SUMMARY

The effects of certain alterations in cellular metabolism on the potency and specificity with which mutations were induced were measured. Two systems were chosen for this study, namely the *trp* operon of *E.coli* and the *r* system of bacteriophage T4.

1. An attempt was made to determine whether the induced mutability of a locus was affected by its state of activity. A number of *trp* auxotrophs of *E.coli* were tested for their reversion response to a number of different mutagens. Two mutants were found to respond strongly to EMS and these alleles were chosen to measure the frequency of reversion induced by EMS when the operon was in a state of derepression and when it was repressed. It was found that for both alleles the induced reversion frequency was greater when the operon was derepressed during EMS treatment but that the state of the operon's activity immediately after the mutagenic treatment had no effect on induced reversion frequency.

2. The effects of various ancillary treatments on the specificity of 5-BU and NA mutagenesis in the T4*r* system were examined.

a). A preliminary study to determine whether the pH at which NA mutagenesis was carried out affected the spectrum of induced rII's and specifically whether pH influenced the frequency with which NA induced deletion mutants. Although the lethal action of NA was found to be strongly pH-dependent, there being much more rapid killing at lower pH's, it was found that NA induced rII deletions at equal frequencies when the treatment was performed at pH 3.7 and at pH 4.0 and that the intragenic rII spectra induced by NA at the

Mutagen Specificity and Potency in Micro-organisms

by

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two pH's were essentially the same.

b). The effects of the antibiotic amino acid analogue, PFPA, on 5-BU and NA mutagenesis in T4 were measured. Under standard conditions for 5-BU mutagenesis, in the presence of PFPA in the pre-treatment growth medium of the host and to a lesser extent in the mutagenic treatment medium, T4 burst size was doubled and 5-BU mutagenesis of both forward and reverse mutations was depressed compared to when PFPA was absent or when PA was substituted for PFPA. A constituent of standard 5-BU treatment medium is the folate antagonist, SU. If another folate antagonist, TM was used in the treatment medium or if no antagonist was used at all, it was found that PFPA resulted in a decreased burst size but it had no effect on 5-BU-induced mutation frequencies. Various models to account for this apparent PFPA-mediated relief of the inhibition of SU were put forward and discussed.

Using NA as a mutagen, it was found that when extracellularly mutagenized T4r phage were used to infect E.coli grown in the presence of PFPA, the induced r frequency was approximately double that found when PFPA was absent. This increased frequency was not accompanied by a change in specificity as measured by the classes of mutations induced and the rII spectra of both point and deletion mutants.

3. Another antibiotic, SM, was used in conjunction with the mutagens 5-BU and NA. In preliminary experiments it was found that when T4 infected E.coli grown in the presence of low concentrations of the drug, adsorbtion was impaired and many infective centres failed to develop. If however, T4 was added at the same time as SM to E.coli, adsorbtion and phage viability were not affected but

the burst size was reduced.

When T4r^+ were treated with 5-BU in the presence of SM or when NA-treated T4r^+ were used to infect E.coli in the presence of SM, no effect in potency or specificity of either mutagen could be demonstrated.

4. A preliminary investigation of a mutant strain of T4, designated hm, which had been shown to have effects on mutation rates was carried out.

Compared to the wild type strain (T4hm^+) the T4hm strain was more resistant to the lethal action of NA and HA, but displayed normal sensitivities to UV, EMS, bisulphite and high temperature.

Reversion analyses on various rII mutants in the hm and hm^+ genetic backgrounds demonstrated that NA, 5-BU and 2-AP were all more potent in reverting rII's in the hm strain but that EMS, HA and bisulphite reversion frequencies were unaffected by the allele at the hm locus. It was found that the DNA repair capabilities of the host bacteria affected neither the survival nor the reversion frequencies of the rII's in both T4hm and T4hm^+ strains after the phage had been treated with any of the agents used in this study.

NA-induced forward mutation frequency was found to be about twice as high in T4hm and as in T4hm^+, but the spectra of induced r mutants were the same in both strains.

The spontaneous r frequency of hm was also higher than in hm^+ and there were also some differences in the rII spectra of spontaneous mutants in the two strains.

It was shown that all the alterations in phenotype in the hm strain compared to the hm^+ strain were probably due to a single

mutation.

The possible nature of the hm mutation was discussed.

INDEX

ABBREVIATIONS

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
AT	Adenine-Thymine Base Pair
GC	Guanine-Cytosine Base Pair
U	Uracil
2-AP	2-Aminopurine
5-BU	5-Bromouracil
BUdR	5-Bromodeoxyuridine
DEB	Diepoxybutane
e.o.p.	efficiency of plating
EDTA	Ethylene Diamine Tetracetic Acid
EMS	Ethyl Methanesulphonate
5-FU	5-Fluoruracil
HA	Hydroxylamine
5-HMC	5-Hydroxymethylcytosine
IP	Indole-3-Propionic Acid
LTS	Low Titre Stock
MM	Minimal Medium
MMS	Methyl Methanesulphonate
m.o.i.	multiplicity of infection
NA	Nitrous Acid
NTG	N-Nitro-N-Methyl-N-Nitrosoguanidine
PA	Phenylalanine

PABA	p-Aminobenzoic Acid
PFPA	p-Fluorophenylalanine
p.f.u.	plaque forming units
SM	Streptomycin
SU	Sulphanilamide
SUM	Sulphanilamide Medium
TM	Trimethoprim
TMM	Trimethoprim Medium
UV	Ultraviolet

INTRODUCTION

General Introduction

The science of Genetics may be defined as the study of mutant organisms. It is not surprising therefore to find that attempts to unravel the mechanisms whereby these tools of the trade are generated have been, and continue to be, a major aspect of research in genetics.

Apart from the cases of mutation mediated by cortical inheritance reported for some Protozoa (see for example Sonneborn 1964), the origin of a mutant form demands a heritable alteration of the genetic material, which on this earth means a change in the structure, or more specifically the sequence of nucleic acid.

Since the time of the first reports of physical (X-rays: Muller 1927) and chemical (sulphur mustard: Auerbach and Robson 1946) mutagens, a whole galaxy of such agents is now available exerting their mutagenic powers by a variety of diverse mechanisms.

It might well have been thought that by the early sixties the study of mutagenesis was over bar the shouting for, by this time, thanks mainly to the work of Benzer, Freese, Crick and Brenner, there existed an elegant classification of microlesion mutations *viz* transitions, transversions and frameshifts. The classification of macrolesions had of course already been made.

Not only had the analysis of the types of mutation been done, but also models which could explain why particular mutagens favoured the production of specific classes of mutations on the basis of the mutagens' chemical actions on the nucleic acid were being put forward.

Although the correctness of the classifications and the essence of the mechanisms by which a substantial number of mutagens act are not now in doubt, there is a large body of evidence which indicates that there is a great deal more to mutagenesis than can be elucidated by a study of the strictly chemical reactions between DNA and mutagens.

To a large degree such evidence comes from studies of mutagen specificity. There are many examples of such specificity which simply cannot be attributed to the specificity of action of the mutagen at the level of the DNA, the implication being that cellular processes have a role in the generation of mutations.

Freese's finding (1959a&b) that classes of T4rII mutations induced to revert by base analogues and by proflavine were mutually exclusive is certainly due to the fact that the base analogues induce base pair substitutions, while proflavine promotes the generation of frameshifts. Thus generation of mutagen specificity is not the exclusive prerogative of mechanisms mediated by cellular processes.

Particularly in the case of induced reverse mutation, where as a rule a specific mutational change is required, the most obvious interpretation of a finding of mutagen specificity, would be in terms of differential revertibility by the mutagens under study of the specific molecular classes of mutation at the different mutant alleles. Nevertheless several cases of mutagen specificity in reverse mutation studies show that explanations in terms of molecular specificities are inadequate.

Probably the most intensively studied reverse mutation system

has been Kølmark's (1953) K3/17 diauxotrophic adenine$^-$ inasitol$^-$ strain of Neurospora crassa. A consideration of some of this work will indicate some of the ways in which cellular metabolism can be manipulated to produce changes in mutagen specificity.

The ratio of adenine to inositol ($^a/_i$) revertants has been measured after treatment with a wide variety of mutagens, both chemical and physical (Auerbach and Ramsay 1968). Not only do different mutagenic treatments result in different $^a/_i$ ratios, but modification of certain parameters concerning the nature of the mutagenic treatment can alter the specificity of a particular mutagen.

For example Kilbey (1963 & 1969) found that the UV-induced $^a/_i$ ratio was depressed when the temperature of the mutagenic treatment was increased and that a similar reduction was found when the dose of the treatment was increased.

The two alleles also react differently to minute traces of chemical mutagens which remain after the mutagenic treatment proper. Traces of DEB, ethylene oxide and nitrosoethyl urethan all act specifically on the adenine locus (Kølmark and Auerbach 1960; Kølmark and Kilbey 1962; Kilbey and Kølmark 1968; Auerbach and Ramsay 1973a&b).

There have also been studies on this system which employed the joint treatment of more than one mutagen. Auerbach and Ramsay (1970 & 1972) have shown that for certain mutagen combinations the frequency of adenine$^+$ revertants is greater than the additive effects of the individual agents, while for inositol$^+$ revertants the induced frequency may be less than additive.

Recently Kilbey (1973a&b) has shown that the kinetics of DEB-induced reversion at the adenine locus, and the loss of sensitization of adenine^{+} reversion to UV by prior low-level DEB treatment could be modified by the addition of the inhibitor of protein synthesis, actidione.

Although the extensive study of this system has shown unequivocally that mutagen specificity can be generated independently of the molecular specificity of the mutagen, there has been no adequate explanation to account for the way in which the modulation of mutagen specificity could occur.

Other studies on reverse mutation systems have shown that the constitution of the post-mutagenic plating medium can affect mutagen specificity. For example, addition of amino acids, which at first sight would appear to be quite unrelated to the locus under scrutiny, has been shown to have marked effects on the frequency of recovery of mutants at specific loci (Clarke 1962; Queiroz 1973). Addition of inorganic ions to the plating medium has also been shown to affect mutagen specificity (Arditti and Sermonti 1962; Loppes 1970).

Chopra (1967) has found that the presence of an adenine^{-} allele in E.coli depressed the frequency with which UV induced reversion of a tryptophan auxotroph. In Bacillus subtilis, Corran (1969) found a complex interaction effect in which the spontaneous reversion of a histidine^{-} mutant required the joint presence of threonine in the plating medium and a threonine^{-} marker in the genetic background.

Unlike all the examples cited above there is at least one

system where the effect of genetic background and of the constitution of the plating medium on mutant induction has a cogent explanation. Independently, Clarke (1973) and Skavronskaya et al. (1973) found that the UV-induced recovery of ochre suppressors of a tryptophan⁻ allele in E.coli was reduced in SM-resistant strains but that the depressive effect could be relieved by the addition of SM to the plating medium. As will be discussed later, SM-resistant ribosomes restrict the efficiency of suppression but the efficiency can be enhanced by addition of SM.

Mutagen specificity is not however restricted to reverse mutations. In forward mutation, the contribution of the specificity of the changes induced in the DNA by one given agent to the generation of intergenic specificities must, as a rule, be minimal. If for example mutations from prototrophy to auxotrophy are to be measured, under the conditions used for the initial isolation, the gene product should be entirely dispensable. Not only is there the opportunity for mutation at many different sites within a single locus, but the same initial phenotype may be obtained by mutations at quite separate loci.

I know of only two examples where the generation of intergenic forward mutagen specificities can be interpreted on the basis of the specificity of action of a mutagen at the level of DNA.

The first was the finding that SM-resistant mutations in E.coli, although readily obtained after mutagenesis by alkylating agents, could not be induced by the frameshift-inducing mutagen, ICR-191 (Silengo et al. 1967). The explanation for this is that if the ribosomal protein which is the target of SM is completely inactivated

(as would be the case for the great majority of frameshift mutations) the cell is inviable. Only those agents which induce base pair substitutions and cause specific changes in the amino acid sequence of the protein which render it resistant to SM but do not completely destroy its activity will therefore be effective in inducing such mutants.

In a way akin to this is the fact that in bacteriophage T4, when a mutagen which induces base pair substitutions is used to generate r mutants the majority of such mutants are rI's. Mutagens which induce frameshifts, such as proflavine, and spontaneously-arising r mutants (the majority of which are frameshifts) show a preponderance of rII's. This fact is interpreted, though there is no direct evidence for the hypothesis, as being due to the fact that more amino acid substitutions can occur in the rII gene product without causing a detectable change in phenotype than in the rI protein. The great majority of frameshift mutations will completely inactivate the protein and hence the tolerance of the proteins to amino acid substitution is no longer a factor (Drake 1970).

Other cases of forward mutation specificities cannot be accommodated into a rational framework as readily as these two examples.

Zetterberg (1961 & 1962) found that UV failed to induce any histidine auxotrophs of Ophiostoma when the fungus was plated on complete medium after irradiation, Nitrosomethylurethan on the other hand induced histidine-requirers quite readily. On closer examination it was found that if, after UV treatment, plating was done on minimal medium supplemented with histidine, UV could induce

histidine-requirers quite efficiently. This was a case of a plating medium effect for forward mutations.

Recently it has been found that in the Basidiomycete, Ustilago, the frequencies with which a large range of different auxotrophs were induced by UV were enhanced when the organisms were starved of inositol (Thomas 1972), and in addition the spectra of the different mutations induced by UV alone and UV combined with inositol starvation were quite different.

At a different level a striking example of forward mutation intragenic specificity has been demonstrated. The distribution of mutations within the rII locus of T4 was shown to be highly non-random and the spectra obtained after different mutagenic treatments were quite distinct (Benzer 1961). This topic of intragenic mutagen specificity is a major part of this work and will be discussed more fully later.

This catalogue of reports of mutagen specificity did not pretend to be a comprehensive one but was meant simply to demonstrate that the phenomenon exists and that the influences of cellular physiology may be complex. For a fuller exposition of mutagen specificity in eukaryotes, see the review by Auerbach and Kilbey (1971).

It is, on reflection, not too surprising that cellular processes have a role in the determination of mutagenic potency and specificity since the expression of mutants does not occur in a vacuum but in a cellular environment; and this applies even in cases where extracellular phage or naked DNA are mutagenized *in vitro*.

It should also be always borne in mind that mutagens are by definition biologically active agents and that it is not surprising that their effects on organisms need not be restricted to their attack on the genetic material. Only when pure DNA is mutagenized can one be sure that this complication will be avoided. Related to this is the fact that the interaction between mutagen and DNA can itself trigger off changes in cellular physiology not directly connected with the mutagenic powers of the particular agent.

The catalogue of reports pertaining to such effects of mutagens is legion and only a sample of the more recent ones and those with special reference to the mutagens used in this study or else those which are pertinent to questions of mutagen specificity will be enumerated below.

Even bacteriophages treated extracellularly with mutagen are not immune from the action of mutagens or non-genetic material. Protein-DNA crosslinks have been shown to be induced after coliphage have been treated with UV (Yamada et al. 1973), NA (Dussault et al. 1970), and O-methylhydroxylamine (Tikchonenko et al. 1973).

Since virtually all mutagens act by directly causing changes in the nucleic acid of the genetic material, it is not surprising to find that there is a tendency for them also to attack other nucleic acids involved in the transcriptional and translational machinery.

For example in an exhaustive investigation of the effects of UV on various components of the protein-synthesizing apparatus of wheat, it was found that mRNA, tRNA and ribosomal RNA were all inactivated (Murphy, Kuhn and Murphy 1973). UV also inactivates

some of the enzymes involved in nucleic acid metabolism; e.g. ribonuclease (Aktipis and Iammartino 1971), DNA polymerase (Sauerbier et al. 1970).

When BUdR is incorporated into bacteriophage lambda DNA, the substituted DNA is some seven times less efficient as a template for transcription as is untreated DNA (Jones and Dove 1972).

BUdR has also been shown to suppress differentiated function in various tissue types. Since such suppression is reversible it cannot be due to mutations induced by the base analogue though it has to be incorporated into the DNA to be effective and the cause of the suppression has been postulated as being due to the inhibition of transcription of the substituted DNA. In hepatoma cells BUdR inhibited the production of some enzymes but not of others (Stellwagen and Tomkins 1971) and it specifically inhibited pigment synthesis in melanoma cells (Wrathall et al. 1973). These two examples illustrate intergenic specificities mediated by a mutagen wearing its non-mutagenic hat.

There is a report of an interactive effect between UV and another halogenated analogue of thymidine, IUdR, in which it was shown that UV-induced inactivation of thymidine kinase was greater when IUdR rather than thymidine was used as substrate. The explanation is that the UV induces photolysis of the analogue and that the resultant free radical disturbs the specificity of the enzymes' active site (Cysyk and Prusoff 1972).

HA which is a highly specific mutagen when applied to phage extracellularly (inducing C → T transitions exclusively) has however the unusual ability to suppress certain rII mutants when

administered to the phage in the intracellular state (Levisohn 1967 & 1970). The classes of mutants which were genotypically and phenotypically reverted by HA were mutually exclusive. The nature of the mechanism of suppression is not really known but a reasonable explanation is that HA induces compensatory changes in the base sequence of the mRNA opposite the mutant site.

It has been demonstrated that methoxyamine, which is closely related to HA, can act on tRNA, converting cytosine residues to uracil (Cashmore, Brown and Smith, 1971).

At a somewhat different level it has been found that HA can inhibit electron transport in Chlorella (Katch et al. 1970).

The case of HA is a good one for illustrating the importance of cellular processes in the modification of mutagen specificity. In contrast to the high specificity with which the C → T transition is induced when phage are mutagenized extracellularly, when phage S13 is treated intracellularly all four transitions are induced (Tessman, Ishiwa and Kumar 1965). Indeed it was found that pretreatment of the host with HA was sufficient to induce phage mutations, but the mechanism of this type of mutagenesis is not known. This type of host-mediated mutagenesis of phage is not restricted to HA, but has also been found with UV (Jacob 1954) and NTG (Kondo and Ichikawa 1973; quoted in Kondo 1973).

NA, another mutagen used extensively in this work has been shown to induce drastic changes in the mitotic apparatus of Vicia fabia which are probably not due to the agents mutagenic action (Boar et al. 1972).

It might be appropriate to mention here some mutagens whose

actual mutagenic abilities may be solely mediated by their effects on non-genetic material.

Amino acid analogues have been found to be mutagenic in Ustilago (Lewis and Tarrant 1971). It was postulated that the analogues were incorporated into such enzymes as DNA polymerase and hence altered their specificity causing increased inaccuracies in DNA replication.

A similar explanation may account for the mutagenicity of divalent manganese. This ion has been known to be mutagenic in E.coli for over twenty years (Demerec and Hanson 1951) and in T4 it has been shown to induce mainly transitions (Orgel and Orgel 1965), but still the mechanism of its action is unknown. However, Mn^{2+} has been shown to alter the specificity of DNA polymerase (Berg et al. 1963) and the hypothesis that the ion's mutagenic property depends on its causing a reduction of the fidelity of DNA polymerase is an attractive one. It is perhaps surprising that it has not been so far tested.

For a mutation to be scored, whether it be concerned with eye colour in Drosophila, growth requirement in E.coli or plaque morphology in T4, or whatever, the mutation is not recognized by a change in the DNA sequence and less still by the presence of a premutational lesion in the nucleic acid, but by the final manifestation of such changes in a living, growing organism. This is an obvious statement of fact but it does emphasize the importance of cellular metabolism in the production of what we see as a mutation. The initial lesion must be replicated, transcribed and translated for the end result to be scored. Auerbach (1969) has graphically

described such steps in the pathway from premutational lesion to the fully fledged newly mutant individual as mutational sieves at which the potential may exist for various cellular systems to act differentially on the mutational pathways and hence mutagen specificity may be generated.

When this allegorical description was first put forward by Auerbach, it was in response to the results obtained in the sort of approaches to mutagen specificity which were illustrated above, where the nature of the ancillary treatments (i.e. genetic background, plating medium and treatment by a second mutagen) may have had general, and in nearly all cases, uncharacterized effects on cellular metabolism. The use of the metaphor is therefore more in terms of a conceptual framework than as a precise model for any particular example of mutagen specificity.

There is now however an increased interest in the detailed examination of the specific roles of certain enzymes in the generation of mutations, both spontaneous and induced. Most of the studies on comparative mutation rates and the specific classes of mutations which arise have been done in T4 and E.coli, using strains which are deficient and proficient in a step in the general area of DNA metabolism. The terms of reference of such an approach are pitched at a less empirical level than those of the examples already cited.

A large number of different systems involving DNA repair and synthesis have been shown to play a part in the production of mutations and in some cases an understanding of the mechanisms involved is available. As will be illustrated throughout the course of this

thesis, this approach has already yielded dividends in the elucidation of some of the complexities of mutagenesis. Whether the examples of mutagen specificity discussed above will be able to be interpreted on the basis of modification by the ancillary treatments of the activity of enzymes concerned with DNA metabolism or whether qualitatively different systems are the culprits in the determination of this type of mutagen specificity remains to be seen.

The work in this thesis was concerned with two aspects of ancillary treatments on mutagen specificities, both of which, in their different ways, took a somewhat more refined approach to the problem than was used in most of the studies described above.

The first was a comparative study of induced mutation in the trp operon of E.coli which depended on the ability of the ancillary treatment to cause a highly specific change in metabolism viz. repression or derepression of the operon. This was in contrast to the more general effects of the ancillary treatments on cellular metabolism which occurred in the examples of mutagen specificity already given.

The second approach used the r system in bacteriophage T4, and was an attempt to see if the ability of cellular processes to modify mutagen specificity was confined to intergenic specifities, or whether their influence could extend into the realm of intragenic mutagen specificities. Here the fine control was not on the specificity of action of the ancillary treatment, but rather it was the specificity of the induced mutants which was subjected to more rigourous analysis than is usual in mutagen specificity studies.

A description of the systems used in this work will now be

given.

The Systems Used

a). The Tryptophan (trp) Operon of E.coli

For reasons which will be apparent, the work on the trp operon was terminated prematurely, and the section pertaining to this work will to a large extent be considered independently. Accordingly the introduction to the trp operon, along with the results and the discussion of the findings in this system are presented in a single section (Chapter 1 in Results).

b). The r System in Bacteriophage T4

The r loci of T4, and in particular the rII locus have been studied more actively perhaps than any other system in genetics. This intense study has yielded rich rewards in our fundamental understanding of the nature and the organization of genetic information. Thanks to it, such words as cistron, codon, frameshift and suppressor have become commonplace in the vocabulary of geneticists. The rII locus is a genetic system par excellence in that the information gleaned from it has been obtained despite an almost complete lack of understanding of the nature and function of the rII gene product.

Before considering the aspects of the r system which are of particular relevance to mutation studies, I shall briefly review what is known about this rather remarkable system from a physiological point of view.

One of the most obvious, and the first to be noticed (in 1929, according to Adams (1957)) phenotypes of r mutants is the alteration

in plaque morphology. Wild type T4 have plaques which are small and which have turbid haloes around the edges. The r plaques are larger and have sharp edges. The reason for this difference is connected with the phenomenon known as lysis inhibition. When an E.coli cell is infected with T4r^+ and is later subjected to further infection the latent period is greatly extended and the yield of phage at the end of the infectious cycle is greatly elevated (Doermann 1948). Such lysis inhibition does not occur in r (r stands for rapid lysis) mutants (Hershey 1946). The halo around r^+ plaques is due to the proportion of bacteria which have not been lysed, due to lysis inhibition.

Rapid lysis mutants arise at several loci, scattered around the T4 genome (Hershey and Rotman 1948; Doermann and Hill 1953). Mutants at the different r loci can be most readily distinguished on the basis of plaque morphologies on different strains of E.coli. The three classes of r mutants considered in this work (rI, rII and rIII) all give rise to r plaques on E.coli B. On E.coli BB, rI's alone make r plaques, the other two being characterized by wild type plaques on this strain. On E.coli K-12(λ) (or any other strain carrying a lamboid lysogen), rI's have r plaques, rIII's have r^+ plaques and rII's fail to develop (Benzer 1955). Benzer (1957) also showed that the rII region comprised two adjacent cistrons, rIIA and rIIB.

The studies on the physiological effects of r mutants have shown that they cause a bewildering array of different changes of phenotype.

Certain mutants of T4 (designated z) adsorb poorly to E.coli

due to disorientation of the tail fibres (Rutberg 1969 & 1970). The inability of these mutants to adsorbed is greatly relieved when any r mutation is introduced into the T4 genome which carries the z mutation (Rutberg 1970 & 1971).

The altruism shown by r mutants towards their tailless brethoren does not stop there. Equally unfortunate T4 which have mutations in gene 30 (DNA ligase) are also rescued by rII mutations (Karam 1969; Ebisuzaki and Campbell 1969; Berger and Kozinski 1969; Karam and Barker 1971). This relief by rII mutants is mimiced when DNA ligase mutants are grown in the presence of chloramphenicol (Kozinski and Mitchell 1969; Hosoda and Mathews 1971). To account for this similarity in the suppression by rII's and by chloramphenicol, it has been proposed that the rII gene product may affect DNA endonuclease activity by direct or indirect means (Warner 1971; Karam and Baker 1971).

Indeed it has been found that certain E.coli strains, when infected with rII mutants possess less nuclease than when infected with T4r^{+} (Rutberg and Rutberg 1966). With less nuclease, there will be fewer gaps in the DNA to be repaired by ligase. It has been shown that the suppression of gene 30 mutants by rII's requires that the host ligase activity be intact (Gellert and Bullock 1970; Krisch, Shah and Berger 1971), and it has been suggested that in the rII background, the host ligase is sufficient to join up any gaps in the DNA.

More recently another rapid lysis mutant, called m, has been isolated and characterized. This mutation also suppresses T4 DNA ligase mutants, and although it maps near rII, and clearly shares

some of the properties of its eminent neighbour, the two mutants are not identical. For instance high Mg^{2+} concentrations overcome the suppression of gene 30 mutants by rII's (Koch 1973). This effect with Mg^{2+} is not found with the m mutation (Chan et al. 1970; Chan and Ebisuzaki 1973).

The nature of the suppression of gene 30 mutants by rII's assumes greater complexity with the finding that an independent T4 mutation (su30) can enhance the suppression though it cannot itself suppress ligase mutants (Krylov 1972).

One other aspect of the interaction between gene 30 and rII mutants is that the recombination frequency of such double mutants is enhanced compared to wild type (Krisch, Hamlett and Berger 1972).

One more piece of indirect evidence which points to the influence of the rII gene on endonucleolytic activities was reported by Carlson and Kozinski (1969). They found that the transfer of parental DNA to the progeny was very irregular in rII mutants, unlike the case for r^+ phage where parent-to-progeny transfer was very regular (Kozinski and Kozinski 1963; Shahm and Kozinski 1966).

Yet another T4 mutation can be suppressed by rII (but not rI or rIII) mutants. Mutants of the t gene were isolated by Josslin (1970). Such t mutants fail to terminate metabolism at the normal lysis time, and although phage are produced within the cell, no lysis occurs. Double mutants (t rII) do lyse at the normal time (Josslin 1971).

Lest it be thought that the philosophy of the rII locus is all give and no take, it should be pointed out that a locus (si^+) has been identified and mapped. Certain mutants at this locus can

relieve the restriction on the growth of certain rII's in lambda lysogens. The nature of the *si*$^+$ gene product is not known (Freedman and Brenner 1972).

This polyglot of *r* phenotypes would at first sight appear to lack any unifying factor. However several lines of evidence point to the fact that at least the rII protein is a T4-directed component of the host membrane which is involved in the control of transport.

It has been found that extensive hydrolysis of phospholipids, which are components of membranes, occurs in *E.coli* infected with T4rII, but not with *r*$^+$ or rI phage (Bradley and Astrachan 1971).

When *E.coli* K-12(λ) is infected with rII phage, there is extensive leakage of polyamines (Ferroluzzi-Ames and Ames 1965). Such leakage does not occur when T4*r*$^+$ is used as the infecting phage (Dion and Cohen 1971) and it was suggested that this leakage of polyamines is in fact responsible for the abortive infection of rII's in this strain. Certainly these compounds are necessary for successful phage infection (Dion and Cohen 1971 and 1972) and their addition to the medium can relieve the restriction on rII growth lambda lysogens (Brock 1965; Ferroluzzi-Ames and Ames 1965; Buller and Astrachan 1968).

It has also been shown that the addition of Mg^{2+} ions also allow rII mutants to grow in *E.coli* K-12(λ) (Garen 1961). The fact that gene 30 mutants can also be suppressed by divalent cations (Koch 1973) suggests that the rII-mediated suppression of ligase mutants may act through effects of the rII mutant on ionic transport, though the exact nature of such a mechanism remains obscure.

Both the rapid lysis, and the suppression of the *t* gene defect

shown by r mutants are compatible with the hypothesis that they contribute to membrane function. The suppression of the T4g mutation, is however somewhat more difficult to accommodate into such a model.

Also unexplained is the mechanism by which lambda and specifically the rex gene of lambda (Howard 1967) restricts rII growth. After infection of λ rex$^+$-lysogenized strains of E.coli by T4rII mutants, DNA replication is severely inhibited at about eight minutes post-infection (Garen 1961; Nomura 1961; Ferroluzzi-Ames and Ames 1965), and the DNA which is synthesized is much more fragmentary than that following r$^+$ infection (Szargel and Shalitin 1972).

Severe inhibition of lysozyme synthesis also occurs about eight minutes after infection, and inhibition becomes complete some seven minutes later (Mark 1972).

A series of varied experimental approaches to detect the time of rII transcription and translation have given results compatible with the work above, in that it was shown that the rII gene product is synthesized early in the viral programme (Sedgroff et al. 1971; Nesterova and Zapadnaja 1970; Mattson and Russel 1972). The mechanism of the shutoff, and the role which the rex gene of λ plays in it is still a mystery.

In view of the paucity of knowledge of the nature of the rII gene product function, it is not surprising to find that it is so elusive to isolate. Given the welter of genetic information which has been derived from this system a gene product more readily amenable to analysis might be considered as an embarrassment of riches.

Nevertheless, some progress has been made in the isolation of both the rII messenger and protein. Specific mRNA from the rII locus has been isolated by competitive hybridization techniques but this work, though it tells us the time of transcription and which is the transcribed strand is still in its preliminary stages (Jayaraman and Goldberg 1969; Sederoff, Bolle and Epstein 1971).

With regard to the isolation of the protein product of rII, the goal of its purification and characterization (particularly with regard to its amino acid sequence) still seems a distant one. Elegant work by McClain and Champe (1967 and 1970) has resulted in the isolation of a small fragment of the rII B protein. Incidentally, these workers confirmed previous findings (Crick et al. 1961; Champe and Benzer 1962a) that the rII locus has an anticlockwise polarity.

The most telling piece of evidence which implicates the rII gene product as a membrane component is the finding that two T4r^+-directed proteins are found in the bacterial membrane shortly after infection.

If large deletions of either the rIIA cistron (Ennis and Kievitt 1973) or the rIIB (Peterson, Kievitt and Ennis 1972) are carried by the infecting phage the corresponding protein is missing.

rII as a System for Mutation Studies

It was of course the crucial observation of Benzer (1955) that rII mutants failed to grow on lambda lysogens that paved the way for the elevation of this locus from its status as a T4 marker with a rather interesting phenotype to its role as one of the primary foci of study in genetics and molecular biology.

With this observation the r system, and the rII locus in particular, acquired a property, which allied to its other characters rendered it a uniquely useful one for mutation studies. Not only could independently arising r mutants be detected visually quite easily and allocated into three different loci thus allowing the potential to assay changes in intergenic mutagen specificity but, crucially, the inability of rII's to grow on lysogens provided the necessary screening system for revertants of rII's and for wild type recombinants when crosses are performed. The rII system has been used to characterize the specificity of action of several mutagens. The reverse of the coin is that unknown rII's can be classified into mutational type on the basis of their reversion responses to specific mutagens. It should be said that this attribute is not exclusive to the rII locus.

As stated, the screening in favour of r^+ phage allows the facility of scoring recombinants between independent rII's with ease, and in consequence the rII intragenic spectrum as well as the intergenic spectrum of r mutants can be determined.

One of the results of the intense study to which the rII locus has been subjected is that a large number of characterized rII mutants has been amassed, and these can be used to advantage in further analyses of the system. Most notably, the large collection of mapped rII deletion mutants allows rapid and precise preliminary allocation of newly arising rII mutants into small subregions of the locus by means of deletion mapping (see Materials and Methods).

The facilities offered by the rII system have certainly been exploited for various different ends. The aspect of the various

studies which is of most relevance to this work is that pertaining to the characterization of the spectra of rII mutants within the locus. The best illustration of this type of study is shown in Benzer (1961) in which the sites of many hundreds of independent rII mutants (both spontaneous and induced) are given. Various maps of the rII locus, each showing the distribution of mutants derived after a particular mutagenic treatment make two striking phenomena immediately obvious. First, for most of the mutagens (NA, 2-AP, 5-BU, proflavine, diaminopurine and 5-bromodeoxycytidine) and especially for the spontaneous, the mutants are not distributed at random along the locus, but rather certain sites (known as hotspots) are represented by a disproportionate number of multiple occurrences. This is so marked as to make a statistical analysis virtually superfluous. It was shown however, that the distribution of rII mutants among sites did differ greatly from the Poisson distribution which would be expected if the mutants were distributed randomly amongst the sites. Since 1961, the spectra of rII mutants induced by other agents have been obtained e.g. UV (Drake 1966b) and HA (Alikhanian et al. 1970). Both these spectra were of a non-random nature.

The second striking feature is that the spectra of rII's induced by different mutagens and those of spontaneous origin all differ from each other. There are however some similarities between the spectra obtained after treatment by mutagens which induce the same types of mutation e.g. base analogues.

Both these phenomena still have no coherent explanation. Much of the work to be described in this thesis centres around an attempt

to reveal what mechanisms may be responsible for the non-random nature of the rII spectra.

The importance of cellular metabolism in the determination of intergenic mutagen specificity has already been illustrated and discussed. The question which was asked here was whether the influence of cellular physiology also extended to intragenic mutagen specificity.

To this end, r mutants were obtained, either spontaneously arising or after mutagenic treatment, under conditions where either the mutagenesis (in the case of intracellular mutagenic treatment) or the expression period of premutational lesions (in the case of extracellular mutagenic treatment) was carried out in host cells whose metabolism had been altered in some way. The classification of r mutants obtained with the various ancillary treatments was compared with that of r mutants isolated under standard conditions and with those which are in the literature.

Altogether four separate ancillary treatments were used in this study. Two of them involved the treatment of the host cells by drugs and one was concerned with the effect of a specific mutant allele in T4. In addition the effects of the pH at which extracellular treatment of T4 by NA was performed were assayed. The rationales behind the use of each of these ancillary treatments will be found in the introductions to the appropriate chapters in the Results section.

The Mutagens Used

It was decided to compare the effects of the ancillary treatments on the r spectra induced by two different mutagens with

wholly different modes of action and means of administration. Though there is a wide choice of condidates, the two agents which were in fact chosen for most of the studies were NA and 5-BU.

Both have been used extensively as mutagens on the r system and the classes of mutations and the spectra of r mutants induced by them have been characterized. Although there are similarities in the rII spectra induced by the two agents (Benzer 1961), they are distinct from each other and both differ markedly from the spectrum of spontaneous rII's. Both have non-random spectra and the 5-BU spectrum possesses the most striking hotspots of any of the mutagen-induced maps. The NA spectrum on the other hand is characterized by a larger number of less intense hotspots.

It has already been shown that both mutagens have biological effects other than those directly concerned with their mutagenic properties. In addition, the mutagenic potency and specificity of both have been shown to be amenable to modification by a variety of ancillary factors. This will be illustrated in greater detail in the context of the discussion of each of the mutagens below.

Although, as will be seen, NA and 5-BU are quite unrelated in the ways in which they induce mutations, the classes of rII mutants induced by them are not dissimilar. In T4, at least two mutational classes are induced by each mutagen and thus the opportunity exists to examine whether the ancillary treatments affect (if at all) one of the classes of induced mutation in a specific manner. The fact that both mutagens induce approximately the same classes of mutations allows one to estimate whether any alterations in the spectra elicited by secondary treatments act upon the class of

mutation irrespective of its origin, or rather, whether any modifications are dependent upon the mutational mechanism by which the mutants are induced.

The two mutagens will be discussed in some detail paying particular regard to their action on the rII system and on the importance of ancillary factors on their action.

a). NA

The molecular basis of the mutagenic properties of NA is perhaps the most readily understandable of any chemical mutagen.

Transitions comprise the majority class of mutations induced by NA. This specificity of NA mutagenicity extends from a variety of phages (Freese 1959b; Bautz-Freese and Freese 1961; Tessman, Poddar and Kumar 1964; Siegel 1965; Wittmann and Wittmann-Liebold 1966) to E.coli (Yanofsky, Ito and Horn 1966; Weigert and Garen 1966) and Neurospora (Malling and de Serres 1968), though the relative proportions of the various classes of transitions do vary between the various systems which have been tested. In fact in phage S13, NA was shown to induce all four classes of transition i.e. T$\leftrightarrow$C and A$\leftrightarrow$G (Tessman and Vanderbilt 1970).

The mechanisms by which NA induces transitions almost certainly involve oxidative deamination, the most pronounced of the molecular rearrangements induced by NA in the DNA (Schuster and Schramm 1958; Schuster 1960 a and b). The deamination of cytosine leads to the formation of uracil, which will then have a probability of pairing with adenine thus generating a GC $\rightarrow$ TA transition. The deamination of adenine results in the formation of hypoxanthine and this intermediate can pair with guanine, to bring about the AT $\rightarrow$ GC

transition. The deamination product of guanine is xanthine. This reaction usually has lethal consequences, since xanthine is a 'nonsense' base in polynucleotides (Richardson et al. 1963; Michelson and Grunberg-Manago 1964).

In addition to its ability to induce transitions, NA has been reported to be able to generate other classes of mutations. There have been claims that it may induce transversion (Yanofsky, Ito and Horn 1966) and frameshifts (Magni: quoted in von Borstel 1968). However in both reports, the contributions of spontaneous mutants of such classes were substantial and it is not really proven that NA can in fact produce such mutations.

It does seem clear however that under certain conditions NA can induce deletions, both in bacteria (Beckwith, Signer and Epstein 1966) and in T4 (Tessman 1962). Koch and Drake (1970) could not repeat Tessman's findings, and a section of the work to be described involved an attempt to resolve this discrepancy. Since the mutagenic and ancillary treatments (the ancillary treatment being pH) were done on extracellular phage this section was not directed at a study of metabolic influences on mutagenesis. Nevertheless it was approached on the basis of an examination of the effects which a secondary factor exerted on the specificity of NA mutagenesis. It was also thought desirable to try to establish the reason for the discrepancy for its own right.

Apart from NA's ability to cause oxidative deamination, it has also been shown to be able to induce DNA cross-links (Becker, Zimmerman and Geiduschek 1964). It is not implausible that this is the reaction responsible for the production of deletions.

Despite the apparent simplicity of the molecular changes in DNA which result from NA treatment as measured *in vitro*, and the readily understood correlations between such changes and the main classes of mutations induced by it, the mechanism of NA mutagenesis may not be so straightforward as it would first appear. The influence of physiological factors has been shown to play a role in what might have been thought to be as simple a series of chemical reactions as could be wished for from a mutagen.

Clarke (1970) has shown that *E.coli* strains which are deficient in excision repair exhibit a greater sensitivity to the lethal action of NA, and that the frequency of induced mutations is enhanced compared to wild type. He also found that caffeine, which is a dark-repair inhibitor in *E.coli* (e.g. Clarke 1967), when added to the plating medium acted as a stimulus to NA mutagenesis in the repair-sufficient strain but had an antimutagenic effect in the repair-deficient strain.

In *Salmonella*, Rudner (1961) found that the addition of broth to the plating medium markedly enhanced the NA-induced reversion frequency of a tryptophan auxotroph.

Although not directly related to NA's mutagenic powers, it should still be noted that the extent of NA-induced killing in many different species has been shown to be under the control of loci involved in DNA repair. A catalogue of such reports will be found in Chapter 5 of the Results section.

In the already-mentioned K3/17 strain of *Neurospora*, Auerbach and Ramsay (1967) found that when NA-treated spores were incubated at 32°C rather than 25°C, the recovery of *adenine*$^{+}$ revertants was

indifferent to the temperature of incubation.

In this strain, NA induces many more adenine$^+$ than inositol$^+$ revertants (Auerbach and Ramsay 1968). In interaction experiments, the same authors (Auerbach and Ramsay 1972), showed that joint mutagenesis with NA and UV induced many more adenine$^+$ revertants than would be expected by summing the expected frequencies obtained if the mutagens were given separately. In contrast, the frequencies of inositol revertants were substantially less than the sum of the individual treatments. The authors argued strongly that such effects are explicable only by invoking effects of cellular physiology on the mutagenic pathway of NA.

Turning to T4, Drake and Greening (1970) showed that the frequency of NA-induced AT → GC (but not the GC → AT) transitions at the rII locus was depressed when the treated phage carried an antimutator allele at the gene 43 (DNA polymerase) locus. In contrast, Freese and Freese (1967) could not detect mutational synergism between gene 43 mutator alleles and NA.

Since in this work to be described, NA was administered extracellularly, the possible interactions between NA and various cellular components were not a factor in any potential modification of NA's specificity of action.

b). 5-BU

In contrast to NA, it is necessary for 5-BU to be administered intracellularly for it to be effective as a mutagen. The reasons for this requirement will become obvious when its mode of action is described. In consequence, the effects of 5-BU on cellular physiology which were described earlier may come into play in

determining the specificity with which this mutagen acts.

Given that the method of DNA replication involves the accurate recognition of complementary bases (Watson and Crick 1953), it might have been predicted that analogues of the bases would possess mutagenic powers, and indeed the double helical model was further ratified by the finding that certain halogenated analogues of uracil were effective mutagens (Litman and Pardee 1956). Amongst these was 5-BU.

This mutagen induces exclusively transitions in T4 (Freese 1959a) with a predominance of GC → AT mutants (Bautz and Freese 1960; Champe and Benzer 1962b; Drake 1963). In the single stranded phages S13 and ØX174 it is the AT → GC transition which is favoured by the closely related mutagen BUdR (Howard and Tessman 1964). This paradox is one of many problems which are to be found in a study of 5-BU mutagenesis.

Incidentally, base analogue mutagenesis is not restricted to prokaryotic systems. When Drosophila larvae are treated with BUdR, morphogenic changes are induced, and this has been interpreted as being due to somatic cell mutations induced by the analogue (Rizki, Douthit and Rizki 1971; Rizki, Rizki and Douthit 1972).

Since 5-BU can substitute for thymine to a very large extent (e.g. Hotz and Walser 1970) it might have been thought that the analogue would promote the AT → GC transition. The fact that GC → AT transition is the commoner class suggests that it is the rarer incorporation of 5-BU in place of cytosine which is the predominant mutational culprit. It is clear that, as pointed out above, the analogue must be incorporated into freshly synthesized

DNA for it to be mutagenic, making it obligatory for the mutagen to be given to phage which are actively replicating.

In physico-chemical studies on the ionization and tautomerization of 5-BU (see Drake 1970) a much higher efficiency of 5-BU mutagenesis is expected than is observed, given the ease with which it is incorporated into DNA. The implication is that various cellular processes play a role in deciding the fidelity with which base analogue-substituted DNA is replicated.

In the same study (Drake and Greening 1970) which was mentioned in the discussion of NA mutagenesis, it was found that 5-BU mutagenesis was depressed in phage carrying gene 43 antimutator alleles. There have been conflicting results regarding the effects of gene 43 mutator alleles on 5-BU mutagenesis in T4. Freese and Freese (1967) reported no effect but Speyer (1969) and Albrecht and Drake (quoted in Drake 1970, p.183) demonstrated some mutational synergism between 5-BU and the tsL56 mutator allele of gene 43.

More recently, various DNA repair systems have been shown to be implicated in determining the potency of 5-BU mutagenesis (Pietrzykowska 1973). She found that when the host E.coli was deficient in excision repair, 5-BU mutagenesis of phage lambda was enhanced, indicating that the mutational heteroduplex could be recognized and corrected by the excision repair system. On the other hand mutations in loci concerned with recombinational repair, whether they were in the host (recA or lex) or in the phage (red), depressed 5-BU mutagenesis of the phage. This antimutator effect of recombination deficiency is also found with UV as mutagen both in lambda (Defais et al. 1971) and in E.coli (Witkin 1967 & 1969a,b&c);

Miura and Tomizawa 1968). The mechanism by which 5-BU-induced mutations are generated by a recombination event is obscure, but whatever the cause it does add another dimension to the mode of 5-BU mutagenesis.

The role of recombination in 5-BU mutagenesis is supported by the fact that recombination frequencies are enhanced when 5-BU is present in the DNA (Folsome 1960; Pietrzykowska 1973). A long-standing unresolved problem in 5-BU mutation studies stems from the work of Fermi and Stent (1962), in which it was shown that 5-BU mutagenesis of T4 was much more effective in singly than multiply infected cells. Pietrzykowska (1973) found that in lambda, such an effect was reduced in a red⁻/recA background, suggesting that some aspect of recombination which was affected by m.o.i. was responsible for the observation of Fermi and Stent. Here again there is no adequate detailed model.

A rather interesting negative result also indicates the complexity of 5-BU mutagenesis. Chopra (pers. comm,) tried to repeat the work of Cerdo-Olmedo et al. (1968) using 5-BU rather than NTG on synchronous cultures of E.coli. The original work with the latter mutagen elegantly demonstrated that mutants at different loci could be induced with NTG in peaks, the particular array of mutants obtained depending on the position of the DNA replication fork on the chromosome when the mutagen was given. Such a result depends on the fact that the mutagen employed acts primarily at the DNA replication fork. Given the fact that 5-BU must be incorporated into DNA (presumably at the replication fork) for it to be mutagenic one would have supposed 5-BU would behave in a similar way to NTG

when applied to synchronous E.coli cultures. In fact Chopra failed to find any such effect with 5-BU. Once again this apparent anomaly remains unexplained.

One rather indirect piece of evidence which associates repair processes with the action of 5-BU is given by Bishop and Suedka (1972). 5-BU at high concentration is toxic to Bacillus subtilis. Mutants were isolated which were 5-BU-tolerant. These mutants were also resistant to UV and to caffeine, and this is symptomatic of a change in repair capacities. Note however that resistance to BUdR in frog tissue culture cells was obtained by a decreased permeability of the membrane to pyrimidines (Mezger-Freed 1972) and that in Burkitt Lymphoma cells, resistance was achieved by a loss of thymidine kinase activity (Hamper et al. 1971).

MATERIALS AND METHODS

I. Strains of *E.coli* and of Bacteriophage used

(a) Tryptophan auxotrophs of *E.coli* kindly supplied by Dr. W.J. Brammar from the collection of C. Yanofsky.

Strain	Site of Mutation	Molecular Change Responsible for the mutation and pattern of reversion
A3	trp A	GAG (glu) → GUG (val) Reverts to GAG and is also missense suppressed.
A88	trp A	GAG → UAG (amber) Reverts to GAG but does not revert by nonsense suppression (Brammar, pers. comm.)
A11	trp A also *his* *str*^{R}	GAG → CAG (gln) Reverts to GAG and is also missense suppressible.
A23	trp A	GGA (gly) → AGA (arg) Reverts to GGA and is also missense suppressible. It can also revert to AUA (ile), AGC (ser) or ACA (thr)
A187	trp A	GGX → GUX (val) Reverts to GGX. CUX (leu) → CGX (arg) acts as an intragenic second site suppressor

cont'd.

Strain	Site of Mutation	Molecular Change Responsible for the mutation and pattern of reversion
A98R3	trp A	frameshift (-)
A21	trp A	frameshift (-)
T3	trp E	missense mutation. The molecular change involved is unknown.

(b) Plating Bacteria for T4

Strain	Comments
E.coli BB	Used for routine cultivation of T4 stocks.
E.coli B	Used for isolation of r mutants.
E.coli W (Ø80)	Restrictive for rII mutants of T4. Derived from B/r WP2 su⁻ which was lysogenized with bacteriophage Ø80 plus bacteriophage lambda repressor.
E.coli WP-2	Parent of the repair-deficient strains below.
E.coli CM571 exrA	Obtained from WP-2 by conjugation
E.coli CM561 exrA	Obtained from WP-2 by transduction
E.coli WP2 uvrA	
E.coli CM611 uvrA exrA	Obtained from WP2 uvrA by transduction.

The WP-2 strain and its derivatives were kindly supplied by Dr. B.A. Bridges. The other three strains were obtained from Dr. N. Symonds.

(c) Strains of T4 (apart from those obtained from this work)

(i) r^+ Strains

T4B

T4D

T4B hm

(ii) rII deletions

Figure 1. demonstrates the position of each of the following deletion mutations. The deletions were derived from various sources, and were kindly supplied by Dr. N. Symonds.

1272	638	B78	196
1241	NB2327	1364	W8-33
J3	NB3157	PT163	187
PT1	NB3034	1368	1519
PB242	164	1605	NB5060
A105	924	1589	NB7006
145	H88	1299	

(iii) rII point mutations

These were representatives of some of the hotspots obtained by Benzer (1961). The list of these mutants includes the region in which they lie and the mutagen used to obtain them.

Strain	rII region	Mutagenic Origin	Type of Mutation (if known)
117	B4	Spontaneous	Frameshift
131	A6C	"	Frameshift
114	B4	"	

cont'd.

Figure 1.

Diagrammatic representation of the rII region, showing the linear sequence of regions (from Benzer 1961) and illustrating the dimensions of the rII deletion mutants used for deletion mapping.

