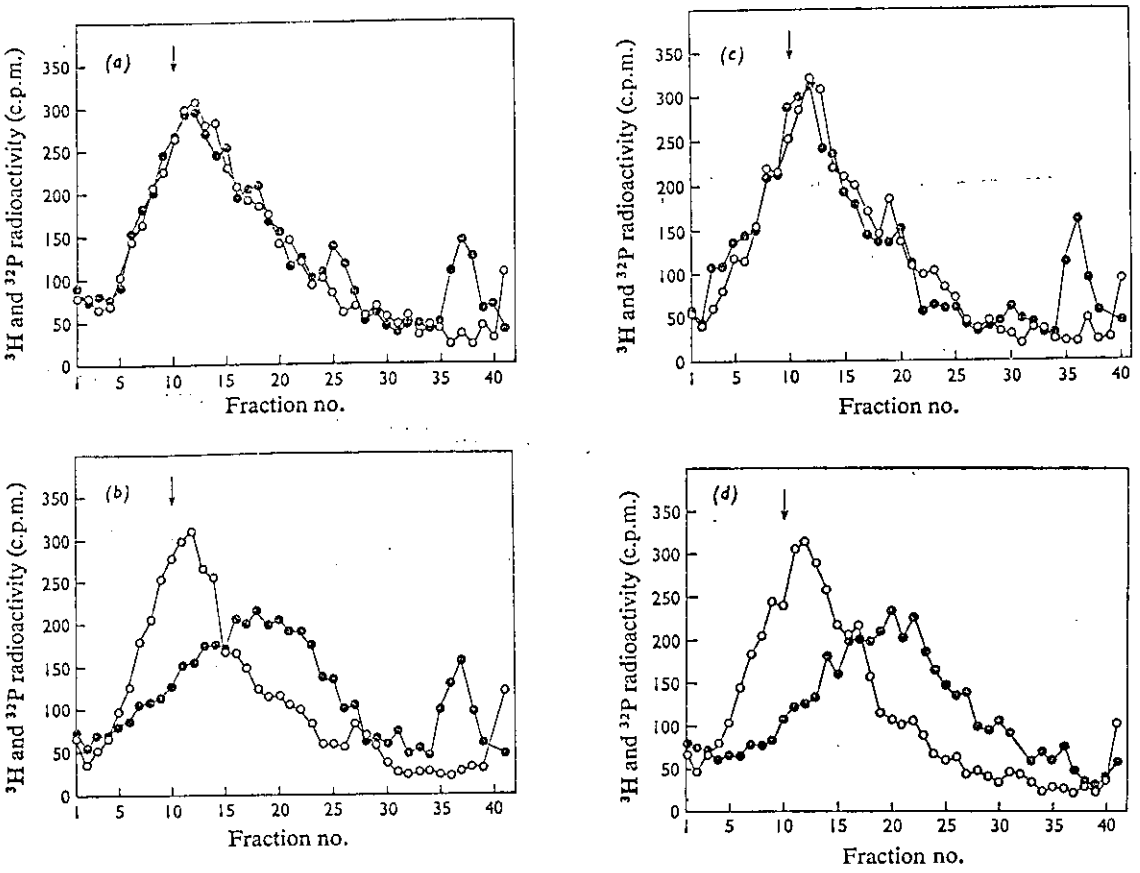

Fig. 7. *Zone sedimentation in neutral sucrose gradients of the products of restriction endonuclease reactions with modified bacteriophage λ DNA*

Methylation reactions. Reaction mixture 1 (350 μl) contained 0.05 M-2-(*N*-morpholino)ethanesulphonic acid, pH 6.0, 0.25 mM-EDTA, 5 mM-2-mercaptoethanol, 4 μM-[*methyl*-^{3}H]-*S*-adenosyl-L-methionine (8.9 Ci/mmol), 20 μg of bacteriophage λ.C DNA, 2×10^4 c.p.m. of ^{32}P-labelled bacteriophage λ.K(P1) DNA and 225 μl of enzyme. Reaction mixture 2 (115 μl) contained 6.0 μg of bacteriophage λ.C DNA, 6×10^3 c.p.m. of ^{32}P-labelled bacteriophage λ.K(P1) DNA and 5.5×10^3 c.p.m. of ^{3}H-labelled bacteriophage λ.C DNA (1 μg), 75 μl of enzyme in addition to 2-(*N*-morpholino)ethanesulphonic acid, EDTA and mercaptoethanol as for reaction mixture 1. After incubation for 3.0 h at 30°C, both reactions were extracted three times with an equal volume of freshly distilled phenol. The aqueous layers were dialysed twice against 1 litre of 10 mM-tris–HCl–0.1 mM-EDTA–0.4 M-NaCl, pH 7.4, then twice against 1 litre of the same buffer without NaCl. After both samples had been kept at 60°C for 10 min to dissociate concatenates, 10 μl of reaction mixture 1 (volume after dialysis 510 μl) was analysed by acid hydrolysis followed by t.l.c. (see the Materials and Methods section). The volume of reaction mixture 2 after dialysis was 154 μl.