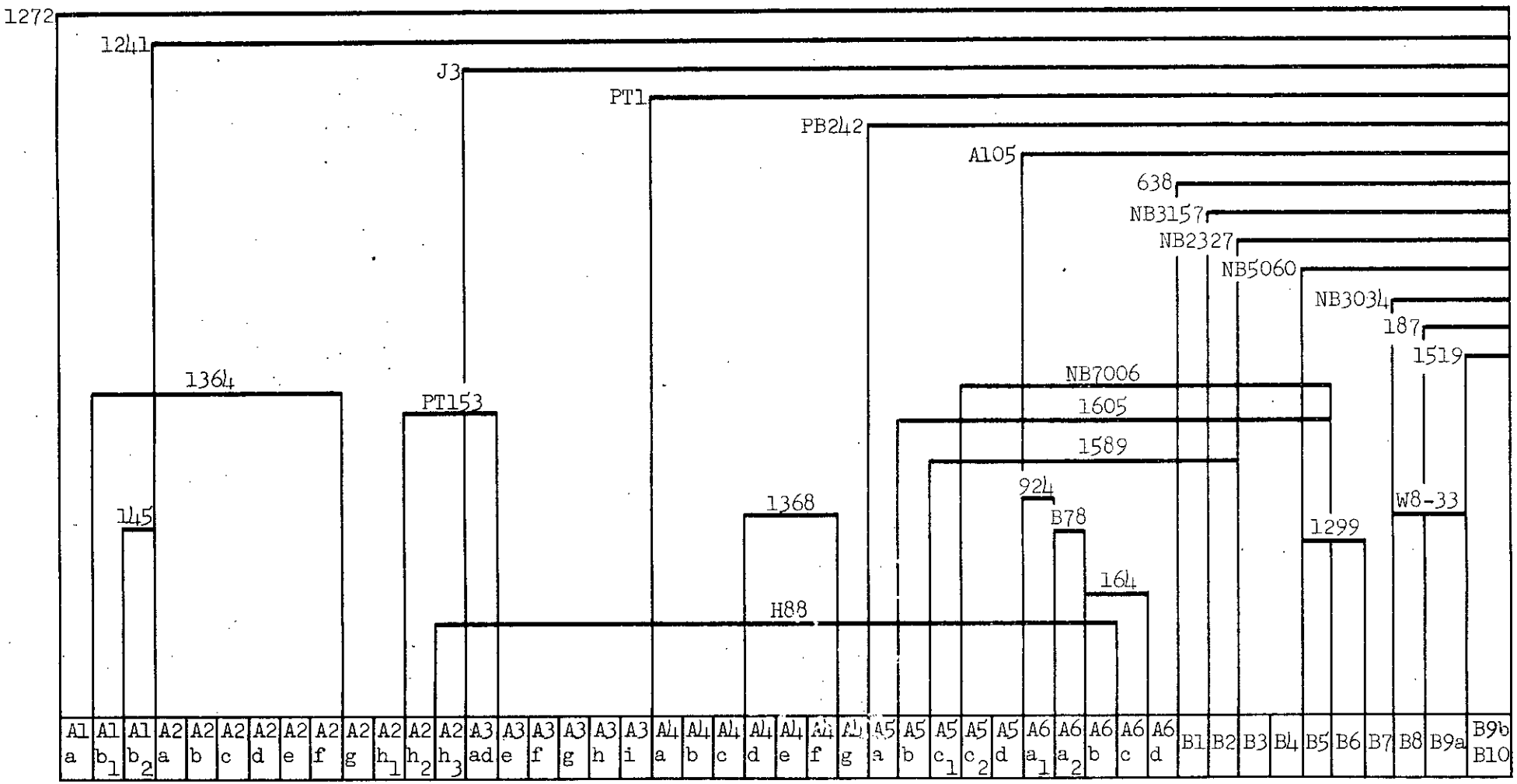

1272
1241
J3
PT1
PB242
A105
638
NB3157
NB2327
NB5060
NB3034
187
1519
1364
PT153
NB7006
1605
1589
924
1368
B78
145
1299
W8-33
164
H88
A1 a
A1 b1
A1 b2
A2 a
A2 b
A2 c
A2 d
A2 e
A2 f
A2 g
A2 h1
A2 h2
A2 h3
A3 ad
A3 e
A3 f
A3 g
A3 h
A3 i
A4 a
A4 b
A4 c
A4 d
A4 e
A4 f
A4 g
A5 a
A5 b
A5 c1
A5 c2
A5 d
A6 a1
A6 a2
A6 b
A6 c
A6 d
B1
B2
B3
B4
B5
B6
B7
B8
B9a
B9b B10

Figure 1.

Strain	rII region	Mutagenic Origin	Type of Mutation (if known)
N11	A4d	5-BU	
N19	$A6a_1$	"	amber
N21	A6d	"	ochre
N24	B1	"	
N97	A3a-d	"	
106	A6c	Spontaneous	

II. MEDIA

(a) Media used primarily for work on the trp operon of E.coli

(i) M9 buffer

This was used routinely for dilution of both T4 and E.coli.

It contained/litre:-

6g Na_2HPO_4

3g KH_2PO_4

1g NH_4Cl

0.5g NaCl

(ii) M9MM

10 mls 0.1M $MgSO_4$

10 mls 0.01M $CaCl_2$

20 mls 20% Glucose

Made up to 1l with M9 Buffer.

M9MM was used as the minimal medium for the culturing of E.coli. For the growth of E.coli trp auxotrophs, MM was supplemented with casein hydrolysate (0.5g/l) plus L-tryptophan (30mg/l). Tryptophan supplementation was required because hydrolyzation breaks down the

tryptophan present in the casein. Where the E.coli strain carried an additional amino-acid auxotrophic marker the appropriate amino acid was added at 30mg/l.

(iii) Solid Minimal Plating Media contained M9MM plus casein plus agar (15g/l). For the work on the E.coli trp mutants L-tryptophan was added to this medium when total cell counts were to be made at 30mg/l. In the plates used for scoring reversion to tryptophan independence tryptophan was present at 0.5mg/l.

(iv) Solid Agar Slants for maintaining bacterial stocks consisted of:-

15g	Difco Bacto Nutrient Broth
5g	NaCl
15g	Agar
1 Litre	Water

(b) Media used in the work on T4

(i) Nutrient Growth Media

T4 was routinely grown in cultures of E.coli in liquid broth medium containing 25g/l Nutrient Broth No.2 (OXOID) supplemented with L-tryptophan at 30mg/l. This amino acid is required as an adsorbtion cofactor for T4B (Anderson 1948). Phage could be stored for long periods at 4°C in this medium without appreciable loss of titre.

(ii) Solid Plating Media for T4

Bottom Layer contained /l. 25g Nutrient Broth No.2

12.5g Agar

30mg L-tryptophan

Each plate contained about 25mls.

Top Layer contained /l. 6g/agar

30mg/L-tryptophan

Unless otherwise stated the soft agar was dispensed into small bottles in 2.5ml aliquots. Prior to use they were melted and equilibrated to 46°C before addition of phage. The agar was poured onto the nutrient plate and allowed to set before the plates were put into the incubator.

In those experiments where T4 was treated with UV, the concentration of agar in the top layer was reduced to 3g/l (See below).

(iii) Phosphate Buffer

Stock Solutions A 0.2M KH_2PO_4

B 0.2M Na_2HPO_4

0.2M, pH 7.0 phosphate buffer. Used in EMS treatment of T4.

330mls A

560mls B

0.1M, pH 6.0 phosphate buffer. Used in HA treatment of T4.

303mls A

32mls B

335mls Water

(iv) Acetate Buffer. Used for NA treatment of T4.

Stock Solutions A 0.2M acetic acid

B 0.2M sodium acetate

pH 4.6 Buffer: 255mls A

245mls B

pH 4.0 Buffer: 410mls A

90mls B

pH 3.7 Buffer: 463mls A

37mls B

(v) Sulphanilamide Medium (SUM). Litman and Pardee (1956) modified by Benzer and Freese (1958). It was used as the medium in which 5-BU mutagenesis of T4 was carried out.

It consists of M9MM supplemented with following /litre:-

2g Sulphanilamide

1g Casein Hydrolysate

30mg L-tryptophan

25mg Xanthine

2mg Uracil

1mg Thiamine

1mg Pyridoxine

1mg Calcium Pantothenate

III. Methods Used

(a) Induction of reverse mutations in E.coli to tryptophan independence by various mutagens.

(i) EMS

E.coli was grown up overnight at 37°C in aerated M9MM plus 30mg/l tryptophan. The cells were spun down at 9,000rpm in an M.S.E. 18 centrifuge. The pellet was resuspended in M9 buffer and was then respun. The cells were resuspended in aliquots of M9MM prewarmed to 37°C. At t = 0, EMS was added to each aliquot at different concentrations. Treatment time was 15 minutes and the

reaction was carried out at 37°C. It was stopped by diluting the cells 1/10 into 6% cold sodium thiosulphate. The cells were centrifugally washed in M9MM containing thiosulphate at 1%. Finally the cells were resuspended in M9MM to give a cell density of about 4 x 10^9/ml.

The washed cells were then plated undiluted on minimal plates for reversion estimates or appropriately diluted on minimal plates supplemented with tryptophan. The plates were incubated at 37°C and after 24hrs were scored for colonies.

EMS concentration:- 0.66mls EMS (Eastman Kodak) dissolved in 10mls water gave a concentration of 0.5M

(ii) MMS

Exactly the same technique was used as that described for EMS.

0.17mls MMS dissolved in 20mls gave a 0.1M concentration of MMS.

(iii) DEB

Again, the protocol was identical to that used for EMS except that thiosulphate was omitted from the stopping medium. Thiosulphate breaks down residual EMS and MMS but is not active against DEB.

(iv) NA

After washing the cells after overnight growth, the cells were resuspended in 0.05M KH_2PO_4 (pH 5.4) to which was added sodium nitrate to a concentration of 0.25M. The treatment was stopped by dilution (1/10) into M9 buffer. The cells were plated for survival and reversion as described above.

(v) UV

The cells were prepared as described above. Aliquots were

put into an irradiation dish. The UV source was a Phillips medium pressure vapour lamp, the major emitted wavelength of which was 2537Å. Samples were taken at intervals and were plated in the usual way. Whilst being irradiated the cells were stirred and after irradiation all work was done under dim yellow light to prevent photoreactivation of UV-induced photoproducts.

(b) Culturing and Plating of T4

(i) The use of the soft agar overlay method was already described. The plates were incubated for 12-16hrs at 37°C by which time the T4 plaques have reached their maximum size.

(ii) Growth of T4 Lysates

The original lysate of T4 was plated on E.coli as described and after six hours incubation a young plaque was picked with a sterile paper strip (10x3cm). The inoculum was added to a broth-grown culture of E.coli BB at about 10^7 cells/ml. The culture was incubated at 37°C under vigourous aeration for five hours, at the end of which time a few drops of $CHCl_3$ were added to complete lysis. The lysate was spun at 6,000rpm to precipitate the bacterial debris and the supernatant was decanted and stored in the cold. The concentration of phage was assayed by plating appropriate dilutions of the lysate on E.coli. The reason for the choice of E.coli BB as the host for lysate growth was that this strain is unselective for r mutants.

It should be noted that where lysates were to be used for mutation experiments, five parallel lysates of the same strains were grown up and each lysate was assayed for spontaneous mutation

frequency:- either $\underline{r}^+ \rightarrow \underline{r}$ or $rII \rightarrow \underline{r}^+$ mutations, depending on the genotype of the lysate.

This procedure was necessary as a safeguard against 'jackpots' which can arise through mutations which arise early in the life of the lysate. The progeny of such early mutations will be represented in the final lysate at a high frequency and will lead to considerable overestimation of the frequency of independent spontaneous mutations. Accordingly the lysate with the lowest incidance of mutants was the one chosen for further use.

(c) Mutagenic Treatments of T4

(i) NA:- Forward Mutation, (Tessman 1962; Koch and Drake 1970).

Acetate buffer was adjusted to the appropriate pH. Immediately before the treatment a fresh solution of $NaNO_2$ was made up and was added to the acetate to a final concentration of 0.1M. The phage were diluted into the NA and the treatment was stopped by a 1/100 dilution of the phage into cold broth. The treated phage could be stored overnight in these broths at $4^{\circ}C$ without loss of titre.

Several of the experiments involved the use of many plates so that many NA-induced r mutants could be collected. For such experiments the protocol was as follows. After the NA treatment, the survival of the phage was assayed by plating on E.coli B. The treated T4 were stored in the cold.

The next day, the viable titre having been ascertained, an appropriate volume of the phage was added to an exponential culture of E.coli B. The actual volume of phage added was that which would result in about 1000 viable particles being delivered on each plate. This was the optimum number, since at densities above this, the

plaques tended to overlap and scoring of r plaques was impaired.

After five minutes which were allowed for phage adsorbtion the culture was poured into soft agar at 46°C (20 volumes agar: 1 volume culture). Using a Struers automatic dispenser calibrated to 2.5mls., the agar was squirted onto nutrient plates which were then incubated overnight and inspected for r mutant plaques.

(ii) NA: Reverse Mutation

An rII mutant was treated with NA exactly as was described for forward mutation. The phage were then plated on E.coli B and on E.coli WØ80 to obtain estimates of survival and of reversion to r^+ respectively.

In certain experiments the phage were allowed a round of replication in a permissive host before being plated on the restrictive E.coli WØ80. This was done by adding the treated phage to a culture of E.coli B in the presence of $4x10^{-3}$M NaCN at m.o.i. of < 1. The cyanide allowed phage adsorbtion but prevented further phage development (Doermann 1952). The complices were diluted into a culture of E.coli WØ80 and were plated. The dilution relieved the cyanide inhibition.

(iii) 5-BU: Forward Mutation (Benzer and Freese 1958)

A culture of E.coli B was prepared by a 1/50 dilution of an overnight culture into SUM followed by aerated incubation for 3.5 hours at 37°C by which time the cells had reached a density of about $4x10^8$/ml.

At this time 5-BU was added to a concentration of 0.05mg/ml followed by T4r^+ at a density which would give about 1000 plaques/plate when the phage were eventually plated out. 10 minutes after

the addition of the phage the culture was diluted into fresh SUM + 5-BU and was then distributed into many aliquots which were incubated for a further hour at the end of which time the contents of each aliquot were plated on E.coli B. The plates were incubated and inspected for r plaques.

The culture was split into many aliquots before the phage had gone through their infective cycle to ensure the independent origin of the r mutants on the different plates. This was important when 5-BU-induced r mutants were to be used for analysis of the mutant spectra. For such work it was imperative that the mutants were of independent origin. Accordingly only one rII mutant from each plate was used for such analysis.

It should be noted here that the combined action of 5-BU and light has a lethal effect when the analogue is incorporated into the DNA (see for example Kvelland 1972; Puck and Kao 1967) caused by light-induced photodynamic transformation of 5-BU (Kazimierczuk and Shugor 1971). This lethality is quite unrelated to the mutagenic action of 5-BU. Accordingly all work with this mutagen was done under a dim light.

(iv) 5-BU: Reverse Mutation

The method was very similar to that employed for 5-BU forward mutagenesis. After allowing time for adsorbtion the phage were diluted into fresh SUM containing 5-BU. The treatment mixture was split into aliquots so that differences in reversion frequencies due to jackpots could be estimated. After one hour in the fresh medium one or two drops of $CHCl_3$ were added to complete lysis. The phage were then plated on E.coli WØ80 and E.coli B.

(v) 2-AP: Reverse Mutation

The protocol was the same as that used for 5-BU-induced reversion except that 2-AP (0.5mg/ml) was used instead of 5-BU.

(vi) 5-BU and 2-AP: Spot-test Reversion Adaption of Freese (1959a)

This was used as a rapid means of determining which rII mutants were transitions.

Cultures of E.coli WØ80 and B were mixed in a ratio of 10:1 and were plated with an agar overlay along with about 10^7 of the rII phage which was to be tested. After the agar had set, each plate was spotted with solutions of 2-AP (10mg/ml) and 5-BU (2.5mg/ml) on different parts of the plate. The plates were stored in the dark until the spots had soaked in and were then incubated. If there was any increase in the number of plaques at either place where mutagen had been spotted compared to the background level, the rII mutant was scored as a transition.

(vii) HA: Reverse Mutation (Tessman 1968)

One part phage lysate was added to five parts 0.1M phosphate buffer plus four parts of a 1.0M filter-sterilized solution of Hydroxylamine Hydrochloride adjusted to pH 6.0 with NaOH. EDTA was added to the reaction mixture at 10^{-3}M. As will be discussed later, EDTA was required to chelate heavy metal ions which may catalyse the breakdown of HA into more noxious products (Bautz-Freese and Freese 1964).

The phage were incubated at 37°C, samples being taken at intervals. The reaction was stopped by a 1/100 dilution into broth + 10^{-3}M EDTA, and the phage were then plated on E.coli WØ80

for reversion estimates to r^+ and on E.coli B to estimate survival.

(viii) EMS: Reverse Mutation (Ray, Bartenstein and Drake 1972)

The rII phage were diluted into 0.2M phosphate buffer (pH 7.0) to which EMS had been dissolved to a concentration of 0.4M. The reaction was carried out at 37°C and samples were taken which were diluted 1/100 into cold broth plus 0.16M sodium thiosulphate. The phage were plated against E.coli WØ80 and B.

(ix) Bisulphite: Reverse Mutation (Summers and Drake 1971)

rII phage were diluted 1/10 into a solution of 0.9M sodium bisulphite dissolved in water. Samples were taken by 1/50 dilution into broth and the phage were again plated against E.coli B and WØ80.

(d) Inactivating Treatments of T4

(i) High Temperature

Phage were diluted 1/100 into M9 buffer preheated to 70°C. Samples were taken at intervals by diluting 1/10 into cold buffer and the phage were then plated against E.coli B for the estimation of survival.

(ii) UV

T4 was diluted into M9 buffer and was treated with UV in the way described for E.coli. Samples were taken and plated against E.coli B for the estimation of survival.

It was mentioned earlier that 0.3% rather than 0.6% agar was used for the top layer when UV-treated T4 was plated. UV-irradiated T4 tend to form small plaques and with agar at 0.6% may be missed altogether. Use of a lower concentration expands such plaques and thus reveals the 'missing' ones (Speyer and Rosenberg 1968).

(e) One-Step Growth Experiment (Ellis and Delbruck 1939)

T4 was added to exponentially growing E.coli at m.o.i. < 1. in the presence of $4x10^{-3}$M NaCN. After 12 minutes which were allowed for preadsorbtion two aliquots were taken. One was plated directly to estimate the number of infective centres. The cells in the other were lysed by the addition of $CHCl_3$ so as to estimate the % phage which were unadsorbed.

At t=0 the complices were diluted at least 1/1000 into fresh prewarmed medium which was incubated at 37°C with vigourous aeration. Samples were taken at intervals and were plated either directly or after lysis with $CHCl_3$. Thus both the release of mature phage and the intracellular formation of mature particles was measured.

It should be noted that in the course of the work with SM, it was found that the antibiotic had an effect on the adsorbtion of the phage. Therefore the time course of adsorbtion was studied and t=0 for this work corresponded to the time at which phage was added to the bacteria rather than when the complices were diluted out of cyanide. Indeed cyanide was not used in the experiments which dealt with the effect of SM on single-step growth of T4.

(f) Characterization of r mutants after their isolation

Since much of the work involved classification of r mutants, the step-wise procedure used in such classification will be described here.

(i) Purification

The plates containing about 1000 plaques from the mutagenized phage were scored for the appearance of r plaques. Purification

of the r mutants was required so as to separate the mutant from the wild type particles. This was clearly necessary when the r mutation was scored in a mottled plaque. Each r plaque was picked lightly with a paper strip which was put into 0.5ml M9 buffer in a tube. The strip was then pulled lightly over the surface of a plate seeded with E.coli B. After incubation separated plaques were formed at the end of the streak.

(ii) Classification of r mutants into genotype

One isolated r plaque from each streak was picked and again suspended into buffer and restreaked onto plates seeded with E.coli W@80. The plates were incubated and scored for plaque formation. The stocks of r's which failed to grow were classified as rII's and were retained. Those which gave r plaques (rI) or wild-type plaques (rIII) were discarded.

(iii) Growth of LTS

This was done by adding 2mls of an early exponential culture of E.coli BB growing in broth + tryptophan to each of the tubes which contained rII mutants. The tubes were incubated for five hours at $37^{o}C$ and lysis was completed by the addition of a drop of $CHCl_3$ to the tubes. Typically, a titre of about $4x10^{9}$/ml was achieved.

(iv) Reversion analyses of rII's

Each rII LTS was spot-testdd with 2-AP and 5-BU. Those mutants which showed an increased reversion in response to either base analogue were classified as transitions. Those which reverted at very high frequency were discarded since fine scale mapping of such mutants would have been impossible. The mutants which gave rise to no revertants were tentatively scored as deletions (though see below

for a more rigourous diagnosis of deletions). The mutants which reverted spontaneously but were not induced with 5-BU or 2-AP were classified as 'other': transversions or frameshifts.

In some experiments the transition mutants were further analysed by testing their response to HA. Those which were responsive to HA were scored as having GC at the mutant site.

(v) Mapping of rII mutants

The facility with which rII mutants can be mapped with high resolution has already been mentioned and is due largely to the ability to perform deletion mapping. The rationale behind this technique is that a mutant cannot recombine with a deletion which spans its position. By using spot-test recombination assays it is relatively simple to allocate an unknown rII to a smaller region of the rII locus on its qualitative recombination pattern in crosses to a number of deletions whose dimensions have been characterized. The collection of deletions in this work allowed the unknown rII to be unambiguously allocated to any one of 30 such subregions. All the rII mutants which fell into a given region were intercrossed and any pairs of mutants which gave no recombinants were assigned to the same site.

Finally where it was possible, a representative from each site was crossed to rII point mutations derived from other sources (See Strains above), so that the sites in this work could be aligned with the sites in the maps in the literature.

The procedure for spot-test recombination was as follows: 0.1ml of each rII parent each at about 2×10^9/ml was added to 0.25ml of E.coli B. After 10 minutes the mixture was spotted onto a plate

seeded with E.coli WØ80. Any clearing at the spot greater than that found for the controls indicated recombination between the parents.

It was mentioned that those rII's which failed to generate any revertants were initially classified as deletions. Such mutants were crossed to a series of separate point mutations whose position was known. If the non-reverting mutant failed to recombine with two or more of the point mutants it could be unambiguously classified as a deletion. The dimensions of the deletions could be estimated from the positions of the different sites with which it failed to recombine.

RESULTS

1. EFFECTS OF GENE ACTIVITY ON INDUCED MUTATION FREQUENCIES IN E.COLI

It will have been noted in the Introduction that several of the reports of mutagen specificity attributable to cellular processes have been discovered by chance observation or else by the use of ancillary treatments whose effects on the cellular metabolism are either imprecisely known, or are of a non-specific nature. This approach has meant that in many of the cases there has been no real understanding of the cellular factors which play a role in governing mutagen specificity.

The work to be described here relied on the obverse approach from that delineated above in that a highly specific cellular parameter was modulated and the effect upon mutation frequency was measured.

A suitable candidate for such modulation is the state of activity of the gene whose mutation frequency is being measured. Bacterial operons provide an ideal system for such work since one can rapidly and reversibly dictate the state of activity of the locus under study without disturbing cellular metabolism to any great extent.

The particular operon used here was the tryptophan (*trp*) of *Escherichia coli*. The anatomy and physiology of this operon is well known.

It contains five structural genes, *trp* E, D, C, B and A which code for the enzymes involved in the biosynthesis of tryptophan (Yanofsky and Lennox 1959). In addition, a large collection of

mutants exists and for many of those which are located within the trpA gene the actual DNA base change can be inferred and the routes by which reversion can occur is known (Brammar, Berger and Yanofsky 1967; Berger, Brammer and Yanofsky 1968). For mutation studies this knowledge of the exact nature of mutant under study is very useful.

The trp operon is repressed when L-tryptophan is present at 30mg/l. The amino acid, or one of its derivatives acts as co-repressor (Mostellier and Yanofsky 1971). The repression can be relieved by tryptophan starvation or by addition of the tryptophan analogue, IP, (Morse, Baker and Yanofsky 1968).

Shortly after the cells have been placed in derepression conditions one RNA polymerase molecule begins to transcribe the operon, the E gene being the first to be transcribed (The trp operon operator is located close to the E gene (Hiraga 1969).) The polymerase transcribes at a rate of about 1000 nucleotides/minute, the transcription of the whole operon requiring some ten minutes. RNA polymerases transcribe the operon with a periodicity of about three minutes ie. they are separated by about 3,000 base pairs. At any one time there are likely to be between two and three RNA polymerase molecules traversing the operon (Imamoto 1968; Baker and Yanofsky 1968).

The nascent chain of mRNA is rapidly charged with about 60 ribosomes very shortly after the start of a round of transcription and the appearance of enzyme occurs almost immediately after synthesis of the mRNA which encodes the particular enzyme (Ito and Imamoto 1968; Morse, Baker and Yanofsky 1968).

Almost immediately after all the ribosomes have translated a particular section of the messenger, the 'naked' mRNA is degraded by nucleases (Morikawa and Imamoto 1969).

In addition to having all the translational machinery in close proximity to the derepressed operon, the structure of the DNA is also physically altered by the transcription. The existence of a transient DNA-RNA hybrid during transcription has been demonstrated in bacteriophage ØX174 (Hayashi and Hayashi 1966 & 1968), Neurospora (Schulman and Bonner 1962) and in Drosophila (Mead 1964).

A recent model (Bick, Lee and Thomas 1972) postulates that at the points of transcription the DNA helix is opened up into a 'bubble' which is of greater dimension than the actual region of hybridization. The RNA hybridizes only with the sense strand of DNA (Marmur et al. 1963; Hayashi, Hayashi and Spiegelman 1964) and further, only the sense strand is involved in the selection of the incoming complementary nucleotide (Doerfler and Hogness 1968; Wetekam 1972). Nevertheless both strands are quite clearly modified in stereochemistry at the point of transcription.

Given all this flurry of activity around the derepressed gene it was thought quite reasonable that the physical differences between a gene in its repressed and derepressed state might be reflected in a difference in induced mutation frequencies under the two regimes.

In fact some parameters, not entirely unconnected with mutagenesis, have been shown to be affected by the state of gene activity.

A decrease in intragenic recombination frequency in the lactose operon of E.coli (Herman 1968) and in the histidine operon of

Salmonella (Savic 1972) was found when the operons were derepressed compared to when they were inactive. In contrast, the opposite result was found in the arabinose operon of E.coli (Helling 1967). It is not clear why this different result was found.

Kölsch and Starlinger (1965a and b) reported that there was a difference in the UV sensitivity of genes in the repressed and derepressed state for galactokinase-forming ability in E.coli. There was also a difference in the photoreactivability of the UV-induced impairmant of galactokinase formation under the two states of gene activity. They found that part of the UV-induced inactivation could be reversed by visible light only when the inducer was absent after UV-treatment.

If, as was the case in this study, it is reverse mutation which is to be measured, two stipulations regarding the nature of the chosen auxotrophic mutants must be made.

a) Obviously the muhation must be induced to revert by a mutagen at sufficient frequency for a valid comparison of the mutation frequencies under the two regimes to be made.

b) The reversion must not be mediated by an extracistronic suppressor, or at least those revertants which arise by such means should be readily distinguishable from true revertants. The reason for this restraint is that the state of activity of any suppressor locus will almost certainly be independent of the transcriptional state of the operon. Thus any revertants which arise through extracistronic suppression will confuse the estimation of the effects of gene action actually at the locus within the operon.

Accordingly the trp mutants used here were, with the exception

of the unsuppressible A88, all frameshift or missense mutants.

It has recently been shown that frameshift suppression can occur through mutational alteration of species of tRNA. Such suppression is restricted to those sites where the frameshift occurs in a run of GC base pairs (Yourno 1971 & 1972; Yourno and Kohno 1972; Riddle and Roth 1970, 1972a & b). However such suppressed strains grew very poorly and could be distinguished from true revertants.

Extracistronic missense suppression can also occur, in some cases with high efficiency, but the mutants used here which were missense-suppressible were suppressed with low efficiency and grew more slowly than true revertants. The latter thus could be distinguished by their larger colony size (Brammar, pers. comm.).

Intracistronic suppression is also a means of reversion for frameshift (Crick et al. 1961) and of missense mutations (Yanofsky et al. 1963). However, false revertants which arise by such means will not interfere with the interpretation since the second-site mutation will be in the same state of activity as the original auxotrophic mutation.

One other point concerning the choice of missense mutations is that in their presence the whole operon is transcribed and translated in a normal fashion (Imamoto and Yanofsky 1967a and b). In contrast, translation stops prematurely at a nonsense codon due to the release of ribosomes from the messenger which in turn is very rapidly degraded (Morse and Yanofsky 1969). Thus the conditions of gene action are more 'natural' with a missense than a nonsense mutation.

Mutagenic Treatments

A number of mutagens were used in this study: UV, NA, DEB, MMS and EMS.

Each tryptophan auxotroph was treated with each mutagen in turn as described in Materials and Methods so as to obtain a suitable mutagenesis system before proceeding to the comparison of the induced frequencies in the different transcriptional states.

(a) UV

Unlike the chemical mutagens, it was thought to be unlikely that there would be a differential physical accessibility of UV to the DNA in the repressed and derepressed state. However the different stoichiometric conformation might have been expected to alter the nature and the potency with which UV induced its lesions.

Quite a lot of evidence now suggests that UV-induced mutations occur through an error-prone recombination-repair system (Witkin 1969a,b and c). It was therefore thought that the state of activity of the operon after irradiation rather than during it, might have more effect on the frequency of UV-induced reversions. In fact Witkin (1966) has postulated that for suppressor loci at least,the repair-mediated UV-induced mutation frequency is greater in genes in the active than in the repressed state.

Each mutant was treated with UV for 60 seconds and the bacteria were plated and reversion and survival were assayed. From Table 1 it can be seen that none of the mutants was induced to revert to any significant extent and hence UV was not suitbale for this study.

(b) NA

The details of NA mutagenesis will be discussed at greater

TABLE 1. UV-Mutagenesis of Tryptophan Auxotrophs

STRAIN	UV Dose (Secs.)	% Surv.	Try^+ Revertants	Reversion Frequency ($x10^{-9}$)
A3	0	100	43	6.1
	30	11.2	9	5.6
	60	0.16	0	-
T3	0	100	32	2.8
	30	18.4	11	2.1
	60	0.31	1	6.4
A187	0	100	50	3.5
	30	10.1	14	4.1
	60	0.23	0	-
A11	0	100	47	4.4
	30	6.4	11	6.5
	60	0.09	0	-
A88	0	100	85	7.5
	30	21.1	19	8.1
	60	0.07	0	-
A21	0	100	103	8.1
	30	17.5	25	11.1
	60	0.12	2	14.1
A23	0	100	56	6.6
	30	12.4	9	7.0
	60	0.20	1	17.0
A9813	0	100	41	4.2
	30	14.2	6	3.4
	60	0.11	1	8.7

length in relation to the work on T4. It is sufficient at this point simply to note that it can be mutagenic in E.coli (e.g. Clarke 1970).

Each mutant was treated with NA, but as can be seen in Table 2 again none was induced to revert.

(c) DEB

This bifunctional alkylating agent has rarely been used in bacterial systems and the exact nature of the molecular changes induced by it are unknown. There is some evidence that it can form crosslinks between guanines which are almost opposite in the DNA and that it can alkylate in a fashion similar to that of better studied alkylating agents. The classes of mutation induced by it are also unknown although there is indirect evidence that it can induce base-pair substitutions.

Table 3 shows that only one mutant, T3, responded quite strongly to DEB mutagenesis. None of the other trp mutants responded at all to DEB.

(d) EMS and MMS

These two monofunctional alkylating agents are chemically closely related and will be considered together.

The major classes of lesion induced by these agents are alkylation of guanine at the 7 position and of adenine at the 1 position (Lawley 1966).

In E.coli, EMS can induce a comprehensive array of mutational classes; AT → TA and GC → CG transversions and AT → GC transitions (Yanofsky, Ito and Horn 1966). Similar information of the classes of mutation induced by MMS in E.coli is not available.

TABLE 2. NA-Mutagenesis of Tryptophan Auxotrophs

STRAIN	NA Dose (Mins.)	% Surv.	Try^+ Revertants	Reversion Frequency ($x10^{-9}$)
A3	0	100	35	4.8
	30	62.4	24	5.6
	60	21.6	21	9.1
T3	0	100	40	3.5
	30	57.0	23	3.3
	60	17.1	4	2.1
A187	0	100	61	2.7
	30	74.8	39	3.6
	60	15.1	4	1.1
A11	0	100	60	5.1
	30	63.6	44	4.8
	60	19.9	9	4.8
A88	0	100	92	7.2
	30	53.7	46	6.5
	60	11.4	11	6.8
A21	0	100	87	7.6
	30	64.1	56	7.1
	60	20.4	21	8.2
A23	0	100	37	4.1
	30	50.9	19	4.0
	60	14.6	19	7.3
A9813	0	100	44	5.2
	30	71.1	35	6.4
	60	24.1	14	5.7

TABLE 3. DEB Mutagenesis of Tryptophan Auxotrophs

STRAIN	DEB Dose (Molarity)	% Surv.	Try^+ Revertants	Reversion Frequency ($x10^{-9}$)
A3	0.0	100	51	4.1
	0.05	42.6	29	3.9
	0.10	20.0	12	4.3
T3	0.0	100	25	2.5
	0.05	51.4	152	36.1
	0.10	23.2	138	54.6
A187	0.0	100	64	4.4
	0.05	48.[illegible]	50	7.6
	0.10	19.0	9	3.8
A11	0.0	100	38	3.2
	0.05	50.0	11	2.5
	0.10	17.6	6	2.6
A88	0.0	100	99	6.9
	0.05	62.6	41	7.3
	0.10	18.5	11	3.7
A21	0.0	100	68	7.5
	0.05	31.9	29	8.2
	0.10	15.2	24	10.9
A23	0.0	100	44	7.5
	0.05	68.4	34	8.5
	0.10	21.5	7	6.7
A9813	0.0	100	61	5.8
	0.05	52.3	48	7.1
	0.10	17.0	10	5.2

Tables 4 and 5 show that three of the auxotrophs were induced to revert with both agents.

Both A11 and T3 showed a strong response to EMS but a much smaller one to MMS. On the other hand A23 was induced to revert moderately with MMS but only very slightly with EMS.

The positive response of A11 to both mutagens confirms that both agents can indeed induce transversions since this mutant can revert only by such means.

Any similar conclusions with regard to A23 and T3 are not tenable since the former can revert by a number of mutational pathways and the base pair change in the T3 mutation is not known (see Strains in Materials and Methods).

EMS Mutagenesis of A11 and T3 under Conditions of Repression and Derepression

Of all the mutant-mutagen systems tested above, the two which gave the strongest responses were those between EMS and the mutants T3 and A11. Accordingly these systems were chosen for the first estimates of the effect of gene action on induced mutagenesis.

The experiments were performed in two ways both of which gave similar results.

(a) Repression of the operon by the addition of tryptophan

Each strain of bacteria was grown overnight in fully supplemented minimal media. The cells were washed in buffer three times by centrifugation in order to deplete them of tryptophan. The cells were finally resuspended in fresh M9 media at 37°C supplemented with casein , and each suspension was then split into two aliquots. To

TABLE 4. MMS Mutagenesis of Tryptophan Auxotrophs

STRAIN	MMS Dose (Molarity)	% Surv.	Try^+ Revertants	Reversion Frequency ($x10^{-9}$)
A3	0.0	100	59	5.4
	0.05	62.9	41	5.0
	0.10	35.1	19	5.2
T3	0.0	100	41	3.4
	0.05	71.0	82	10.1
	0.10	40.2	176	32.4
A187	0.0	100	37	4.0
	0.05	51.8	22	6.1
	0.10	22.4	14	5.3
A11	0.0	100	62	4.9
	0.05	81.6	111	9.2
	0.10	39.9	104	17.6
A88	0.0	100	136	9.4
	0.05	69.1	49	7.6
	0.10	38.2	45	10.2
A21	0.0	100	81	6.2
	0.05	50.2	47	7.1
	0.10	21.0	24	9.3
A23	0.0	100	69	5.8
	0.05	46.8	216	30.4
	0.10	23.7	329	52.6
A9813	0.0	100	30	6.4
	0.05	60.5	17	5.1
	0.10	34.6	17	9.8

TABLE 5. EMS Mutagenesis of Tryptophan Auxotrophs

STRAIN	EMS Dose (Molarity)	% Surv.	Try^+ Revertants	Reversion Frequency ($x10^{-9}$)
A3	0.0	100	28	6.9
	0.25	81.9	12	4.7
	0.50	25.8	10	8.1
T3	0.0	100	17	3.7
	0.25	90.2	3416	886
	0.50	24.6	3628	4830
A187	0.0	100	73	4.0
	0.25	77.6	57	4.0
	0.50	30.1	35	4.9
A11	0.0	100	30	2.1
	0.25	80.5	274	28
	0.50	17.5	961	98
A88	0.0	100	134	9.5
	0.25	81.8	145	11.4
	0.50	27.9	42	9.1
A21	0.0	100	67	6.2
	0.25	72.1	51	6.1
	0.50	30.5	12	5.5
A23	0.0	100	67	4.7
	0.25	70.0	83	8.2
	0.50	41.3	93	16.2
A9813	0.0	100	18	1.8
	0.25	92.3	29	2.5
	0.50	25.0	11	3.9

one of these aliquots was added L-tryptophan at 30mg/l. This was the repressed aliquot (+ TRP). To the other was added L-tryptophan at 0.5mg/l. This concentration relieves the starvation but is not sufficient to repress the operon. This aliquot was designated -TRP.

Five minutes were allowed to elapse so as to allow transcription to get under way in the derepressed aliquot. EMS was then added to each of the aliquots at various concentrations and the mixtures were incubated for 15 minutes at 37°C. The reaction was stopped by dilution into thiosulphate. In addition to thiosulphate, L-tryptophan was present at 30mg/l in all the stopping mixtures so that all the aliquots contained cells in the repressed state after mutagenic treatment. Tryptophan was omitted from the final wash so as to prevent its carry-over onto the plating media.

Figure 2 and Table 6 show that although there was no difference in either survival or spontaneous mutation frequency in both A11 and T3 under the two regimes, the induced reversion frequencies were higher when mutagenesis was carried out in the absence than in the presence of tryptophan. The ratio of the increase at the highest EMS dose was greater in T3 (9.1) than in A11 (2.2). This result suggests that EMS is a more potent mutagen when it acts on genes which are engaged in transcription. However, for reasons which will be discussed below this method of dictating the operon's transcriptional state is not altogether satisfactory for a mutational study of this type. The protocol to be described below is a more appropriate one.

(b) Derepression of the operon by the addition of IP

As mentioned earlier the tryptophan-analogue, IP, has the

TABLE 6. EMS Mutagenesis of A11 & T3 in the Presence and Absence of Tryptophan

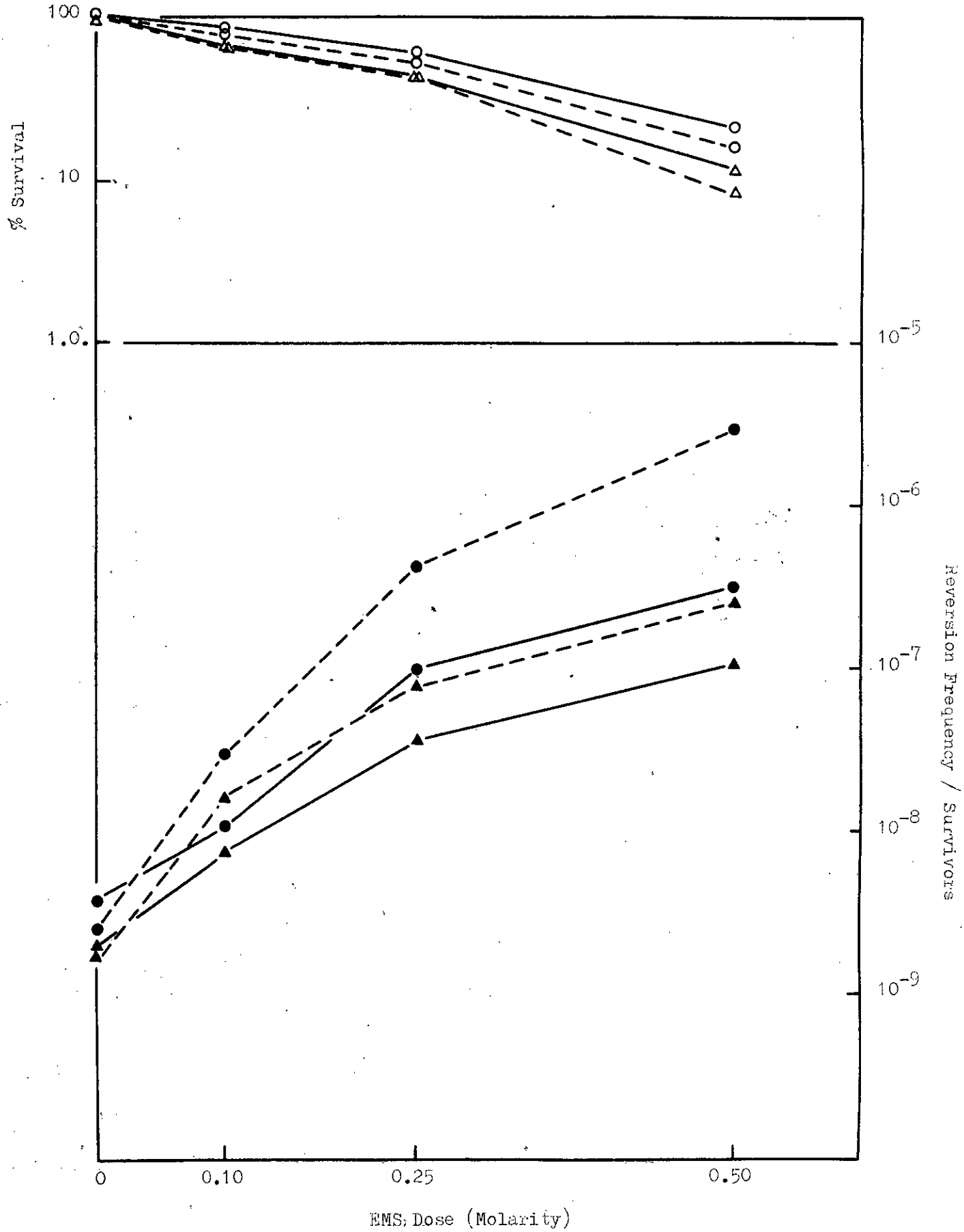
STRAIN	EMS Dose (Molarity)	Reversion Frequ. ($x10^{-9}$)		Ratio of $\frac{-TRP}{+TRP}$ Reversion
		-TRP	±TRP	
A11	0.0	1.8	2.2	0.82
	0.10	17.1	8.1	2.1
	0.25	78.1	39.4	2.0
	0.50	248	112	2.2
T3	0.0	2.8	3.6	0.78
	0.10	27.1	11.4	2.4
	0.25	462	106	4.4
	0.50	3160	346	9.1

Figure 2.

EMS Inactivation and Induced Reversion of T3 and All in the Presence and Absence of Tryptophan

Circles	:-	T3
Triangles	:-	All
Dotted Lines	:-	Tryptophan absent from mutagenic treatment media.
Continuous Lines	:-	Tryptophan present in mutagenic treatment media.
Open Symbols	:-	Survival
Closed Symbols	:-	Reversion frequency to *Try*$^+$

Figure 2.


100
10
1.0
% Survival
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
Reversion Frequency / Survivors
0
0.10
0.25
0.50
EMS: Dose (Molarity)

ability to derepress the trp operon even in the presence of low concentrations of tryptophan without itself being used as a metabolite.

The protocol was largely similar to that described above. After growing up the E.coli and washing them in buffer they were suspended into M9 minimal medium. This suspension was split into two aliquots, A and B. To A (the derepressed aliquot) was added L-tryptophan at 5mg/l plus IP at 30mg/l. To B (the repressed aliquot) was added L-tryptophan at 30mg/l. Figure 3 and Table 7 +IP and -IP correspond to aliquots A and B respectively. The EMS muhagenesis, and termination of treatment was precisely the same as in protocol (a) above. The advantage of this protocol over that in (a) is as follows. The already-stated aim of this work was to modulate a parameter with highly specific effects. In the first protocol although certainly the transcriptional state of the operon could be controlled by adding or withdrawing tryptophan, one is in addition in the former case supplying the metabolite required by the auxotroph. This may mean that the cells under the two regimes differ in their general metabolism quite apart from the local differences concerning the state of activity of the trp operon.

When IP was used however, the required amino acid was present in both regimes albeit at a lower concentration for the derepressed aliquot. Thus with this protocol the general metabolic differences due to the effects of amino acid depletion will be less than in the first protocol.

Figure 3 and Table 7 show a very similar pattern to that found when the first protocol was employed, ie. identical survival and

TABLE 7. EMS Mutagenesis of A11 and T3 in the Presence and Absence of IP

STRAIN	EMS Dose (Molarity)	Reversion Frequ, ($x10^{-9}$)		Ratio of $\frac{+IP}{-IP}$ Reversion
		+IP	-IP	
A11	0.0	4.1	3.2	1.3
	0.10	9.6	5.9	1.6
	0.25	48.9	27.6	1.8
	0.50	216	90.9	2.4
T3	0.0	2.1	1.7	1.2
	0.10	29.1	4.0	7.3
	0.25	307	38.9	7.9
	0.50	4164	486	8.6

Figure 3.

EMS Inactivation and Induced Reversion of T3 and A11 in the Presence and Absence of IP

Circles :- T3

Triangles :- A11

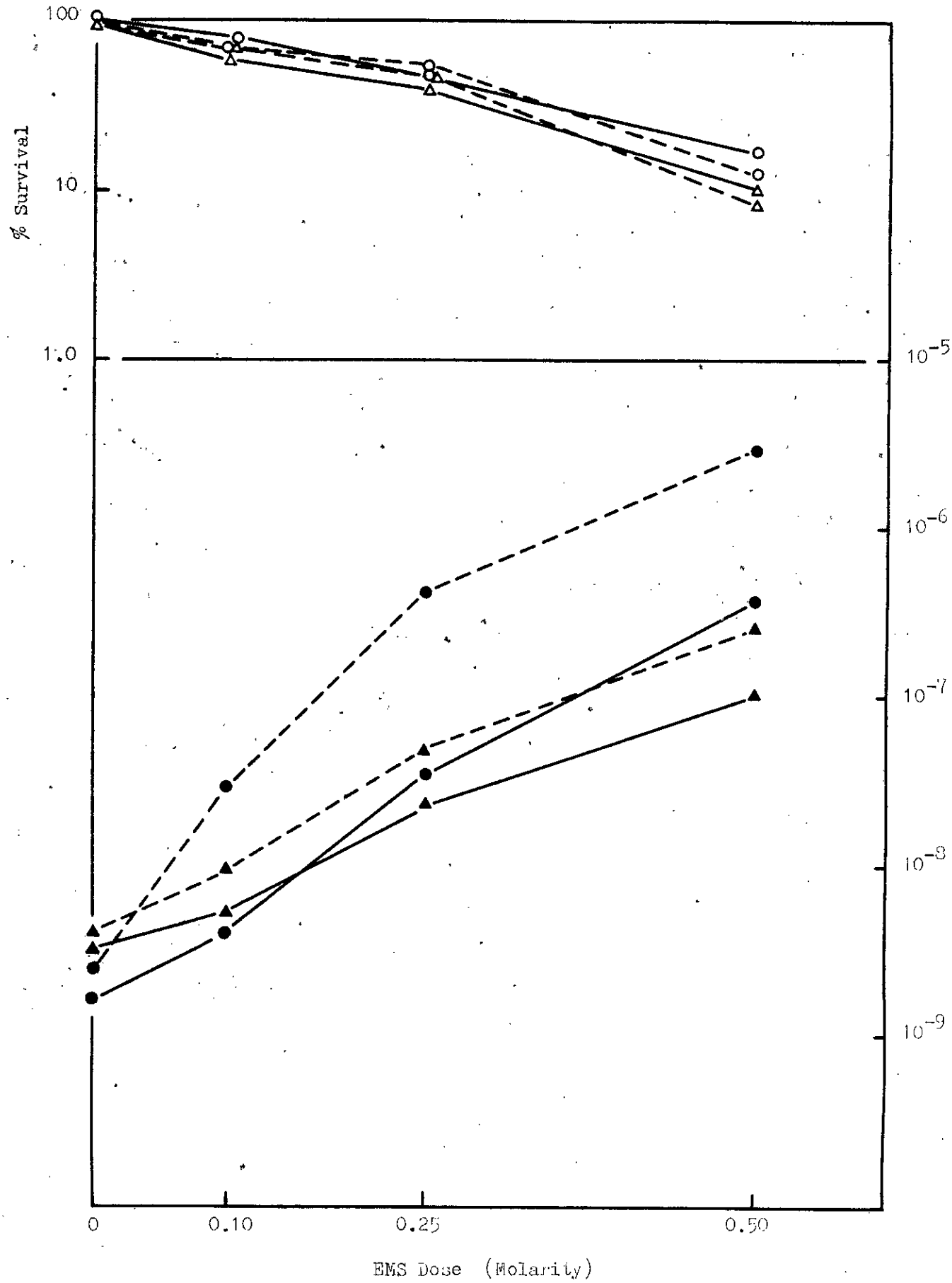
Dotted Lines :- IP present in mutagenic treatment media.

Continuous Lines :- IP absent from mutagenic treatment media.

Open Symbols :- Survival

Closed Symbols :- Reversion frequency to Try^+

Figure 3.


100
10
1.0
% Survival
10^{-5}
10^{-6}
10^{-7}
10^{-8}
10^{-9}
Reversion Frequency / Survivors
0
0.10
0.25
0.50
EMS Dose (Molarity)

spontaneous mutation frequencies but higher induced reversion frequencies in the presence than in the absence of IP and a greater factor of increase in T3 (8.6) than in A11 (2.4).

Effect of Gene Activity after EMS . Mutagenic Treatment

It was mentioned earlier that inaccuracies in repair may well be responsible for the generation of UV-induced mutations and it is possible that EMS mutagenesis may also be mediated *via* such means.

Certainly EMS-induced lethal damage is amenable to repair processes (Ray, Bartenstein and Drake 1972) but the actual implication of repair processes in EMS mutagenesis has not been shown. However Strauss (1962) has shown that in *E.coli* EMS-induced mutations can accumulate during the post-treatment period.

Accordingly a comparison was made between the EMS-induced mutation frequencies of A11 and T3 when the *trp* operon was repressed and derepressed after the actual mutagenic treatment.

The experiment was done in two ways.

(a) *trp* operon derepressed during EMS treatment

The cells were prepared as described already and were treated with 0.5M EMS in M9 medium in the presence of IP.

The reaction was stopped by washing in sodium thiosulphate. The cells were then resuspended into prewarmed M9 medium ± IP. Aliquots were taken immediately and plated for mutation and survival. The cells were incubated a further 20 minutes before being plated. See Table 8a.

(b) *trp* operon repressed during EMS treatment

The procedure was the same as that above except that IP was

omitted during EMS treatment and L-tryptophan at 30mg/l was added in its place. See Table 8b.

For both strains the state of gene activity after the EMS treatment had no effect on the induced mutation frequency.

Reconstruction Experiment

Is the difference in the EMS-induced reversion frequency due to the preferential selections of prototrophs in the treatments where the trp was derepressed? To test such a possibility a reconstruction experiment was performed.

In such an experiment a mixture of the two relevant genotypes (in this case trp auxotroph and prototroph) is subjected to the procedures involved in the original mutagenesis experiment. Any differences in the relative frequencies of the two genotypes before and after the mutagenic treatment is suggestive of differential selection on the two genotypes.

The procedure was as follows. A mixture of a trp^- auxotroph (A88) which was not induced to revert with EMS and a trp^+ strain was made in a ratio of about 10^5:1. The mixture was plated immediately on complete and on minimal plates to estimate the total cell density and the frequency of prototrophs.

The mixture was then split into two aliquots each of which was treated with EMS, one in the presence, one in the absence of IP. After termination of treatment the mixture was again plated so as to estimate the proportion of trp^+ individuals amongst the survivors. Only one dose of EMS was used; 0.5M for 15 minutes. See Table 9.

It can be seen that the relative frequency of the trp^+ E.coli

TABLE 8. EMS Mutagenesis of All and T3 with IP Present or Absent during Post Treatment Incubation

a). Operon Derepressed During EMS Treatment

STRAIN	EMS Dose (Molarity)	Reversion Frequency ($x10^{-9}$) on immediate plating	Reversion Frequency ($x10^{-9}$) after post-treatment incubation	
			+IP	-IP
All	0.0	1.9	2.1	1.7
	0.5	192	176	218
T3	0.0	2.9	3.6	2.5
	0.5	3765	4216	3871

b). Operon Repressed During EMS Treatment

STRAIN	EMS Dose (Molarity)	Reversion Frequency ($x10^{-9}$) on immediate plating	Reversion Frequency ($x10^{-9}$) after post-treatment incubation	
			+IP	-IP
All	0.0	2.3	4.2	3.8
	0.5	24.6	27.5	29.1
T3	0.0	3.7	3.4	4.9
	0.5	628	712	598

TABLE 9. Reconstruction Experiment for EMS Mutagenesis of *trp* Operon in Repressed and Derepressed States

EMS Dose (Molarity)	Initial Proportion of try^+	Proportions of try^+ after treatment	
		+IP	-IP
0.0	2.8×10^{-5}	2.4×10^{-5}	3.0×10^{-5}
0.5M	-	2.7×10^{-5}	2.1×10^{-5}

stayed constant before and after treatment whether the operon was repressed or derepressed during EMS treatment. This means that differential selection of prototrophs was not responsible for the increased induced reversion frequency under conditions of derepression.

The complaint which can always be levelled against reconstruction experiments is that they do not, indeed cannot, measure selection on newly-formed mutants but only on fully-established mutations. Indeed Clarke (1962) provided a salutory example of a case where selection clearly acted on the mutagenic pathway but not on the fully expressed mutation.

Discussion

It should be said that during this work two independent papers appeared (Brock 1971; Herman and Dworkin 1971) both of which dealt with essentially the same problem. In fact the only real difference between Brock's work and mine was that he studied the effect of gene action on the frequency of EMS-induced reversion at the lactose operon in E.coli.

Herman and Dworkin used ICR-191 as their mutagen for inducing reversion of mutants, also in the lac operon. This mutagen induces frameshift mutations almost exclusively so this system was somewhat different from mine where the two mutants reverted by base pair substitution.

The findings of both these sets of authors were the same as mine, viz that some chemical mutagens are more effective when acting on derepressed genes.

More recently it has been shown (Savic and Kanazir 1972) that the frequency of UV-induced reversion of ochre and frameshift mutations in the histidine operon of Salmonella is higher in a strain where the operon is transcribed constitutively than in wild-type strains in which the operon is repressed. This result conflicts with that of Brock (1971) who reported that the state of activity of the lac operon did not affect the frequency of UV-induced reversion.

The fact that the trp and the lac operons of E.coli can be fused (Miller et al. 1970; Michels and Reznikoff 1971) and that such fused strains show co-ordinate control of transcription suggests that these two regions of the E.coli chromosome have very similar transcriptional mechanisms.

Thus it was thought not to be worthwhile pursuing this work, given the similarities between the lac and the trp operons, and since some of the further experiments which I had intended doing had already been carried out in one or more of the reports cited above. I had intended to perform the following additional experiments.

(a) It was intended to see if MMS also proved to be more mutagenic for strains T3, A11 and A23 under conditions where the trp operon was derepressed. T3 also showed a moderate response to DEB, and the effect on the mutagenicity of this agent of gene action was also going to be measured.

(b) I wanted to test the effects of a chain-terminating codon operator-proximal to the EMS-revertible mutation so as to measure the effect of polarity on induced mutagenesis of operons in the

repressed and the derepressed states. Herman and Dworkin (1971) did just this and found that the enhancement of the ICR-191-induced reversion frequency in the derepression conditions disappeared in a strain carrying a polar mutation.

(c) It was intended to repeat the experiments on EMS mutagenesis of T3 and A11 in repair-deficient strains to see if impaired repair enzymes had any influence on the induced mutation frequencies under the two transcriptional states. However both Herman and Dworkin (1971) and Savic and Kanazir (1972) found that repair-deficient strains had no effects on the differential induced mutation frequencies in the active and repressed state. This suggests that the difference in frequencies is due to the differential induction of lesions rather than differential repair in the two regimes. The fact that I could find no difference in the EMS-induced reversion when the operon was repressed or derepressed after the mutagenic treatment supports this idea.

Had I continued this work I would have ascertained that under the conditions used, the trp operon was in fact repressed or derepressed as appropriate by the manipulations which I used. It was always possible that the EMS treatment itself interfered with the control of gene activity and it would have been more rigourous to perform the enzyme assays when the cells were assumed to be in the repressed and derepressed states for the trp operon. It should be said that this criticism also applies to the reports cited above.

The control of a particular operon's state of transcription whether by the use of mutants defective in control (e.g. Herman and Dworkin 1971; Savic and Kanazir 1972) or by experimental manipulation

(Brock 1971 and this work) is highly specific. Although it is a somewhat circular argument, it is not unreasonable to suppose that if a difference is found in the induced mutation frequency when the operon is believed to be repressed or derepressed it is indeed the state of activity which is responsible for the difference in mutation frequency.

One other check on the interpretation that the difference in the mutability under the two regimes is due to the difference in gene activity would be to measure the induced mutation frequency at an unrelated locus when the trp operon was repressed and derepressed. This control was carried out by Brock (1971), Herman and Dworkin (1971) and Savic and Kanazir (1972). In no case was the mutability at such independent loci affected by the activity of the particular operon under study.

This suggests that the increase in induced mutability in active operons is a local one and is not mediated by a general change in cell metabolism.

Why should EMS be more mutagenic in active than repressed genes? It has already been mentioned that EMS preferentially alkylates guanine at the N7 and adenine at the N1 positions. It is not unreasonable to suppose that the access of EMS to these sites is facilitated when the DNA is being transcribed and is temporarily in a single-stranded state. This may be especially true for the attack on adenine where the N1 atom is involved in hydrogen bonding. These bonds are broken during transcription because of the destabilization of the helix. Although the guanine N7 atom is not involved in hydrogen bonding it will still be more exposed in the single-

than in the double-stranded configuration.

If this hypothesis of differential accessibility of EMS to the target sites in DNA in the single- and double-stranded states is correct, then one can explain the increase in the induced mutation frequency in the active gene simply in terms of an increased probability of EMS inducing premutational lesions in DNA which is being transcribed.

The causes for the difference in the ratio of enhancement of T3 and All is a matter of some conjecture. Herman and Dworkin (1971) found quite a range in the degree of enhancement amongst different frameshift mutants within the lac operon. Indeed one of the alleles was induced to revert at lower frequency when the operon was derepressed. They give no explanation for this variation in the behaviour.

We know that All reverts by transversion but all that is known of T3 is that it is a base pair substitution. It may simply be that if T3 reverts by other than CG → GC transversion, that the class of mutation by which T3 reverts is more amenable to enhancement by derepression during EMS mutagenesis.

Incidentally it should be noted that for All the guanine is on the nonsense strand. Since guanine is much more amenable to EMS-induced alkylation this indicates that the lesion need not be induced on the transcribed strand for the effects of derepression on the reversion frequency to be felt.

One other speculative reason for the greater ratio of increase in T3 than in All comes to mind.

It has been known for some time that the trp operons of both

Salmonella (Bauerle and Margolin 1966 and 1967) and of E.coli (Morse and Yanofsky 1968) possess internal promoters which are not subject to the normal control mechanisms of the operon. This promoter is located within the trpD structural gene (Jackson and Yanofsky 1972) which codes for phosphoribozyl anthranilate transferase. It initiates low level constitutive transcription at a rate about 3% the level of the derepressed operon, of the genes (C, B and A) which are operator-distol to it. Thus unlike T3, which is situated proximal to the internal promoter, the All mutant is never fully repressed and this may be reflected in the smaller ratio of increase in the EMS-induced reversion frequency on full derepression of All compared to T3. It is questionable whether this low level constitutive transcription of the All can be responsible for the difference in behaviour of the two alleles which is much greater than 3%

A survey of the literature concerning mutagen specificity by ancillary factors acting on cellular processes reveals no example where the specificity can be attributed to differential transcriptional states at different loci during mutagenic treatment.

However, the fact that the induced mutation frequency is higher in active genes may be turned to advantage if isolation of mutants at a particular locus is desired if the locus in question can be unilaterally derepressed during mutagenesis. If no selection system for the desired class of mutants exists then any means of increasing the proportion of these amongst the total mutant yield is to the good. Thus it might be possible to increase the yield of mutations which may be desired for mutation plant breeding or

for obtaining strains of microorganisms with increased production of a particular metabolite through mutagenesis.

This approach is in a way reminiscent of the use of NTG on synchronous cultures of bacteria. This mutagen acts almost exclusively at the DNA replication fork (Cerdo-Olmedo et al. 1968) with the result that high specificity in the induction of mutants is obtained, the actual array depending on the position of the replication fork on the bacterial chromosome during mutagenesis (Hirota et al 1968). There is in fact a case where a similar rationale has been put to use for obtaining higher specificities for EMS-induced mutations in barley (Natarajan and Shivasankar 1965).

2. EFFECTS OF pH ON THE PRODUCTION OF MUTATIONS IN T4 BY NA

The reason for wishing to determine whether the pH of the NA treatment buffer had any effect on the specificity of NA mutagenesis in T4, and in particular on the production of deletions, stemmed from an apparent discrepancy in the literature.

Tessman (1962) reported that NA induced deletions at the rII locus and this agent has also been shown to induce deletions in E.coli (Beckwith, Signer and Epstein 1966).

Koch and Drake (1970), on the other hand, found no more deletions amongst their collection of NA-induced rII's than would have been expected on the basis of spontaneously arising ones. In addition Benzer (1961) did not report any induction of deletions.

An inspection of the protocols of Tessman and of Koch and Drake, reveals a number of differences. One such was the pH during NA treatment; pH 3.7 by Tessman, pH 4.0 by Koch and Drake.

At first sight this difference would seem to be a minor one. However the influence of pH on the potency of NA action has been demonstrated on several occasions. The rates of NA-induced deamination of cytosine and adenine increases some 90-fold as the pH of the treatment medium drops from 5.0 to 4.2. The rate of deamination of guanine increases by a smaller factor (35-fold) over the same drop in pH. In addition the rates of bacteriophage inactivation and mutagenesis are also inversely correlated with pH (Schuster 1960a and b; Vielmetter and Schuster 1960a and b; Schuster and Vielmetter 1961).

Another fact concerning the influence of pH on the response of

bacteriophage to NA should be mentioned. It has already been said that NA can induce crosslinks in DNA (Geiduschek 1961; Becker et al. 1964). Burnotte and Verly (1971) assayed the numbers of crosslinks induced by NA in bacteriophage T7 DNA. They found that the lower the pH of the buffer into which the treated DNA was diluted to stop the reaction, the higher the number of crosslinks generated, e.g. at pH 6.0, the mean number of crosslinks formed per DNA molecule was 0.76. If the stopping buffer was at pH 10.0, a mean of 0.62 was obtained. It was deduced that the crosslinks were produced after NA treatment, and in the model put forward it was proposed that NA-induced apurinic sites formed crosslinks due to hydrolysis of aldehyde-β-phosphodiester bonds and that this reaction is encouraged by H^+ ions. This is analogous to the model proposed by Freese and Cashel (1964) to account for the induction of crosslinks in DNA by acid alone.

The pH of the stopping buffer used by Koch and Drake was 8.0. Tessman gives no details of his method of terminating the NA treatment. If he used buffer of pH less than 8.0 it could be that more crosslinks were induced. If these lesions are responsible for the production of deletions it may be that a difference in the pH of the stopping buffer could have contributed to the lack of agreement between the two sets of workers. However the extent of the difference in the frequency of deletions is greater than could be accounted for on the basis of differential crosslinking at different stopping pH, even if Tessman used a buffer of pH as low as 6.0 to stop the reaction.

Experimental

As was mentioned in Materials and Methods it is necessary to work with a lysate with as low a frequency of r mutants as possible if mutagenesis experiments are to be done.

Accordingly five lysates of T4r^+ were grown up and the incidence of r mutants was assayed for each. See Table 10. The lysate with lowest r frequency (number 5) was reassayed using a larger number of plates so as to obtain a more accurate estimate. 8.2×10^4 plaques were inspected, out of which 17 were r mutants, giving a frequency of 2.1×10^{-4}. This is a suitably low frequency and all further work which entailed forward mutagenesis from r^+ to r employed this stock.

It should be noted that the titre and the spontaneous r frequency was checked regularly throughout the course of this work.

Inactivation of T4 by NA at Different pH's

T4r was treated with 0.1M $NaNO_2$ in acetate buffer at pH's 3.7, 4.0 and 4.6. Samples were taken and assayed for survival, see Figure 4a. The differences in the rates of NA inactivation at the three pH's were dramatic. There was also an inactivating effect of acid alone which was quite marked at pH 3.7 but only slight at pH 4.0. To account for the lethal effect of acid alone the data were replotted as the ratio of survivors after NA treatment over the survivors in the buffer alone. After this adjustment the lethality was still far greater when NA was given at pH 3.7 than at pH 4.0. At pH 4.6, NA exhibited no lethal action after treatment up to four minutes (Figure 4b).

TABLE 10. Measurement of Spontaneous r Frequencies in T4 Lysates

Lysate	Plaques Inspected	No. of r plaques	r Frequency
1.	1.7×10^{4}	25	1.5×10^{-3}
2.	2.2×10^{4}	19	8.6×10^{-4}
3.	1.9×10^{4}	13	7.1×10^{-4}
4.	1.8×10^{4}	17	9.3×10^{-4}
5.	2.0×10^{4}	9	4.4×10^{-4}

Figure 4a.

Inactivation of T4 by NA at Different pH's

Dotted Lines	:-	Buffer Alone
Continuous Lines	:-	NA in Buffer
Triangles	:-	pH 4.6
Crosses	:-	pH 4.0
Circles	:-	pH 3.7

Figure 4a.

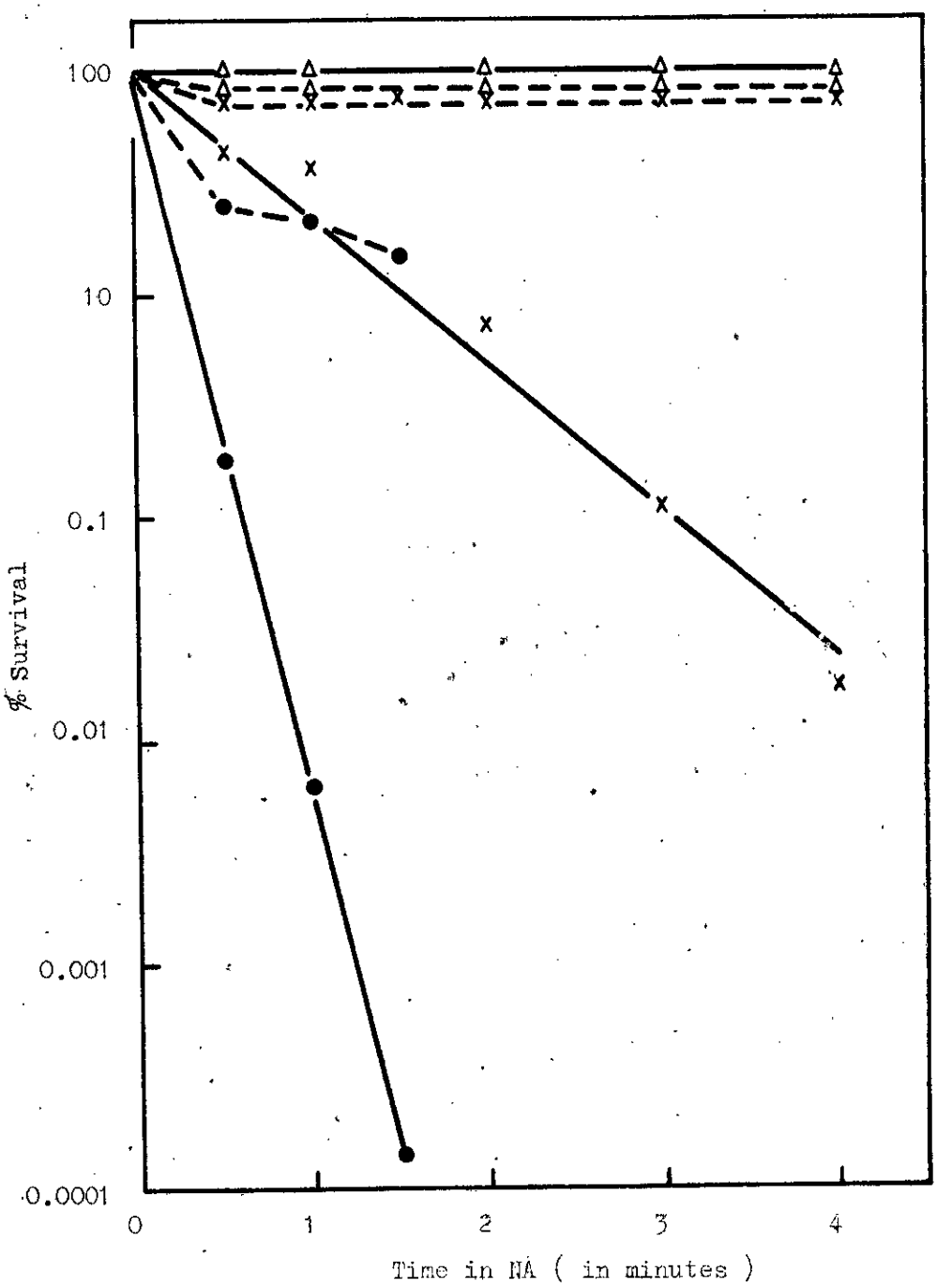
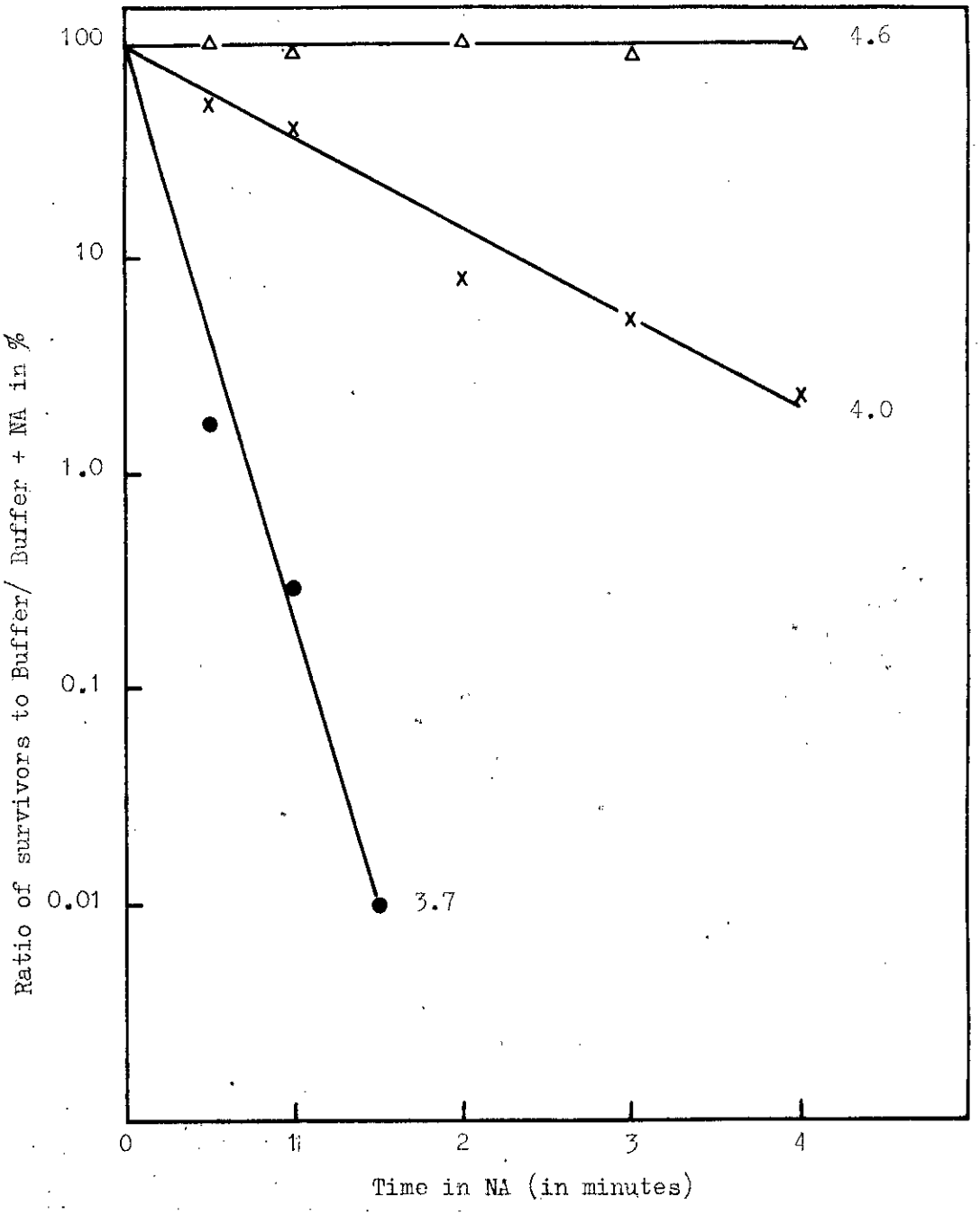


Figure 4b.

Inactivation of T4 by NA at Different pH's (Corrected for Inactivation by Buffer alone)

For each time point and for each pH value, the ratio of survivors after treatment with NA to the survivors after treatment in buffer was calculated and in the figure the ratios are expressed in percentages. The pH's for the corrected survival curves are given in the figure.

Figure 4b.


100
10
1.0
0.1
0.01
Ratio of survivors to Buffer/ Buffer + NA in %
4.6
4.0
3.7
0
1
2
3
4
Time in NA (in minutes)

The most likely explanation for the increase in lethality with decreasing pH is that there is more NA at the lower pH's, the pK of NA being 3.37. This implies that it is molecular NA rather than the nitrite ion which is the inactivating agent.

It was however thought possible that the increase in lethality effected by NA at the lower pH's might be due to some sensitizing effect whereby acid facilitates the lethal action of NA. An experiment was performed to test this idea.

The method involved separation of the effects of pH and of NA. 0.1ml of a T4$\underline{r}^{+}$ lysate was added to 5mls of the solutions designated "1st Treatment" in Table 11 and kept there for the time indicated. A 0.1ml sample was then taken and added to 10mls of the solutions in "2nd Treatment". The phage were finally diluted into broth to end the treatment and were assayed for survival. The rationale of the experiment can be illustrated by consideration of Treatment 6 (in Table 11). NA at pH 4.6 caused no killing after 30 secs (Treatment 9). However it is possible that exposure to pH 3.6 acid prior to NA at pH 4.6 may sensitize the phage to NA killing. A comparison of the survivals after Treatments 5 and 6 shows that this was not so. In both cases all the lethality can be accounted for by acid killing (compared to Treatment 7). Similarly a comparison of the survival after Treatments 1 and 2 shows that exposure to NA at pH 4.6 did not sensitize the phage to subsequent killing by acid alone.

It can therefore be concluded that there was either no sensitization or else it was very transient and was immediately abolished on transfer to the new medium.

TABLE 11. Interaction of pH and NA on T4 Killing

Treatment Code	1st Treatment	2nd Treatment	% Surv.
1.	pH 4.6 + NA (30secs)	pH 3.7 (120secs)	7.9
2.	pH 4.6 (30secs)	pH 3.7 (120secs)	6.8
3.	pH 4.6 + NA (30secs)	pH 4.6 (120secs)	100.8
4.	pH 4.6 (30secs)	pH 4.6 (120secs)	95.4
5.	pH 3.7 (120secs)	pH 4.6 (30secs)	8.8
6.	pH 3.7 (120secs)	pH 4.6 + NA (30secs)	6.9
7.	pH 3.7 (120secs)	M9	7.5
8.	pH 4.6 (30secs)	M9	97.2
9.	pH 4.6 + NA (30secs)	M9	94.3
10.	Stock Lysate	M9	100

Treatment 10 was used as control.

Mutagenesis of T4r$^+$ by NA at pH 3.7 and pH 4.0

Because of the requirement of plating an optimum number of pfu/plate (about 1000) in a large-scale forward mutation experiment it was necessary to estimate the surviving fraction accurately before plating. This was especially relevant in the case of the NA treatment at pH 3.7, where the steepness of the killing curve meant that the margin of error was extremely small.

The protocol for the forward mutation experiment was given in Materials and Methods and it involved storage of treated phage overnight at 4^{o}C. For this to be tenable it had to be shown that NA-treated phage did not lose titre on storage nor did the frequency of r mutants change. To this end the phage were treated with NA at pH's 3.7, 4.0 and 4.6 and were plated either immediately or after periods of storage in cold broth and were assayed for survival and for the incidence of r plaques. Table 12 shows that neither parameter changed with storage.

The mutation experiment was then carried out. The phage were treated with 0.1M NA at pH 3.7 (for 15 secs) and at pH 4.0 (for 4.5 minutes) which resulted in phage survivals of 0.85% and 1.25% respectively. The phage were plated against E.coli B and inspected for r plaques (see Table 13a).

It should be noted that the survival was slightly lower after NA treatment at pH 3.6 than at pH 4.0 and this may be contributory to the slightly higher induced r frequency obtained at the lower pH.

The r mutants were picked and purified and were then streaked onto E.coli WØ80 to allocate them into genotypes. See Table 13b.

For both sets of the untreated controls, the ratio of rI:rII

TABLE 12. Effect of Storage on NA-Treated T4

(a) Survival (pfu/ml)

Treatment	Time of Storage (hours)		
	0	24	48
pH 3.7 (30secs)	1.1×10^8	1.2×10^8	1.5×10^8
pH 3.7 + NA (30secs)	2.0×10^5	1.9×10^5	2.0×10^5
pH 4.0 (4mins)	4.0×10^8	4.0×10^8	4.0×10^8
pH 4.0 + NA (4mins)	7.9×10^6	9.0×10^6	7.7×10^6
pH 4.6 (10mins)	5.2×10^8	4.9×10^8	4.7×10^8
pH 4.6 + NA (10mins)	5.2×10^8	5.0×10^8	5.2×10^8

(b) Mutation to r

Treatment (including time of storage)	Plaques Inspected ($\times 10^4$)	No.of r Plaques	r Frequency ($\times 10^{-4}$)
pH 3.7	1.4	3	2.1
pH 3.7 + NA	6.8	66	9.7
pH 3.7:- stored 24 hrs.	3.2	9	2.8
pH 3.7 + NA:- stored 24 hrs.	3.5	30	8.6
pH 4.0	2.5	4	1.6
pH 4.0 + NA	6.0	36	6.0
pH 4.0:- stored 24 hrs.	2.7	5	1.8
pH 4.0 + NA:- stored 24 hrs.	5.8	50	8.6
pH 4.6	3.6	8	2.2
pH 4.6 + NA	3.6	8	2.2
pH 4.6:- stored 24 hrs.	5.0	16	3.2
pH 4.6 + NA:- stored 24 hrs.	3.9	14	3.6

TABLE 13. NA Mutagenesis of T4 at pH 3.7 and pH 4.0

a) Isolation of r Mutants

Treatment	Plaques Inspected	r Mutants	r Frequency ($x10^{-4}$)
pH 3.7	$8.0x10^4$	19	2.4
pH 3.7 + NA	$1.3x10^5$	245	19.1
pH 4.0	$3.4x10^5$	69	2.0
pH 4.0 + NA	$1.5x10^5$	180	12.0

b) Classification of r's into Genotypes

Treatment	rI		rII		rIII	
	No's	%	No's	%	No's	%
pH 3.7	5	38.5	8	61.5	1	7.7
pH 3.7 + NA	110	54.9	66	32.0	27	13.1
pH 4.0	10	17.2	43	74.1	5	8.6
pH 4.0 + NA	60	40.0	69	46.0	21	14.0

c) Reversion Analysis of rII Mutants

Class of Reversion	Treatment							
	pH 3.7		pH 4.0		pH 3.7 + NA		pH 4.0 + NA	
	No's	%	No's	%	No's	%	No's	%
No Reversion	0	-	3	7.0	11	16.7	8	11.8
High Frequency	0	-	2	4.6	7	10.6	14	20.6
Spontaneous Only	8	100	36	83.7	14	21.2	18	26.5
With 2-AP	0	-	1	2.3	23	34.8	19	27.9
With 5-BU	0	-	0	-	4	6.1	7	10.3
With 2-AP & 5-BU	0	-	1	2.3	7	10.6	2	2.9

d) Frequencies of rII Deletions $\left(\frac{\text{Number of Deletions}}{\text{Total Phage Inspection}}\right)$

Treatment	Deletion Frequency
pH 3.7	$< 1.3x10^{-5}$
pH 3.7 + NA	$3.2x10^{-5}$
pH 4.0	$< 2.9x10^{-6}$
pH 4.0 + NA	$6.0x10^{-5}$

was in accord with Drake (1970); ie. an excess of rII's. It has been mentioned that transition-inducing mutagens favour the production of rI's more than rII's. This was true for NA at pH 3.6 but at pH 4.0 the ratio of ^{rI}/rII was slightly less than unity.

This anomolous result was checked by performing another mutant isolation experiment. The analysis of the mutants induced was taken only as far as the classification into genotypes. Tables 14a and b show the results of this experiment. It can be seen that the ^{rI}/rII ratios of NA-induced mutants was close to 2 at both pH's. The reason for the difference between this experiment and the previous one is not known.

Although Tessman (1962) did not analyse his rII's other than classifying and measuring the extent of the deletions, it was thought possible that if the difference in pH was responsible for the differential recovery of NA-induced deletions, then other changes in mutational specificity might be observed.

Accordingly all the rII mutants isolated from the first mutagenesis experiment were subjected to reversion analysis and they were mapped within the rII locus.

Table 13c gives the results of the reversion analysis. It can be seen that the proportions of transitions (ie. those that responded to base analogues in spot-tests) was much greater amongst the NA-induced rII's than amongst those of spontaneous origin. Also the fraction of presumed deletions (ie. those that failed to give any revertants) was higher in the induced rII's than in the spontaneous ones, whether mutagenesis was carried out at pH 3.7 or pH 4.0.

The rII mutants were mapped within the rII locus. Figure 5a

TABLE 14. NA Mutagenesis of T4 at pH 3.7 and pH 4.0

a) Isolation of r Mutants

Treatment	Plaques Inspected	r Mutants	r Frequency ($x10^{-4}$)
pH 3.7	$3.0x10^5$	75	2.5
pH 3.7 + NA	$4.7x10^4$	89	19.1
pH 4.0	$5.6x10^4$	14	2.5
pH 4.0 + NA	$1.2x10^5$	196	17.0

NA treatment was for 15 secs. at pH 3.7 and for 4.5 mins. at pH 4.0. The survival levels were 1.8% and 2.1% respectively.

b) Classification of r's into Genotypes

Treatment	rI		rII		rIII	
	No's	%	No's	%	No's	%
pH 3.7	26	34.7	47	62.7	2	2.7
pH 3.7 + NA	54	61.4	29	33.0	5	5.6
pH 4.0	8	57.1	6	42.9	0	-
pH 4.0 + NA	120	61.2	71	36.2	5	2.6

illustrates the NA-induced rII spectra of point mutants. The spectra are based on comparatively few numbers of mutants and hence there are to be expected some differences between them due to sampling. However comparison of the spectra shows no marked differences between the two nor between them and the NA-induced spectrum given by Benzer (1961).

Turning to the deletions, a total of nine unambiguous (on the basis of the criteria given in the Materials and Methods) deletions were induced by NA at pH 4.0 and five at pH 3.7. Two rII's induced by NA at pH 4.0 and both mapping within the A1a region were never seen to revert but could not be rigourously shown to be deletions, since I did not possess two point mutants in the appropriate region.

The frequencies of deletions are given in Table 13d and their dimensions in Figure 5b. At both pH's the frequency of rII deletions was enhanced after NA treatment in agreement with Tessman rather than Koch and Drake. The actual frequencies of induced deletions were slightly lower than the value reported by Tessman. As was the case for Tessman, the majority of the deletions extended to the right of the rIIB cistron. The reason for this may be that the locus immediately adjacent to the rIIB cistron is entirely dispensable (Dove 1968; Sederoff et al. 1971). The region appears to have a minor role in DNA breakdown (Bruner et al. 1972; Souther et al. 1972). If however mutations in the locus to the left of the A cistron are lethal then automatically there will be a bias in favour of deletions entering the rII cistron from the right. Unfortunately the nature of the locus adjacent to the rIIA cistron is not known.

Figure 5a.

Representation of the Spectra of rII Point Mutations Induced by NA at pH 3.7 and pH 4.0

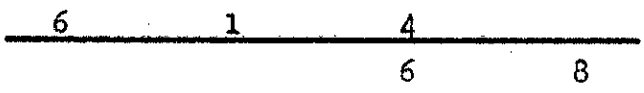
Each line represents a region of the rII locus (see Fig.1 for actual position of each region). The designation of each region is given below each line. Each number represents a site and the value of the number indicates the number of independently-arising mutants at each site. Within any one region the sites are not placed in order.

The numbers above the lines:- Induced by NA at pH 3.7
The numbers below the lines:- Induced by NA at pH 4.0

If mutants isolated at pH 3.7 and pH 4.0 mapped at the same sites this is indicated by the numbers above and below the line being aligned.

The figures in brackets above the line represent the codes of the known hotspots (See Strains in Materials and Methods). Where the figure in brackets is aligned with one of the sites found in this work, this means that no recombination between that site and the known hotspot could be detected.

e.g. (rII106) (rII131)



This represents the A6c region and shows that 6rII mutants induced by NA at pH 3.7 were found to coincide with rII106 site of Benzer and that 8 rII's induced at pH 4.0 mapped at the rII131 site. At another single site 4 rII mutants induced at pH 3.7 and 6 rII's at pH 4.0 were found to be coincident and a still further site was represented by a solitary rII induced by NA at pH 3.7. This example is a hypothetical one chosen merely to illustrate the rationale of the figure.

Figure 5a.

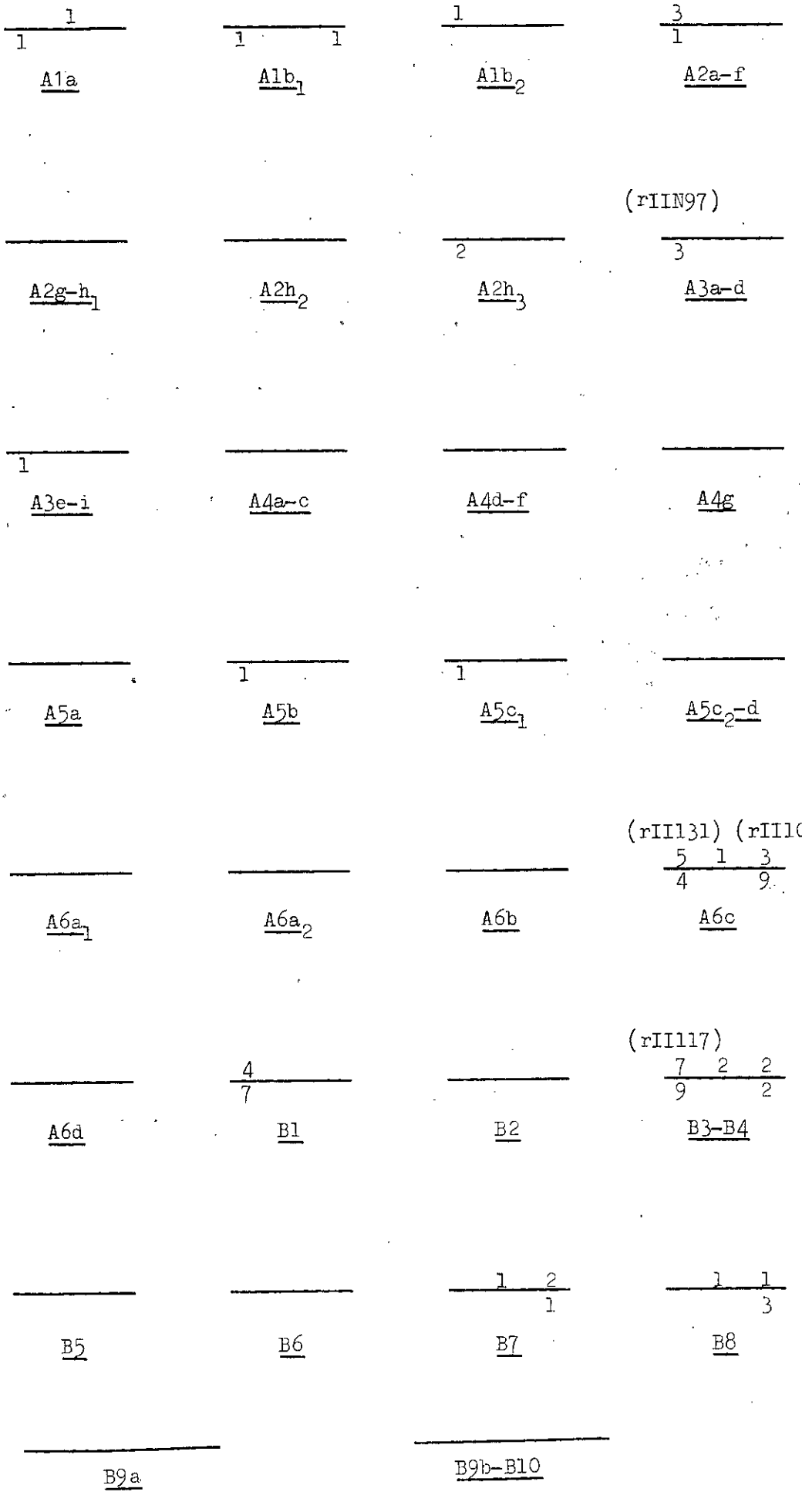

1
1
A1a
1 1
A1b1
1
A1b2
3
1
A2a-f
A2g-h1
A2h2
2
A2h3
(rIIN97)
3
A3a-d
1
A3e-i
A4a-c
A4d-f
A4g
A5a
1
A5b
1
A5c1
A5c2-d
A6a1
A6a2
A6b
(rII131) (rII106)
5 1 3
4 9
A6c
A6d
4
7
B1
B2
(rII117)
7 2 2
9 2
B3-B4
B5
B6
1 2
1
B7
1 1
3
B8
B9a
B9b-B10

Figure 5b.

Representation of the Dimensions of rII Deletions Induced by NA at pH 3.7 and at pH 4.0

The sequence of subregions of the rII locus which could be obtained by deletion mapping with my collection of known deletions mutants is shown in the middle of the figure.

Above and below this representation of the locus the lines delineate the extent of the deletions induced by NA.

Above :- Deletions induced with NA at pH 3.7

Below :- Deletions induced with NA at pH 4.0.

Figure 5b.

A1 a	A1 b_1	A1 b_2	A2 a-f	A2 g-h_1	A2 h_2	A2 h_3	A3 a-d	A3 e-i	A4 a-c	A4 d-f	A4 g	A5 a	A5 b	A5 c_1	A5 c_2-d	A6 a_1	A6 a_2	A6 c	A6 d	B1	B2	B3	B4	B5	B6	B7	B8	B9a	B9b B10

The results point to the fact that the NA-induced spectrum of r mutants is not influenced by the pH of the treatment over the range measured here and so the anomaly between Tessman's results and that of Koch and Drake remains. If anything the protocol used in this work was more similar to that used by Drake, ie. $NaNO_2$ rather than KNO_2 and a higher concentration of nitrite. One difference between the protocol of Koch and Drake and of this work is that the T4 treated with NA already carried a leaky rII mutation. However this was probably not responsible for the failure of Koch and Drake to induce deletions with NA since even in normal T4 strains no deletions were induced (Drake; pers. comm.). It is of course possible that the difference in the two sets of work is due to T4 strain differences though there is no evidence for this.

It should be said that in subsequent work in which NA was used as a mutagen, deletions were again found to be induced. (See Chapters 3 and 5 of Results).

3. EFFECT OF P-FLUOROPHENYLALANINE ON INDUCED MUTATION IN T4

PFPA is a structural analogue of PA, and the choice of this agent as an ancillary treatment was prompted by two reports, one of which showed that it was mutagenic in Ustilago (Lewis and Tarrant 1971) and the other that it could hasten the onset of ageing in Neurospora (Lewis and Holliday 1970). These findings were interpreted by the authors as being due to the incorporation of PFPA into enzymes, with a resultant loss of catalytic specificity. If such lack of accuracy affected proteins in the translational machinery this could cause a more rapid onset of the error catastrophe proposed by Orgel (1963) to account for ageing. Similarly it was suggested that PFPA incorporation into such enzymes as DNA polymerase could result in loss of fidelity of the enzyme causing an increase in mutation frequencies. Certainly genetic alteration of DNA polymerase can affect mutation rates (Speyer, Karam and Lenny 1969; Drake et al. 1969).

In addition Kilbey (unpublished results) has found that in the K3/17 strain of Neurospora, the UV-induced reversion frequency of the adenine allele was depressed some two-fold if the spores had been pregrown with PFPA present. The inositol allele however was induced to revert by UV at a higher rate after pregrowth with the analogue. The reason for the differential effect at the two loci has no explanation, nor has the fact that Kilbey found no mutagenic effect of PFPA alone, in contrast to the findings on Ustilago mentioned above.

Another amino acid analogue, canavanine, has been shown to

enhance EMS-induced mutation rates in barley (Khalatar et al. 1971).

There is a certain irony attached to the findings of mutagenic and co-mutagenic properties of amino acid analogues since Auerbach has reminisced that prior to the demonstrations of DNA as the genetic material, such compounds were considered as potential mutagens, the rationale being based on the concept of the proteinaceous gene. Thus amino acid analogues might be expected to change the structure and specificity of the gene; in other words, mutation. Plus ca change ...!

Summaries of the range of biological effects of amino acid analogues in microorganisms are given by Richmond (1962) and a catalogue of such agents is to be found in Richmond (1969). Some of the specific effects of PFPA will now be given.

It slows down the rate of protein synthesis and the general rate of E.coli growth (Cohen and Munier 1959) and also increases the rate of protein turnover (Cohen et al. 1958). The presence of exogenous PA relieves the inhibition on growth rate imposed by PFPA showing that PFPA is a biological as well as a chemical analogue of PA.

PFPA is accepted by phe-tRNA at a rate about 10% that of PA (Nisman and Hirsch 1958). The analogue can replace up to 75% of PA residues in proteins of E.coli (Munier 1959; Munier and Cohen 1959) the replacement probably being at random throughout the population of proteins (Cowie et al. 1959).

One well-known property of PFPA is its ability to haploidize a variety of diploid fungi and it is routinely used for this purpose. (Lhoas 1961 and 1968; Da Cunha 1970; Day and Jones 1968, 1169 and 1971). Haploid cells of higher plants have been shown to be

at a selective advantage in the presence of PFPA (Gupta and Carlson 1972). The mechanisms involved in these phenomena are not known.

In the bacteriophage MS2, PFPA decreased the infectivity of the RNA of phage which had been grown in its presence and this was interpreted as being due to the incorporation of the PFPA into an enzyme such as RNA replicase with a resultant increase in the frequency with which errors are produced in the genetic material of the phage (Abdel-Hady and Leach 1972). This is of course analogous to the interpretation of Lewis and Tarrant (1971) to account for their results.

PFPA was used in conjunction with two mutagens, 5-BU and NA.

I. 5-BU Mutagenesis

The preliminary experiments were designed to determine the appropriate concentration of PFPA to be used for subsequent work. Both Lewis and Tarrant (1971) and Kilbey (pers, comm,) used concentrations of the analogue which cut growth rate by 50%. These were 1.0 and 8.0mg/l respectively. The difference may be due to differential sensitivity of the two species or to the constitution of the growth media.

(a) Effects of PFPA on Growth of E.coli B in SUM

Since 5-BU was to be used as a mutagen, SU was present in the growth medium of the E.coli prior to and during the mutagenic treatment. SU is an analogue of PABA, a cofactor required for the synthesis of folate, which in turn is needed for methylation of many compounds, one of which is a precursor of thymine (Friedkin and Kornberg 1957). SU inhibits de novo synthesis of thymine, resulting

in increased incorporation of 5-BU into DNA and thus 5-BU mutagenesis is encouraged.

An overnight culture of E.coli B was diluted into aliquots of SUM containing PFPA at various concentrations. The controls were either unsupplemented SUM, or SUM with PA added at the same concentration as the analogue. The cultures were aerated at 37°C and samples were taken at intervals and plated to estimate the viable cell count.

From Figure 6 it can be seen that PFPA at all concentrations used had a marked inhibitory effect on the bacterial growth rate. The kinetics of the growth curves of the analogue-treated cultures were closer to linear, in contrast to the more exponential growth in the control. This was in agreement with the findings of Cohen and Munier (1959) and Munier and Cohen (1956 and 1959).

The concentration chosen for further work was 300mg/l as this caused a 50% reduction in the viable cell number at the end of the growth period compared to the control. Cohen and Munier (1959) used a concentration of 100mg/l but comparisons are not really valid since the media used by these workers was quite different from SUM.

It should be noted that PA at all concentrations tested had no effect on the growth rate compared to the unsupplemented culture.

(b) Effects of PFPA on Plaque Formation of T4

Since it was intended to plate 5-BU-mutagenized phage on plates containing PFPA, it was necessary to determine whether the presence of the analogue in the plates affected e.o.p. or plaque morphology.

A mixture of T4r^+ and T4rII 1272 in approximately equal proportions was added to cultures of E.coli B grown in SUM in the presence

Figure 7.