Restriction reactions. (*a*) (Challenge with endonuclease R.P.) The reaction mixture (360 μl) contained 0.1 M-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid, pH 8.0, 1.2 mM-EDTA, 8 mM-$MgCl_2$, 60 mM-2-mercaptoethanol, 2.5 mM-ATP, 10 μM-*S*-adenosyl-L-methionine, 150 μl of DNA from reaction mixture 1 (5500 c.p.m. of ^{3}H and 6300 c.p.m. of ^{32}P), 150 μl of endonuclease R.P. (*b*) (Challenge with endonuclease R.K.) The reaction mixture (360 μl) contained DNA, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid, EDTA, $MgCl_2$, 2-mercaptoethanol and ATP as for (*a*), but 20 μM-*S*-adenosyl-L-methionine and 100 μl of endonuclease R.K instead of endonuclease R.P. (*c*) (No restriction enzyme.) As (*a*) except that water (150 μl) replaced endonuclease R.P. (*d*) (Endonuclease R.P control.) As (*a*) except that 150 μl of DNA from reaction mixture 2 (5200 c.p.m. of ^{3}H and 6000 c.p.m. of ^{32}P) replaced DNA from reaction mixture 1.

All reaction mixtures were incubated at 30°C for

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20min; reactions were terminated with 10μl of 0.5M-EDTA, pH8.0, and the mixture was layered on 6–20% (w/v) sucrose gradients in 0.01M-tris–HCl–1mM-EDTA–0.04% (w/v) sodium dodecyl sulphate, pH7.4 (volume 4ml). After centrifugation (MSE Superspeed 65) in a 6×5ml swing-out rotor at 50000rev./min for 130min at 20°C, 5 drop fractions were collected into vials and counted in a Beckman liquid scintillation spectrometer after the addition of 1.5ml of scintillant (30g of naphthalene, 2g of 2,5-diphenyloxazole, 0.1g of dimethyl 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, 50ml of methanol and 500ml of *p*-dioxan). The arrows in (*a*)–(*d*) indicate the position of sedimentation of whole molecule ^{32}P-labelled bacteriophage λ.K (P1) DNA and ^{3}H-labelled bacteriophage λ.C DNA. The small peak of ^{3}H at the top of the gradient in (*a*), (*b*) and (*c*) is [*methyl*-^{3}H]-*S*-adenosyl-L-methionine remaining after dialysis. Sedimentation was from right to left. ●, ^{3}H; ○, ^{32}P.

unglucosylated bacteriophage T4 DNA *in vivo* against bacteriophage P1 restriction (Hattman, 1970). For reasons that are unclear, bacteriophage P1 modification does not operate *in vivo* against bacteriophage P1 restriction in this latter system (Revel & Luria, 1970).

The rather slow kinetics of methylation (Fig. 4) were also observed with *E. coli* B modification activity on bacteriophage fd DNA (Kühnlein & Arber, 1972). In collaboration with Dr. R. Yuan we have been studying the binding of the bacteriophage P1 modification enzyme to bacteriophage λ DNA by the filter-binding method (Riggs & Bourgeois, 1968; Yuan & Meselson, 1970). This technique indicates that in the presence of *S*-adenosyl-L-methionine the enzyme binds to both its substrate and its product (i.e. to both modified and unmodified DNA) and is thus released very slowly after methylation (R. Yuan, unpublished work). This effect may well provide a basis for the slow kinetics.

The investigation of the nucleotide sequences around the methylated base, and of the structure of the enzyme, may provide some insight into the recognition reaction.

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