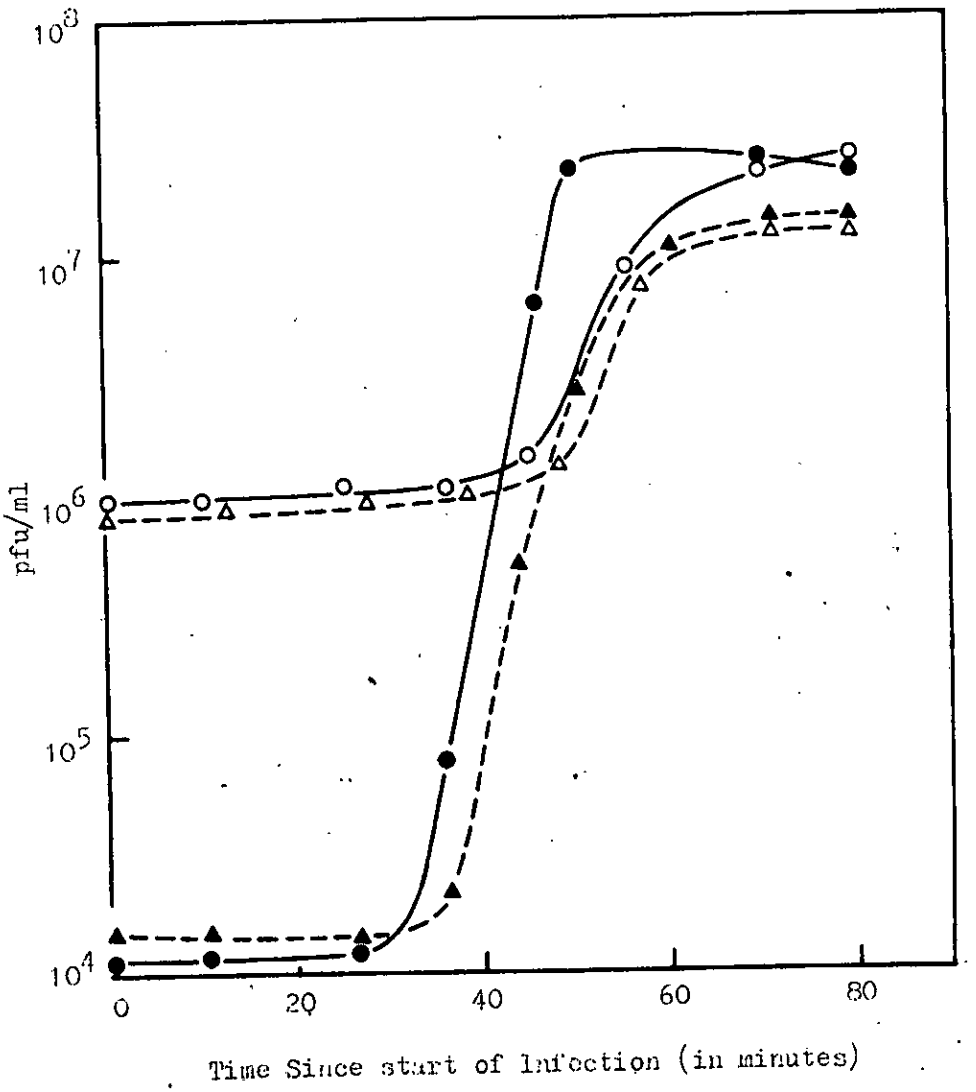

10^8
10^7
10^6
10^5
10^4
pfu/ml
0
20
40
60
80
Time Since start of Infection (in minutes)

Figure 7.

One-Step Growth Experiment with T4 in SUM in the Presence or Absence of PFPA at 300mg/l

Symbols:- Filled Symbols - Lysed Prematurely with $CHCl_3$

Open Symbols - Plated Directly

Circles - PFPA present

Triabgles - PFPA absent

or absence of PFPA at 300mg/l. After 15 minutes samples from each aliquot were plated out. Where the bacterial growth medium had contained PFPA, the analogue was also present in the soft agar at 300mg/l. After incubation, it was found that the absolute frequencies of r^+ and r plaques were the same whether PFPA was present or not and thus such a concentration of PFPA would seem to be appropriate in that it had a marked effect on E.coli growth but did not interfere with e.o.p. or T4 plaque morphology.

(c) Effect of PFPA on One-Step Growth of T4

Since 5-BU has to be administered to phage while they are growing intracellularly it was necessary to determine if PFPA had any effects on phage growth within PFPA-grown E.coli. To do this, a one-step growth experiment was performed.

T4r^+ was added at low m.o.i. in the presence of cyanide to cultures of E.coli B which had been grown in SUM in the presence or absence of PFPA at 300mg/l. The complices were diluted into fresh warm SUM supplemented with PFPA in the case of the aliquot in which the bacteria were grown in the presence of the analogue, and samples were taken at intervals which were either plated directly or after premature lysis. The plating bacteria were grown in the absence of PFPA and the soft agar was unsupplemented.

Figure 7 shows the results of this experiment. For both treatments the burst size was low compared to that typical of T4 grown in normal medium (see Table 18). The reason for this is the presence of SU which as has been said inhibits thymine and hence DNA synthesis. In addition tetrahydrofolate, whose synthesis is also inhibited by SU, is used as a cofactor for a T- even phage-

Figure 6.

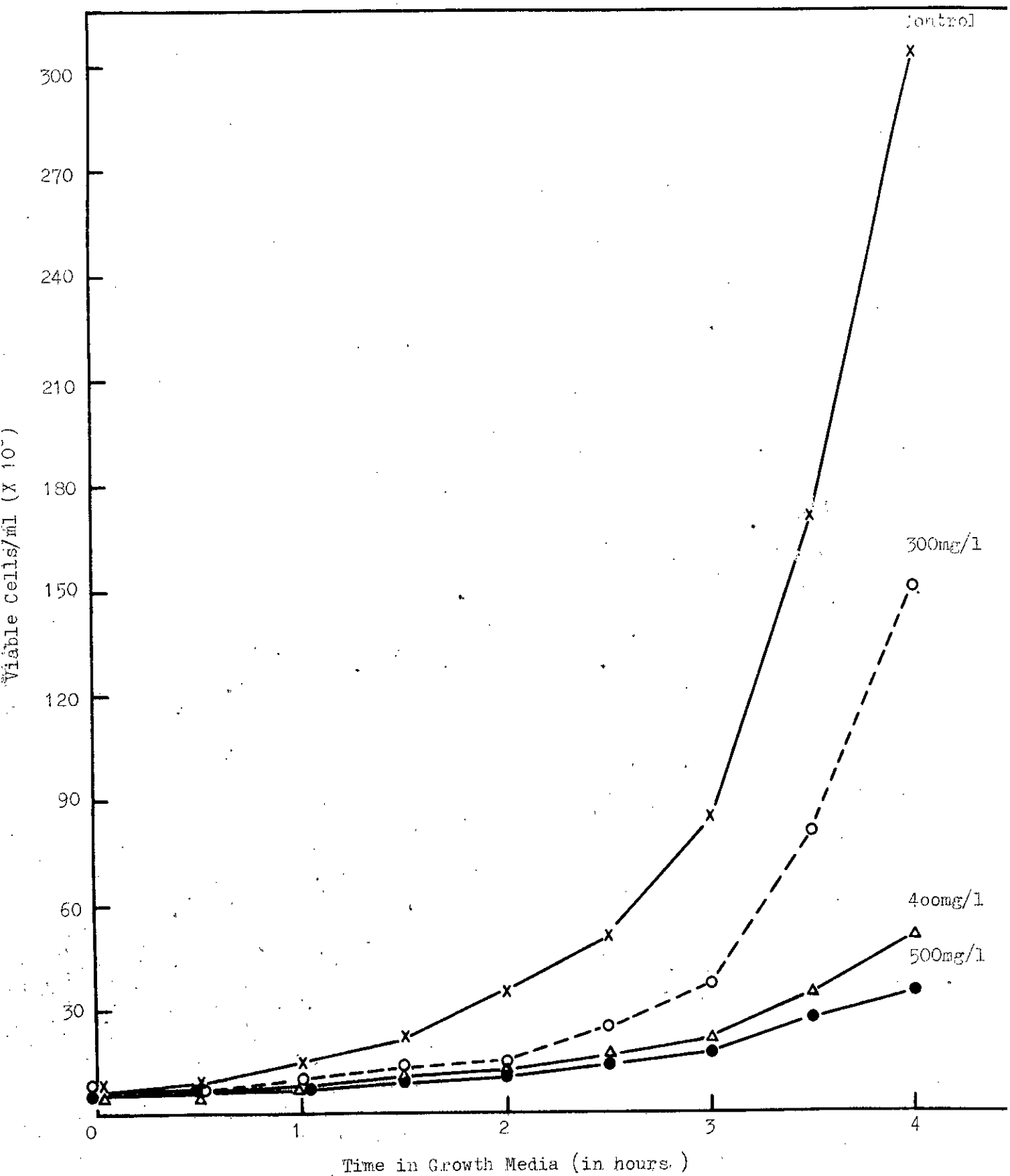

Control
300mg/l
400mg/l
500mg/l
Viable Cells/ml (X 10⁻)
300
270
240
210
180
150
120
90
60
30
0
1
2
3
4
Time in Growth Media (in hours)

Figure 6.

Effect of PFPA on Growth Rate of E.coli B in SUM

The concentrations of PFPA in the different growth media are shown in the figure.

directed enzyme which methylates deoxycytidylic acid (Flaks and Cohen 1957) to form 5-hydroxymethylcytidylic acid which is found in T4 DNA rather than cytidylic acid. Thus SU inhibits T4 DNA synthesis in two ways.

Benzer and Freese (1958) reported a burst size of about 1 in SUM which is some tenfold lower than the value I obtained in the absence of PFPA. This may be due to the fact that they plated out 30 minutes after the start of infection. It can be seen from Figure 7 that lysis in SUM did not occur until 40 minutes had elapsed. Thus Benzer and Freese may well have based their estimate on the number of infective centres rather than the progeny of a burst.

It can be seen from Figure 7 that the presence of PFPA almost doubled the burst size. In a further similar experiment the burst size was measured in SUM in the presence and absence of PA. No difference was found, the respective burst sizes being 11.3 and 12.8. Thus the increase caused by PFPA is not due simply to a general effect caused by a high concentration of amino acid. The length of the latent period and the efficiency of adsorbtion was not affected by the presence of PFPA.

The fact that an agent which inhibited the metabolism of the bacteria actually enhanced the burst size of the phage was an unexpected result and will be discussed later.

Nevertheless, the fact that PFPA did not drastically interfere with phage development meant that 5-BU mutagenesis could be feasible.

(d) 5-BU-Induced Forward Mutation in the Presence of PFPA

Cultures of E.coli B were grown up in SUM and in SUM supplemented with PA or PFPA. The cells in each culture were adjusted

to equal cell density so as to normalize the m.o.i.. The experiment was then performed as described in the Materials and Methods, with PFPA or PA being present throughout the course of the mutagenic treatment. Three independent estimates (a, b & c in Table 15) were made for each treatment. Where appropriate the growth media of the plating bacteria and the top agar contained PA or PFPA, both at 300mg/l.

In Table 15 it can be seen that although PFPA had no effect on the spontaneous mutation frequency compared to either of the control treatments, it reduced the 5-BU-induced r frequency some six-fold compared to the treatments where the SUM was unsupplemented or when PA was present.

It was decided to see whether the inhibitory effect of PFPA on 5-BU-induced forward mutation frequencies also occurred for reverse mutagenesis.

(e) 5-BU-Induced Reversion of Two T4rII Mutants in the Presence of PFPA

Two T4rII mutants (coded L1 and L5) which had been induced with NA and which had been shown by reversion spot-tests to be 5-BU-revertible were chosen. Lysates of each mutant were grown and tested for spontaneous reversion frequency, and the lysate of each mutant with the lowest incidence of revertants was chosen for further study.

In separate treatments, the rII phage from each strain were used to infect, in the presence or absence of 5-BU, E.coli grown in SUM, SUM + PA or SUM + PFPA, the E.coli in each culture having been adjusted to the same cell densities. The experiment was then done

TABLE 15. Induction of r Mutants by 5-BU in SUM in the Presence and Absence of PFPA and in the Presence or Absence of PA

Treatment Medium	Aliquot	No. of r Mutants	Mutation Index ($\times 10^{-4}$)
SUM	a	29	2.3
	b	23	2.5
	c	53	2.9
SUM + 5BU	a	136	48.1
	b	124	41.5
	c	158	54.9
SUM + PFPA	a	11	2.2
	b	17	2.4
	c	15	2.1
SUM + 5BU + PFPA	a	16	8.6
	b	21	8.1
	c	19	9.3
SUM + PA	a	31	2.4
	b	38	3.0
	c	45	2.6
SUM + 5BU + PA	a	141	47.1
	b	306	86.2
	c	127	37.5

as described in Materials and Methods. Three estimates of the reversion frequency after each treatment were made. There was good agreement for the estimates within each group and Table 16 gives the mean reversion index of the three values.

The results show that PFPA, though not mutagenic by itself, did depress the 5-BU-induced reversion indices of both rII mutants by a factor of about ten, compared to the other two treatments.

The fact that PFPA inhibited both 5-BU-induced forward and reverse mutagenesis is good evidence against the effect of PFPA being due to differential selection of r genotypes under the conditions used in 5-BU mutagenesis.

Timing of the Effects of PFPA on 5-BU Induced Reversion

Since in all the experiments, PFPA was present both in the growth media of the host bacteria, and during the actual mutagenic treatment, it is reasonable to ask which of the two components is responsible for the depression of 5-BU mutagenesis. It could be that the presence of free PFPA during mutagenic treatment is responsible or else the physiology of the host may be altered by growth in the presence of the analogue such that 5-BU is rendered a less effective mutagen.

In an attempt to resolve these possibilities the two components were separated. This was done by growing E.coli B in SUM either in the presence of PA or PFPA. After 3.5 hour's growth the cells were washed so as to remove free PA or PFPA. Each culture was split into two, one aliquot being suspended in fresh SUM + PA and the other in SUM + PFPA. Where appropriate, 5-BU was added to aliquots of each of these four cultures and T4rII25 was added at low m.o.i. to

TABLE 16. 5-BU-Induced Reversion of T4 rII's L1 and L5 in SUM in the Presence of PFPA or of PA

STRAIN	Treatment Medium	r^+ Revertants	Mean Reversion Index
rIIL1	SUM	27	5.3×10^{-6}
	SUM + 5BU	2216	4.7×10^{-4}
	SUM + PA	17	3.2×10^{-6}
	SUM + PA + 5BU	2041	4.9×10^{-4}
	SUM + PFPA	37	8.3×10^{-6}
	SUM + PFPA + 5BU	176	4.0×10^{-5}
rIIL5	SUM	7	3.5×10^{-7}
	SUM + 5BU	3142	8.0×10^{-4}
	SUM + PA	12	2.8×10^{-7}
	SUM + PA + 5BU	3233	7.7×10^{-4}
	SUM + PFPA	10	3.1×10^{-7}
	SUM + PFPA + 5BU	246	5.9×10^{-5}

all cultures and the reversion indices were measured in the usual way. Three independent estimates for each treatment were made and Table 17 shows the mean reversion index of the three. Where the post-washing medium contained PFPA, so too did the plating agars and the growth medium of the plating bacteria. In Table 17, treatments 1., 2., 7. & 8. were analogous to the original experiments in that the media were constant throughout the experiment. Again PFPA can be seen to have decreased the mutagenicity of 5-BU. A study of the reversion indices where the media were different for the E.coli growth and the mutagenic treatments shows that the major component of its depressing effect on 5-BU mutagenesis was exerted by its presence in the bacterial growth medium rather than in the mutagenesis medium. The slight decrease in 5-BU-induced reversion frequency when PFPA was present only during and after mutagenesis (ie. Treatment 3) indicates that a minor component of the inhibition may be due to the presence of the free analogue during infection.

A general explanation to account for the facts that PFPA depressed 5-BU mutagenesis and enhanced T4 burst size is that it in some way relieves the inhibitory effects of SU. Such a relief would result in enhanced de novo synthesis of folate and hence of DNA. If thymine were present in higher concentrations, then 5-BU incorporation into DNA would be discouraged. The increased burst size could also be accounted for if folate starvation were not so acute in the presence of PFPA.

This hypothesis was investigated in two ways. The experiments were repeated using MM instead of SUM and in addition a different antagonist of folate was used instead of SU.

TABLE 17. Estimation of the Timing of the Inhibitory Effect of PFPA on 5-BU-Induced Reversion of T4 rIIL5

Treatment Code	E.coli Growth Medium	Infection Medium	r^+ Revertants	Reversion Index
1.	SUM+PA	SUM+PA+5BU	4263	6.4×10^{-4}
2.	"	SUM+PA	144	2.4×10^{-7}
3.	"	SUM+PFPA+5BU	2963	4.7×10^{-4}
4.	"	SUM+PFPA	185	2.9×10^{-7}
5.	SUM+PFPA	SUM+PA+5BU	1284	1.1×10^{-4}
6.	"	SUM+PA	52	4.3×10^{-7}
7.	"	SUM+PFPA+5BU	541	8.2×10^{-5}
8.	"	SUM+PFPA	85	1.3×10^{-7}

Effects of PFPA in Minimal Medium

(a) Effect of PFPA on Growth of E.coli B in MM

A culture of E.coli B was diluted into MM with or without PFPA at 300mg/l. The cultures were aerated at 37°C and samples were taken and plated. Figure 8 shows the results. PFPA had a more marked effect on the growth rate in MM than in SUM in that the viable cell density at the end of 4.0 hours growth was about 25% that of the control in MM than in SUM. The growth rate of the culture with PFPA present was similar whether the medium was MM or SUM.

(b) Effect of PFPA on T4 One-Step Growth in MM

The experiment was performed as describedibefore, and was done using both T4r$^+$ and T4rII1272. E.coli B was grown up in SUM or MM either in the presence or absence of PFPA at 300mg/l. In addition the effects of 5-BU on the burst sizes was measured by adding the base analogue (0.05mg/ml) to some of the aliquots during the infection.

Figure 9 shows only the effect of PFPA in MM on the one-step growth parameters of T4r$^+$. The results for rII1272 were essentially the same as the wild type. Table 18 shows the mean (of two experiments) burst size of both T4r$^+$ and T4rII1272 when grown in the various media.

The results show that in the absence of SU, PFPA, far from enhancing the burst size, reduced it to some 25% that of the control. Analogous to the results of the bacterial growth experiments, this difference could be attributed to a marked increase in the burst size of the control when SU was omitted whereas the size of the

Figure 8.

Effect of PFPA (300mg/1) on Growth Rate of E.coli B in MM

The constitution of the growth media are shown in the figure.

Figure 8.

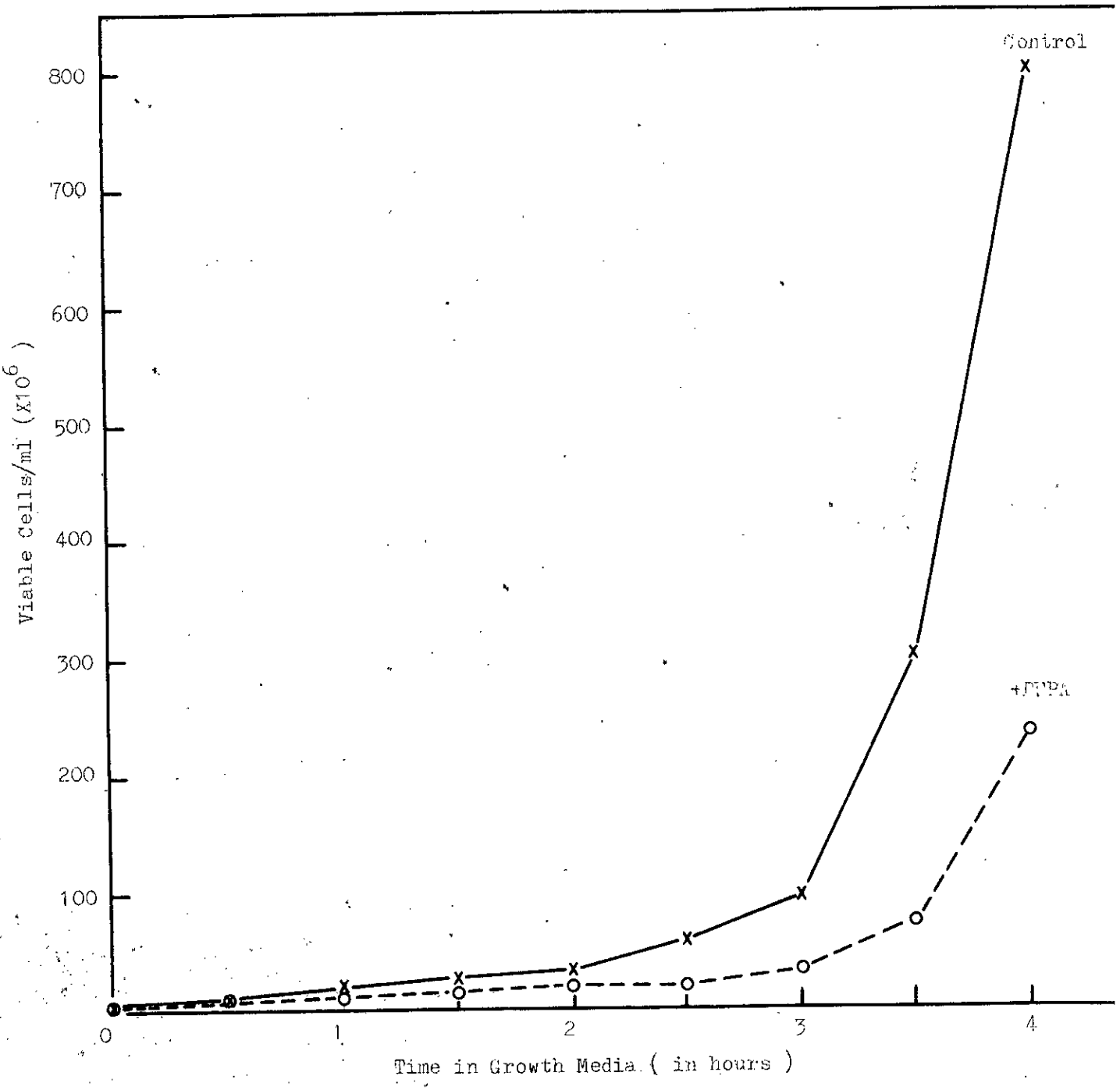

Control
800
700
600
500
400
300
200
100
Viable Cells/ml (X10^6)
0
1
2
3
4
Time in Growth Media (in hours)

Figure 9.

One-Step Growth Experiment with T4 in MM in the Presence or Absence of PFPA at 300mg/l

Symbols as for Figure 7.

Figure 9.

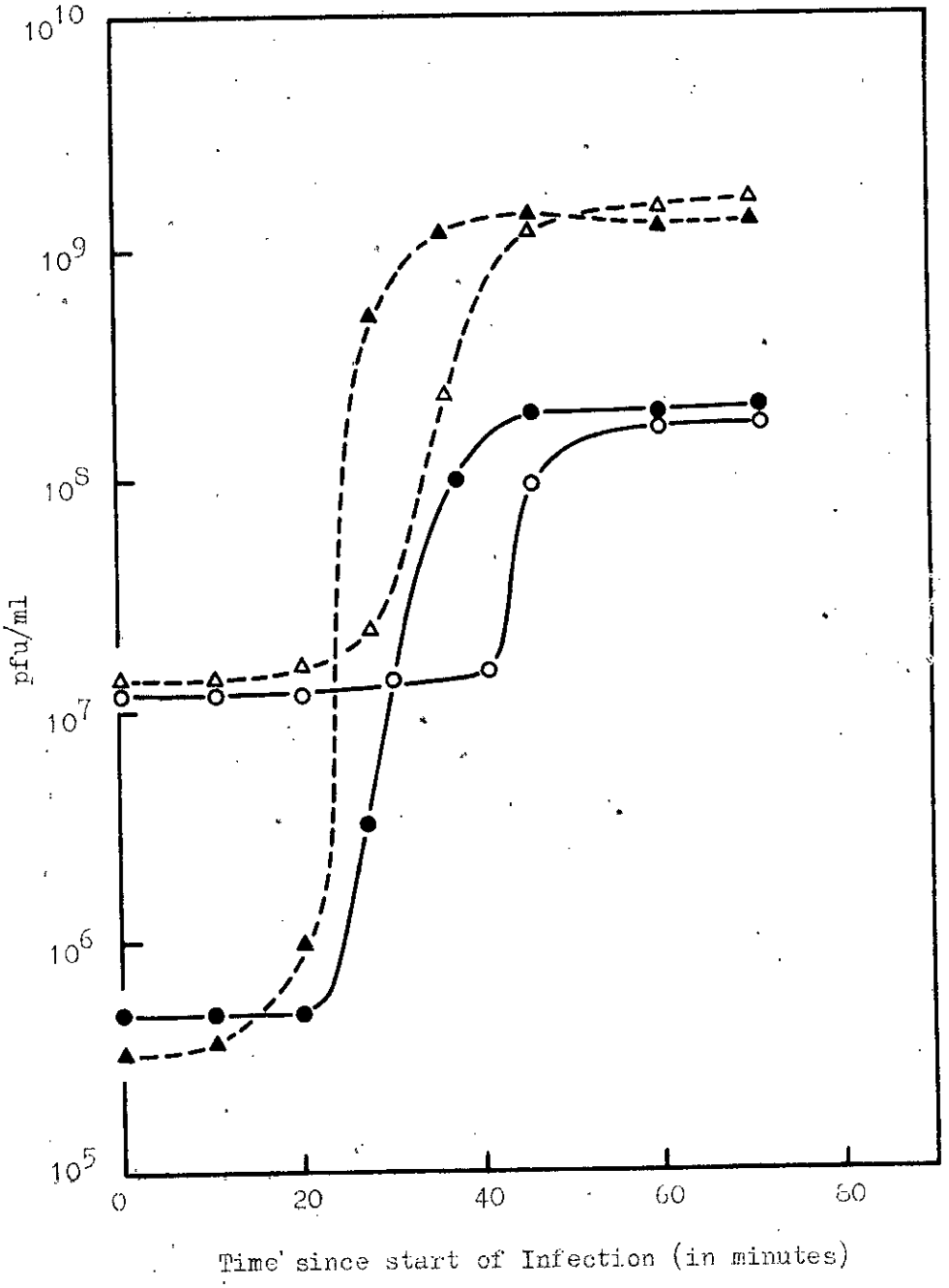

10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
pfu/ml
0
20
40
60
80
Time since start of Infection (in minutes)

TABLE 18. Comparison of the Effects of PFPA on Mean T4 Burst Size in the Presence and Absence of SU and in the Presence and Absence of 5-BU

Growth Medium	Mean Burst Size	
	T4r$^+$	T4rII1272
SUM	12.6	10.5
SUM+5BU	10.5	11.6
SUM+PFPA	26.5	24.3
SUM+5BU+PFPA	23.5	24.3
MM	122.1	92.0
MM+5BU	126.3	90.6
MM+PFPA	29.6	23.5
MM+5BU+PFPA	28.6	21.9

burst in the presence of PFPA was very much the same in MM and SUM.

In none of the growth conditions did the presence of 5-BU have any significant effect on burst size.

(c) Effect of PFPA on 5-BU-Induced Reversion of T4rII L5 in MM

The experiment was performed in the same way as described before except that SU was omitted from one of the treatment media. Table 19 gives the mean (of three estimates) reversion indices of L5 after the different treatments.

As was to be expected, in the absence of SU, 5-BU mutagenesis was depressed but was nevertheless quite strongly mutagenic. It is clear however that when 5-BU was administered in MM, PFPA had no effect on the induced reversion index, in contrast to the situation when SUM was used as the treatment medium.

The findings that in MM, compared to the controls, PFPA had a more inhibitory effect on E.coli growth, depressed the size of the burst, and had no effect on 5-BU mutagenesis supports the notion that PFPA can indeed relieve the inhibition which is imposed by SU on bacteria and phage and that this relief can mask the general inhibitory action of PFPA.

It is pertinent to ask whether this postulated relief by PFPA was specific to SU inhibition, or whether it was a more general phenomenon such that PFPA could relieve blocks imposed by other drugs at other points in the folate biosynthetic pathway.

To this end the experiments concerning the effects of PFPA on growth rate of E.coli, T4-burst size and 5-BU mutagenesis were done, using the drug TM (2,4-diamino-5(2'4'5'trimethoxybenzyl)-pyrimidine) rather than SU.

TABLE 19. Effect of PFPA on 5-BU-Induced Reversion Frequency of T4 rII L5 in the Presence and Absence of SU

Growth Medium	r^+ Revertants	Mean Reversion Index
MM	41	6.7×10^{-7}
MM+PFPA	37	6.2×10^{-7}
MM+5BU	678	1.8×10^{-5}
MM+5BU+PFPA	554	1.5×10^{-5}
SUM	69	6.9×10^{-7}
SUM+PFPA	82	7.5×10^{-7}
SUM+5BU	1391	4.5×10^{-4}
SUM+5BU+PFPA	315	7.7×10^{-5}

Effects of PFPA in the Presence of TM

TM is a potent inhibitor of dihydrofolate reductase in bacteria (Burchall and Hitchings 1965) and this inhibition results in a depletion of tetrahydrofolate (Bertino and Stacey 1966; Wilson, Farmer and Rothman 1966) with a consequent depletion of synthesized thymine. TM also has been shown to inhibit RNA (Then and Angehrn 1972) and protein synthesis (Dale and Greenberg 1972) but both these inhibitions are less than that imposed by TM on DNA synthesis, which is probably caused by thymine starvation (Dale and Greenberg 1972) rather than by the more indirect method mediated by the inhibition of protein synthesis proposed by Eisenstadt and Langyel (1966).

(a) Effect of PFPA on Growth of E.coli B in TMM

The extent of the inhibition caused by TM on bacterial growth rate has been shown to depend on the constitution of the growth medium (Bushby and Hitchings 1968). In particular, the exogenous addition of endproducts of folate metabolism, such as certain amino acids, purines and pyrimidines, were found to relieve the inhibition.

Accordingly it was necessary to estimate the concentration of TM in the MM used here which would inhibit E.coli growth to the same extent as SU before going on further to measure the effects of PFPA in TMM.

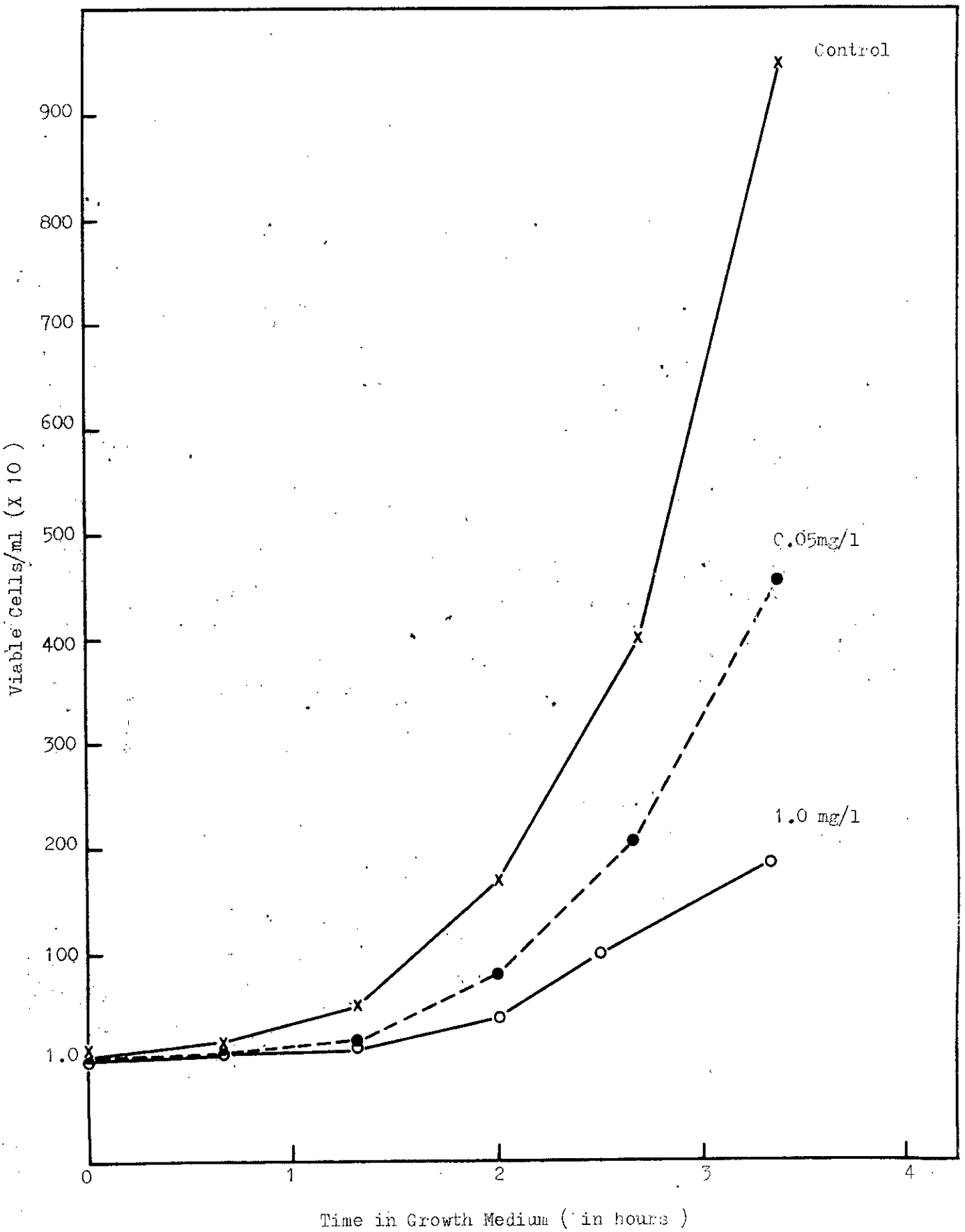
E.coli B was diluted into MM to which TM had been added at different concentrations. The cultures were grown up and sampled. It was found that TM at 0.1mg/l inhibited E.coli growth to about the same extent as did SU in the earlier experiments (see Figure 10). Concentrations of TM greater than 0.2mg/l were found to be bacterio-

Figure 10.

Effect of TM on Growth Rate of E.coli B in MM

The concentrations of TM in the different growth media are shown in the figure.

Figure 10.


Control
0.05mg/l
1.0 mg/l
900
800
700
600
500
400
300
200
100
1.0
Viable Cells/ml (X 10)
0
1
2
3
4
Time in Growth Medium (in hours)

cidal.

Having chosen 0.1mg/l as the appropriate concentration of TM the effect of adding PFPA (300mg/l) to this medium on E.coli B growth rate was estimated. This was done in the usual way, and the E.coli viable cell densities after 3.5 hours growth in the various media are given in Table 20.

In TMM, the presence of PFPA at 300mg/l reduced the final viable titre to about 25% the value in the TMM alone. This is a similar reduction to that found when PFPA was used in MM and compares with the 50% reduction found in SUM.

(b) Effects of PFPA on T4 One-Step Growth in TMM

The experiment was performed as already described. E.coli B was grown in MM, TMM (TM at 0.1mg/l) and TMM + PFPA. The cultures were equilized for cell density and were each infected with $T4r^+$. Figure 11 shows the growth curves obtained for the three growth conditions. The mean burst sizes of T4 in the presence of MM, TMM and TMM + PFPA were 168.4, 43.5, and 15.6 respectively. The presence of PFPA therefore reduced the burst size some 60-70% in the TMM in contrast to the enhancement of burst size in SUM caused by PFPA. TM itself reduced the burst size to about 25% of the value found in MM. This reduction was not as great as that caused by SU in a physiologically equivalent concentration (as determined by the inhibition of E.coli B growth).

Why this difference exists is not clear. It has in fact been shown that E.coli treated with TM at quite high levels can still support phage growth (Miovic and Pizer 1968) but the media in that study and in this one were quite different.

TABLE 20. Effects of PFPA on the Growth of E.coli in the Presence and Absence of TM (0.1mg/l)

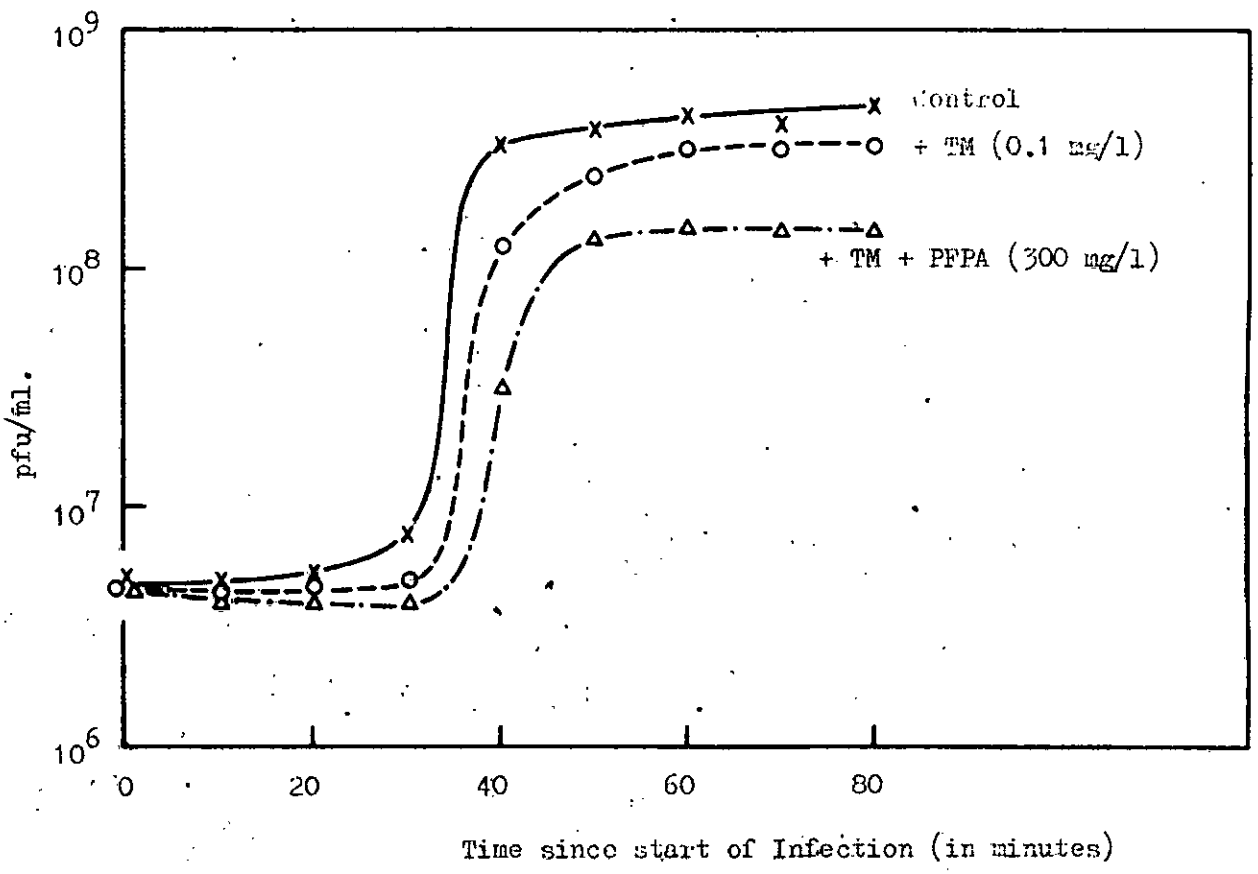
Growth Medium	Viable cells/ml After 3.5 Hours Growth
MM	8.8×10^8
MM+TM (0.1mg/l)	4.5×10^8
MM+PFPA	2.4×10^8
MM+PFPA+TM	7.9×10^7

Figure 11.

One-Step Growth Experiment with T4 in the Presence of PFPA and of TH

The constitutions of the different growth media are given in the figure. The curves obtained after premature lysis are not given.

Figure 11.


10^9
10^8
10^7
10^6
pfu/ml.
0
20
40
60
80
Control
+ TM (0.1 mg/l)
+ TM + PFPA (300 mg/l)
Time since start of Infection (in minutes)

Effects of PFPA on 5-BU-Induced Reversion of T4rII L5 in TMM

Aminopterin, another folate antagonist which acts in a very similar fashion to TM (but is a great deal more expensive) has been found to enhance base analogue mutagenesis (Freese 1959a) for reasons analogous to those proposed for the increase in mutagenesis caused by SU. In the light of the similarities between TM and aminopterin it is not unreasonable to suppose that TM should also enhance 5-BU mutagenesis.

The estimation of the effect of TM on 5-BU mutagenesis was done using T4rII L5 in reversion studies. The protocol was as already described for reversion experiments except that TM (0.1mg/l) was used instead of SU. In addition, the effects of PFPA on 5-BU mutagenesis in TMM and in MM were measured in the usual way. Table 21 shows the reversion indices of T4rII L5 under the various conditions. (Mean of three estimates.) The presence of TM enhanced the induced reversion index about ten-fold compared to when 5-BU was used in MM. This compares with the approximately forty-fold increase when SU was used as a folate antagonist. It can be seen that PFPA had no effect on 5-BU-induced reversion both in MM and in TMM.

It should be noted that TM was not mutagenic by itself. It is a pyrimidine analogue and might thus have been thought to possess mutagenic powers. Freese (1959a) has shown that only a minority of base analogues are mutagenic and it would seem that TM falls into the category of non-effective base analogues.

One other point which may be considered here is that thymine deprivation imposed by both TM and SU might have been expected to

TABLE 21. Effect of PFPA on the Frequency of 5-BU-Induced Reversion of T4 rII L5 in the Presence and Absence of TM

Treatment Medium	r^+ Revertants	Mean Reversion Index
MM	41	6.3×10^{-7}
MM+5BU	371	1.7×10^{-5}
MM+TM	26	3.5×10^{-7}
MM+TM+5BU	2941	1.4×10^{-4}
MM+PFPA	57	6.8×10^{-7}
MM+PFPA+5BU	250	1.4×10^{-5}
MM+TM+PFPA	51	6.9×10^{-7}
MM+TM+PFPA+5BU	2714	1.2×10^{-4}

have mutagenic consequences. Drake (1973) has demonstrated that mutations in T4 can be induced by thymine deprivation. There are two possible reasons why neither SU or TM were shown to be mutagenic in this study.

(a) Drake (1973) found that only AT mutants were induced to revert by thymine deprivation. The rII mutants, L1 and L5, which I used were both strongly revertible with 5-BU and were therefore likely to be GC at the mutant site (Champe and Benzer 1962b; Drake 1963), and hence may be refractory to reversion caused by thymine starvation.

(b) Drake employed conditions of total starvation unlike the conditions used here where the thymine starvation was not complete.

In a very different system, Stevenson et al. (1973) have shown that neither TM nor sulphamethoxazole (an analogue of PABA, related to SU) could induce chromosomal damage in human patients treated with these drugs.

Discussion of the Effects of PFPA on 5-BU Mutagenesis

The absence of any interaction between TM and PFPA on any of the parameters measured in this study indicates that PFPA cannot relieve all inhibitory blocks in folate metabolism and that there may be a specific interaction between SU and PFPA.

The fact that the burst size in the joint presence of SU and PFPA was higher than that found in SU alone but only slightly less than that found in MM + PFPA suggests that PFPA relieved SU inhibition rather than vice versa. This view is supported by the fact that PFPA in MM had no effect on 5-BU-induced mutagenesis but only depressed it in the joint presence of SU.

The fact that virtually all the depression of 5-BU mutagenesis

in SUM could be accounted for by pregrowth of E.coli B suggests that the preinfection host physiology was responsible for the effect.

Some possible explanations for the phenomenon are discussed below. It should be born in mind that at this time such models must be a little speculative.

(a) SU acts as a competitive inhibitor of the condensation reaction between PABA and 2-amino-4-hydroxy-6-methylpyrophosphate-dihydropteridine in the formation of dihydropteroic acid (Woods 1940; Griffin and Brown 1964).

If PFPA was incorporated into the primary sequence of the enzyme which catalyses this reaction, it might paradoxically, increase the specificity of the recognition properties of the enzyme such that it can more readily distinguish PABA and SU, and in consequence may have a preferential affinity for PABA compared to the unmodified enzyme. Certainly the extent of inhibition elicited by incorporation of PFPA has been shown to vary greatly between different enzymes (Yoshida 1960; Richmond 1962; Westead and Boyer 1961). Indeed the last authors could trace no decrease in the activities of certain enzymes into which PFPA had been incorporated.

The concept of incorporation of the wrong amino acid actually increasing enzyme specificity is somewhat hard to envisage. This postulated mode of action of PFPA can be thought of as a phenotypic mutation. Certain genotypic mutations do in fact result in quite marked differences in specificities of enzymes in the utilization of different substrates (see Harris 1970). In addition, the existence of T4 gene 43 antimutator alleles demonstrates the possibility of mutations actually being able to increase enzyme

specificity.

In order to account for the lack of interaction between T4 and PFPA, one would have to state that an increase in discriminatory properties of dihydrofolate reductase does not occur when PFPA is incorporated into it. Since T4 itself directs the synthesis of a dihydrofolate reductase (Mathews 1967a & b; Hall 1967) one has to apply this proviso to phage and bacterial enzyme alike. Incidentally the T4 enzyme has the unusual feature of being incorporated into the tail plate of the phage (Kozloff et al. 1970; Mathews 1971).

(b) The fact that mutants of Neurospora (Tatum et al. 1954) and of E.coli (Davis 1955) can be isolated which show multiple requirements for PABA; tyrosine, PA and tryptophan indicates that these compounds are metabolically related. This is not surprising since all are aromatic amino acids and indeed they all share a common precursor, shikimic acid (Davis 1955).

The control mechanisms of aromatic amino acid synthesis are complex and are poorly understood. Brown (1970a and b) has shown that there are at least two sets of permeases for each of the amino acids, one specific for each, and a general one, amenable to feedback inhibition by any of the end-products. In addition an E.coli gene (ptyR) jointly regulates all the enzymes in tyrosine synthesis and one of enzymes in the PA pathway. The repressor product of this gene can be activated by any of the amino acids such that the target enzyme in the PA pathway is repressed while the enzymes in the tyrosine pathway are derepressed.

It has already been mentioned that the tryptophan analogue, IP, has just the opposite effect from tryptophan in deciding the state

of activity of the tryptophan biosynthetic pathway.

If in an analogous way, PFPA possesses the ability to disorientate the control of aromatic amino acid synthesis, a possible explanation for the interaction between SU and PFPA can be postulated.

Given that shikimic acid is common to all the pathways, if PFPA could affect the overall control of aromatic amino acid synthesis such that PABA was preferentially synthesized, the consequence of the increased flux to PABA would be a larger pool size of the co-factor which could then alleviate the competitive inhibition of SU.

Given the nebulous state of knowledge of the control mechanisms of aromatic amino acid synthesis in the normal state, let alone in the presence of PFPA, the actual mechanism of such a postulated interference of control by PFPA must be a matter of conjecture.

(c) This point again relates to the similarity of structure between PA and PABA, and consequently of their respective analogues PFPA and SU. This third model proposes that because of the similarity of shape and charge between PFPA and SU, the former can prevent the formation of the complex between SU and enzyme without itself acting as an inhibitor. One would need to say that this shielding effect by PFPA does not, or at least does, but to a lesser extent, apply to the situation where PABA is presented as substrate.

The protein in which this shielding occurs need not necessarily be the enzyme involved in the condensing reaction with PABA but could be any protein which uses PABA as substrate. This point applies equally to model (a) above.

One would predict that in model (a) the effect would require E.coli to be grown in the presence of PFPA so as to allow the

analogue to be incorporated into the relevant bacterial enzyme.

Since bacterial protein synthesis stops on phage infection (Benzer 1953; Bilizikian et al. 1967; Levin and Burton 1961) due to the exclusion of host mRNA from the bacterial ribosomes (Kennel 1968 & 1970), it is to be expected on this model that the presence of PFPA during infection would have no further effect on the size of the burst or 5-BU mutagenesis. For the latter parameter, this was in fact the case.

Models (b) and (c) require that the pools of the required products of folate metabolism are so depleted during the growth of the bacteria in SUM, that the addition of PFPA during the mutagenic treatment is not sufficient to cause an increase in the thymine pool to an extent sufficient to depress 5-BU mutagenicity.

All three models invoked somewhat unorthodox mechanisms to account for the interaction between PFPA and SU, but on the other hand the phenomenon to be explained is of an unusual nature. The validity of the hypotheses could however be tested.

In model (a) assays of the condensation reaction with PABA as substrate using extracts of E.coli grown in the presence or absence of PFPA would show whether there was differential competitive inhibition by SU on enzyme from the two extracts. On the basis of model (a), PFPA need not be present in the free state during the assay.

If model (c) were valid, free PFPA would be required during the assay for any relief of SU inhibition by PFPA to be observed and the bacteria need not have been grown in the presence of the analogue.

With regard to the testing of these two models it has already been mentioned that the target protein need not necessarily be the enzyme which catalyses the condensation of PABA. Therefore if the assays were done on this enzyme, and PFPA was found to have no effects, this in itself would not preclude the basic validity of the models. An exhaustive series of assays of all enzymes which use PABA as a substrate would have to be carried out before any definite conclusion could be reached.

If model (b) were true, this would be reflected in an alteration of pool sizes and enzyme activities concerned with at least some of the intermediate steps in the synthesis of aromatic amino acids, when the cells are grown in the presence of PFPA. To determine whether this in fact occurs would be a task of sizeable proportion.

Since the phenomenon involving the interaction between PFPA and SU is somewhat peripheral to mutagenesis studies, I decided not to investigate vigourously the underlying mechanisms involved, though I believe the problem to be of no small interest.

If then one can consider the antimutator effect of PFPA on 5-BU mutagenesis in the presence of SU to be of a trivial (from the mutation worker's point of view) nature, it would seem that PFPA has no effect on the efficiency of 5-BU as a mutagen in T4. This lack of effect will be discussed at the end of this chapter in relation to the findings on the effect of PFPA on NA mutagenesis in T4.

II. NA Mutagenesis

The antimutator action of PFPA on 5-BU mutagenesis in the presence of SU was interpreted as being due to a dose reduction effect whereby less mutagen was able to reach its site of action.

If phage are mutagenized extracellularly, as is the case with NA as mutagen, in the absence of PFPA, then clearly the dose of mutagen administered cannot be modified in the way proposed for 5-BU. When mutagenized particles are used to infect E.coli grown in the presence or absence of PFPA, any differences which may be observed will be due to modifications of resolution and expression of pre-mutational lesions.

NA Induced Forward Mutation Experiments

The NA treatment was performed in the way described, phage being treated with 0.1M $NaNO_2$ in pH 4.0 acetate buffer to about 1% survival. After treatment the phage were used to infect E.coli B which had been grown in MM supplemented with either PA or PFPA (both at 300mg/l) and the complices were plated, using soft agars to which PA or PFPA had been added as appropriate. The plates were incubated and inspected for r plaques. Table 22a shows that there was a slight enhancing effect of PFPA on NA-induced (but not spontaneous) r frequency. The effect was only a small one and would not be valid in its own right, but Table 22b, c and d shows the results of three repeats of the experiment. In all experiments the induced mutation frequency was about twice as high when PFPA was present than when PA was added.

The fact that PFPA was present in the soft agar prompted me to

TABLE 22. NA-Induced r Frequencies in the Presence and Absence of PFPA

NA treatment was for 4.5 mins. in all experiments.

	Growth Medium and Treatment	No. of Plaques Inspected	No. of r Plaques	r Frequency
Experiment a)	+PA	4.2×10^4	14	3.3×10^{-4}
	+PA +NA	9.0×10^3	16	1.8×10^{-3}
	+PFPA	3.5×10^4	12	3.4×10^{-4}
	+PFPA +NA	8.3×10^3	22	2.7×10^{-3}
		% Surv.:- 2.3%		
Experiment b)	+PA	1.1×10^4	3	2.7×10^{-4}
	+PA +NA	2.3×10^4	18	7.8×10^{-4}
	+PFPA	9.9×10^3	2	2.0×10^{-4}
	+PFPA +NA	2.2×10^4	24	1.5×10^{-3}
		% Surv.:- 9.4%		
Experiment c)	+PA	5.3×10^4	15	2.8×10^{-4}
	+PA +NA	2.8×10^4	48	1.7×10^{-3}
	+PFPA	5.7×10^4	15	2.6×10^{-4}
	+PFPA +NA	1.0×10^4	29	2.9×10^{-3}
		% Surv.:- 1.6%		
Experiment d)	+PA	5.0×10^4	13	2.6×10^{-4}
	+PA +NA	2.1×10^4	32	1.5×10^{-3}
	+PFPA	4.3×10^4	12	2.8×10^{-4}
	+PFPA +NA	3.2×10^4	82	2.6×10^{-3}
		% Surv.:- 2.2%		

think of a possible explanation for the increased NA-induced mutation frequency.

The ability of the rII protein to tolerate mutational change without actually causing a change in phenotype has been mentioned. If phage carrying such inocuous mutations are plated in the presence of PFPA and the analogue is incorporated into r proteins, then the combined effect of the two components could reduce the activity of the protein to such an extent that an altered phenotype is generated.

This idea is analogous to that of Koch and Drake (1970) who found that in T4 semi-rII strains, full rII mutants could be induced by NA at sites hitherto unreported. Rather than the genotypic sensitization suggested by these authors, I wanted to test whether what might be termed phenotypic sensitization occurred.

This was done simply by taking all the r mutants which had been induced by NA and plated in the presence of PFPA in the experiment whose results were given in Table 22d and restreaking them on E.coli B on plates with PFPA absent. For all the 82 r mutants so tested, r plaque morphology was found on plates lacking PFPA. This shows that the idea of sensitization of the r proteins by PFPA was not responsible for the increase in mutation frequency.

Reconstruction Experiment

A reconstruction experiment was done to determine if r mutants are at a selective advantage after NA-treated phage are plated in the presence of PFPA.

A phage mixture of T4r^+, rII1272 and an rI mutant was made up in an approximate ratio of 50:1:1. The mixture was treated with NA for 4.5 minutes, stored overnight and plated against both E.coli B

and E.coli BB both of which had been grown in the presence or absence of PFPA. The top agars also contained PFPA where appropriate. Counts were made of total plaques and of the incidence of r plaques on both strains of bacteria. Any r plaques on E.coli BB are rI whereas on E.coli B the mutant plaques will be a mixture of both genotypes. Table 23 shows that PFPA had no effect on the relative frequencies of either r genotype whether the phage were treated with NA or not.

Effect of PFPA on NA-Induced r Frequency in Joint Presence of SU

The models put forward to account for the depressive effect of PFPA on 5-BU mutagenesis in SUM all predicted that this antimutagenic effect would be specific to base analogue mutagens. This prediction was tested by repeating the NA mutagenesis experiments but using E.coli hosts which had been grown in SUM ± PFPA rather than in MM ± PFPA.

The results of two such experiments are shown in Table 24 and they can be seen to be virtually the same as were found in the experiments where MM was used. The fact that in the presence of SM, PFPA still increased the NA-induced r frequency is in keeping with the prediction made above.

NA Treatment of T4 Grown in the Presence of PFPA

The protocol of this experiment can be considered as the mirror-image of the first series of experiments.

Lysates from single T4r$^+$ plaques were grown up on E.coli BB in MM supplemented with PA or PFPA, both at 300mg/l. Although it was not checked, it was thought that phage grown in the presence of the analogue would contain PFPA in the somatic proteins.

TABLE 23. Reconstruction Experiment:- NA Treatment of a Mixture of T4r^+, rII 1272 and an rI Mutant

Growth Medium and Treatment	Mean pfu/plate	Mean % of r's on E.coli B (ie. rI & rII)	Mean % of r's on E.coli BB (ie. rI)
PA	524.7	3.24	1.86
PA+NA	294.5	2.51	1.50
PFPA	592.3	3.10	1.62
PFPA+NA	268.1	2.86	1.41

TABLE 24. Effect of PFPA on NA-Induced r Frequency when Host Cells are Grown in SUM

Experiment 1.

Growth Medium and Treatment	No. of r Plaques	r Frequency
SUM+PA	18	2.8×10^{-4}
SUM+PA+NA	46	1.4×10^{-3}
SUM+PFPA	12	2.5×10^{-4}
SUM+PFPA+NA	93	2.9×10^{-3}

NA treatment time:- 4.5 mins.

Survival :- 1.8%

Experiment 2.

Growth Medium and Treatment	No. of r Plaques	r Frequency
SUM+PA	11	2.9×10^{-4}
SUM+PA+NA	69	1.5×10^{-3}
SUM+PFPA	14	2.8×10^{-4}
SUM+PFPA+NA	126	3.0×10^{-3}

NA treatment time:- 4.5 mins.

Survival :- 2.7%

The two sets of phage were treated with NA in the usual way and were scored for survival and for both spontaneous and NA-induced r frequencies. Table 25 shows that these parameters were very similar for both lysates.

This experiment involved NA treatment in the presence of PFPA (if in fact the analogue has been incorporated) but allowed mutant expression to occur in the absence of PFPA, since the analogue was omitted from the growth medium of the plating bacteria and the plating medium and to all intents and purposes only the phage DNA is injected into the host (Hershey and Chase 1952; Hershey 1953). Thus the protocol contrasts with that used in the first series of experiments in which NA was administered in the absence of PFPA and where it was the expression of the mutants which was subjected to PFPA-treatment.

The lack of any effect of PFPA when it was administered in this fashion is not too surprising. It had been thought however that the incorporation of PFPA might alter the structure of the head such that reactivity of DNA to NA might be modified. It has been shown that protein-nucleic acid interactions do have some importance in the action of NA on viruses (Schuster and Schramm 1958; Schuster and Wilhelm 1963; Dussault et al. 1970). Clearly under the conditions used here the effect of PFPA incorporation was not sufficient to modify the reactivity between NA and the DNA.

Effect of PFPA on the Spectrum of NA-Induced r Mutants

The enhanced recovery of r mutants when NA-treated T4 infect E.coli grown with PFPA seems to be a genuine co-mutagenic effect. I wished to determine if there was a change in the specificity of

TABLE 25. NA-Treatment of T4 Grown up in Medium Containing PFPA

a) Survival

Time of Treatment (mins.)	% Survival	
	PFPA-Grown T4	Control Phage
0	100	100
1.0	59.3	67.1
2.0	18.6	22.0
3.0	5.1	8.1
4.0	1.5	1.7
5.0	0.86	0.65

b) r Mutation Frequency (NA treatment was for 5.0 minutes).

Phage Grown in PFPA	NA	Plaques Inspected	r Plaques	r Frequency
No	No	6.4×10^{4}	12	1.9×10^{-4}
No	Yes	4.2×10^{4}	54	1.3×10^{-3}
Yes	No	4.8×10^{4}	8	1.7×10^{-4}
Yes	Yes	4.6×10^{4}	61	1.3×10^{-3}

NA mutagenesis as well as in its potency.

A large scale mutant isolation experiment was performed, using the same protocol described for the preliminary experiments in this section.

The induced r mutants were purified and ascribed to the different r genotypes. The rII's were retained and were mapped within the rII region. In addition reversion analyses were carried out on the rII mutants.

It is evident that though PFPA increased the NA-induced r frequency it had no effect on the specificity of the mutants so induced. Both the relative proportions of the different r genotypes and the distribution of the rII mutant classes were essentially the same whether PFPA was present or not (Table 26). Figures 12a and 12b show that the maps of point mutations and deletions did not differ significantly from the control when PFPA was present.

The fact that there was no change in specificity is perhaps surprising since it suggests that there is a common step in the pathway via which all NA-induced premutational lesions are fixed and that this step is altered by PFPA. The other possibility is that independent mechanisms in the NA mutagenic pathway are all equally affected by PFPA to the same extent. This alternative would seem *a priori* to be somewhat improbable.

Effect of PFPA on NA-Induced Reversion of an rII Mutant

Five rII transition mutants which had been induced by NA were tested for their reversion response to NA. One mutant, designated L4, showed a strong response to NA and was chosen for this reversion

TABLE 26. Isolation and Characterisation of NA-Induced r Mutants Plated in the Presence and Absence of PFPA

a) Isolation of Mutants

Treatment	Plaques Inspected	r Plaques	r Frequency
+PA	6.2×10^{4}	21	3.4×10^{-4}
+PA+NA	1.7×10^{5}	227	1.3×10^{-3}
+PFPA	5.8×10^{4}	26	4.5×10^{-4}
+PFPA+NA	1.5×10^{4}	402	2.6×10^{-3}

NA treatment was for 4.5 minutes and gave a survival of 0.91%.

b) Classification of r's into Genotypes

Treatment	rI		rII		rIII	
	Nos.	%	Nos.	%	Nos.	%
+PA	12	56.2	7	33.3	2	9.4
+PA+NA	126	63.1	62	30.9	12	6.0
+PFPA	11	43.8	14	56.2	0	-
+PFPA+NA	224	60.0	140	35.0	20	5.0

c) Reversion Analysis of rII Mutants

Class of Reversion	Origin of rII's			
	+PA	+PFPA	+PA+NA	+PA+PFPA
High Reversion	1	1	6 (9.7)	14 (10.3)
No Revertants	0	1	8 (12.9)	18 (13.2)
Background Reversion Only	5	10	14 (22.6)	30 (22.1)
With 2-AP	0	1	18 (29.0)	38 (27.9)
With 5-BU	0	0	12 (19.4)	28 (20.6)
With 2-AP & 5-BU	1	1	4 (9.7)	8 (5.9)

Figures in brackets are the % of each class.

Figure 12a.

Representation of the Spectra of rII Point Mutations Induced by NA with Post-treatment Infection in the Presence and Absence of PFPA

Legend:- As for Figure 5a.

The numbers above the lines:- PFPA present in post-NA-treatment medium.

The numbers below the lines:- PFPA absent from post-NA-treatment medium.

Figure 12a.

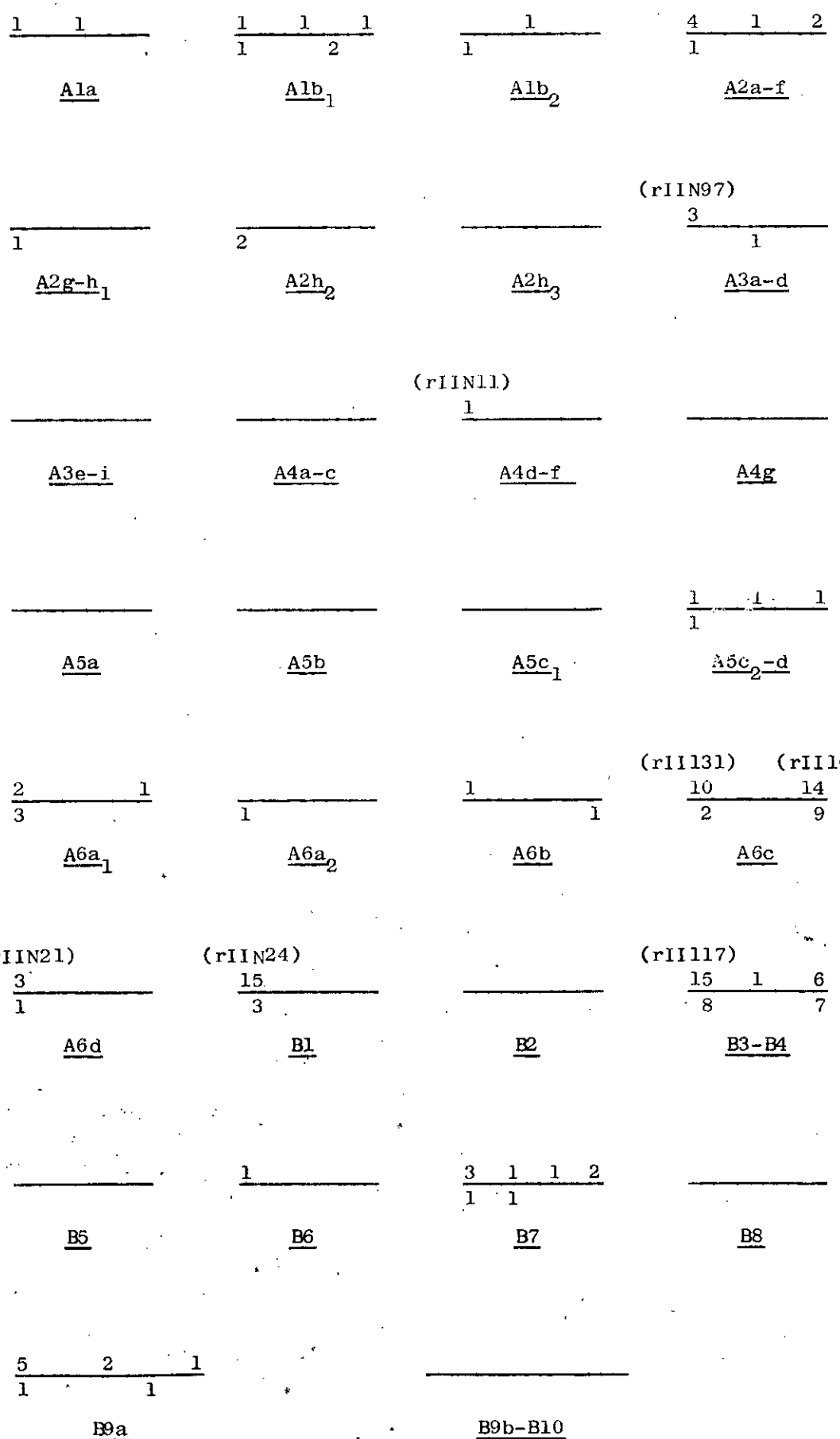

1 1
A1a
1 1 1
1 2
A1b1
1
1
A1b2
4 1 2
1
A2a-f
1
A2g-h1
2
A2h2
A2h3
(rIIN97)
3
1
A3a-d
A3e-i
(rIIN11)
1
A4a-c
A4d-f
A4g
A5a
A5b
A5c1
1 1 1
1
A5c2-d
2 1
3
A6a1
1
A6a2
1 1
(rII131) (rII106)
10 14
2 9
A6b
A6c
(rIIN21)
3
1
A6d
(rIIN24)
15
3
B1
B2
(rII117)
15 1 6
8 7
B3-B4
B5
1
B6
3 1 1 2
1 1
B7
B8
5 2 1
1 1
B9a
B9b-B10

Figure 12b.

Representation of the Dimensions of rII Deletions Induced by NA with Post-treatment Infection in the Presence and Absence of PFPA

Legend:- As for Figure 5b.

Above:- PFPA present in post-NA-treatment medium

Below:- PFPA absent from post-NA-treatment medium.

A1 a
A1 b_1
A1 b_2
A2 a-f
A2 g-h_1
A2 h_2
A2 h_3
A3 a-d
A3 e-i
A4 a-c
A4 d-f
A4 g
A5 a
A5 b
A5 c_1
A5 c_2-d
A6 a_1
A6 a_2
A6 b
A6 c
A6 d
B1
B2
B3
B4
B5
B6
B7
B8
B9a
B9b B10

Figure 12b.

study. In spot tests L4 failed to revert with 2AP but it did respond to 5-BU, suggesting that it carries GC at the mutant site.

The reversion experiment was done by treating L4 with NA for 4.5 minutes in the usual way. The treated and the control phage were then plated on E.coli B and E.coli WØ80 in order to estimate survival and reversion respectively. Where the effect of PFPA was to be measured, the analogue was present in the top agar and in the growth medium of the plating bacteria.

In addition aliquots of treated and control phage were adsorbed to E.coli B grown in the presence or absence of PFPA before being diluted into fresh NM ± PFPA. The cultures were incubated for 60 minutes before lysis was completed by the addition of $CHCl_3$. The phage were then plated against E.coli B and E.coli WØ80 which had been grown in the absence of PFPA.

Thus reversion was measured either after a cycle of growth in the presence of the analogue or after direct plating on the restrictive host. See Table 27.

No difference in the reversion frequency was detected when NA-treated phage were plated directly on the restrictive host in the presence or absence of PFPA. However when the phage were first allowed a round of growth in the presence of PFPA the reversion frequency of L4 was increased by a factor of about four compared to when this growth occurred in the presence of PA. Thus it would appear that replication or at least some parameter of growth must occur for PFPA to exert its co-mutator effect on NA mutagenesis.

Discussion

As was already mentioned, the fact that PFPA causes a general

TABLE 27. Effect of PFPA on NA-Induced Reversion of T4 rII L4

a) Immediate Plating on E.coli WØ80

Growth Medium and Treatment	r^+ Revertants	Reversion Frequency
+PA	12	3.6×10^{-7}
+PFPA	17	4.0×10^{-7}
+PA+NA	45	7.1×10^{-6}
+PFPA+NA	49	6.0×10^{-6}

NA treatment was for 4.5 mins. and the surviving fraction was 0.92%.

b) Plating after Allowing a Cycle of Growth in E.coli B

Growth Medium and Treatment	r^+ Revertants	Reversion Frequency
+PA	305	1.0×10^{-6}
+PFPA	330	1.2×10^{-6}
+PA+NA	61	8.9×10^{-6}
+PFPA+NA	242	3.7×10^{-5}

increase in NA mutagenicity points to the fact that a single step is common to the production of both classes of NA-induced transitions and perhaps to deletions. It should be said that the frequency of deletions was based on a small sample so that the increase in their frequency found when PFPA was present may not be real.

Since in the absence of SU, PFPA was found not to affect the frequency of 5-BU-induced mutations it would seem that there is a PFPA-sensitive step in the NA mutagenic pathway which does not have a role in 5-BU mutation production.

Can we postulate any likely candidates for this PFPA-sensitive step in NA mutagenesis?

Alleles of T4 DNA polymerase (gene 43) have been shown to have marked mutator (Speyer, Karam and Lenny 1966) or antimutator (Drake et al. 1969) behaviour. In some instances the effects of such alleles on induced mutation frequencies have been measured. Freese and Freese (1967) found no evidence of mutational synergism between the mutator allele tsL56 and NA. Drake and Greening (1970) on the other hand used two antimutator alleles and reported a depression of NA mutagenesis, specific for the AT→GC transition when EMS is the mutagen and the AT→GC mutation when NA is used.

Mutations at other loci in the T4 genome have also been shown to alter 'spontaneous' mutation frequencies. Mutants of gene 32 which encodes Albert's protein (Alberts 1970; Alberts and Frey 1970) have been shown to depress mutations at GC sites but to enhance mutation rates where AT is the mutant site (Drake 1973).

The reports concerning the mutator effects of gene 30 (DNA ligase) mutants are in some conflict. Sarabhai and Lamfrom (1969)

reported that proflavine-induced reversion of an rII frameshift was enhanced when the phage carried a mutation in gene 30. This finding was not confirmed by other workers (Bernstein 1971; Koch and Drake 1973). A report of a very large mutator effect of a ligase mutant of T4 on spontaneous mutation rates (Campbell and Rowe 1972) is also in conflict with the findings of Koch and Drake (1973).

In E.coli, mutant alleles at several loci have been shown to have mutator behaviour; mut T (Treffers et al. 1954), ast (Goldstein and Smoot 1955), mut S (Siegel and Bryson 1967), mut V4 (Siegel 1973) and several others (e.g. Hill 1968 and 1970; Liberfarb and Bryson 1970) have been reported in the literature.

Hill (1972) has shown that there is mutational synergism between mut H1 and UV, EMS and MMS. Whether the mut H locus codes for a DNA polymerase is not known. This study of Hill's was exceptional, since studies on mutational synergism between mutagens and bacterial mutators are rare.

It should be said that the effects of PFPA on NA mutagenesis have some points in common with the effects mediated by a T4 mutation, hm. A preliminary study of this mutation formed the subject matter for Chapter 5 of Results and the similarities between the PFPA-effect and the effect of hm will be more appropriately discussed after the consideration of hm.

Probably the most reasonable explanation of the synergism between PFPA and NA is that a mutator enzyme is transiently generated because of the incorporation of the analogue into its sequence. Such an enzyme with altered specificities could be termed a phenotypic, as opposed to genotypic mutator.

Of course it is possible that it is not a single mutational mechanism which is so changed, but rather that each NA-induced premutational lesion class has its own independent pathway and that each of these mutational pathways is equally affected by PFPA.

The failure of PFPA to cause a change in the NA-induced spectrum may be considered a necessary corollary of the lack of alteration in the relative proportions of the classes of mutant induced by NA in the presence or absence of the analogue. The relationship between the spectrum and the classes of mutants of which it is composed will be discussed later.

The fact that PFPA enhanced the NA-induced mutation frequency but not the spontaneous nor (in the absence of SU) the 5-BU-induced frequencies may be interpreted in one of two ways. The first is to say that the mutagenic pathways of NA-induced mutations are different from the spontaneous and 5-BU pathways and that it is only the step(s) in the former which are PFPA-sensitive. The second possibility is that the incorporation of PFPA into the mutator protein changes the accuracy of this protein only when it encounters NA-induced lesions, and that when the lesions are 5-BU-induced the error-proneness of the protein is unaffected by PFPA incorporation. Clearly at this stage it is impossible to distinguish between these possibilities.

4. EFFECT OF STREPTOMYCIN ON INDUCED MUTATION IN T4

The thinking behind the choice of the antibiotic SM as an ancillary agent in mutagenesis was basically two-fold.
(a) SM has been shown to intercalate into DNA (Lerman 1964) but this author failed to demonstrate any mutagenicity with SM. There is one report of mutation induction by SM (Fernandez, Haas and Wyss 1953), but the measurements in this study were open to considerable error. SM at very high concentrations has been shown to induce achromatic lesions in human chromosomes (Obe 1970) but not in those of Vicia fabia (Obe 1972). Whether the effect in the former case is due to intercalation is not known.

Erythromycin, which is related to SM both in structure and in mode of action has been shown to induce petites in yeast with very high efficiency (Carnevali et al 1971; Williamson et al 1971) but in all probability this effect is an indirect one mediated by the inhibition of mitochondrial protein synthesis and not by a direct mutagenic effect of erythromycin.

It was thought possible that intercalation of an agent into DNA would result in localized stereochemical alteration of DNA. The possible contribution of DNA stereochemistry to the positioning of hotspots will be discussed later. By altering the stereochemistry it was considered to be a possibility that the rII spectrum of mutants could be changed. It has in fact been shown that ethidium bromide, an agent which readily intercalates with DNA, results in differential reading of treated templates by DNA polymerases from a variety of vertebrates, the extent of the infidelity of reading

depending on the base composition of the template (Fridliner and Weissbach 1971).

(b) SM is of course best known as an antibiotic which acts by inhibition of protein synthesis. A great deal of evidence now exists to show that SM binds to bacterial ribosomes and inhibits their function. (See review by Weisblum and Davis 1968). It should be noted that in the already-mentioned study of Fermi and Stent (1962), it was found that in the presence of another protein synthesis inhibitor, chloramphenicol, the 5-BU-induced intergenic specificity in T4 was altered. The cause of this phenomenon is still unknown.

Apart from the actual inhibition of protein synthesis, the interaction of SM and bacterial ribosomes results in two other related phenomena which make the use of this agent of greater relevance to a study of this type.

Low levels of SM have been shown to cause phenotypic suppression of some nonsense codons in E.coli (Gorini and Kataja 1964), bacteriophage T4 (Scarfati 1967; Karam and O'Donnell 1973) and several RNA bacteriophages (Kuwanō and Endo 1969).

Complementary to this is the finding that StrA mutants of E.coli, which are resistant to SM, diminish the efficiency of pre-existing nonsense suppressors (Gartner and Orias 1966; Gorini 1970). This restriction applies also to missense suppressors (Biswas and Gorini 1972). This restriction probably accounts for the recent findings (Clarke 1973; Skavrōnskaya et al. 1973) that the frequencies of UV-induced ochre suppressors were depressed in SM-resistant strains of E.coli. This antimutator effect could be relieved by

addition of SM to the plating medium.

Although the nature of the phenotypic suppression and of the restriction of suppression is imprecisely understood, it seems clear that translational ambiguity is involved. This interpretation is in accord with the findings of *in vitro* experiments where the addition of SM resulted in increased levels of misreading of synthetic polyribonucleotides (e.g. Davies, Gilbert and Gorini 1964) and of naturally occurring messengers (Schwartz 1965). Although SM-mediated suppression is fairly unspecific in that it can act on all three nonsense codons and on a variety of missense codons, the misreading is not totally at random. If homopolynucleotides are used as messenger, only one of the base-pairs within a codon is subject to misreading (Davies, Gorini and Davis 1965) and it has also been shown that certain triplets are refractory to misreading *in vitro* (Davies, Jones and Khorana 1966). The demonstration of mistranslation *in vivo* has been somewhat harder, but Strignini and Gorini (quoted in Gorini 1970) have shown it to occur.

If the hypothesis put forward concerning the ability of PFPA to cause misreading is to be believed, then one could consider PFPA and SM as mediators of the same end result, though each agent achieves this end by very different means.

If SM could be shown to have an effect on the specificity of the induced mutant spectra of T4, then the possibility exists of dissecting the two components of the action of SM enumerated above to see which is responsible.

The relative importance of intercalation could be tested by the use of another compound, such as spermine, which also intercalates

into DNA (Lerman 1964). This compound has no effect on mutation rates in T4 (Drake 1970) but it is antimutagnic for E.coli (Johnson and Bach 1965 and 1966).

The relative importance of the phenotypic suppression and misreading could be determined by the use of another agent, such as 5-FU, which mimics SM in these parameters. 5-FU is an analogue of uracil and is incorporated into RNA, and may pair with either A or C. This results in the suppression of nonsense codons (Benzer and Champe 1961; Champe and Benzer 1962b; Garen and Siddiqui 1962). Concomitant with this loss of accuracy, as measured by phenotypic suppression, is the fact that 5-FU also causes the production of defective proteins as a result of misreading (Naono and Gros 1960).

However 5-FU is a potent inhibitor of nucleic acid synthesis (e.g. Osawa 1965) and hence the mutagenicity of 5-BU (one of the mutagens used in this study) would be increased for reasons which are analogous to those used to account for the fact that other inhibitors of DNA synthesis (e.g. SU, TM, Aminopterin) also increase the mutagenic powers of base analogues.

A better choice might well have been a ram mutant of E.coli, which has similar effects to those obtained by the addition of SM. It suppresses nonsense and missense codons (Gorini 1969) and it increases the level of in vitro misreading, to a level equivalent to that found when wild type ribosomes in the presence of SM are used to direct the synthesis (Gorini 1970).

EXPERIMENTAL

I. 5-BU Mutagenesis

(a) Effect of SM on Growth of E.coli B in SUM

Just as in the work with PFPA, it was necessary to estimate the dose of SM which would cut the growth rate of E.coli B grown in SUM by about 50% since the initially intended protocol for 5-BU mutagenesis was to mutagenize the phage in the presence of SM and in cells which had been grown in the presence of the drug. The procedure for estimating the appropriate concentration of SM was the same as that used in the preliminary experiment on PFPA. It can be seen from Figure 13 that SM at 2mg/l resulted in a viable cell density after four hours growth in its presence which was about half that found in the control.

(b) Effect of SM in the Plating Medium on T4 Plaque Formation

This preliminary experiment was necessary because it was intended to plate mutagenized phage on plates containing SM.

E.coli B was grown up in SUM supplemented with various concentrations of SM. After 3.5 hours growth T4r^+ was added at low m.o.i. and the complices were plated on plates to which SM had been added at different concentrations.

Table 28 gives the number of T4 plaques/plate after the various treatments. The results show that pregrowth of E.coli with SM at 2mg/l was not very satisfactory since T4 showed a drop in e.o.p. even when the plates lacked the drug. In addition those plaques which did form were not well defined and it would have been very difficult to recognize plaque morphology mutants. When E.coli was

Figure 13.

Effect of SM on Growth Rate of E.coli B in SUM.

The concentrations of SM in the growth media are given in the figure.

Figure 13.

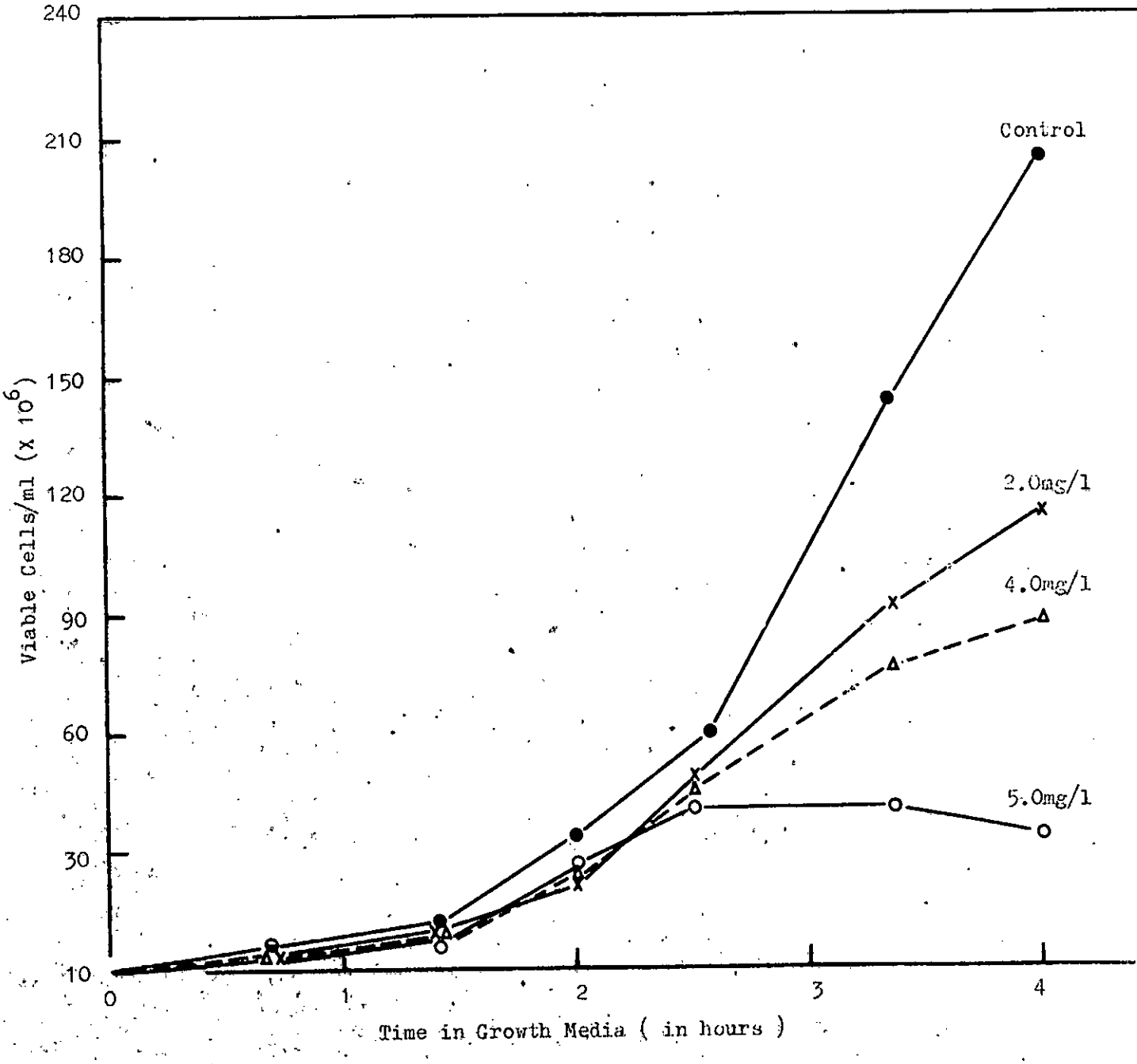

240
210
180
150
120
90
60
30
10
Viable Cells/ml (X 10^6)
0
1
2
3
4
Time in Growth Media (in hours)
Control
2.0mg/l
4.0mg/l
5.0mg/l

TABLE 28. Effect of SM in E.coli Growth Media and Plating Media on T4 Plaque Formation

Conc. of SM in E.coli Growth Media (mg/l)	Conc. of SM in Plates (mg/l)			
	0	0.1	0.5	1.0
0	200	211	195	199
1.0	142	130	74°	30°
2.0	44°	36°	?	?
3.0	?	?	?	?

? - No plaques discernible because of very poor bacterial lawn formation.

o - Plaques poorly formed on thin lawn.

grown in SM at 1.0mg/l, the e.o.p. was still less than the control but the plaques were quite distinct so long as the plates contained SM at a concentration no higher than 0.1mg/l. The effects of SM on e.o.p. will be returned to after consideration of the one-step growth experiments.

(c) Effect of SM on One-Step Growth of T4

A culture of E.coli B was grown in SUM with or without SM at 1.5mg/l. This lower dose of SM was chosen because of the drastic effect which SM at 2mg/l had on T4's plaque-forming ability.

After 3.5 hours growth, the bacteria were resuspended in fresh SUM $\pm$ SM, the viable cell density of each aliquot having been adjusted so that they were the same.

T4r$^+$ was then added. SM was omitted from the plates and from the growth medium of the plating bacteria. Because the effect of SM on e.o.p. hinted at the possibility of impaired T4 adsorbtion to SM-grown E.coli, it was decided to do the experiment in the absence of NaCN so that the kinetics of adsorbtion could be measured. Accordingly the zero times in Figures 14 and 15 correspond to the times at which the phage was added, rather than the times at which the complices were diluted out of NaCN. In these experiments, dilution into fresh medium was done twelve minutes after the addition of the phage.

Figure 14 shows that under the conditions used here, SM had a drastic effect on a number of parameters of T4 growth. The adsorbtion of the phage was greatly impaired, as shown by the fact that in those aliquots prematurely lysed, the number of viable phage was similar to the values found in the unlysed aliquots.

Figure 14.

One-Step Growth Experiment with T4 in SUM in the Presence or Absence of SM at 1.5mg/l

Symbols:- Open Symbols - Prematurely lysed with $CHCl_3$

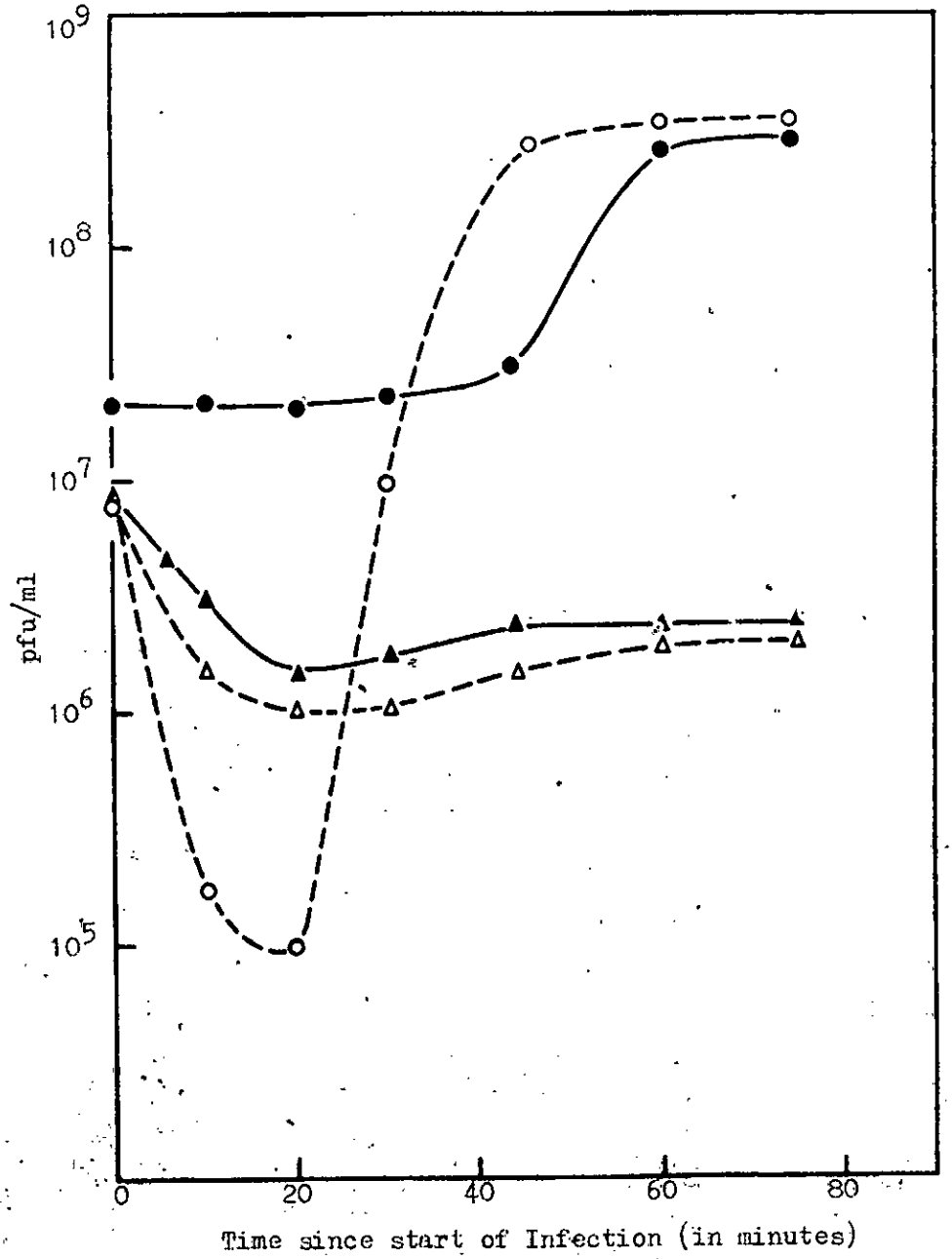
Closed Symbols - Plated directly

Triangles - SM present

Circles - SM absent

N.B. t = 0 was the time at which phage were added, in the absence of cyanide.

Figure 14.


10^9
10^8
10^7
10^6
10^5
pfu/ml
0
20
40
60
80
Time since start of Infection (in minutes)

The fact that the titre of phage in the unlysed aliquots fell during the latent period pointed to the failure of a proportion of the phage which had been adsorbed to develop. The fact that the lysed and the unlysed aliquots paralled each other so closely suggested that the large majority of adsorbed phage failed to develop.

This experiment was repeated, using a lower concentration of SM (1.0mg/l). See Figure 15. The effects which were seen with SM at 1.5mg/l also occurred at 1.0mg/l but to a lesser degree. Adsorbtion was still impaired. Approximately 70% of the phage were adsorbed compared with more than 95% in the control. There was also a fall in the titre of phage in the unlysed aliquots such that the titre of viable phage was reduced about three-fold. It should be noted however that the final titre (after 80 minutes) reached a value of about twice that at the start of the infection. Clearly then, there were some successful infections. If one divides the final titre by the number of viable adsorbed phage (this latter function can be obtained by subtracting the lysed titre from the unlysed titre 20 minutes after the start of the infection) one obtains a corrected burst-size of 13.0. This compares with a value of 14.1 in the control. If the interpretation of the behaviour of the T4 growth parameters is correct, then SM had no effect on the burst size of those phage which adsorb and develop successfully but it completely inhibited the growth of the majority of phage.

Freeda and Cohen (1966) found that intracellular protein synthesis directed by T4 was in general inhibited less than that of bacteria, though the synthesis of certain proteins such as lysozyme

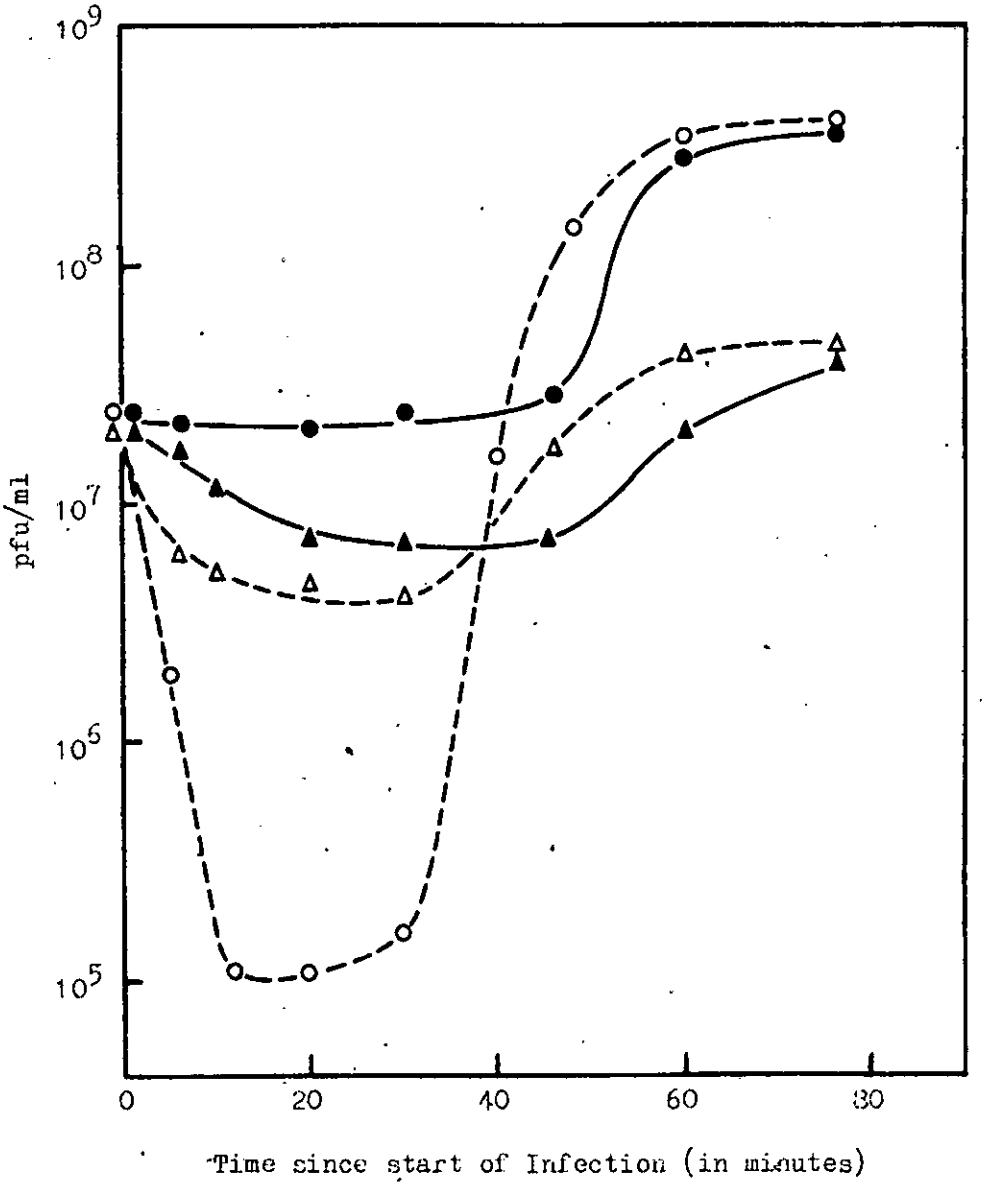
Figure 15.

One-Step Growth Experiment with T4 in SUM in the Presence of Absence of SM at 1.0mg/l

Symbols as for Figure 14.

As in Figure 14, t = 0 was the time at which phage were added.

Figure 15.


10^9
10^8
10^7
10^6
10^5
pfu/ml
0
20
40
60
80
Time since start of Infection (in minutes)

were peculiarly sensitive to inhibition by SM.

Older work, with another aminoglycoside antibiotic, Aureomycin, showed that this drug inhibited adsorbtion of phage through a still unknown mechanism (Altenbern 1953). It has also been reported that SM has a lethal effect on free phage (Edlinger 1949; Jones 1945; Cohen 1947; Bourke, Robbins and Smith 1952), but the doses used in all these studies were massive. However to check this possibility I exposed free T4 to SM at 1.5mg/l for two hours. At this concentration no loss of titre was observed. The fact that bacteria containing mature T4 can be lysed prematurely by addition of SM at high concentration (Symonds 1957) could not have been responsible for the drop in titre since in a subsequent single-burst experiment with an amended protocol (see below), SM was present throughout the course of infection but no loss of titre was observed.

The finding of a reduced mean burst size as measured by dividing the final by the initial titre (burst size = 2.1 in the presence of SM at 1.0mg/l), which was interpreted as being due to a minority of individuals giving rise to full bursts, while the remainder contribute little or nothing to the final titre is reminiscent of an aspect of the phenomenon of capacitance (Benzer and Jacob 1953). UV-killed E.coli can efficiently support the growth of T4 so long as infection is done immediately after irradiation. However if time is allowed to elapse before infection the mean phage burst is reduced. It was found on closer analysis that some infective centres yielded normal bursts while others gave no progeny (Boyle and Swenson 1971). However the ability of the phage to adsorb was not impaired under the conditions causing loss of capacitance.

It is clear that the effects of SM on the development of T4 are such as to render its use as an ancillary agent in 5-BU mutagenesis most unsuitable, at least under the conditions used above. The most obvious drawback is the fact that a substantial fraction of the phage remains unadsorbed and hence is impervious to the mutagenic action of 5-BU.

A second protocol was found to be more acceptable. Here the E.coli was grown in SUM in the absence of SM and after 3.5 hours growth phage and drug (2.0mg/l) were added contemperaneously, again in the absence of cyanide, and the experiment was performed in the usual way. Under this regime SM did not interfere with adsorbtion nor did it inactivate any infective centres during the latent period. It did however reduce the burst size by about 50% compared to the control and it extended the latent period some ten minutes. See Figure 16.

(d) Effect of SM on 5-BU Forward Mutagenesis

T4r^+ phage, and where appropriate SM at 2.0mg/l and 5-BU at 0.05mg/ml were added to a culture of E.coli B which had been grown in SUM. The experiment was carried out as described in Materials and Methods. The results are given in Table 29a. Estimates of the induced mutant frequencies were made in two ways.

(a) By dividing the total number of r's by the total number of plaques inspected. This value will be an overestimate since it treats all r's as if they were of independent origin. Given the large number of independent estimates (i.e. one for each plate), on which this mean is based, the extent of the overestimation should be

Figure 16.

One-Step Growth Experiment with T4 in SUM in the Presence or Absence of SM at 2.0mg/l. (SM absent from E.coli growth media.)

Symbols as for Figure 14.

t = 0 was the time at which phage were added.

Figure 16.

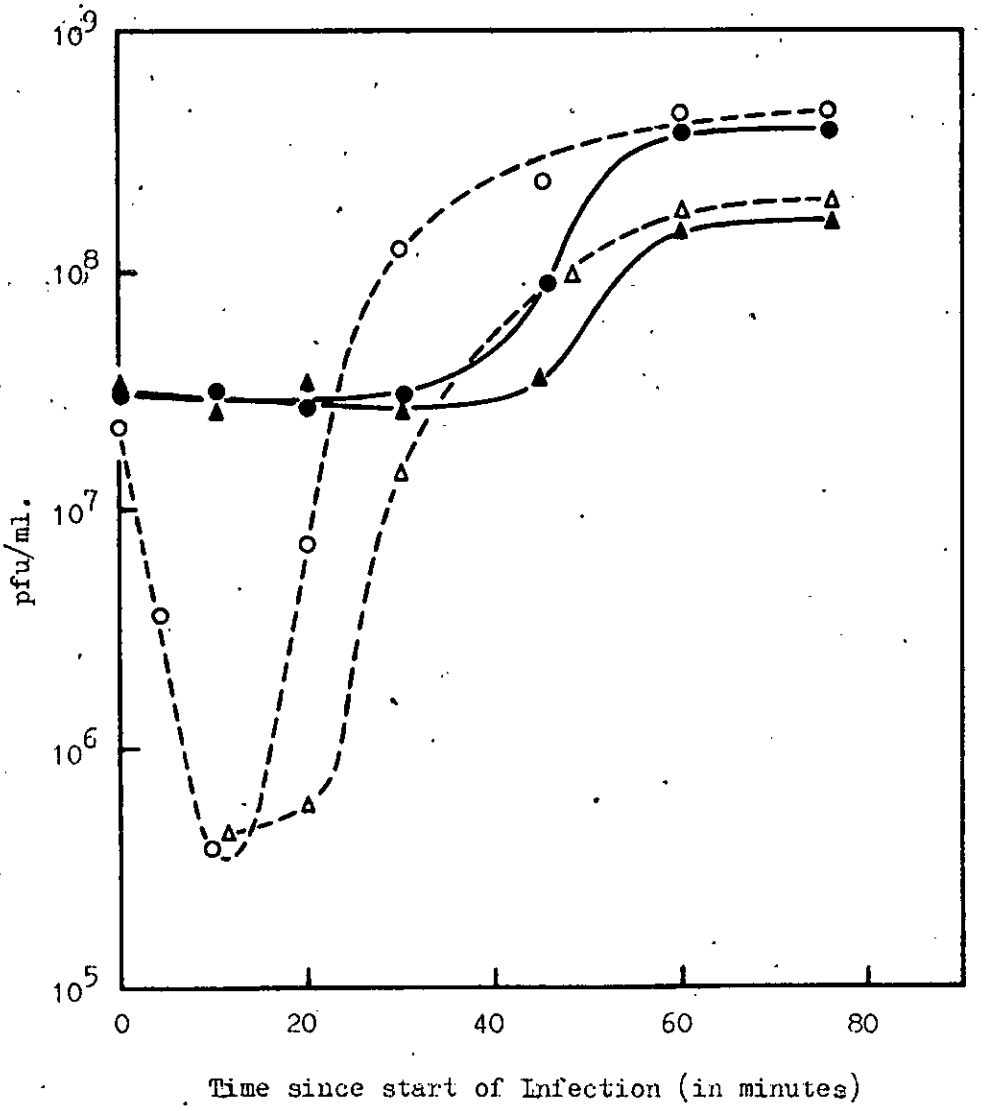

10^9
10^8
10^7
10^6
10^5
pfu/ml.
0
20
40
60
80
Time since start of Infection (in minutes)

TABLE 29. Effect of SM on 5-BU-Induced Frequency of T4 r Mutation (1st Isolation)

a) Isolation of Mutants

	TREATMENT MEDIA			
	Control	+SM	+5-BU	+5-BU+SM
Total Plaques Inspected	6.0×10^3	8.1×10^3	2.3×10^4	7.1×10^4
Total of r Plaques	19[o]	1	153	310
% of plates with at least 1 r	5	2.5	77.1	64.2
Mean pfu/plate	918	834	786	815
Mutation Index($\frac{\text{Total r's}}{\text{Total Plaques}}$)	3.3×10^{-3}	1.3×10^{-4}	6.1×10^{-3}	4.3×10^{-3}

[o] - 18 of the r's obtained in the control were on one plate. On testing, all 18 were found to be rI's and were in all probability the result of a jackpot.

b) Classification of r's into Genotypes

(i) Totals

Treatment	rI		rII		rIII	
	Nos.	%	Nos.	%	Nos.	%
5-BU+SM	157	57.3	108	39.4	9	3.3
5-BU	97	81.5	17	14.3	5	4.2

(ii) Number of Plates Carrying at Least One Plaque of the Relevant Genotype

Treatment	rI		rII		rIII	
	Nos.	%	Nos.	%	Nos.	%
5-BU+SM	86	58.9	51	34.9	9	6.2
5-BU	52	75.4	12	17.4	5	7.2

similar for the SM-treated phage and the control.

(b) By calculating the percentage plates with at least one mutant plaque. For this to be legitimate the pfu/plate in the two treatments should be the same. In this experiment (and in a subsequent repeat experiment) this was the case. This method will give rise to an underestimate of frequency since it assumes that all r mutants on a single plate are the progeny of one mutant parent. That this assumption is not true could be shown by demonstrating that on some plates a mixture of r genotypes occurred.

Both methods of calculation show that 5-BU-induced forward mutation frequency was marginally lower in the presence than in the absence of SM, but given the errors involved in the calculations, there is no justification for considering the difference to be a real one.

All the r mutants induced by 5-BU were purified and then allocated into the r genotypes in the usual way. Table 29b(i) shows the classification of all the r's, irrespective of whether they came from the same plate or not. In Table 29b(ii) the classification was based on the number of plates on which there was at least one representative of the relevant genotype.

It is questionable whether the higher proportion of rII's found in the 5-BU + SM treatment is a real one, given the already-mentioned errors involved. In fact in a subsequent experiment (see below) no real difference was found in the relative frequencies of the different r genotypes induced by 5-BU in the presence and absence of SM.

The rI and rIII mutants were discarded and no more than one rII

was taken from each plate for further analysis. This precaution ensures their independent origin. There was therefore a total of 12 rII's induced by 5-BU in the absence of SM and 59 rII's induced in the presence of the drug.

I considered these numbers too low for any valid comparison between the two classes to be made. Accordingly the collection was supplemented with more rII's isolated in the way described above.

Table 30 shows that the results were essentially the same as in the first experiment viz:- no effect of SM on 5-BU mutation frequencies and lack of mutagenicity of SM itself. As was mentioned above when the r's were classified into genotypes no difference in the relative frequencies of the r genotypes of mutants induced in the presence and absence of the drug was found.

Independently arising rII's were taken from this isolation, giving a grand total of 71 rII's induced by 5-BU and 80 induced by 5-BU in the presence of SM.

Although there appeared to be no effect of SM on 5-BU-induced mutation frequency this did not necessarily preclude the possibility of modification of the induced spectrum, though to be sure it made it less likely than if the frequency had been changed. Accordingly the rII's were mapped within the rII region. It is clear from Figure 17 that there was no essential difference in the 5-BU-induced spectra in the presence and absence of SM. Of course for those sites with only single representatives there can be no exact overlap but every hotspot contained representatives induced by 5-BU in the presence and absence of SM.

TABLE 30. Effect of SM on 5-BU-Induced Frequency of T4 r Mutations (2nd Isolation)

A). Isolation of Mutants

	TREATMENT MEDIA			
	Control	+SM	+5-BU	+5-BU+SM
Total Plaques Inspected	3.3×10^4	2.7×10^4	4.8×10^4	2.6×10^4
Total of r Plaques	7	9	381	258
% of Plates with at least 1 r	6.2	8.1	69.4	82.8
Mean pfu/plate	745	816	564	731
Mutation Index($\frac{\text{Total r's}}{\text{Total Plaques}}$)	2.1×10^{-4}	3.5×10^{-4}	7.8×10^{-3}	9.8×10^{-3}

b). Classification of r's into Genotypes

(i) Totals

Treatment	rI		rII		rIII	
	Nos.	%	Nos.	%	Nos.	%
5-BU+SM	168	65.1	72	27.9	18	7.0
5-BU	214	56.2	143	37.5	24	6.3

(ii) Number of Plates Carrying at Least One Plaque of the Relevant Genotype

Treatment	rI		rII		rIII	
	Nos.	%	Nos.	%	Nos.	%
5-BU+SM	54	51.4	29	27.6	18	17.1
5-BU	86	50.9	59	34.9	24	14.2

Figure 17.

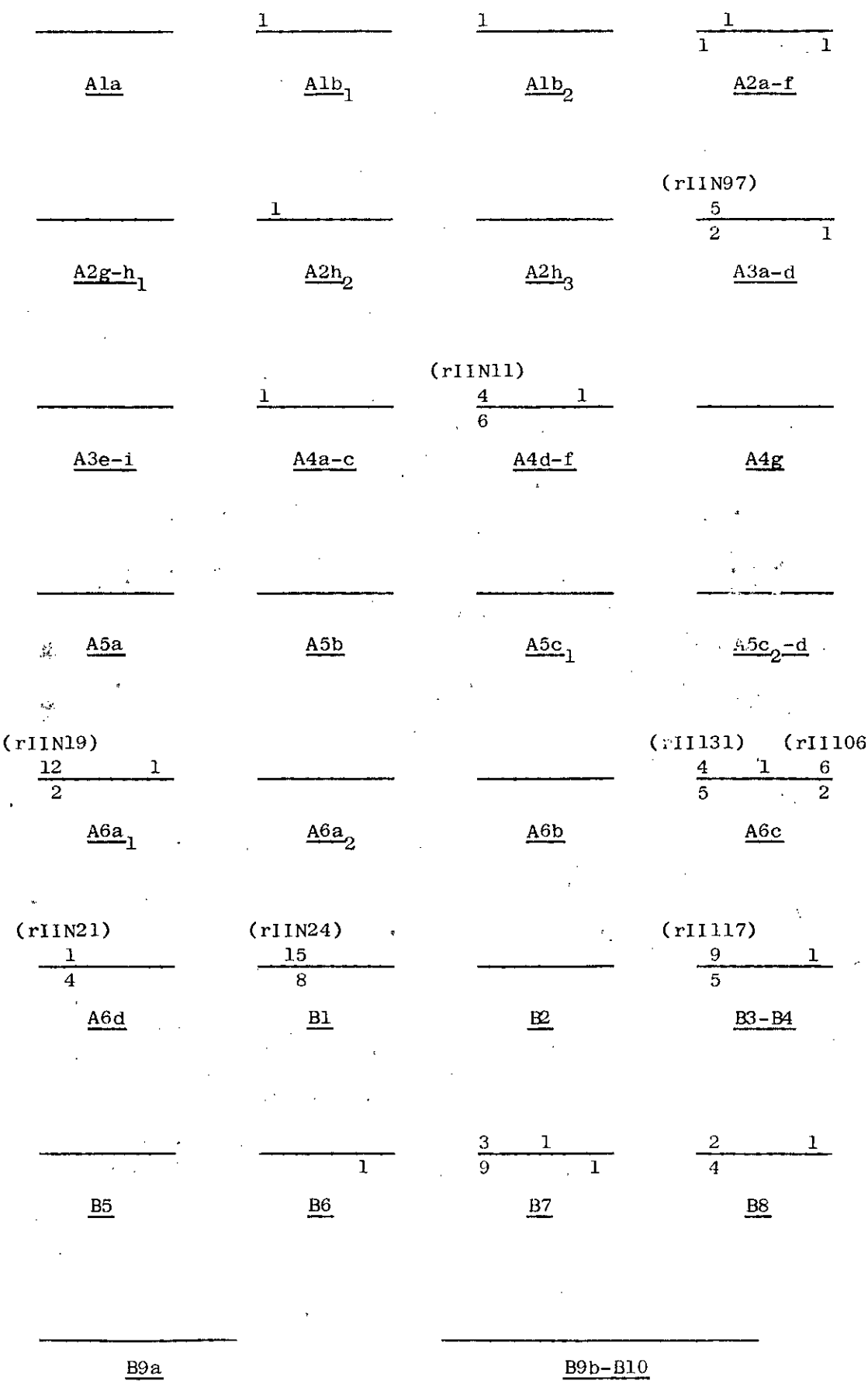
Representation of the Spectra of rII Point Mutations Induced by 5-BU in the Presence and Absence of SM

Legend:- As for Figure 5a.

The numbers above the lines:- Induced by 5-BU in the Presence of SM.

The numbers below the lines:- Induced by 5-BU in the Absence of SM.

Figure 17


A1a
A1b1
A1b2
A2a-f
A2g-h1
A2h2
A2h3
(rIIN97)
A3a-d
A3e-i
(rIIN11)
A4a-c
A4d-f
A4g
A5a
A5b
A5c1
A5c2-d
(rIIN19)
A6a1
A6a2
A6b
(rII131)
(rII106)
A6c
(rIIN21)
A6d
(rIIN24)
B1
B2
(rII117)
B3-B4
B5
B6
B7
B8
B9a
B9b-B10

In view of the lack of any difference in the rII spectra and the 5-BU-induced mutation frequencies in the presence and absence of SM it was decided not to take the analysis further by classifying the patterns of reversion of the rII's.

II. NA Mutagenesis

Effect of SM on NA Forward Mutagenesis

In a forward mutation experiment T4r^+ phage were treated with NA in the usual way (o.1M $NaNO_2$ in pH 4.0 acetate buffer for 4.5 minutes). The mutagenized phage were then added to a culture of E.coli B which had been grown in MM and to which cyanide had been added ten minutes earlier. After allowing time for adsorbtion the complices were diluted into two fresh aliquots of MM prewarmed to 37°C. To one of these aliquots had been added SM at 2.0mg/l. The cultures were incubated for 15 minutes before being diluted further and plated against E.coli B. Neither the top agar nor the growth media of the bacteria had had SM added.

After overnight incubation the plates were inspected for r plaques. From Table 31 it can be concluded that SM had no effect on NA-induced r frequency and on further analysis it can be seen that it failed to alter the relative proportions of the different r genotypes. See Table 31b.

Given these negative results, it was decided to take the analysis further only as far as allocating the rII's into cistrons. This was done by crossing the induced rII's with rII638. Those mutants which recombined with this deletion were placed in the rIIA cistron and those that failed to cross were classified as belonging

TABLE 31. Effect of SM on NA-Induced Frequency of T4 r Mutations

A) Isolation of Mutants

Treatment	Total Plaques Inspected	Total r Plaques	r Frequency
Control	7.8×10^{4}	11	2.6×10^{-4}
+SM	6.9×10^{4}	17	2.5×10^{-4}
+NA	9.2×10^{4}	129	1.4×10^{-3}
+NA+SM	2.4×10^{5}	284	1.6×10^{-3}

b) Classification of r's into Genotypes

Treatment	rI		rII		rIII	
	Nos.	%	Nos.	%	Nos.	%
+NA	73	56.6	42	32.6	14	10.8
+NA+SM	160	56.3	92	32.4	32	11.3

c) Classification of rII's into rIIA and rIIB Cistrons

Treatment	rIIA		rIIB	
	Nos.	%	Nos.	%
+NA	25	59.5	17	40.5
+NA+SM	58	66.0	30	34.0

to rIIB. Table 31c shows that the presence of SM had no effect on the relative frequencies of induced rII's of the two cistrons and it was decided to take the analysis no further.

Rather disappointingly then, SM had no effect on the potency or specificity of NA- and 5-BU-induction of r mutants. All one can say is that none of the modifications of cellular metabolism caused by SM are of importance in the mutagenic pathways of either mutagen. This is not to say that all modifications of ribosomal function or all intercalating agents will also fail to have any effect on induced mutagenesis.

5. A PRELIMINARY STUDY OF THE hm MUTATION IN T4

The work to be described in this chapter was concerned with a study of possible changes in mutagen specificity due to an alteration in genetic background rather than by experimental manipulation of the conditions under which mutagenesis was carried out.

The mutant used was designated hm by Dr. J.W. Drake who supplied a stock of the strain. This mutant has a rather unusual history. A mutant of T4 with increased UV sensitivity was isolated by Harm (1963). This mutant, named x, was also shown to have reduced intragenic recombination frequency (Harm 1964) and an increased sensitivity to X-rays and to MMS (Haldy, Strom and Bernstein 1971; Mortelmans and Friedberg 1972). Drake (1973) has established that the x "mutation" is in fact a double mutant and he has designated the two mutants px and hm. He found that px is responsible for the UV and MMS sensitivities and for the reduced recombination frequency. The hm mutation, on the other hand was characterized as having wild-type UV and MMS sensitivities and normal recombination frequency. It does however possess a higher spontaneous frequency and it enhances the frequency with which mutations induced by 2-AP, MMS and UV are induced.

The fact that hm can enhance induced mutagenesis without itself causing a great increase in spontaneous frequency renders it a good candidate for a study on the effects of genetic background on the specificity of induced mutations since there will be less 'contamination' of the induced mutations by those arising spontaneously than would be the case if the effects of an allele which produces

massively elevated spontaneous mutation rates (e.g. gene 43) on induced mutations were measured.

Experimental

NA Treatment of T4 hm and T4 hm^+

Preliminary to any studies on the T4hm strain, lysates were grown up on E.coli BB in the usual way and assayed for the incidence of spontaneous r mutants. The frequencies in five lysates are shown in Table 32 and were some two to three times greater than those found for the hm^+ strain. This increase in spontaneous r frequency was not as great as that reported by Drake (1973) and the reason for this is not known. Of the five, lysate E had the lowest frequency and this was used in the subsequent work.

It was intended initially to isolate a variety of different classes of rII mutants and to compare the reversion frequencies induced by different mutagens when the rII's were in the hm and hm^+ backgrounds.

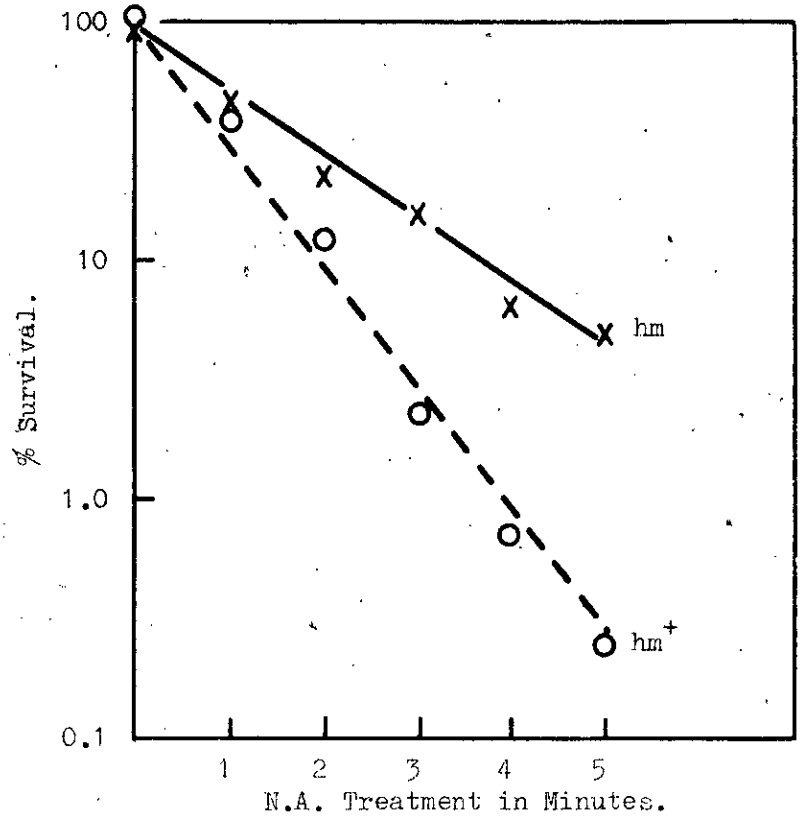
NA was chosen as the mutagen to induce rII mutants in the hm background since it is simple to administer and it induces a variety of mutant classes. It was intended to backcross the rII's so induced into the hm^+ strain and compare the induced reversion frequencies of the same rII in the two strains.

The hm stock lysate was treated for 4.5 minutes with 0.1M $NaNO_2$ in pH 4.0 buffer. From previous experience it was assumed that such a treatment would result in about 1% survival. The dilutions, prior to plating were calculated on this assumption so as to give about 10^3 pfu/plate. In the event however the plates

TABLE 32. Spontaneous *r* Mutant Frequency in Five *hm* Lysates

Lysate	Plaques Inspected	No. of *r* Plaques	*r* Frequency
A	2.6×10^{4}	18	6.9×10^{-4}
B	3.6×10^{4}	24	6.7×10^{-4}
C	2.4×10^{4}	16	6.7×10^{-4}
D	3.2×10^{4}	16	5.0×10^{-4}
E	4.4×10^{4}	20	4.5×10^{-4}

Figure 18.



showed virtually confluent lysis, there being many more survivors than expected. This suggestion of increased NA-resistance in hm was investigated.

Both T4hm and hm^+ were treated with NA (pH 4.0) in the usual way, samples being taken at intervals and plated against E.coli B to estimate phage survival.

From Figure 18, it is clear that there was a marked increase in the resistance of hm compared to the wild-type. Both killing curves were linear and hence the ratio of survivors in the hm over the hm^+ strain increased with increasing NA dose.

To my knowledge this was the first demonstration of a genetically determined effect on the response of T4 to inactivation by NA. Differences in sensitivity due to genetic background have however been found in E.coli (Howard-Flanders et al. 1966; Clarke 1970), Salmonella (Rudner 1961) and various fungi (Chang and Tuveson 1967; Lennox and Tuveson 1967; Zimmerman 1968). In addition Winkler (1965) has shown that to a certain extent, the NA-sensitivity of bacteriophage kappa can be altered by genetic changes in the host, Serratia.

It is of course quite possible that the difference between the two strains was not due to the mutation at the hm locus. Indeed it could be that by chance, my wild-type strain was in fact NA-sensitive due to an independent mutation. To go some way towards testing this possibility, the experiment was repeated, but in addition the responses of two extra wild-type stocks (T4B and T4D; kindly supplied by Dr. J.W. Drake) were measured. It was found that the killing curves of these two strains and of my

original wild-type virtually overlapped, the hm strain being more resistant than all other three strains. This result suggests that my wild-type was not in fact NA-sensitive. It does not however say that it was the mutation in the hm locus itself which was responsible for the resistance in this strain.

Given this difference in the NA-sensitivities of the two strains, it was decided to test whether the strains differed in their response to NA as a mutagen.

When making comparisons of induced mutation frequencies in strains showing different sensitivities to the lethal action of a mutagen there is a problem in deciding the definition of equivalent doses. One can compare equal physical doses, or one can consider equivalent doses as those which give the same level of survival in the two strains.

To a certain extent a prior knowledge of the reasons for the sensitivity difference is required before deciding which of the two definitions of equivalence is appropriate. If in the resistant strain there is some kind of barrier whereby fewer lesions in DNA are induced per unit dose then equivalent survivals should be compared. If on the other hand, the same number of lesions were induced by NA during the treatment of hm and hm^+, but in the former strain a higher fraction of the lethal lesions were repaired, then the criterion for equivalence would be in terms of equal physical dose.

Since the nature of the resistance in the hm strain was not known it was decided to compare the NA-induced forward ($r^+ \rightarrow r$) mutation frequencies in hm and hm^+ both at equal doses and at

equal survivals.

Both hm and hm^+ were treated with NA to 1% survival in the usual way. In addition hm was treated for the same time (4.5 minutes) as gave about 1% survival in hm^+. The treated and control phage of both strains were plated on E.coli B and were inspected for the incidence of r plaques. See Table 33.

Even after allowing for the higher spontaneous r frequency in hm, the frequency of NA-induced mutants was about double that of the frequency in hm^+ at equal doses, and about 2.5 times higher at equal survivals. This experiment was repeated three more times with essentially the same result.

Two very general explanations for the nature of the resistance in hm were mentioned above. One invoked a mechanism by which the number of induced lesions was reduced and the other was in terms of improved efficiency of repair of potentially lethal mutations.

The fact that hm not only had a higher survival at any given NA dose, but also had an enhanced induced r frequency favours the second general model. Although NA-induced lesions leading to lethality and to mutations may be different, it is reasonable to assume that the rates at which both classes are induced will be correlated. Hence one would predict that if the resistance to NA in hm is caused by better protection of the DNA, the hm strain should have a lower induced mutation frequency compared to wild-type.

One other possible explanation was considered. As well as acting on DNA alone, NA can covalently join DNA to protein, and in phage T7 this type of crosslinking is responsible for a significant

TABLE 33. NA-Induced r Frequencies in T4hm and T4hm$^+$

Strain	NA Treatment (mins)	% Surv.	Plaques Inspected	No. of r Plaques	r Frequency
hm$^+$	0	100	5.0×10^4	12	2.4×10^{-4}
	4.5	1.3	2.8×10^4	34	1.2×10^{-3}
hm	0	100	2.4×10^4	16	6.6×10^{-4}
	4.5	12.5	1.9×10^4	54	2.8×10^{-3}
	6.5	1.1	2.1×10^4	71	3.4×10^{-3}

proportion of the lethal action of NA (Dussault et al. 1970). NA has also been shown to crosslink nucleohistone in calf thymus (Potti and Bello 1971). It is not known whether phage can be inactivated by the action of NA solely on protein. If such inactivation could occur and if hm had a somatic protein more resistant to it, then a higher proportion of lethality will be of genetic origin (rather than what might term somatic killing) in hm than in hm^+. At equivalent survivals then on this model the induced mutation frequency would be expected to be higher in hm.

However the model also predicts equal mutation frequencies at equal physical doses. Unless one postulates a strong (and a priori, a very unlikely,) correlation between the probability of a phage's receiving a somatic lethal hit and of its carrying a premutation at one of the r loci, the fact that hm had a higher induced frequency than hm^+ after 4.5 minutes NA treatment argues against this model.

Reconstruction Experiment

One further possible explanation is that r mutants are at a selective advantage in NA-treated hm phage. A reconstruction experiment was performed to see if this was so. There certainly are precedents, as was illustrated in the Introduction, of mutations in the rII locus being at a selective advantage in certain genetic backgrounds, rII mutants being able to suppress a variety of mutations at other loci in T4.

The experiment was done as follows. Twelve spontaneous hm r double mutants were picked, purified, and allocated to genotype. Four of them were rII's and the reversion response of each to NA

was measured. None was induced to revert and the one with the lowest spontaneous reversion frequency (hm rIIA10; with a frequency of 1.6×10^{-8}) was chosen so that the selection on both the major classes of r mutants could be determined.

Mixtures of hm r^+ and hm rIIA10 and of hm r^+ and hm rI were made up in ratios of 20:1 in favour of the hm r^+ phage. The mixtures were treated with NA for 6.5 minutes and were plated on E.coli B. The frequencies of the r mutants were compared with the frequencies when the mixtures were plated before being subjected to NA treatment. Table 34 shows that the frequencies of both r mutants were almost identical before and after NA treatment indicating that selection in favour of fully expressed r mutants in the hm strain was not responsible for the increase in induced mutation frequency.

Effect of NA Treatment on One-Step Growth of T4hm and T4hm$^+$

It has long been known that some mutagenic agents, most notably UV, are not only lethal to bacteriophage but also have marked effects on the physiology of the survivors of the treatment. After UV irradiation the mean burst size is reduced and the latent period is extended (Luria 1944) and this delay is due to effects of UV on the DNA (Setlow et al. 1955).

Phage which survive EMS treatment, have normal burst sizes but the latent period is extended (Ray, Bartenstein and Drake 1972). To my knowledge the effects of NA treatment on the one-step growth parameters of T4 have not been reported, and it was decided to do the appropriate experiment and further, to compare the effects of

TABLE 34. Reconstruction Experiment. Treatment with NA of a Mixture of *hmr*$^+$ and *hmr*II A10 and of *hmr*$^+$ and *hmr*I

a). Mixture of *hmr*$^+$ and *hmr*II A10

Treatment	Plaques Inspected	No. of *r* Plaques	*r* Frequency (%)
0	813	39	4.8
NA (6.5mins)	1089	61	5.6

b). Mixture of *hmr*$^+$ and *hmr*I

Treatment	Plaques Inspected	No. of *r* Plaques	*r* Frequency (%)
0	333	28	8.4
NA (6.5mins)	521	37	7.1

the NA treatment on the burst of hm and hm^+.

It is not unreasonable to suppose that if the hm mutation is implicated in the repair of NA-induced lethal damages, this may be reflected in an amelioration of any physiological damage as well.

Both hm and hm^+ were treated with NA for 4.5 minutes and the survival of each strain was assayed. The treated and control phage of each strain were used to infect E.coli B, grown in MM, in the presence of cyanide at low m.o.i. Multiplicity reactivation was thus prevented.

The experiment was conducted in the usual way. The mean burst size of the NA-treated phage was estimated in the normal manner i.e. as the ratio of viable phage at the end of the infective cycle over the initial viable phage number. Table 35 summarizes the results of the experiment and Figure 19 shows the time-course of the one-step growth, showing only phage plated directly and not after peemature lysis. It can be seen that neither the burst size nor the efficiency of adsorbtion was affected by NA treatment of either strain compared to the controls. The latent period of the treated phage was extended by about eight minutes in hm^+ and by about five minutes in hm. This slight diminution in the extent of the delay in the hm strain was found in two further repeats so it represents a small but real difference between the two strains. The lack of effect on mean burst size, but an increased latent period of NA treated phage is reminiscent of the already-mentioned effects of EMS on one-step growth.

At this stage it is not possible to say what are the reasons for the extension of latent period in NA-treated phage. It may

TABLE 35. Effects of NA-Treatment on the Parameters of the One-Step Growth Experiment with T4hm and T4hm$^+$

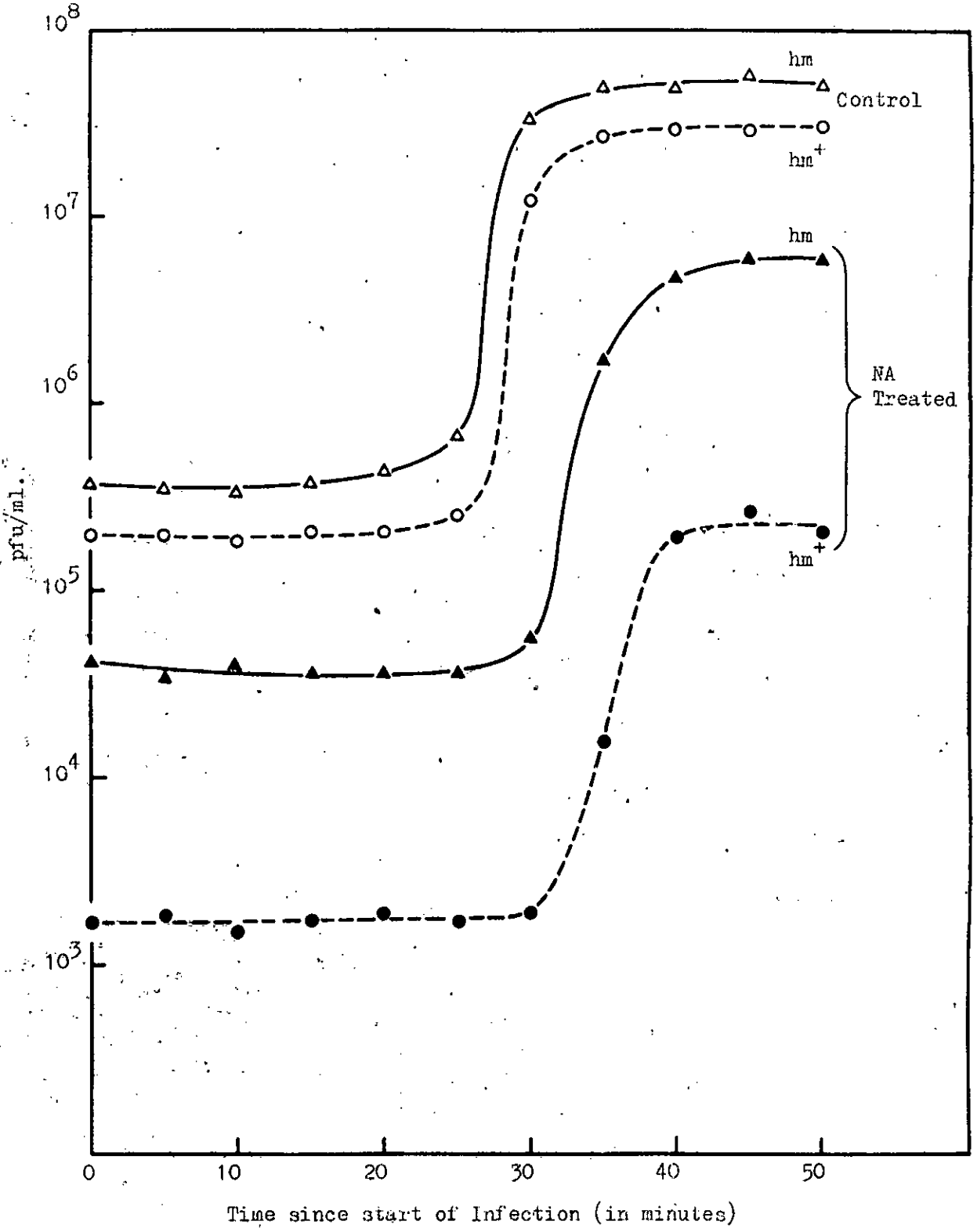
Strain	NA Treatment (mins)	% Surv.	% Adsorbtion	Burst Size
T4hm	0	100	92.4	143.2
	4.5	11.6	90.1	131.8
T4hm$^+$	0	100	89.8	135.6
	4.5	0.97	87.7	144.2

Figure 19.

One-Step Growth Experiment with T4*hm* and T4*hm*$^{+}$ with or without Prior NA Treatment

The strains and the treatments are shown in the figure. The curves obtained after premature lysis are not given.

Figure 19.


10^8
10^7
10^6
10^5
10^4
10^3
pfu/ml.
hm
Control
hm+
hm
NA
Treated
hm+
0
10
20
30
40
50
Time since start of Infection (in minutes)

well be that damage inflicted on the DNA by NA slows down replication and transcription. If the difference in the latent period of NA-treated hm and hm^+ is real, one could hazard that the former strain is in some way better able to cope with the damage brought by NA.

Intracellular NA Treatment of T4hm and $T4hm^+$

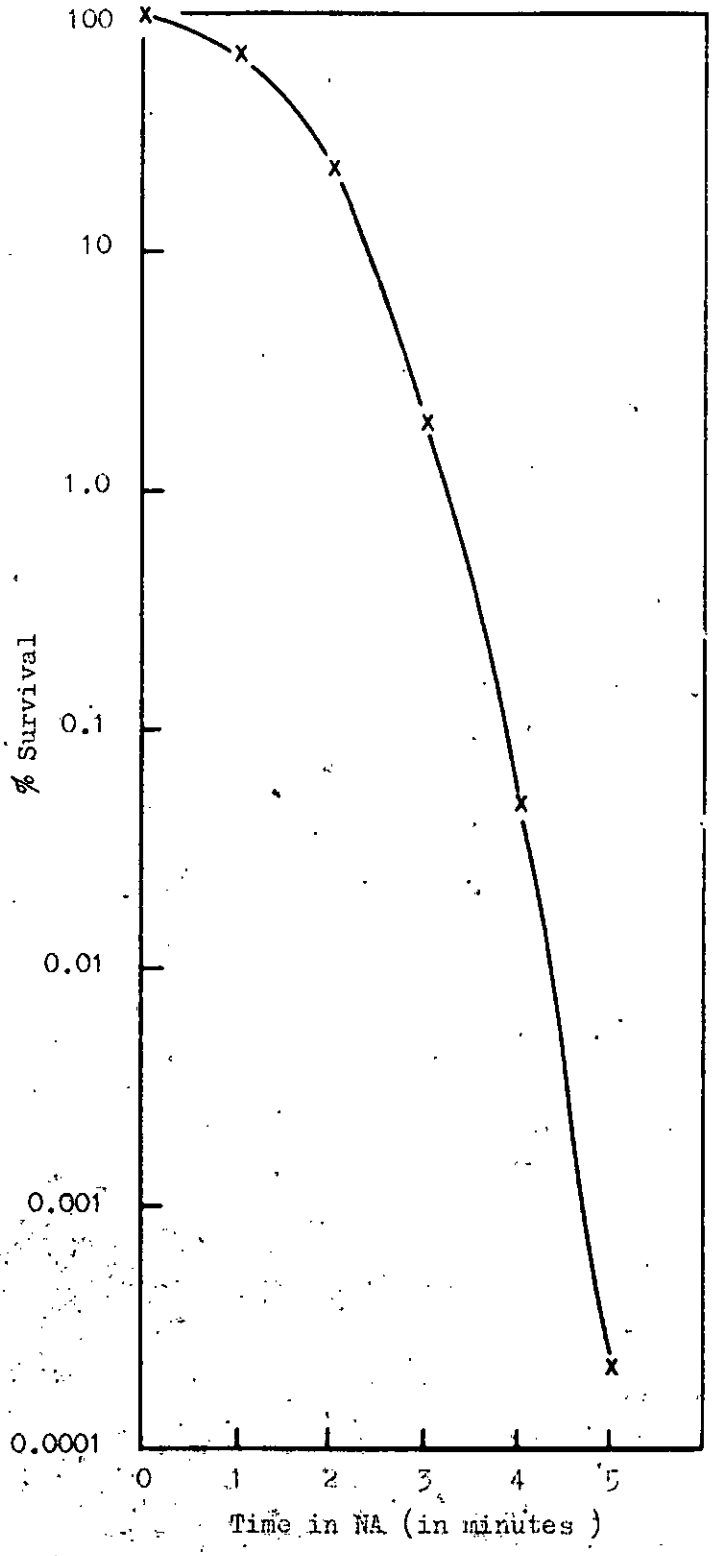
Having demonstrated a marked difference in the sensitivities of hm and hm^+ when they were treated with NA extracellularly it was intended to investigate the relative sensitivities of the two strains to intracellular NA treatment. A number of preliminary experiments were carried out to find the best conditions for such a study. The first was to measure the lethal effects of NA on E.coli B.

A culture of E.coli B was filter-washed and suspended in pH 4.0 acetate buffer to which had been added 0.1M $NaNO_2$. Samples were taken at intervals and the bacteria were plated and assayed for survival. See Figure 20. Comparison with Figure 18, which illustrates T4 inactivation under the same conditions, reveals that the E.coli was very much more sensitive to the lethal action of NA than was T4. The reason for this is not known but it may be that a larger proportion of the killing of bacteria is due to non-genetic inactivation.

Whatever the reason, the greater sensitivity of the bacteria does not augur well for an attempt to treat T4 intracellularly.

The phenomenon of the capacity of UV-killed E.coli to support T4 growth if infection is done immediately after irradiation has

Figure 20.


100
10
1.0
0.1
0.01
0.001
0.0001
% Survival
0
1
2
3
4
5
Time in NA (in minutes)

already been mentioned (Boyle and Swenson 1971). Capacity of X-ray-killed E.coli has also been demonstrated (Laban et al. 1953; Pollard et al. 1958; Whitmore and Pollard 1958; Marsden et al. 1972). The next experiment to be described was designed to test whether capacity is exhibited by NA-killed E.coli.

E.coli B was treated with NA as described previously and immediately after the treatment the bacteria were infected with T4hm$^+$ at m.o.i. of about 0.5 in the presence of cyanide. After 10 minutes the cultures were diluted and plated against untreated E.coli B. In addition aliquots were taken and lysed with $CHCl_3$ so as to assay the fraction of unadsorbed phage. It can be seen from Table 36, that although NA treatment of E.coli did not interfere with adsorbtion, those phage which did adsorb failed to develop. The phage survivors in the unlysed aliquots could be almost completely accounted for by the unadsorbed phage, which would be indifferent to whether the host had been treated or not.

In the final experiment in this section the phage were actually treated with NA during their intracellular growth. T4hm$^+$ was added to a culture of broth-grown E.coli B in the presence of cyanide and at low m.o.i. After 10 minutes the complices were diluted into fresh prewarmed broth and were incubated for 5 minutes at 37^{o}C at the end of which time they were further diluted into the usual NA reaction mixture. Samples were taken and plated against E.coli B either directly or after having been prematurely lysed with $CHCl_3$.

The results in Table 37 show that the extent of T4 inactivation was much greater in the intra- than extracellular state. (The rate

TABLE 36. Measurement of Ability of NA-Killed *E.coli* B to Support T4 Growth

NA Treatment of *E.coli* (mins)	% Survival of Bacteria	No. of T4 (pfu/ml)	
		+$CHCl_3$	no $CHCl_3$
0	100	7.1×10^4	6.7×10^6
2.5	2.5	6.5×10^4	1.0×10^5
5.0	0.0005	7.1×10^4	6.8×10^4

TABLE 37. Survival of T4 Treated with NA Intracellularly

NA Treatment of Complices (mins)	No. of T4 (pfu/ml)	
	+$CHCl_3$	no $CHCl_3$
0	4.4×10^2	5.6×10^4
2.0	8.6×10^1	1.3×10^2
4.0	1.4×10^1	2.9×10^1

of inactivation of extracellular phage can be estimated by the fall in titre of unadsorbed phage.

Given the findings from the preliminary experiments the most plausible explanation is that the killed bacteria cannot support the growth of the phage. Another possibility is that in fact the phage are more NA-sensitive when in the intracellular state.

In any event it was clear that it was not possible to do the intended comparison of hm and hm^+ inactivation when treated intracellularly.

The Effect of hm on the Specificity of Spontaneous and of NA-Induced r Mutants

Because of the enhancing effect of NA mutagenesis in the hm strain it was reasonable to ask whether this synergism was a general non-specific increase, or whether it involved a change in the specificity of the mutational spectrum. Complimentary to this was the need to determine the nature of the spontaneous mutant spectrum in this strain, given the higher spontaneous r frequency in hm. This is of interest in its own right. In addition if there is a difference in the spontaneous spectra of hm and hm^+, then the NA-induced spectrum in hm may need to be adjusted by a correction factor which takes into account the difference in the background.

Two fairly large-scale experiments were performed to isolate and characterize r mutants induced in the two strains. The experimental procedure was the same as that described for the first forward mutation experiments on hm and hm^+. Both strains were treated to around 1% survival and hm was treated for the same time

as gave 1% survival in $\underline{hm}^+$.

The results of the two experiments appear in Tables 38 and 39. These tables show the frequencies of the induced r mutants, the distribution of the r's into genotypes and the reversion analyses of the rII's. The reversion analysis of transition rII mutations was taken a step further compared to previous analyses in that all transitions were tested for their reversion response to HA. As has been mentioned those transitions which respond to 2-AP are mainly AT mutants and among those which are induced to revert with 5-BU there is a preponderance of mutants with GC at the mutant site (Bautz and Freese 1960; Champe and Benzer 1962b; Drake 1963). However a more rigourous diagnosis of the base pair at a transition site can be gleaned from the reversion response to HA; those which fail to be induced are AT at the mutant site and those which do show a response (a positive response was scored if reversion frequency was greater than 6-fold higher than spontaneous frequency) are GC mutants. The fact that HA under the appropriate conditions acts only on cytosine (5-HMC in the case of T4 (Janion and Shugar 1965)) has already been discussed.

In this work, most of the rII's which responded to 5-BU were also HA-revertible.

It should be mentioned here that after HA treatment there appeared to be a larger surviving fraction in $\underline{hm}$ than in $\underline{hm}^+$. This observation will be returned to later.

Inspection of Tables 38 and 39 shows that although $\underline{hm}$ exhibited a higher r frequency (both spontaneous and HA-induced) than $\underline{hm}^+$, the relative proportions of the different r genotypes were not modified,

TABLE 38. Isolation and Characterization of NA-Induced r Mutants in the T4hm and T4hm$^+$ (1st Isolation)

a). Isolation of Mutants

Strain	Time of Treatment (mins)	% Surv.	Plaques Inspected	r Plaques	r Frequency
hm$^+$	0	100	2.5×10^4	8	3.2×10^{-4}
hm$^+$	5.0	1.5	3.6×10^4	48	1.3×10^{-3}
hm	0	100	1.3×10^4	11	8.5×10^{-4}
hm	5.0	8.6	1.2×10^5	326	2.7×10^{-3}
hm	6.5	1.6	1.6×10^5	497	3.1×10^{-3}

b). Classification of r's into Genotypes

Strain	Treatment	rI		rII		rIII	
		Nos.	%	Nos.	%	Nos.	%
hm$^+$	0	3	37.5	5	62.5	0	-
hm$^+$	5.0	28	58.3	16	33.3	4	8.3
hm	0	5	45.5	5	45.5	1	9.1
hm	5.0	165	67.9	61	25.1	17	7.0
hm	6.5	289	63.0	141	30.7	29	6.3

c). Reversion Analysis of rII's

Class of Reversion	Strain and Origin of Mutations				
	hm$^+$0	hm$^+$5.0	hm 0	hm 5.0	hm 6.5
High Reverting	1(20.0)	1(6.7)	0(-)	5(9.8)	9(7.4)
No Reversion	0(-)	3(20.0)	2(18.2)	9(17.6)	17(14.0)
Background Reversion only	3(60.0)	9(60.0)	9(81.8)	9(17.6)	34(28.0)
With 2-AP	0(-)	0(-)	0(-)	10(19.6)	16(13.2)
With 5-BU	1(20.0)	1(6.7)	0(-)	11(21.6)	27(22.3)
With 2-AP & 5-BU	0(-)	1(6.7)	0(-)	3(5.9)	3(2.5)
With HA (only transitions tested)	0(-)	0(-)	0(-)	4(7.8)	15(12.5)

Figures in brackets are the % of each class.

TABLE 39. Isolation and Characterization of NA-Induced r Mutants in T4hm and T4hm$^+$ and Analysis of Spontaneous hmr Mutants (2nd Isolation)

a). Isolation of Mutants

Strain	Time of Treatment (mins)	% Surv.	Plaques Inspected	r Plaques	r Frequency
hm$^+$	0	100	1.1×10^4	4	3.6×10^{-4}
hm$^+$	5.0	1.9	2.0×10^5	200	2.0×10^{-3}
hm	0	100	7.5×10^5	430	5.7×10^{-4}
hm	5.0	6.8	3.2×10^5	1364	4.3×10^{-3}
hm	6.5	2.5	2.1×10^5	921	4.6×10^{-3}

b). Classification of r's into Genotypes

Strain	Treatment	rI		rII		rIII	
		Nos.	%	Nos.	%	Nos.	%
hm$^+$	0	4	100	0	-	0	-
hm$^+$	5.0	247	61.6	122	30.4	32	8.0
hm	0	156	36.3	244	56.7	30	7.0
hm	5.0	781	58.6	450	33.8	101	7.6
hm	6.5	576	64.7	246	27.6	68	7.6

TABLE 39. Continued....

c) Reversion Analysis of rII's

Class of Reversion	Strain and Origin of Mutations			
	$hm^{+}5.0$	hm 0	hm 5.0	hm 6.5
High Reverting	28(23.1)	37(16.0)	79(21.0)	49(22.5)
No Reversion	20(16.5)	12(4.9)	48(12.7)	22(10.1)
Background Reversion only	31(25.6)	13.8(56.7)	76(20.2)	51(23.4)
With 2-AP	20(16.5)	36(14.7)	108(28.6)	74(33.9)
With 5-BU	6(5.0)	6(2.5)	28(7.4)	8(3.7)
With 5-BU & 2-AP	7(5.8)	5(2.4)	10(2.7)	6(2.8)
With HA (only transitions tested)	9(7.4)	7(2.6)	28(7.4)	8(3.7)

Figures in brackets are the % of each class.

d) Absolute Frequencies of the Different Frequencies of NA-Induced and Spontaneous rII Mutants in hm and hm^{+}

Class of Mutation	Strain and Time of NA Treatment				
	$hm^{+}0^{A}$	$hm^{+}5.0$	hm 0	hm 5.0	hm 6.5
Deletion	2.9×10^{-6}	9.0×10^{-5}	1.3×10^{-5}	1.1×10^{-4}	7.1×10^{-5}
Transition[B]	8.3×10^{-6}	1.7×10^{-4}	6.3×10^{-5}	4.6×10^{-4}	4.2×10^{-4}
Frameshift & Transversion[C]	1.1×10^{-4}	1.6×10^{-4}	1.8×10^{-4}	2.4×10^{-4}	2.4×10^{-4}

A - The data for this are taken from the spontaneously arising rII's which were analysed during the work in Chapter 2 in Results and which are shown in Table 13.

B - rII's which were induced to revert with either base analogue.

C - rII's which failed to be induced to revert with base analogues but did revert spontaneously.

there being an excess of rII's in the spontaneously-arising hm r mutants and an excess of rI's in the NA-induced mutants in both strains.

The classification of NA-induced rII mutants with regard to their reversion characteristics shows that the increase in the frequency in the hm strain could not be attributed to a specific increase in one type of mutation but rather there was a general increase in all classes of induced mutations. There were very few spontaneous rII's in the hm^+ strain so no comparison could be made within these experiments of the types of spontaneous rII mutants arising in the hm and hm^+ strains. However a comparison of the patterns of reversion of the hm rII's and the rII's which arose spontaneously in the wild-type strain and which were analysed earlier (see Table 13) shows that the contribution of transitions amongst the spontaneous rII's in hm was greater than in the rII's arising in the wild-type strain. This is in agreement with the findings of Drake (1973). However, when the classification of the NA-induced rII's in the two strains was corrected for this difference in spontaneous spectra this did not alter the initial conclusion i.e. that the increase in the NA-induced frequency was a general rather than a specific one.

Mapping of the rII mutants was done in the usual way. The spontaneous hmrII spectrum comprised mutants obtained in the two experiments and is given in Figure 31 in conjunction with the spontaneous spectrum obtained by Benzer (1961). The two spectra are similar but they do exhibit some differences.

The relative contributions of the two very large hotspots of

Figure 21.

Representation of the Spectrum of Spontaneous rII Point Mutations in T4hm

The numbers above the lines:- T4rIIhm mutants.

This map differs slightly from the previous ones, in that the numbers below the line represent the spectrum of spontaneous rII's shown in Benzer (1961).

The fact that I possessed very few representatives of the sites in Benzer's map meant that in the majority of cases no alignment between his sites and mine could be made. Therefore the fact that most sites in the two spectra are not aligned, does not mean that the sites are in fact different, only that in most cases the comparison was impossible.

Figure 21.

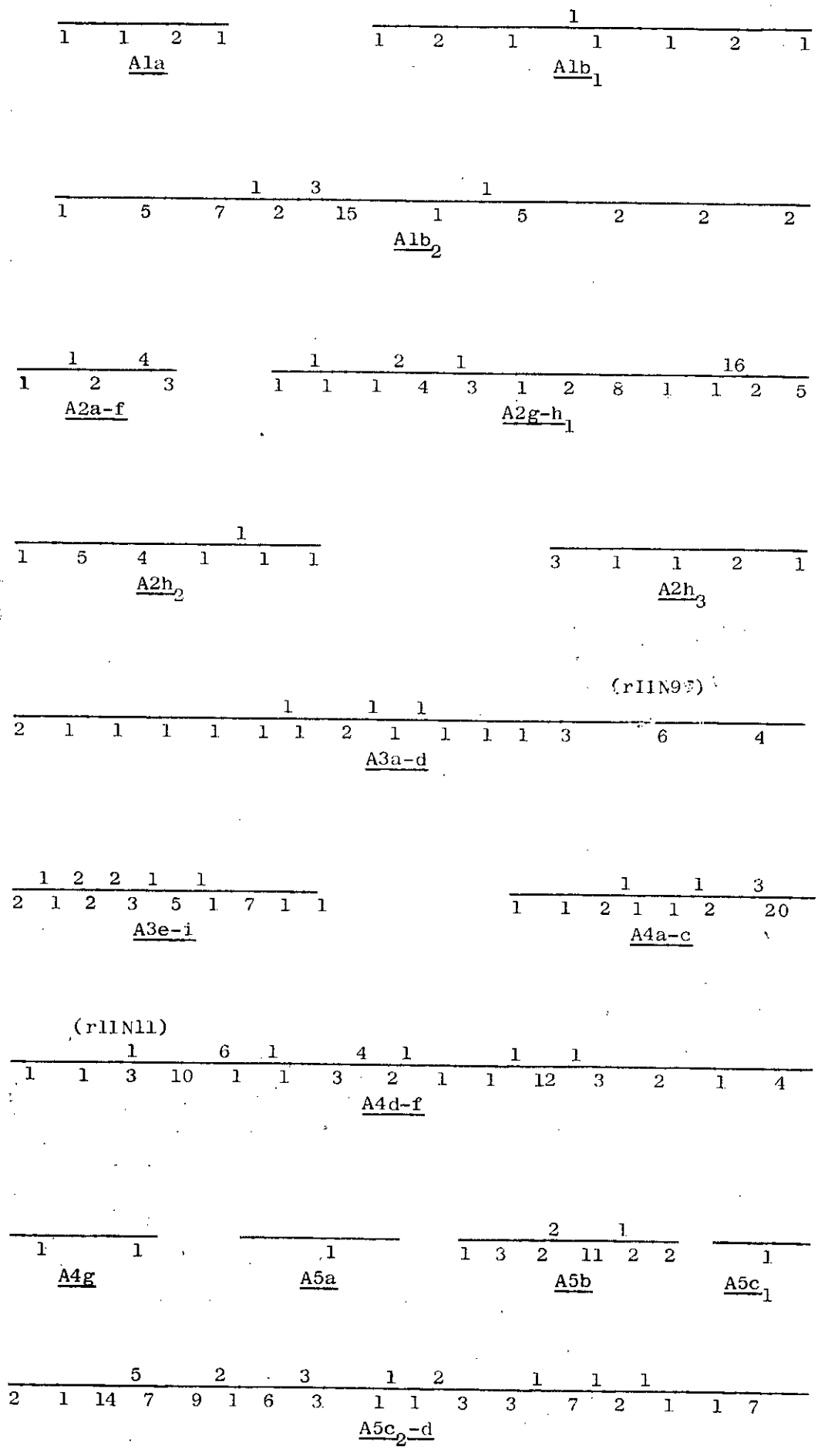
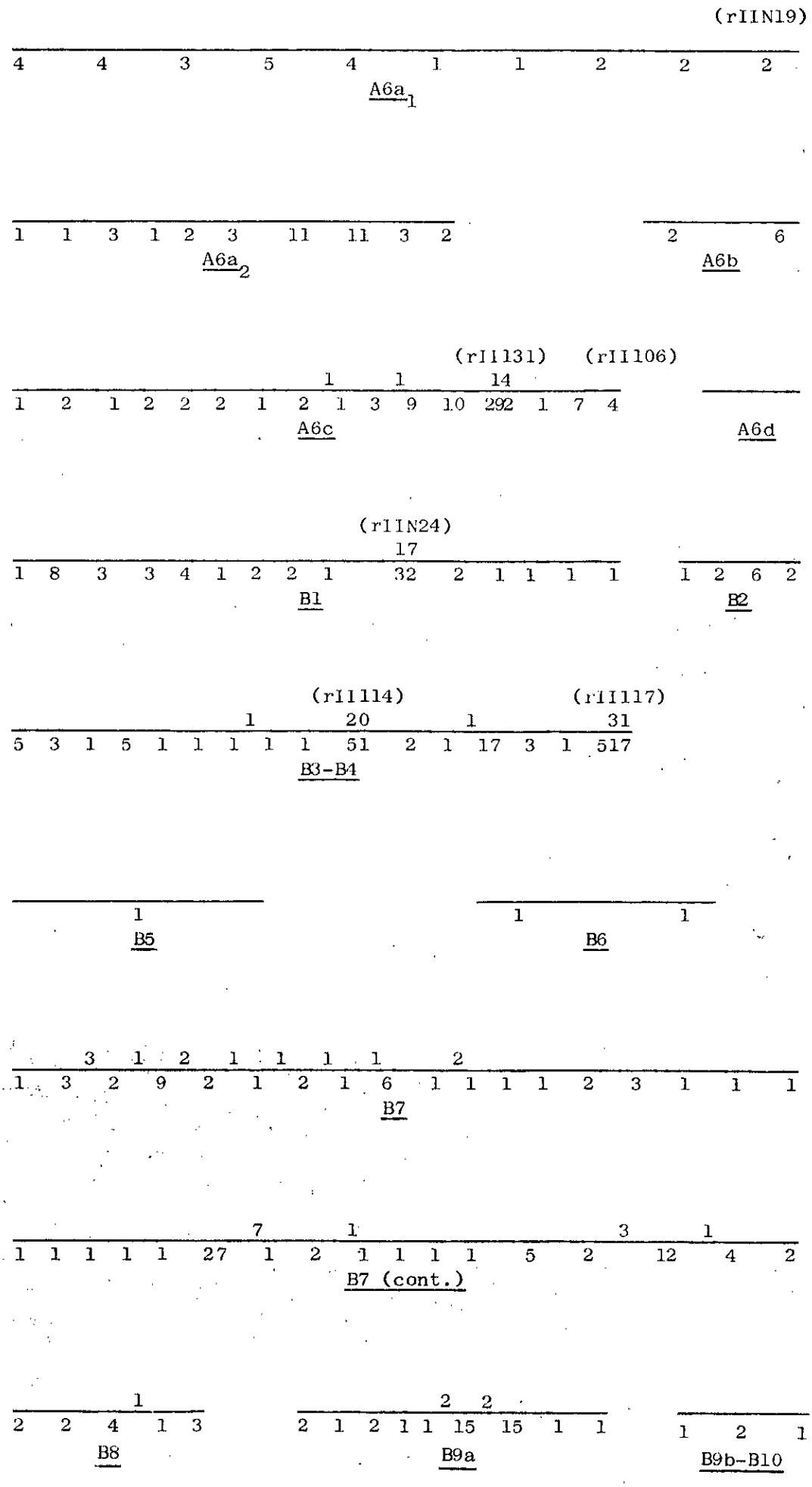

1 1 2 1
A1a
1
1 2 1 1 1 2 1
A1b1
1 3 1
1 5 7 2 15 1 5 2 2 2
A1b2
1 4
1 2 3
A2a-f
1 2 1 16
1 1 1 4 3 1 2 8 1 1 2 5
A2g-h1
1
1 5 4 1 1 1
A2h2
3 1 1 2 1
A2h3
(rIIN93)
1 1 1
2 1 1 1 1 1 1 2 1 1 1 1 3 6 4
A3a-d
1 2 2 1 1
2 1 2 3 5 1 7 1 1
A3e-i
1 1 3
1 1 2 1 1 2 20
A4a-c
(rIIN11)
1 6 1 4 1 1 1
1 1 3 10 1 1 3 2 1 1 12 3 2 1 4
A4d-f
1 1
A4g
1
A5a
2 1
1 3 2 11 2 2
A5b
1
A5c1
5 2 3 1 2 1 1 1
2 1 14 7 9 1 6 3 1 1 3 3 7 2 1 1 7
A5c2-d

Figure 21. (cont.)



Benzer (rII131 in A6c and rII117 in B4) were less in the hm strain and the hotspots rII114 and rIIN24 assumed greater importance. In addition there was a botspot in region A2g-h_1. In Benzer's spontaneous map (based on about 5 times as many mutants) there is one site represented by 8 mutants. The hotspot in the hmrII spectrum at this region is therefore some 10 times hotter than the one reported by Benzer. All the mutants at this site were transitions and were induced to revert with HA. Given this last fact it is not irrelevant to note that Benzer's (1961) 2-AP induced spectrum shows a marked hotspot in A2g. Unfortunately a representative of this site was not available so it was not possible to determine whether this 2-AP hotspot and the hm spontaneous hotspot mapped at the same site.

The maps of the NA-induced rII spectra of the hm and hm^+ strains are found in Figures 22a and b. Each map comprised mutants obtained in the two experiments, and for the map of rII's in the hm strain the mutants induced at both doses of NA were pooled. This was felt to be legitimate since the spectra obtained from the individual treatments were similar. Figure 22a shows that the spectrum of NA-induced rII point mutants in the hm strain did not differ in any striking way from that found in hm^+ and both spectra are in accord with that shown by Benzer (1961). All the NA-induced rII deletions in the hm^+ strain and a sample of 24 deletions induced in hm were mapped. Both maps are in agreement with previous work and with Tessman (1962) in that the majority of the deletions extended to the right of the rIIB cistron.

The implications of these results on the effect of the hm

Figure 22a.

Representation of the Spectra of rII Point Mutations by NA in T4hm and T4hm$^+$

Legend:- As for Figure 5a.

Numbers above the lines:- NA-induced T4rIIhm mutants

Numbers below the lines:- NA-omdiced T4rIIhm$^+$ mutants

Figure 22a.

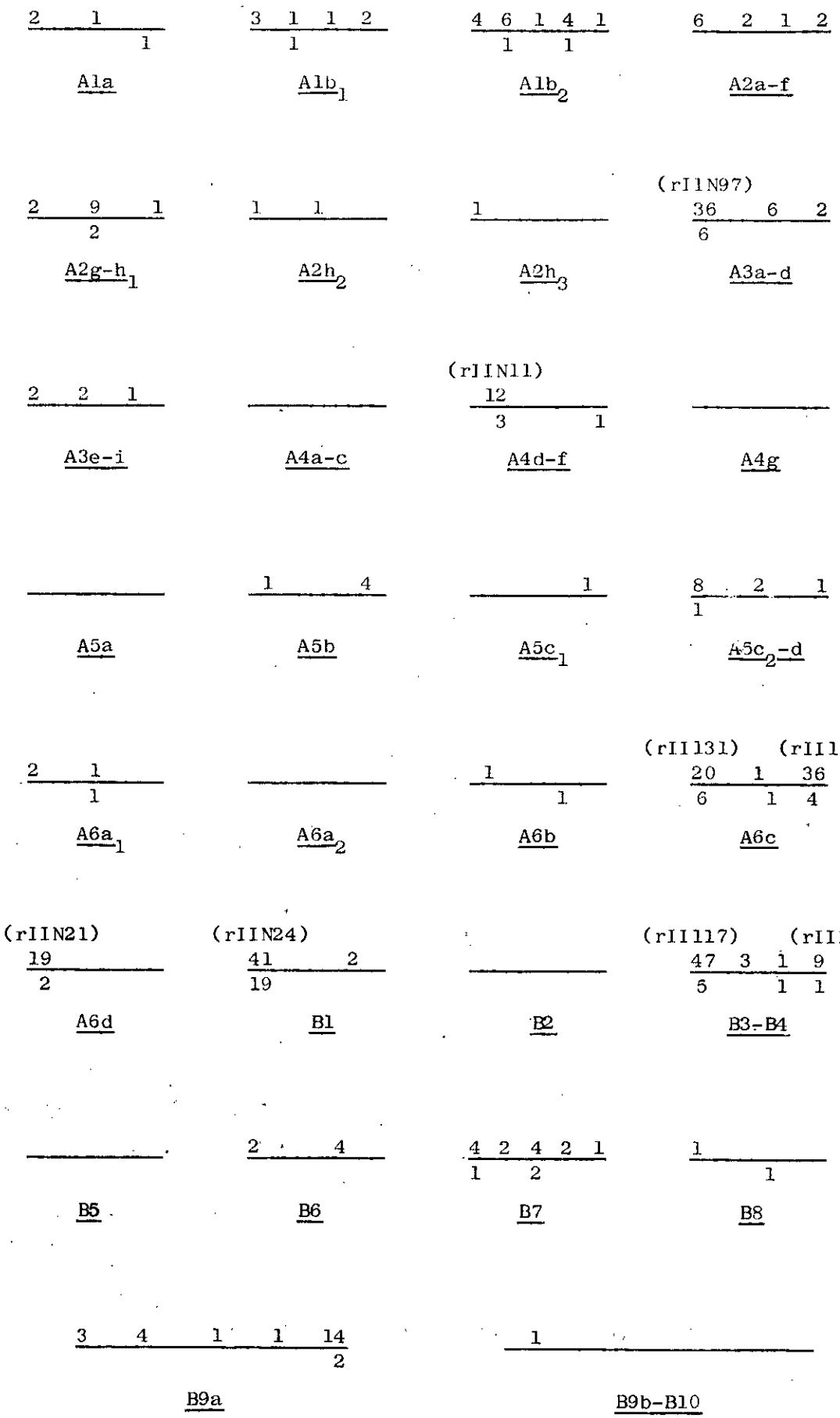

2 1
1
A1a
3 1 1 2
1
A1b1
4 6 1 4 1
1 1
A1b2
6 2 1 2
A2a-f
2 9 1
2
A2g-h1
1 1
A2h2
1
A2h3
(rI1N97)
36 6 2
6
A3a-d
2 2 1
A3e-i
A4a-c
(rIIN11)
12
3 1
A4d-f
A4g
A5a
1 4
A5b
1
A5c1
8 2 1
1
A5c2-d
2 1
1
A6a1
A6a2
1
1
A6b
(rII131) (rII106)
20 1 36
6 1 4
A6c
(rIIN21)
19
2
A6d
(rIIN24)
41 2
19
B1
B2
(rII117) (rII114)
47 3 1 9
5 1 1
B3-B4
B5
2 4
B6
4 2 4 2 1
1 2
B7
1
1
B8
3 4 1 1 14
2
B9a
1
B9b-B10

Figure 22b.

Representation of the Dimensions of rII Deletions Induced by NA in T4hm^{+} and T4hm

Legend:- As for Figure 5b.

Above :- Deletions induced in T4hm

Below :- Deletions induced in T4hm^{+}

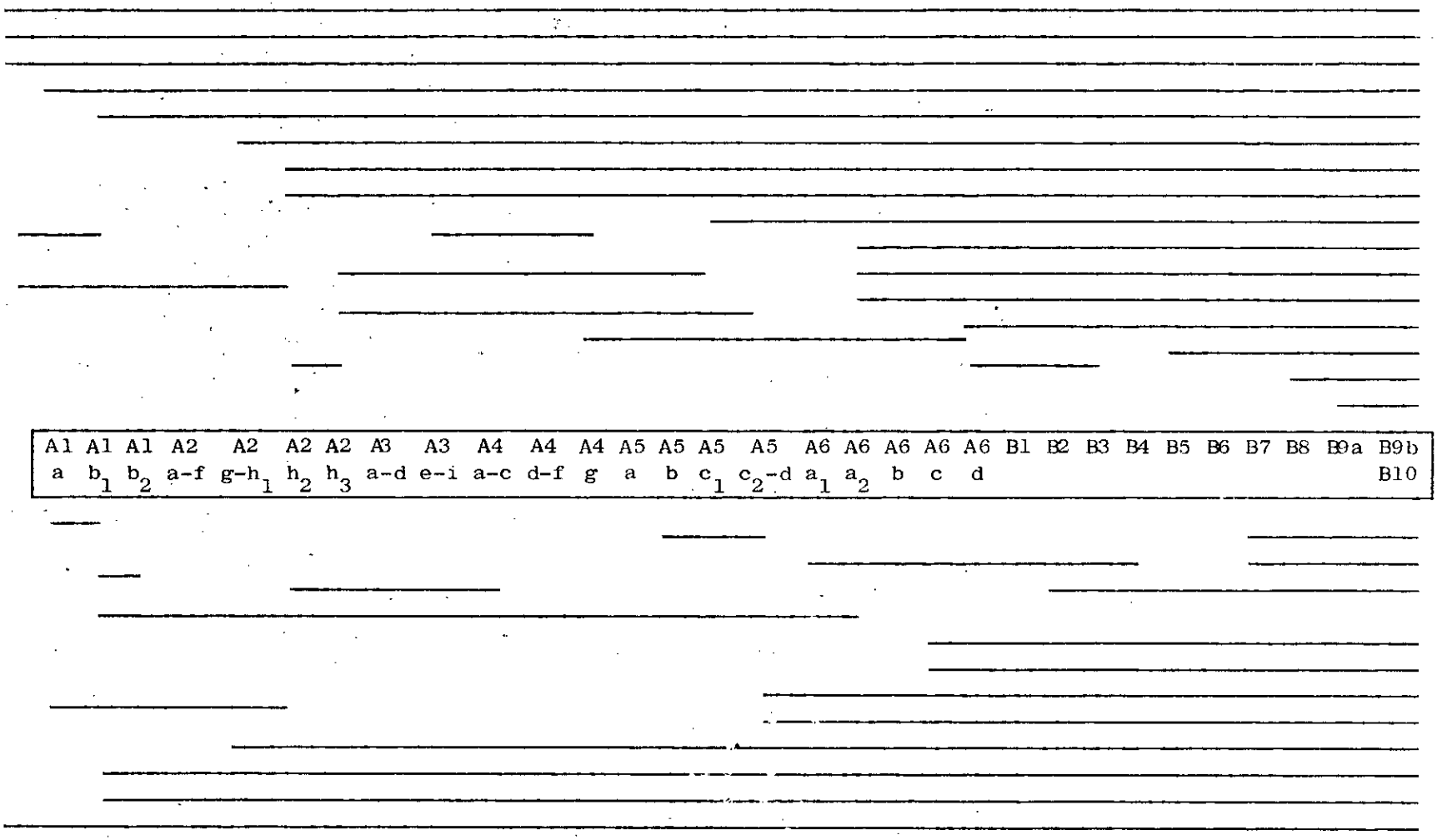

A1 A1 A1 A2 A2 A2 A2 A3 A3 A4 A4 A4 A5 A5 A5 A5 A6 A6 A6 A6 A6 B1 B2 B3 B4 B5 B6 B7 B8 B9a B9b
a b1 b2 a-f g-h1 h2 h3 a-d e-i a-c d-f g a b c1 c2-d a1 a2 b c d B10

Figure 22b.

mutation on NA-mutagenesis will be discussed later after consideration of further work on this strain.

Comparison of Survival of T4hm and T4hm$^+$ after Treatment with Other Agents

Having shown a marked difference in the sensitivities of the two strains to NA inactivation and possibly to HA it was thought to be worthwhile to see if killing of hm and hm$^+$ by other agents also differed in severity.

Only a few of all the possible agents were chosen. All had been shown to be lethal to T4 and most of them are known to induce genetic damage to phage.

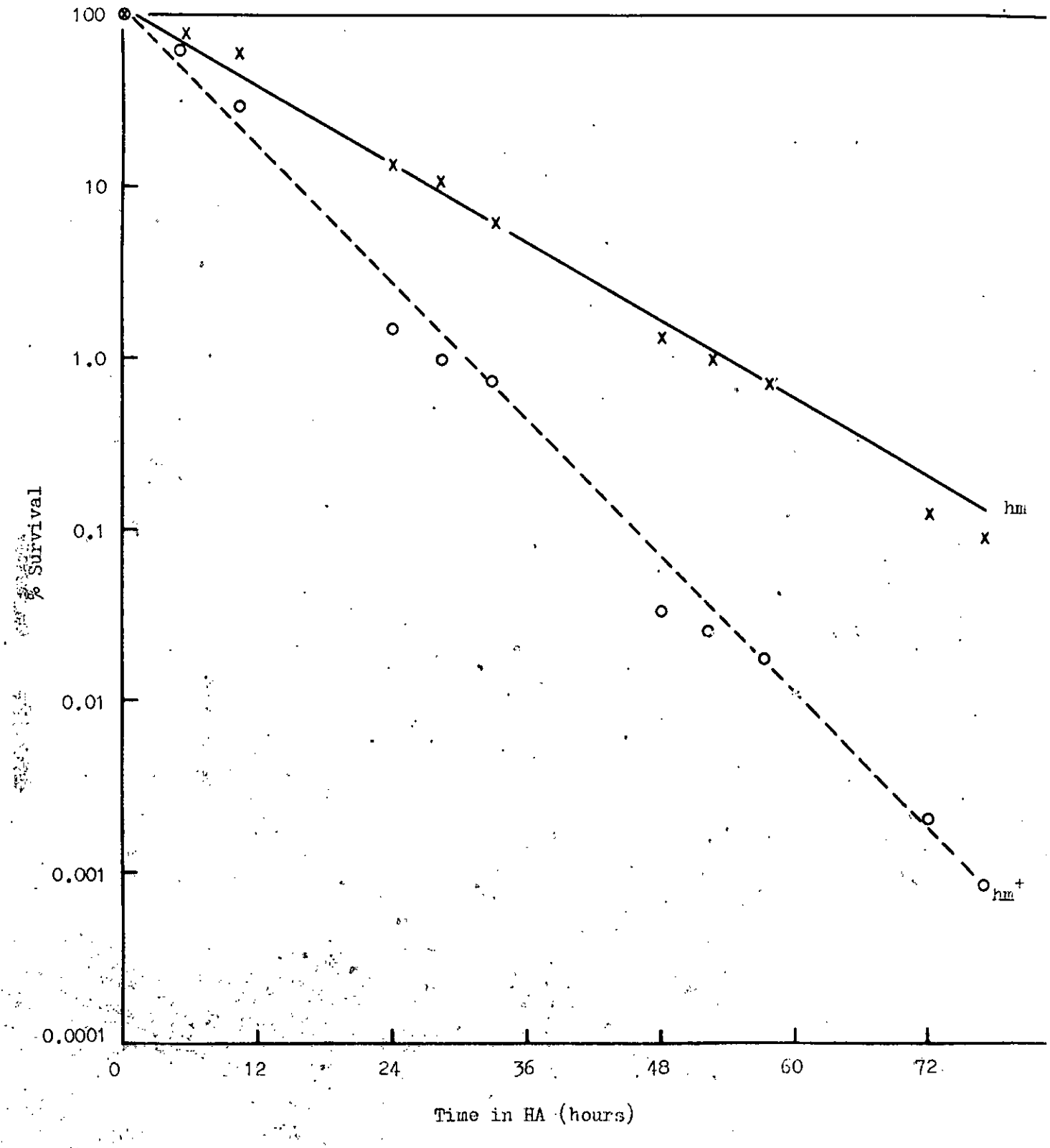
(a) HA

Both hm and hm$^+$ strains were treated with HA as described in Materials and Methods. Samples were taken at intervals and plated against E.coli B to assay survival. From Figure 23 it can be seen there was indeed a difference in the two strains. Both were inactivated exponentially but the slope of the killing curve was greater for hm$^+$ phage than for hm, such that at the highest dose (76 hours) the surviving fraction in the hm strain was about a hundred times greater than in the wild-type. The extent of the resistance to HA found in the hm strain was approximately the same as that found for NA inactivation.

(b) UV

Both strains were treated with UV as described in Materials and Methods. Samples were taken after various times of irradiation and the phage were plated on E.coli B and survivals were

Figure 23.


100
10
1.0
0.1
0.01
0.001
0.0001
% Survival
0
12
24
36
48
60
72
Time in HA (hours)
hm
hm+

estimated. Figure 24 shows that there was no difference in the sensitivities of the two strains towards UV. This is in agreement with Drake (1973).

(c) Heat

The details of the inactivating effects of high temperature on T4 are not known. In bacteria, mutations and depurinations can readily be induced by heating (Zamenhof and Greer 1958; Zamenhof 1960; Greer and Zamenhof 1962). The mutagenicity of high temperature treatmentmof T4 has not been assayed, but it has been demonstrated that medium-level heating (45°C) especially in acid conditions can be mutagenic to T4 (Freese 1961; Drake 1966a).

It is quite likely that a substantial fraction of the inactivation of T4 is due to effects of heating on protein structure. Indeed the rate of phage inactivation fits well with that which would be expected if denaturation of protein were responsible (Adams 1959). There is indirect evidence that such denaturation does not involve the phage tail fibres (Kacser 1957).

The phage of the two strains were subjected to heating at 70°C as described in Materials and Methods. No real difference in the rates of inactivation of the two strains could be detected. See Figure 25.

(d) Bisulphite

It was mentioned in the Introduction that bisulphite can act on cytosine in single-stranded regions tRNA, converting it to uracil (Goddard and Schulman 1972) and in addition it has been shown to modify uridine residues (Shapiro and Braverman 1972;

Figure 24.

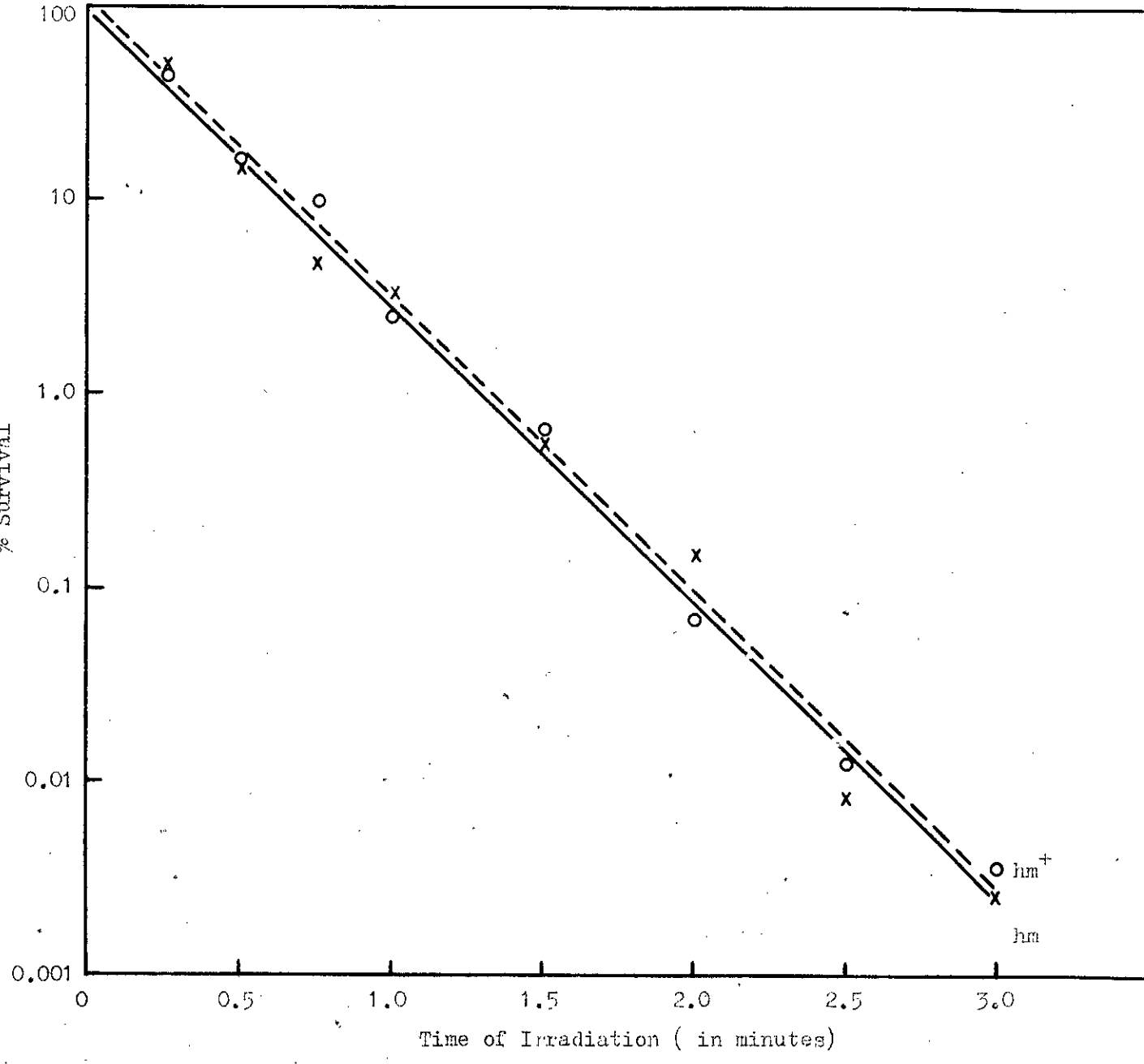
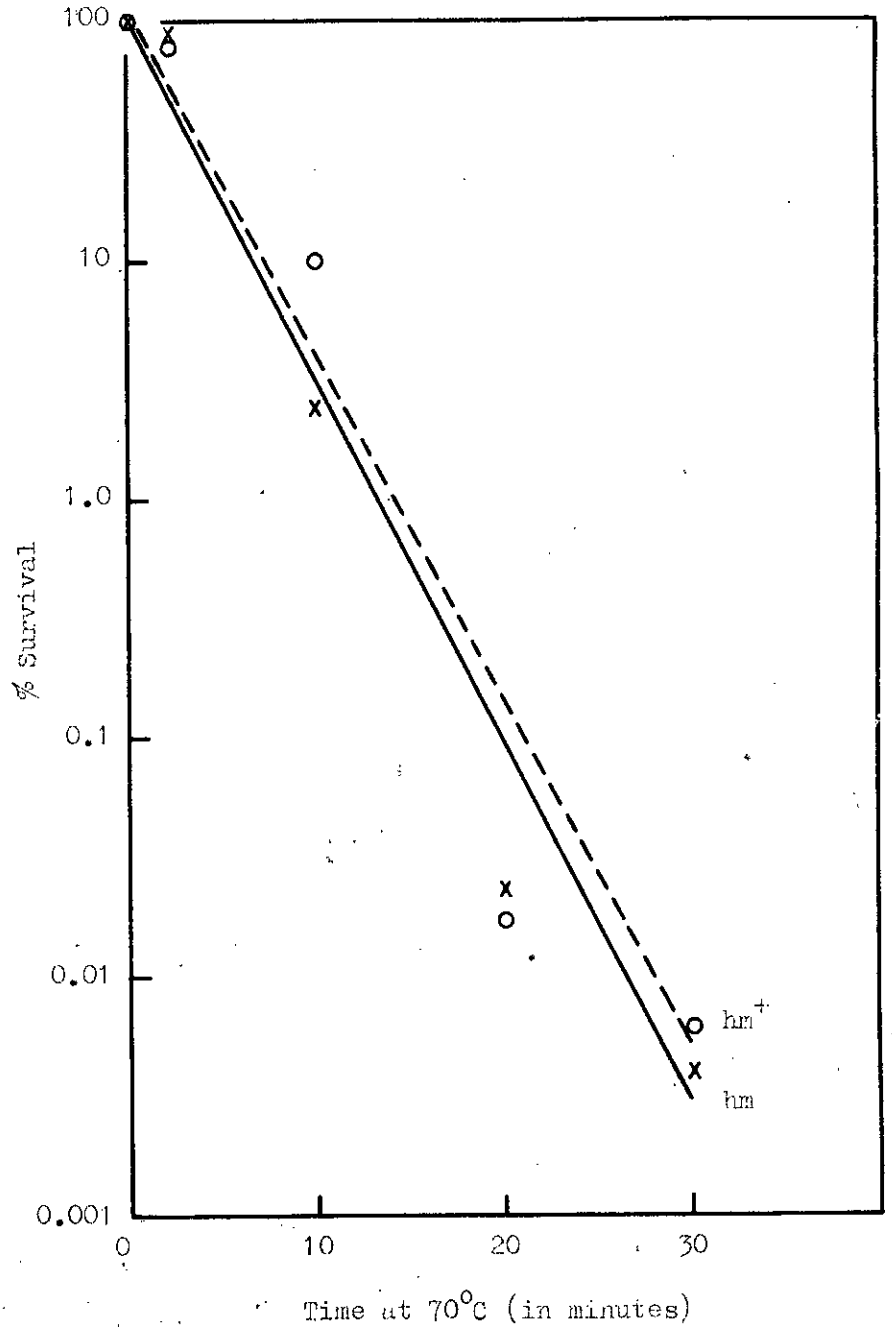

100
10
1.0
0.1
0.01
0.001
% Survival
0
0.5
1.0
1.5
2.0
2.5
3.0
Time of Irradiation (in minutes)
hm+
hm

Figure 25.


100
10
1.0
0.1
0.01
0.001
% Survival
0
10
20
30
Time at 70°C (in minutes)
hm+
hm

Wataya and Hayatsu 1972).

It has also been shown to be mutagenic in bacteriophages lambda (Hayatsu and Miura 1970) and T4 (Summers and Drake 1971) and in E.coli (Mukai et al. 1970) the mutants induced being almost exclusively GC →AT transitions.

The two strains were treated with sodium bisulphite as described in Materials and Methods and were assayed for survival on E.coli B. Figure 26 shows that there was no difference in the sensitivities of the hm and hm^+ strains to bisulphite.

(e) EMS

This alkylating agent is moderately lethal to phage T4. A large fraction of the lethality can be attributed to genetic damage since EMS-inactivated phage show strong multiplicity reactivation (Ray, Bartenstein and Drake 1972). Chemical studies also indicate a genetic target (Brookes and Lawley 1963). In addition the rates at which T4 is inactivated by EMS have been shown to be affected by both viral and host genotypes. The T4x strain shows greater EMS sensitivity than does wild-type. Mutations in both the T4 and the E.coli DNA polymerase loci also render the phage more EMS-sensitive (Ray, Bartenstein and Drake 1972).

However, the hm mutation appears to have no effect on the rate of EMS inactivation as witnessed by the similarities in the killing curves in the hm and hm^+ strains shown in Figure 27. It is hence reasonable to assume that the EMS sensitivity found in the T4x strain is due to the px rather than the hm mutation.

Figure 26.

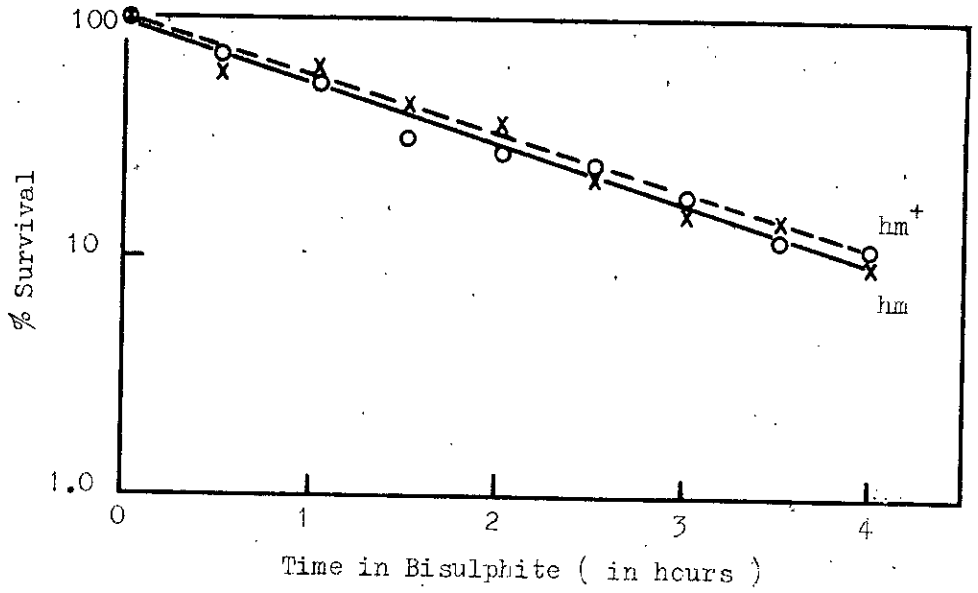
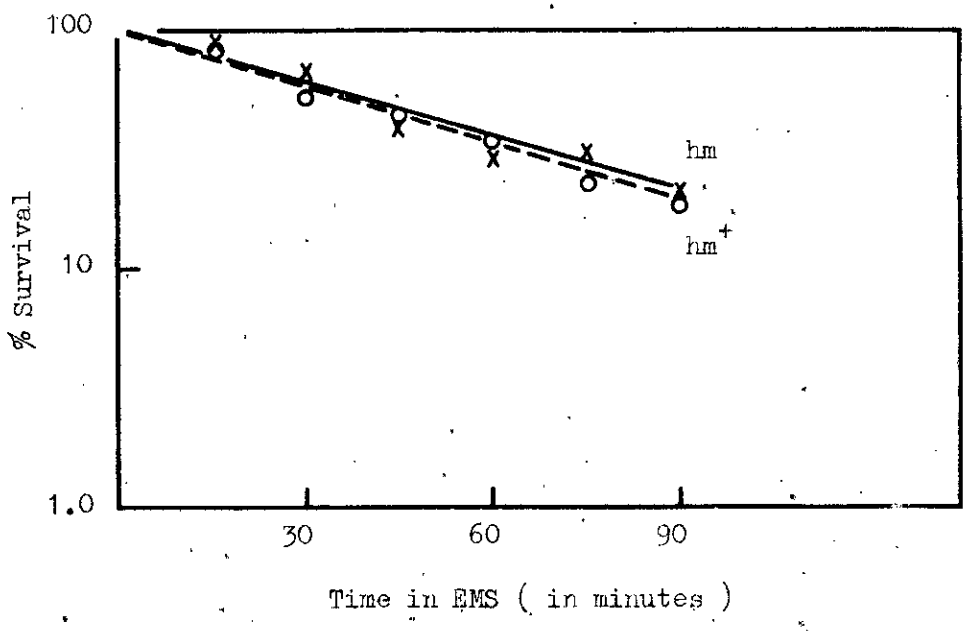


Figure 27.



Effect of Host Genotypeson Inactivation of T4hm and T4hm$^+$

The survival of T4 after treatment by agents such as UV and photodynamic action is influenced by host genotype to a much lesser extent than is most phage. Most notably the sensitivity of irradiated T4 is refractory to host cell reactivation (Harm 1968 and 1973) unlike other phages such as lambda and the T-odd coliphages (e.g. Geissler 1968).

However T4 sensitivity to UV is increased when the host is defective in the Kornberg polymerase (Smith et al. 1970).

As already mentioned host genotype has also been shown to have an effect on the rate at which T4 is inactivated by EMS (Ray, Bartenstein and Drake 1972). These authors also reported a synergistic effect on EMS sensitivity between certain phage mutations and specific mutations in the host.

It was decided to examine the effects of different host genotypes on the survival of the hm and hm$^+$ strains after they had been treated with the agents described above (except heat) and with NA to see if the two phages responded differentially in various repair-deficient hosts.

The strains of repair-deficient hosts were all derivatives of E.coli WP-2 and were as follows.

(a) CM561. This strain carries a mutation at the exr locus (Mattern et al. 1966) and was initially isolated by Hill (1958) as a UV-sensitive mutation. It has slightly reduced recombination frequency (Howard-Flanders and Boyce 1966) and it entirely abolishes UV mutagenesis. Witkin (1969a and b) has rather speculatively suggested

that the wild-type exr gene product attaches to the ends of DNA adjacent to gaps and stimulates recombination repair of UV-induced damage and that it causes errors in the repair.

(b) CM571. This strain possesses a mutation at the recA locus which renders it UV-sensitive due to an inability to carry out recombination repair of UV-induced damage to DNA. The recA mutation is characterized by its UV-sensitivity, an increase in DNA degradation after UV-irradiation, a markedly decreased recombination frequency, and decreased rates of UV-induction of bacteriophage lambda (Clark and Margulies 1965; Howard-Flanders and Theriot 1966; Clark et al. 1966; Clark 1967; Brooks and Clark 1967; Hertman and Luria 1967). Like the exrA mutation the recA mutation also causes a loss of UV mutability (Witkin 1967).

(c) WP2 uvrA. This strain carries the uvrA allele and is UV-sensitive due to an inability to excise damage inflicted on DNA by UV (Boyce and Howard-Flanders 1964).

(d) CM611. This is a double mutant carrying both the exrA and uvrA alleles.

Both hm and hm^+ phage were treated with the same agents as were used in the section above, i.e. NA, HA, UV, bisulphite and EMS. The treated phage were plated against the E.coli repair-deficient strains and also against the parental WP-2 strain and E.coli B. It was found that the killing kinetics of both phage strains in response to all the agents tested was independent of the host genotype used. Thus the hm strain maintained its resistance to HA and NA in all hosts tested.

To my knowledge there are no reports dealing with the effects

of host genotype on the inactivation of T4 by NA, HA or bisulphite. Ray, Bartenstein and Drake (1972) found no effects of exr, hcr or recA mutations on the rate of EMS-inactivation of T4. The refractoriness of the effects of most changes in host genotype on T4 survival after UV has been mentioned.

In the light of the comparative independence of bacterial repair shown by T4 the failure to detect any changes in the survival of the hm and hm^+ strains in the different hosts used here was not too surprising. It should be said here that under exceptional conditions of infection (in the presence of chloramphenicol), nicks in T4 DNA can be repaired by the bacterial repair system. Interestingly, when the complices are released from chloramphenicol inhibition, the T4 system acts on the host-mediated repair (Carlson et al. 1973).

Reversion Studies in T4hm and T4hm^+

Although forward mutation studies on the r system have the greater potential for providing information on mutagen specificity, they are clearly unwieldy compared to reversion experiments. This is especially true if, in addition to measuring the frequency of forward mutants, the mutants are also classified into the r loci, and into sites within the rII locus. Time did not allow forward mutation studies in the hm and hm^+ strains to be done with all the mutagens described below.

It was therefore decided to compare the response to a selection of mutagens of a number of rII mutants in the hm and hm^+ backgrounds. Since all the mutagens to be used induced base-pair substitutions

predominantly, rII transition mutants were used as tester strains.

Four independent rII's isolated after NA mutagenesis of the hm strain were chosen since they had acceptably low spontaneous reversion frequencies and they were judged to be transitions on the basis of their positive responses to base analogue reversion spot-tests. Two of them (coded 11C and 12J) were induced to revert with HA and were classified as possessing GC at the mutant site. The other two (14A and 15I) failed to respond to HA and were typed as AT mutants.

In order to get each rII into the wild-type genetic background repeated backcrossing was carried out. E.coli B at $2x10^8$/ml was infected simultaneously with T4hm^+r^+ at about $5x10^8$/ml and the appropriate T4hmrII at about $5x10^7$/ml in the presence of cyanide. After 12 minutes the culture was diluted into fresh broth and was incubated at 37°C for 60 minutes before lysis was completed by the addition of $CHCl_3$. The phage were then plated on E.coli B.

After incubation, an r plaque was picked from the plates and used to infect a culture of E.coli BB so as to give an LTS. Using the rII's from each LTS a new cross between each mutant and the hm^+r^+ strain was performed, and the whole procedure described above was repeated. Altogether eight serial backcrosses were carried out. After the final backcross five rII plaques from each strain were used to set up LTS's.

To test whether the hm allele had been lost during the backcrossing, each of the original hmrII stocks, the original wild-type hm^+r^+ strain and all the LTS's of the backcrossed rII's were treated with HA and NA and the rates of inactivation of all the strains were

measured. It was found that for both agents, the survival curves of all the backcrossed rII mutants were very similar to that of the original $\underline{hm^+r^+}$ strain and hence were more sensitive than the original hmrII strains, suggesting that the hm allele (or the allele responsible for conferring NA- and HA-resistance in the hm strain) had been lost in all the strains which had been subjected to backcrossing. One LTS of each backcrossed rII mutant was grown up to high titre for further studies.

The general procedure for the reversion studies was to treat the rII's in both the hm and hm^+ backgrounds. The mutagenized phage were then plated on E.coli B to measure survival and on E.coli WØ80. The phage were either plated directly on E.coli WØ80 or else were adsorbed to a permissive host so as to allow the mutagenized phage a cycle of growth before plating on the restrictive host. In order to test whether the genotype of the host modified the reversion frequencies induced in hm and hm^+, a variety of E.coli strains were used as hosts for this growth cycle. These were E.coli B, E.coli WP-2, and the four repair-deficient derivatives of WP-2 catalogued above. In fact the results of the reversion experiments for both phage strains and for all mutagens tested were shown not to be affected by the genotype of the bacteria in which permissive growth was allowed. Accordingly only the results of the experiments in which E.coli B was used as host will be given.

(a) NA

Each rII mutant both in the hm and hm^+ strains was treated for 4.5 minutes with NA in the usual way and the survivors were assayed.

The treatment was stopped by diluting directly into cultures

of E.coli (at about $4x10^8$/ml) in broth and in the presence of cyanide at m.o.i. of about 0.5. Where plating for revertants was done directly, the bacteria in the stopping broth were E.coli WØ80. If a cycle of replication was allowed, E.coli B was used. In this latter case the samples were diluted a further 1/50 once adsorbtion was complete so as to ensure that the vast majority of plating bacteria were E.coli WØ80. In a similar study, Bautz-Freese and Freese (1961) killed the E.coli B with UV prior to phage adsorbtion to ensure that the permissive host contributed very little to the restrictive bacterial lawn. However the technique used here was quite satisfactory although it did entail the need for a larger number of reversion plates because of the lower phage density.

It has already been shown that NA treatment of T4 did not affect adsorbtion ability. Thus one can be sure that the great majority of phage adsorb to the permissive host ensuring that a round of growth of the survivors does indeed occur. In parenthesis it should be mentioned that the effects of the other agents to be described below (HA, EMS and bisulphite) on adsorbtion were measured. None of them had any inhibitory effect on the rate of adsorbtion. It has been postulated that at low concentrations HA attacks T4 tail fibres but that this effect disappears at higher HA concentrations (Kozloff, Lute and Henderson 1957).

Table 40 shows the results of the NA-induced reversion experiments. In general the spontaneous reversion frequencies were higher when replication was allowed before plating on E.coli WØ80. This is to be expected and is in agreement with Bautz-Freese and Freese (1961) and Freese, Bautz and Bautz-Freese (1961). Each

TABLE 40. NA-Induced Reversion of rII's in hm and hm^+ Backgrounds

rII Strain	NA	Cycle of Growth	% Surv.	Revertants	Rev. Freq.
hmrII 11C	-	No	100	47	6.9×10^{-7}
	+	No	10.1	237	8.1×10^{-6}
	-	Yes	100	29	1.8×10^{-6}
	+	Yes	10.1	251	3.1×10^{-5}
hm^+rII 11C	-	No	100	42	3.5×10^{-7}
	+	No	0.91	176	2.8×10^{-6}
	-	Yes	100	36	5.8×10^{-7}
	+	Yes	0.91	212	6.2×10^{-6}
hmrII 12J	-	No	100	78	1.3×10^{-6}
	+	No	9.6	361	1.6×10^{-5}
	-	Yes	100	108	2.4×10^{-6}
	+	Yes	9.6	518	2.5×10^{-5}
hm^+rII 12J	-	No	100	37	9.4×10^{-7}
	+	No	0.86	184	6.6×10^{-6}
	-	Yes	100	62	1.8×10^{-6}
	+	Yes	0.86	300	7.6×10^{-6}
hmrII 14A	-	No	100	25	2.0×10^{-7}
	+	No	15.2	211	3.5×10^{-6}
	-	Yes	100	31	2.6×10^{-7}
	+	Yes	15.2	474	9.1×10^{-6}
hm^+rII 14A	-	No	100	30	1.1×10^{-7}
	+	No	1.6	209	6.1×10^{-7}
	-	Yes	100	51	2.5×10^{-7}
	+	Yes	1.6	315	2.4×10^{-6}

Continued.....

TABLE 40. Continued.....

rII Strain	NA	Cycle of Growth	% Surv.	Revertants	Rev. Freq.
hm rII 15I	-	No	100	37	6.5×10^{-7}
	+	No	12.6	248	7.2×10^{-6}
	-	Yes	100	68	2.1×10^{-6}
	+	Yes	12.6	516	6.2×10^{-5}
hm^{+} rII 15I	-	No	100	28	2.3×10^{-7}
	+	No	1.4	186	2.1×10^{-6}
	-	Yes	100	42	7.1×10^{-7}
	+	Yes	1.4	257	9.8×10^{-6}

E.coli B cell which was infected with a viable phage will contain many progeny at the end of the latent period and it needs only one spontaneously-arising revertant amongst the progeny for a revertant plaque to be scored. However since the infected cells were plated prior to lysis the number of infective centres is the same whether the phage were allowed a cycle of growth or not and thus the reversion frequency as measured by dividing the number of revertants by the number of infective centres should be greater where growth was allowed. The fact that Kreig (1963) and Green and Kreig (1961) found no increase in spontaneous reversion frequencies of rII's after allowing a cycle of growth was probably due to the fact that the phage had lysed before being plated on the restrictive host.

Whether the phage were plated directly or were allowed a cycle of growth, it was found that the spontaneous reversion frequency of any given rII mutant was higher in the hm than in the hm^+ strain. This applied for all four rII mutants and was found in the controls of all the other mutagenic treatments described below.

Turning to the NA-induced reversion frequencies, it was found that here too the frequencies of revertants were increased in those treatments where a round of growth was allowed. This enhancement held after correction for the higher spontaneous reversion frequencies found under such conditions.

This suggested that a proportion of NA-induced lesions required replication before they could be expressed as mutations. This is in agreement with the findings and conclusions of Bautz-Freese and Freese (1961).

In order to see more clearly the effects of the hm mutation

on the NA-induced reversion frequencies, the results have been expressed as the ratio of reversion frequency in the hm strain over that found in the hm^+ strain. The ratios of the induced reversion frequencies have been corrected for spontaneous reversion.

Table 45 gives these ratios as well as those obtained with the other mutagens used in this study.

It can be seen that for all the rII's the reversion frequency induced by NA in the hm strain was greater than that found in the hm^+ strain. In a repeat of this experiment, such synergism was again found, the ratio never being less than 3.0. The extent of the increase for all the strains was approximately the same and was not much influenced by the phages being allowed a cycle of growth.

The fact that the enhancing effect of hm NA mutagenesis operated in both forward and reverse directions is good evidence against selection for or against r mutants being a factor in the synergism.

(b) HA

All the rII's were treated with HA for 36 hours in the way already described. After termination of the treatment the experiment was performed in the way described for NA mutagenesis. Tables 41 and 45 show the results. As was to be expected, only the GC mutants (11C and 12J) were induced to revert with HA. The frequencies of induced reversion of both these rII's were somewhat higher if the phage were passaged through E.coli B. Such an increase was also noted by Freese, Bautz and Bautz-Freese (1961).

The ratios of the corrected HA-induced reversion frequencies of rII's 11C and 12J in the hm and hm^+ strains are given in Table 45,

TABLE 41. HA-Induced Reversion of rII's in hm and hm^+ Backgrounds

rII Strain	HA	Cycle of Growth	% Surv.	Revertants	Rev. Freq.
hmrII 11C	-	No	100	68	8.9×10^{-7}
	+	No	9.3	247	8.3×10^{-6}
	-	Yes	100	52	7.5×10^{-7}
	+	Yes	9.3	315	9.9×10^{-6}
hm^+rII 11C	-	No	100	95	3.0×10^{-7}
	+	No	0.82	186	6.8×10^{-6}
	-	Yes	100	64	9.2×10^{-7}
	+	Yes	0.82	375	1.2×10^{-5}
hmrII 12J	-	No	100	47	9.4×10^{-7}
	+	No	11.6	325	2.7×10^{-5}
	-	Yes	100	71	2.6×10^{-6}
	+	Yes	11.6	218	3.7×10^{-5}
hm^+rII 12J	-	No	100	56	2.1×10^{-7}
	+	No	1.2	95	1.6×10^{-5}
	-	Yes	100	74	7.2×10^{-6}
	+	Yes	1.2	134	3.5×10^{-5}
hmrII 14A	-	No	100	29	1.5×10^{-6}
	+	No	14.1	93	2.1×10^{-6}
	-	Yes	100	41	4.9×10^{-6}
	+	Yes	14.1	172	6.2×10^{-6}
hm^+rII 14A	-	No	100	29	2.7×10^{-7}
	+	No	0.71	74	1.7×10^{-7}
	-	Yes	100	41	7.1×10^{-7}
	+	Yes	0.71	80	8.4×10^{-7}

Continued.....

TABLE 41. Continued.....

rII Strain	HA	Cycle of Growth	% Surv.	Revertants	Rev. Freq.
hmrII 15I	-	No	100	37	8.2×10^{-7}
	+	No	11.1	94	7.1×10^{-7}
	-	Yes	100	41	1.4×10^{-6}
	+	Yes	11.1	152	9.9×10^{-6}
hm^{+}rII 15I	-	No	100	19	2.0×10^{-7}
	+	No	0.53	28	1.7×10^{-7}
	-	Yes	100	65	4.5×10^{-7}
	+	Yes	0.53	74	6.9×10^{-7}

and can be seen to be close to unity. It is clear that the hm mutation had no effect on HA-induced reversion of GC mutants. The fact that the two AT mutants in the hm strain were not induced to revert shows that no change in mutational specificity was engendered by hm. It should be pointed out that the rII's were initially classified as AT or GC on the basis of their response to HA.

(c) Bisulphite

The rII's in the two strains were all treated with bisulphite for five hours. After stopping the treatments the phage were added to the appropriate bacteria and plated as already described.

In agreement with Summers and Drake (1970), only the GC mutants were induced to revert by this agent. As in the case of HA, the fact that the AT mutants failed to respond in both hm and hm^+ strains points to the failure of hm to change the specificity of bisulphite mutagenesis. See Table 42.

As was found for NA and HA, the frequency of induced revertants of rII's 11C and 12J was increased after a cycle of growth in E.coli B.

From Table 45, it might be inferred that if anything, hm antimutated the action of bisulphite, since three of the four ratios were around 0.5. The ratios found when growth was allowed on the repair-deficient strains were around unity. In fact when the experiment was repeated (twice) using only E.coli B as the host for the cycle of growth the ratios were found to be about one, suggesting that the apparent antimutagenic effect of hm on bisulphite was due to sampling.

TABLE 42. Bisulphite-Induced Reversion of rII's in hm and hm^+ Backgrounds

rII Strain	Bisulphite	Cycle of Growth	% Surv.	Revertants	Rev. Freq.
hmrII 11C	-	No	100	42	6.4×10^{-7}
	+	No	2.4	341	3.2×10^{-6}
	-	Yes	100	28	1.2×10^{-6}
	+	Yes	2.4	471	5.1×10^{-6}
hm^+rII 11C	-	No	100	37	2.1×10^{-7}
	+	No	1.6	214	4.0×10^{-6}
	-	Yes	100	48	9.4×10^{-7}
	+	Yes	1.6	371	8.1×10^{-6}
hmrII 12J	-	No	100	56	7.1×10^{-7}
	+	No	3.9	238	9.8×10^{-6}
	-	Yes	100	71	2.3×10^{-6}
	+	Yes	3.9	383	1.9×10^{-5}
hm^+rII 12J	-	No	100	71	3.1×10^{-7}
	+	No	5.8	464	1.1×10^{-5}
	-	Yes	100	103	8.1×10^{-7}
	+	Yes	5.8	432	1.5×10^{-5}
hmrII 14A	-	No	100	29	2.8×10^{-6}
	+	No	3.0	38	3.6×10^{-6}
	-	Yes	100	42	8.2×10^{-6}
	+	Yes	3.0	57	7.4×10^{-6}
hm^+rII 14A	-	No	100	36	7.8×10^{-7}
	+	No	1.8	28	5.8×10^{-7}
	-	Yes	100	41	3.4×10^{-6}
	+	Yes	1.8	26	5.2×10^{-6}

Continued.....

TABLE 42. Continued.....

rII Strain	Bisul-phite	Cycle of Growth	% Surv.	Revertants	Rev. Freq.
hmrII 151	-	No	100	142	5.8×10^{-7}
	+	No	2.4	148	7.1×10^{-7}
	-	Yes	100	136	2.8×10^{-6}
	+	Yes	2.4	95	2.6×10^{-6}
hm^{+}rII 151	-	No	100	72	1.2×10^{-7}
	+	No	5.1	88	1.9×10^{-7}
	-	Yes	100	182	7.3×10^{-7}
	+	Yes	5.1	231	9.2×10^{-7}

(d) EMS

The phage were treated with EMS for 90 minutes as described. After stopping the reaction the phage were added to the bacteria in the same way as was done for the previous mutagenic treatments. See Table 43. In agreement with Kreig (1963) the GC mutants were much more amenable to EMS-induced reversion than were the AT mutants though the latter group did show some response to this agent. In addition the GC mutants profited most (in terms of increased reversion frequency) from the cycle of growth in E.coli B.

The effectiveness of EMS mutagenesis for any given rII mutant was the same in both the hm and hm^+ strains whether a cycle of growth was allowed or not. See Table 45.

(e) 5-BU

Each of the phage strains was adsorbed to SUM-grown E.coli B. After 10 minutes the complices were diluted into fresh SUM containing 5-BU. Each aliquot was immediately split into three (aliquots a, b and c in Table 44) and the experiment was performed in the normal fashion for 5-BU reversion studies. Clearly the option of direct or indirect plating which was available for those mutagens administered extracellularly did not apply for 5-BU. For the calculations of the ratios in Table 45 the mean value of each set of three estimates was used. This was felt to be legitimate since no obvious jackpot was found in any of the aliquots.

Although all four rII's in both the hm and hm^+ strains were 5-BU-revertible, it was the two GC mutants which showed the strongest responses. This is in keeping with several reports on specificity of 5-BU mutagenesis in T4 (Bautz and Freese 1960;

TABLE 43. EMS-Induced Reversion of rII's in hm and hm^+ Backgrounds

rII Strain	EMS	Cycle of Growth	% Surv.	Revertants	Rev. Freq.
hmrII 11C	-	No	100	64	4.9×10^{-7}
	+	No	21.8	486	8.6×10^{-6}
	-	Yes	100	126	7.1×10^{-7}
	+	Yes	21.8	571	3.5×10^{-5}
hm^+rII 11C	-	No	100	31	1.2×10^{-7}
	+	No	14.6	248	7.0×10^{-6}
	-	Yes	100	59	2.1×10^{-6}
	+	Yes	14.6	414	4.6×10^{-5}
hmrII 12J	-	No	100	62	7.1×10^{-7}
	+	No	17.9	288	1.2×10^{-5}
	-	Yes	100	131	2.4×10^{-6}
	+	Yes	17.9	389	7.9×10^{-5}
hm^+rII 12J	-	No	100	34	1.6×10^{-7}
	+	No	26.4	61	9.4×10^{-6}
	-	Yes	100	79	4.2×10^{-7}
	+	Yes	26.4	71	3.8×10^{-5}
hmrII 14A	-	No	100	77	2.4×10^{-6}
	+	No	20.1	304	1.2×10^{-5}
	-	Yes	100	136	4.6×10^{-6}
	+	Yes	20.1	322	1.9×10^{-5}
hm^+rII 14A	-	No	100	45	3.5×10^{-7}
	+	No	16.2	186	1.9×10^{-5}
	-	Yes	100	73	6.5×10^{-7}
	+	Yes	16.2	290	1.9×10^{-5}

Continued.....

TABLE 43. Continued.....

rII Strain	EMS	Cycle of Growth	% Surv.	Revertants	Rev. Freq.
hmrII 15I	-	No	100	34	6.1×10^{-7}
	+	No	17.4	281	2.7×10^{-6}
	-	Yes	100	41	5.8×10^{-7}
	+	Yes	17.4	341	2.6×10^{-6}
hm^{+}rII 15I	-	No	100	53	1.9×10^{-7}
	+	No	15.6	64	1.6×10^{-6}
	-	Yes	100	206	4.1×10^{-7}
	+	Yes	15.6	356	3.5×10^{-6}

TABLE 44. 5-BU-Induced Reversion of rII's in hm and hm^+ Backgrounds

rII Strain	5-BU	Aliquot	Revertants	Rev. Index
hmrII 11C	-	a	68	8.2×10^{-7}
	-	b	31	4.0×10^{-7}
	-	c	60	8.1×10^{-7}
	+	a	568	1.8×10^{-4}
	+	b	407	8.7×10^{-5}
	+	c	503	1.1×10^{-4}
hm^+rII 11C	-	a	21	1.6×10^{-7}
	-	b	31	2.2×10^{-7}
	-	c	31	2.7×10^{-7}
	+	a	236	3.6×10^{-5}
	+	b	195	2.8×10^{-5}
	+	c	404	5.6×10^{-5}
hmrII 12J	-	a	62	1.6×10^{-6}
	-	b	40	8.7×10^{-7}
	-	c	44	9.5×10^{-7}
	+	a	723	4.2×10^{-4}
	+	b	648	3.9×10^{-4}
	+	c	936	5.6×10^{-4}
hm^+rII 12J	-	a	51	3.8×10^{-7}
	-	b	106	5.5×10^{-7}
	-	c	158	1.6×10^{-6}
	+	a	286	7.8×10^{-5}
	+	b	533	1.2×10^{-4}
	+	c	371	9.4×10^{-5}

Continued.....

TABLE 44. Continued.....

rII Strain	5-BU	Aliquot	Revertants	Rev. Index
hmrII 14A	-	a	24	2.3×10^{-7}
	-	b	52	4.8×10^{-7}
	-	c	47	5.6×10^{-7}
	+	a	309	8.6×10^{-6}
	+	b	586	1.3×10^{-5}
	+	c	352	9.8×10^{-6}
hm^{+}rII 14A	-	a	12	1.1×10^{-7}
	-	b	27	2.8×10^{-7}
	-	c	30	3.7×10^{-7}
	+	a	186	2.5×10^{-6}
	+	b	98	1.1×10^{-6}
	+	c	271	3.9×10^{-6}
hmrII 15I	-	a	71	6.8×10^{-7}
	-	b	83	8.1×10^{-7}
	-	c	36	4.4×10^{-7}
	+	a	203	6.2×10^{-6}
	+	b	299	9.4×10^{-6}
	+	c	271	8.1×10^{-6}
hm^{+}rII 15I	-	a	42	3.1×10^{-7}
	-	b	21	1.2×10^{-7}
	-	c	28	1.8×10^{-7}
	+	a	126	2.4×10^{-6}
	+	b	171	3.1×10^{-6}
	+	c	87	1.6×10^{-6}

TABLE 45. Ratios of Induced Mutation Frequencies (Corrected for Spontaneous Reversion) of the rII's in the hm Strain over the Frequencies in the hm^+ Strain

Mutagen	Strains							
	11C		12J		14A		15I	
	D	C	D	C	D	C	D	C
NA	3.1	5.2	2.6	4.0	6.7	4.2	3.4	6.6
HA	1.1	0.84	1.6	1.2	-	-	-	-
EMS	1.2	0.81	1.2	2.3	0.67	0.83	1.5	0.71
Bisulphite	0.68	0.54	0.54	1.2	-	-	-	-
5-BU[A]	-	3.3	-	4.7	-	4.5	-	3.3

D - Directly plated.

C - With cycle of growth before plating.

A - The figures for calculating the ratio of 5-BU mutagenesis in the two strains were taken by averaging the three aliquots for each treatment.

Champe and Benzer 1962b; Drake 1963; Drake and Greening 1970).

Table 45 shows that the 5-BU-induced reversion frequencies of all four rII mutants were higher in the hm than in the hm^+ strain. A replicate of this experiment gave essentially the same result i.e. an approximately five-fold increase in the induced reversion frequencies of both the AT and the GC mutants in the hm strain compared to hm^+.

In summary the reversion experiments performed here have shown that of the mutagens tested, only 5-BU and NA showed an increased potency in the hm strain. The enhancement applied to both classes of transitions and in the case of NA, did not depend on a cycle of growth before plating on the restrictive host.

The implications of the synergism between hm and these two mutagens will be discussed later in conjunction with the other aspects of the phenotype of the hm strain.

Is One Locus Responsible for all the Phenotypic Changes in T4hm?

Throughout the work on the hm strain, it has been tacitly assumed that a single mutation was responsible for all the changes in phenotype observed in this strain, and further that this mutation was at the hm locus. Finally and perhaps a little belatedly, this assumption was tested.

The procedure was to cross the hm strain to the wild-type and then choose those progeny which possessed one of the characters of the hm strain. The appropriate product of the cross was then used to repeat the procedure so that a series of backcrosses to wild-type was performed.

None of the parameters which distinguished the two strains was amenable to screening, so the protocol was somewhat laborious. The character chosen was the comparative resistance of the hm strain to NA as this was reasonably easy to measure and also because the difference between the two strains in this character was striking. The actual protocol used was as follows.

E.coli B was jointly infected with hm and hm^+ (both r^+) at multiplicities of about 3.0 each in the presence of cyanide. The complices were diluted into fresh broth, and after an hour's incubation at 37°C, lysis was completed with $CHCl_3$ and the phage were plated on E.coli B. After incubation, 12 isolated plaques were picked and each was used to infect an early exponential culture of E.coli BB and high titre lysates were grown up. Each lysate was then treated with NA for 4.5 minutes and the phage, both treated and untreated were plated on E.coli B to assay survival. The survival levels of the lysates fell into two classes (about 15% and about 1%) which corresponded to those expected for hm and hm^+ respectively. One of the lysates with the higher survival level was chosen as one of the parents in the second backcross to the wild-type strain. The whole procedure was repeated a total of nine times.

After the final backcross, one of the lysates of NA- resistant phage was plated on E.coli B and five young plaques were picked and used to initiate fresh high titre stocks. Each of these five lysates was then assayed for spontaneous r frequency and the one with the lowest was chosen for further study.

A comparison was made between this strain (designated hm bc),

the original hm strain, and the hm^+ wild-type strain, in which the lethal action of HA, the spontaneous and the NA-induced r frequencies were measured.

Table 46 shows that the hm bc strain behaved in almost identical fashion to the original hm strain in all these parameters, suggesting strongly that one mutation was responsible for them, as well of course for the NA-resistance which was the selected character in the backcrossing, although the possibility of a series of very closely linked mutations is not completely excluded.

If however it is to be believed that a single mutation was responsible for the phenotypic changes which I observed in the hm strain, the question of whether the mutation is in fact at the hm locus still remains. Drake (1973) used the increased levels of 2-AP mutagenesis as one of the criteria for diagnosing the presence of the hm mutation, and this parameter was chosen as the one to link Drake's observations with mine.

In the section which dealt with the effects of hm on induced reversion of rII mutants, the procedure for getting the rII's into different genetic backgrounds was described. It will be remembered that two of the rII's (14A and 151) were judged to be AT transitions since they failed to respond to HA but did respond to 2-AP in reversion spot-tests. These two rII's were serially backcrossed eight times from the original hm strain to the hm bc strain. Thus these rII's existed in the hm, the hm^+ and the hm bc backgrounds.

The 2-AP-induced reversion frequencies of both rII's in all three backgrounds were measured, the experiment being performed in the way described in Materials and Methods. Table 47 shows the

TABLE 46. Comparison of the Strains T4hm, T4hm$^+$ and T4hm bc

a). Inactivation by NA

NA Treatment (mins)	Strain % Survival		
	hm	hm$^+$	hm bc
0	100	100	100
4.5	12.1	0.95	13.2

b). Spontaneous and NA-Induced r Frequencies

Strain	NA Treatment (mins)	% Surv.	Plaques Inspected	r Plaques	r Frequency
hm	0	100	2.2×10^4	15	6.8×10^{-4}
	4.5	12.1	2.2×10^4	65	2.9×10^{-3}
hm$^+$	0	100	3.9×10^4	11	2.8×10^{-4}
	4.5	0.95	4.2×10^4	54	1.5×10^{-3}
hm bc	0	100	3.3×10^4	24	7.1×10^{-4}
	4.5	13.2	2.7×10^4	82	3.0×10^{-3}

c). Inactivation by HA

HA Treatment (hours)	Strain % Survival		
	hm	hm$^+$	hm bc
0	100	100	100
48	2.4	0.04	4.1

TABLE 47. 2-AP-Induced Reversion of rII 14A and rII 15I in the hm, hm^+ and hm bc Strains

a). rII 14A

Strain	2-AP	No. of Revertants	Rev. Index
hm	-	26	6.2×10^{-7}
	-	21	5.1×10^{-7}
	-	42	7.4×10^{-7}
	+	286	3.9×10^{-4}
	+	359	5.4×10^{-4}
	+	244	6.5×10^{-4}
hm bc	-	39	8.1×10^{-7}
	-	52	6.2×10^{-7}
	-	61	5.0×10^{-7}
	+	483	3.5×10^{-4}
	+	614	6.8×10^{-4}
	+	449	4.4×10^{-4}
hm^+	-	37	2.8×10^{-7}
	-	29	1.9×10^{-7}
	-	35	3.1×10^{-7}
	+	281	8.9×10^{-5}
	+	304	1.2×10^{-4}
	+	296	1.0×10^{-4}

Continued.....

TABLE 47. Continued.....

b). rII 15J

Strain	2-AP	No. of Revertants	Rev. Index
hm	-	28	1.6×10^{-7}
	-	37	1.9×10^{-7}
	-	39	2.0×10^{-7}
	+	286	8.4×10^{-5}
	+	261	9.3×10^{-5}
	+	302	7.9×10^{-5}
hm bc	-	35	2.1×10^{-7}
	-	40	2.3×10^{-7}
	-	41	1.8×10^{-7}
	+	443	7.9×10^{-5}
	+	416	8.8×10^{-5}
	+	479	8.1×10^{-5}
hm^+	-	61	8.2×10^{-8}
	-	54	9.1×10^{-8}
	-	72	1.1×10^{-7}
	+	644	2.0×10^{-5}
	+	586	1.6×10^{-5}
	+	702	2.3×10^{-5}

results. The induced reversion frequencies of both rII's were some four-fold higher in the hm and hm bc strains compared to the hm^+ strain. This was good evidence for the hypothesis that it was indeed the hm mutation which was responsible, not only for the effects found by Drake in his work, but also for the additional changes in phenotype uncovered in this study.

Having obtained the two rII's in the three backgrounds, the reversion response of each rII to 5-BU was measured. It will be remembered that the two AT transitions responded only moderately to 5-BU. However, it was found that the reversion indices of both rII's in the hm and the hm bc strains were about five times as great as the values found in the hm^+ strain. Thus this mutational synergism with 5-BU is added to the catalogue of changes attributable to the hm mutation.

Discussion on the Nature of the T4hm Strain

Is it possible to put forward a coherent model to account for all the changes in phenotype in the hm mutant strain?

First of all it seems clear that the hm locus does not dictate a somatic protein, but rather that it is involved with some aspect of the phage's intracellular metabolism. Two aspects of the hm phenotype strongly support this view.

(a) The higher rates of base analogue mutagenesis found in the hm strain almost certainly must be due to changes in the intracellular physiology since these mutagens are administered intracellularly. One would have to invoke a highly improbable model involving a mutagenic interaction between base-analogue-substituted DNA and the

coat protein to reconcile the mutational synergism with base analogues with a somatic function for hm.

(b) This second point has already been mentioned. It is the finding that the hm strain is not only resistant to NA but also exhibits mutational synergism with this agent.

The differential resistance and (or) induced mutation frequencies in response to a number of agents whose primary target is DNA suggests that the hm function is concerned with some aspect of DNA metabolism and perhaps, more specifically with repair of damage to DNA.

The study and understanding of repair processes in T4, have so far lagged behind the spectacular progress made in this field in bacteria. The v gene of T4 is really the only one which has been characterized. A mutant at this locus was isolated by Harm (1963) which was UV-sensitive. It was later shown that this gene encodes an endonuclease which specifically recognizes pyrimidine-dimers (Friedberg and King 1971; Friedberg 1972; Friedberg and Clayton 1972) and v gene mutants are unable to perform excision repair of UV-induced damage. The UV-induced mutation frequency, as measured by the number of mutants per survivor is however unaffected by defects in the v gene (Meistrich and Drake 1972).

The UV-sensitive px mutation in T4 (Drake 1973) has several properties which are compatible with the px gene product being involved in recombination repair. It has in addition to an increase in UV-sensitivity, a reduced recombination frequency and a lowered UV-induced mutation frequency. Such characters are reminiscent of some of the phenotypes found in bacterial mutants

which are defective in recombination repair. These bacterial mutants include recA (Witkin 1969c), recB and recC (Witkin 1969c and 1972) and lex (or exrA) (Witkin 1967; Mount et al. 1972). Another T4 mutant, designated y is also UV-sensitive (Boyle and Symonds 1969) and like px, the y gene product may be involved in recombination repair. The y gene is unlinked to px and complements with it with regard to the UV sensitivity (Boyle 1969).

Of course it would be rash to assume exact parallels between T4 and E.coli repair systems. Indeed several differences have been found in the excision repair mechanisms of the two organisms. For example the T4v endonuclease is indifferent to inhibition by caffeine and acriflavine (Harm 1973), two agents which can strongly inhibit bacterial excision repair (Metzger 1964; Feiner and Hill 1963). One other difference is that the v gene product displays a higher specificity than does the bacterial excision repair system in that it acts solely on pyrimidine dimers, whereas the E.coli system can excise different damages inflicted by various agents, e.g. Friedberg (1972) and Kondo (1973).

Despite such provisos, the well characterized E.coli repair systems do stimulate general hypotheses which may well accommodate findings pertaining to specific viral processes.

Apart from the highly specialized photoreactivation system of repair, which can monomerize UV-induced pyrimidine dimers in situ, there are two basic ways by which bacteria can deal with damage to their DNA. Very briefly they are as follows.

(a) The first involves excision of the damaged region of DNA followed by the resynthesis of DNA to fill the gap so formed (Boyce and

Howard-Flanders 1964).

(b) Recombinational, or post-replicative repair circumvents damages which escape excision by a mechanism whereby the daughter strands of replicated DNA which contain gaps opposite the lesions in the parental DNA can recombine with intact portions of the parental DNA so as to generate a full length genome. There is now a large array of bacterial mutants which are defective in specific steps of these two general modes of repair.

Let us first compare and contrast the hm mutation with the bacterial and T4 mutants with altered excision repair capacities.

Unlike hm, all such bacterial mutants are very sensitive to UV. The UV-sensitivity of the T4 v mutation has been mentioned. Such mutants also differ from hm in that they exhibit the same UV-induced mutant yield per lethal hit (Witkin 1967; Meistrich and Drake 1972) whereas hm shows mutational synergism with UV.

In E.coli, Clarke (1970) found that an excision-defective strain was NA sensitive and that the NA-induced reversion frequency per lethal hit was substancially increased. The situation with hm differs from this in that the increase in NA-induced mutation frequency is accompanied by an enhanced resistance to this agent's lethal action.

If the hm mutation is to be implicated into an excision repair system one would need to say that such a system in the hm strain is more efficient at removing lethal damage inflicted by HA and NA but in the case of the latter mutagen such repair must be error-prone to explain the synergism between NA and hm. If one pursues such an idea several severe discrepancies occur.

To account for the fact that hm is not hyper-resistant to UV, MMS, bisulphite or EMS one would have to say that the hm repair system either does not act on damages inflicted by these agents or else acts on them with the same efficiency as the hm^+ allele. If it is postulated that UV- and MMS-induced damages are not amenable to hm repair, it becomes rather difficult to account for the enhanced mutation frequency induced by both mutagens in the hm strain.

It will be remembered that 5-BU mutagenesis of bacteriophage lambda was enhanced in strains of E.coli deficient in excision repair (Pietrzykowska 1973) suggesting that a mechanism other than excision repair is involved in 5-BU mutagenesis. The fact that 5-BU was found to be more potent in the hm strain therefore again suggests that hm is not involved in excision repair.

Setlow and Carrier (1968) provided biochemical evidence to suggest that hm is not involved in excision repair. They found that in the T4 x strain (which harbours the hm mutation) the rates of UV-induced dimer excision was the same as in the wild-type.

Any model put forward for the hm gene function, must account, among other factors, for the fact that the hm strain is resistant only to HA and NA of all the inactivating agents so far tested.

Can this fact be due to the lethal lesions induced by HA and NA being qualitatively different from those induced by the other agents? It can be argued that this may be the case.

The model of Burnotte and Verly (1971) was discussed in Chapter 2 of the Results. Briefly, they proposed that certain classes of NA-induced damage to DNA could lead to either single

strand breakage or to DNA crosslinking, both of which are greatly more lethal than other classes of damage, such as deaminations which NA inflicts on DNA.

Turning to HA, it will be remembered that EDTA was added to the reaction mixture to prevent catalysis of the breakdown of HA into toxic byproducts. It may be, however, that despite this precaution there is still residual formation of such products and that a proportion of the lethality is due to their action rather than that of HA. The actual rate of inactivation of T4 by HA treatment is low, the 1% survival level in hm and hm^+ taking about 50 and 30 hours treatment respectively. It may be that this postulated spontaneous breakdown of HA could account for a fraction of this low rate of killing. Among the breakdown products of HA is H_2O_2.

Peroxide can cause DNA backbone breakage which results in high lethality but no mutagenicity (Freese et al. 1967; Rhaese and Freese 1968; Rhaese, Freese and Metzer 1968; Freese and Freese 1965).

Both these accounts of the types of damages induced by HA and NA are speculative, but let us for a moment accept them and further let us postulate that the hm strain is better able to cope with single-strand breaks than is wild-type. What are the implications of such an hypothesis?

It effectively precludes the hm system from having any role in excision repair. In E.coli the sensitivities of mutants defective in excision repair to agents (such as X-rays) whose primary induced lesions are single-strand breaks, are only marginally increased compared to wild-type and this suggests that excision

repair is not involved in the repair of such damage. Strains of bacteria which are very sensitive to X-rays are known. They are not excision repair mutants but rather they are deficient in recombination repair.

The attempt to assign to the hm system a role in excision repair was fraught with difficulties. Will an allocation of hm to a step in recombination repair fare any better? The fact that the hm strain has wild-type UV-sensitivity and recombination frequencies and displays mutational synergism with UV does not augur well for such a model since as was mentioned all the E.coli mutants deficient in this type of repair are UV-sensitive, and are refractory to UV-mutagenesis, and most of them have reduced recombination frequencies.

However, despite such differences the fact that bacterial recombination repair has been shown to have a role in the generation of mutations means that the characters of the hm strain are less at variance with this type of model.

Apart from the importance of the recombination repair system in UV-mutagenesis, it has also shown, as has been mentioned, to be involved in EMS and 5-BU mutagenesis (Pietrzykowska 1973). The model put forward by Clarke (1970) also invoked a role for recombination repair in NA-mutagenesis in E.coli. In contrast to these mutagens Kondo et al. (1970) found that EMS and NTG could still be mutagenic in recA strains of E.coli. As far as I know the effects of recombination-repair deficiency on the potency of the other mutagens used on hm (i.e. HA, bisulphite and 2-AP) have not been measured. Nevertheless for those mutagens which do show an

enhanced effect in the hm strain, models which invoke recombination repair in their mutagenic pathways have been put forward.

The difficulties which were met when the possible role of the hm system in excision repair was considered also occur in a model which proposes that the hm mutation dictates a recombination-repair enzyme which can act more rapidly to repair certain types of lethal damages (i.e. those induced by HA and NA) but is more error-prone while performing this service. One again has to propose that it is specifically the classes of damage induced by HA and NA which are dealt with more rapidly in the hm strain.

Speculative proposals as to the ways in which HA and NA could generate single-strand breaks were made above. One could consider such breaks as being primary lesions. In the case of an agent such as UV, where under certain conditions the initial primary lesions are exclusively thymine dimers (Meistrich 1972; Meistrich and Lamola 1972) single strand gaps are formed, bot as primary lesions, but as gaps in the DNA in the daughter strand opposite the initial lesions in the parental strand.

It has already been stated that amongst UV-induced transitions in T4, the majority are GC $\rightarrow$ AT transitions (Drake 1963 and 1966b; Meistrich and Drake 1972). This mutational specificity may be a reflection of the specificity of the siting of the regions of DNA destined to be involved in the recombination repair event.

Let us for a moment suppose that the hm system is involved with the dictation of the accuracy of the pairing of DNA strands during recombination.

It could be postulated that in the case of recombination-

repair of UV-induced damage, some system other than hm is involved in the siting and (or) the setting up of the recombination event. Very tentatively px could be put forward as a candidate for such a system in T4. A system which dictates the fidelity of the process might then be considered an added bonus and hence the hm mutation would not affect the rate of recombination repair of UV damage, only its accuracy. If so, this would account for the normal UV-sensitivity in the hm strain and the enhancement of UV-mutagenesis.

Turning to the more random primary breaks induced by HA and NA one could argue that the more fastidious hm^+ gene product could reject the possibility of repair at such unscheduled sites of breakage but that the hm gene product could tolerate and perhaps modify the break-points so as to render them more readily amenable to repair by some other system.

To account for the fact that NA, but not HA-mutagenesis is enhanced in the hm strain one has to postulate that HA-induced mutations do not arise via a recombination-repair pathway whereas HA-induced ones do.

The fact that there is such a good correlation between the molecular changes induced by HA in the DNA (i.e. it acts solely on cytosine of 5-HMC when given under the appropriate conditions (see Phillips and Brown 1967)) and the very high degree of specificity with which it induces GC →AT transitions (Freese, Bautz and Bautz-Freese 1961; Tessman, Poddar and Kumar 1964; Howard and Tessman 1964; Brenner, Stretton and Kaplan 1965) supports the view that the HA mutagenic pathway is independent of repair-mediated mechanisms.

The model tentatively proposed for hm has certain characteristics of that proposed for exrA function in E.coli. Mutants at this locus are UV-sensitive, and the UV-induced mutation frequency is reduced to zero (Witkin 1967; Mount et al. 1972). They are also sensitive to X-rays (Howard-Flanders and Boyce 1966) and to NTG (Witkin 1966).

Derivatives of the exrA mutations have been isolated and characterized. These so-called exrA(W) mutants have their radiation-sensitivity restored to wild-type level but the antimutator effects on UV remains (Sedgwick and Bridges 1972; Bridges et al. 1973).

In contrast to the exrA(W) mutants, hm exhibits increased rather than decreased UV-induced mutation frequency. However one need only remember that different gene 43 alleles may be mutator or antimutator to realise that opposite phenotypes (compared to wild-type) may be obtained by different alleles at a single locus.

Witkin (1969a and b) has suggested that the $exrA^{+}$ gene product may modify terminal DNA bases adjoining gaps and although rendering such bases available for recombination repair, also encourages the likelihood of mispairing.

Quite obviously the state of knowledge of the nature of the hm function is such that it is not realistic to say that the hm and exrA functions are totally analogous, though the properties of hm can be compared to exrA with slightly more justification than with most other bacterial systems.

There is however one aspect of the phenotype of the hm strain which is very difficult to fit into such a model and that is that the sensitivities of hm to EMS and to MMS are identical to those

of the hm$^+$ strain (Drake 1973). Alkylating agents have been shown to induce depurination in DNA (Bautz and Freese 1960) resulting in single-strand breaks in a way essentially the same as postulated for HA- and NA-induced breaks. The induction of most of the depurinations by alkylating agents is a slow process (Lawley and Brooks 1963) occurring after the treatments have been terminated. If then the phage are plated immediately after treatment, then such lesions may not contribute so much to the induced lethality.

It is clear however that no simple model of hm, based on analogies with better characterized repair systems will account for all the ranges in phenotype found in this strain. If hm is indeed involved in recombination repair, then such an hypothesis could be evaluated by performing the following experiments.

(a) Other agents which induce single-strand breaks should be used to inactivate the hm and hm$^+$ strains. The model predicts that hm would be more resistant than hm$^+$ in response to treatment by such agents at H_2O_2, ionizing radiation or UV-treatment of 5-BU-substituted DNA.

(b) The lethality of NA in strains of E.coli known to be recombination-repair deficient should be tested. It would be predicted that such mutant strains should be NA-sensitive. In addition, the strains of T4, px and y, have, as has been mentioned, both been implicated in recombination processes. If both strains were sensitive to HA and to NA, this would give greater credance to the model.

(c) A rigourous biochemical analysis of the rate and efficiency of repair of the hm and hm$^+$ strains after NA and HA treatment should

be made. If in the hm strain gaps in the DNA induced by either agent are repaired more rapidly, this could be detected by measuring the mean length of the DNA fragments. In the hm strain one would predict that the mean DNA length would be greater.

Of course one can readily think of quite different and novel roles for hm. For example one could postulate that it is involved in the scheduled synthesis of DNA, perhaps as an adduct of the DNA polymerase machinery. The hm gene product might be less fastidious when it encounters lesions in the DNA induced by NA or HA which may result in an increased probability of the altered bases being used as templates for replication, unlike the hm gene product which may reject such bases as templates. Again one has to say that for lesions induced by the other mutagens tested in this system, the hm and hm^+ gene products are equally recalcitrant about allowing replication of the lesions. If spontaneously-arising lesions are also preferentially replicated in the hm strain, this could account for the higher spontaneous mutation frequency found in this strain. If specifically the lesions were those which potentiated base-pair substitutions this might explain the fact that it is the transitions whose frequency is enhanced in hm. The problems which applied to the other possible models for hm also apply to such an hypothesis, the main general problem being the fact that some mutagens (MMS and UV) have normal sensitivities but enhanced mutation frequencies, some (EMS and bisulphite) are unchanged in both parameters, and that although both HA and NA are less lethal to hm, than to hm^+, only the latter exhibits higher induced mutation frequencies in the hm strain.

This last model is similar to one of the tentative hypotheses put forward by Boyle (1969) to account for the T4*y* gene function. At the moment however there is not enough information on the *hm* strain to allow a critical evaluation of any single model.

Apart from the experiments mentioned above, which were designed to test the validity of a specific model of *hm*, there are a number of other avenues along which studies of the *hm* system might profitably proceed. These include:

(a) A study of the dominance relationships of the *hm* mutant might give information on the nature of the *hm* gene product. Sinha and Snustad (1971) reported that mutants of T4 gene 32 (Alberts Protein) inhibited the action of wild-type alleles at the same locus, whereas mutants deficient in a number of different genes dictating the production of enzymes were recessive to wild-type. They concluded from this that the gene 32 product had a stoichiometric role.

(b) If complex genotypes carrying *hm* and a mutation at another locus concerned with DNA metabolism were constructed, the survival and mutational responses of such double mutants might tell us whether *hm* cooperates with any other known system in an epistatic manner.

(c) It would be useful to know whether the *hm* system is dispensable. A start could be made by measuring the effects of nonsense suppressors on the phenotype of the *hm* mutant. If the phenotype was restored to wild-type in the presence of a nonsense suppressor this would show almost certainly that *hm* gene function is not essential for T4 growth. A failure to demonstrate the suppression of the *hm* mutation would not prove the opposite conclusion. For

rigourous study of the hm locus it would be very useful to develop a conditional lethal system. It may be that certain hm alleles could be conditionally lethal. This brings us to point (d).

(d) An effort should be made to isolate further independent alleles at the hm locus. Such a task will be very arduous, given the absence of any simple detection procedure for hm mutants, but it would be interesting to see to what extent different alleles vary in phenotype.

It is certainly not impossible that a rigourous study of the hm locus, both at the genetic and biochemical level, will allow it to join the list of loci in T4 whose analyses have given us a fuller understanding of some of the basic mechanisms involved in the role of cellular physiology in the mutagenic process.

DISCUSSION

The work in this thesis has been focussed on the study of the effects of various ancillary treatments on the specificity and potency of mutagenesis. With the exception of the work in which the effects of pH on NA-mutagenesis of T4 were examined such ancillary treatments involved the modification of the physiological state during, or subsequent to the mutagenic treatments.

The discussion of the work and the results on the *trp* operon are to be found in Chapter 1 in the Results. The major part of this final discussion will be devoted to the work carried out on T4. It will have been noted that each chapter in the Results section carried its own relevant discussion. This section will be concerned with a more general consideration of the problem of intragenic mutagen specificity in which the findings made in this work and those of other workers will be referred to and assessed.

It must be said at the outset that, to a great extent, the original aim of trying to estimate which, if any, physiological factors determine the spectra of induced and spontaneous *r* mutants of T4 did not succeed. Where an effect of ancillary treatment was found, it was, with one exception, the general potency rather than the specificity of mutant yield which was altered. Such non-specific effects were found with the co-mutagenic effects on NA-mutagenesis by PFPA and by the presence of the *hm* allele in the genetic background. It was argued that the antimutator effect of PFPA on 5-BU mutagenesis in the presence of SU could be thought of as being of an artifactual nature from a mutagenesis

point of view and in reality it appeared that PFPA had no effect on 5-BU mutagenesis. Two other ancillary treatments, pH on NA mutagenesis, and SM on either NA or 5-BU mutagenesis also gave negative results.

Really the only change in the specificity of mutations was that found in the spontaneous rII spectrum of the T4 hm strain, in which a higher proportion of transitions, and a somewhat different rII map were obtained. Despite the lack of change in the NA-induced spectrum in the hm strain it might still be worthwhile to test whether the hm mutation causes a change in the spectra induced by mutagens whose potencies are enhanced in this strain viz base analogues, MMS and UV. Unfortunately, time did not allow such analyses, the work on hm being the last to be undertaken.

Supposing a change in the rII spectrum had been elicited by any of the ancillary treatments, it is justifiable to ask what contribution this would have made to our understanding of the underlying causes of intragenic mutagen specificity.

The very demonstration that the spectrum could be altered would have been of interest in its own right since such modification has not so far been demonstrated apart from those cases (e.g. Campbell and Rowe 1972) where the changes in physiology result in such a massive increase in mutation frequency in their own right that the ancillary treatment can be thought of as the primary mutagen which may well have its own characteristic spectrum.

The two drugs which were used as ancillary agents, SM and PFPA, were chosen deliberately because, as was discussed in the relevant chapters each has fairly drastic, and as far as one can tell, rather

general effects on cellular metabolism. The choice of such agents might be criticised on the grounds of their being sledgehammers to crack a mutagenic nut. However their use was felt to be justified for the very reason that they had wide-ranging effects. Had a change in rII spectrum been observed with either agent, this in itself would not have indicated which mechanisms were responsible for generating intragenic mutagen specificity. However it would have given encouragement to use smaller nutcrackers. For example in Chapter 4 in the Results it was mentioned that if SM had been effective in altering the rII spectra, this could have been followed up by testing the effects of other agents which mimic specifically one of the components of the effects which SM exerts on cellular metabolism.

In the event, the fact that neither drug did modify the spectra, is paradoxically a partial vindication of the choice of such agents since one could argue that if they can't do it, nothing will.

Unfortunately such as assertion is not really justified. It may well be that the nature of the rII spectra is indeed influenced by cellular physiology, but simply that such physiological mechanisms as may be involved happen to be refractory to all the ancillary treatments used in this study.

It would be appropriate now to discuss in some detail some of the possible reasons for the non-random nature of the rII spectrum, to see whether the phenomenon can be explained without recourse to the invokation of mechanisms involving cellular physiology.

Let us first consider some explanations which might be considered trivial from a mutagenesis point of view.

(a) One obvious hypothesis to account for the non-random distribution of mutants within a locus, is that only at certain sites will an amino acid substitution cause a detectable change in phenotype.

Certainly it is clear that only a fraction of mutational events are in fact detected in forward mutation experiments. Specifically, the rII region has been estimated to contain about 2,000 base pairs (Edgar et al. 1962). Therefore 2000 sites are available for base pair substitution mutations alone. Add to this the 4,000 sites which can be generated just by (+1)and (-1) frameshifts and it will be realised that the 250 sites shown in Benzer's (1961) arduously obtained spectra are only a very small fraction of potential sites.

There is another way of demonstrating the fact that the majority of base-pair substitution mutations are never detected. The number of substitutions which generate nonsense codons should be minor compared to those which result in missense mutations. Yet in the rII locus, Drake (1970) has calculated that of 71 base pair substitution mutants, all mapping at different sites, no fewer than 44 were nonsense mutations.

It should be noted that a lower proportion than this was found for HA-induced rIIA nonsense mutants. Out of 239 mutations, 54 were classified as being nonsense (Schwartz and Bryson 1969).

In the β-galactosidase gene of E.coli the knowledge of the enzyme's amino acid sequence and consequently the nucleotide sequence of the gene, has allowed more precise formulation of the expected contribution of base substitutions which cause nonsense codons amongst all such mutants. In contrast to the expected value of

6%, Langridge and Campbell (1968) found that 93% of the mutants were nonsense.

It is not surprising to find that the deviation between observed and expected varies from gene to gene. Whitfield et al. (1966) showed that missense mutants were about one third of all mutants isolated in the aminotransferase gene of Salmonella.

As if further evidence for the invisibility of most mutants were not required, Koch and Drake (1970), as was already mentioned, found that new rII sites could be revealed if phage already carried a semi-r sensitizer mutation.

Also, it was discussed in the Introduction, that those mutagens which induce base pair substitutions induce a preponderance of rI's, in contrast to those agents which generate frameshifts where rII's are preferentially idduced. This fact has been used as indirect evidence that the rII protein will tolerate more amino-acid substitutions without causing an altered phenotype than will the rI gene product. Even if this is true, it does not tell us whether the rII proteins are exceptional in their ability to tolerate mutations without altering the phenotype.

Clearly then the number of sites amenable to analysis is severely restricted and the spectrum will be non-random. However, are the restrictions on the allocation of sites due to protein structure sufficient to account for the hotspots?

Langridge in his studies on the distribution of mutants along the β-galactosidase locus in E.coli did not possess a mapping system of as high a resolution as that available for rII in T4, and so he could only ascribe the mutants to regions rather than

to sites. He found that mutants were distributed non-randomly within the gene but that the spectrum could be attributed to differential sensitivity of the enzyme at different sites to mutational change since he combined genetic studies with *in vitro* enzyme studies (Langridge 1963). In another study, Crawford et al. (1970) mapped many mutants of different origin within the *trpA* gene of *E.coli*. Their distribution along the gene was highly non-random. There were clusters of particular classes of mutants in specific regions of the gene, and this was interpreted as being a reflection of the differential ability of the protein to tolerate different classes of mutational changes at different regions.

A glance at the rII spectra shows that the distribution of sites along the length of the cistrons, if not random, is fairly regular. This is particularly true of the spontaneous spectrum (Benzer 1961). It is rather, the striking differences in the intensity of different sites which leaps to the eye. Clearly, even at those sites with only a single representative, a mutation at such a site will generate an altered phenotype. Therefore, although one can say that the restriction placed on the number of detectable sites, due to the tolerance of most sites in proteins to mutational change, will go some way to causing a non-random spectrum, it does not explain the difference in intensity of hot- and cold-spots.

This consideration of the restriction placed upon the number of sites perhaps would seem to diminish the impressiveness of the non-random nature of the rII spectrum.

There is however another consideration which points to just

the opposite conclusion and is worth mentioning here. In reverse mutation experiments in the rII system, where far lower mutation frequencies can be estimated than is practicable in forward mutation experiments, true spontaneous mutation frequencies of 10^{-8} and lower are not uncommon. There is no a priori reason to suppose that at certain sites in rII, forward mutation frequencies of this order will not occur. The task of isolating rII mutants which arise at such frequencies would have been beyond the capacity of even Benzer. In his apparently exhaustive (and certainly exhausting) spectrum of spontaneous rII's, I estimated that the absolute frequency of the occurrence of a site with one representative was around 2×10^{-7} since he must have looked at about 5×10^{6} plaques. It may be therefore that the cold-spots (i.e. those sites with single representatives are still ten, a hundred or even a thousand times more mutable than the lowest limit for spontaneous forward mutation frequencies. In other words, the difference in the 'heat' of different sites may in fact be a great deal greater than might appear to be the case from inspection of the rII spectra, both spontaneous and induced, which are in the literature.

Other lines of evidence point to the fact that the non-random spectra in rII cannot be due solely to the restriction on the numbers of detectable sites caused by the nature of the protein.

Although different mutagens which induce approximately the same classes of mutations have related spectra, (Benzer 1961) there are several sites which are strongly represented in one mutagen's spectrum but very weakly, or not at all in that of a related agent. For example, NA, 2-AP and 5-BU which all induce

both GC → AT and AT → GC transitions (albeit in different relative proportions) each have quite distinct spectra. The differences are greater than would be expected from the differences in the relative contribution of each class of mutant induced by each agent.

Also, EMS, which induces mainly GC → AT transitions has a spectrum with no striking hotspots (Benzer 1961). 5-BU on the other hand induces striking hotspots though it too has the same affinity for inducing GC → AT transitions in T4 as does EMS. HA, which induces GC → AT transitions exclusively in T4 also gives a spectrum quite different from those found for 5-BU and EMS (Alikhanian et al. 1970).

Such differences in the spectra of different agents which induce similar classes of mutations suggest that the differential tolerance of the rII gene product to mutation is not the crucial factor in the generation of hotspots since the mutational origin of a particular mutation will be immaterial in deciding its effect on the protein function once the mutation is fixed.

Finally, the fact that frameshift mutants also exhibit hotspotting argues against the hypothesis that the protein structure is the sole arbiter of the siting of hotspots, since a frameshift, irrespective of its intragenic position is very likely to destroy protein function. Yet proflavine, which induces frameshifts in T4 (Orgel and Brenner 1961) does have a hotspot in its spectrum (Benzer 1961; Alikhanian et al. 1970). In the spontaneous spectrum, the two giant hotspots, r117 and r131, are both composed of frameshifts (Drake 1970).

(b) The second hypothesis which really does not need recourse to

explanations in terms of mutagen action is that hotspots are not in fact single sites but rather that they are larger regions which for some reason are refractory to recombination and hence the mutants within these regions are inseparable.

Certainly Tessman (1965), using very high resolution recombination assays claimed to show that some rII's which apparently mapped at coincident sites could recombine with each other at frequencies of about 10^{-8}. This is some 10,000 times lower than would be expected, on other criteria, for recombination between adjacent base pairs. In all cases where hotspots could be dissected, the mutants could also be distinguished by phenotypic differences, such as their suppressability. However, despite his ultrafine resolution, he still found hotspots induced by NA, whose mutants could not be distinguished either by recombination or by phenotypic differences.

Examples of variation in recombination frequencies have been reported for T4. Beckendorf and Wilson (1972) reported a gradient of recombination frequencies along gene 34 but the differences in the frequencies were not nearly great enough to generate hotspots. At a finer level, Ronen and Salts (1971) found a wide range of intracodon recombination frequencies at different sites in rII.

The obvious criticism of suchaan hypothesis is that it cannot account for the uniqueness of the spectra induced by different mutagens. It is the recombination between fully established mutations which is measured, and hence mutational origin will be irrelevant in the determination of the recombinogenicity in any given region.

Both these hypotheses fail then to give a satisfactory account

of the causes of hotspots and alternatives must be considered.

For those mutagens which are given extracellularly, an initially attractive hypothesis is that the DNA is arranged within the T4 head in such a way as to expose preferentially certain sites for mutagenic attack. Such an hypothesis is rendered unlikely for at least two reasons.

Firstly the packaging of DNA into the T4 head varies from individual to individual. The 'headful' model proposes that although a constant length of DNA is packaged into each head the actual linear sequence varies randomly (Streisinger et al. 1964 and 1967; Sechaud et al. 1966). Consequently no one site will regularly be exposed to attack because of its position in the head.

Secondly, it has been found that UV-induced mutation spectra in rII are essentially the same whether UV is delivered to phage in the intra- or extracellular state (Drake 1963 and 1966b).

While this work was in progress, a fairly steady flow of reports appeared, concerning the role of neighbouring bases in the determination of mutation frequencies at specific sites.

The most striking exposition came from Okada et al. (1972) who found that a frameshift mutation of spontaneous origin in the e (lysozyme) gene of T4 reverted spontaneously to wild type at very high frequency. On analysis of the protein (and hence of nucleotide) sequence it was found that the mutant was sited in a run of 6 AT base pairs and that it reverted to wild-type by a (-1) frameshift. This finding is in nice accord with the model put forward by Streisinger et al. (1966) which predicted that frameshift mutations would tend to arise spontaneously in regions where a run of bases occurred, due to an increased probability of strand slippage

during DNA synthesis at such regions.

In Salmonella a technique is available for the selection of polar frameshift mutations in the histidinol dehydrogenase gene (Fink et al. 1967). Of six such mutants, all were found to reside at a single site, and on analysis this site was shown to comprise two or three consecutive GC base pairs (Yourno, Ino and Kohno 1971).

Turning to induced frameshift mutations, in the same Salmonella system, it seems that ICR-191 has a propensity for inducing frameshifts in runs of GC base pairs (Yourno 1971). The influence of neighbouring base pairs on substitution mutations has also been demonstrated.

The first conclusive report of such an influence came from Koch (1971). He used a system in rII, in which he could manipulate the identity of the base pair adjacent to the one to be mutagenized with 2-AP. He compared the frequency of induced AT → GC transitions at the central position of a triplet when the third position was occupied by AT or GC. He found that the frequency was up to 20-fold higher when AT was present than when GC was the neighbour, but that the identity of the base pair two away from the mutated site had virtually no effect on the induced frequency.

Salts and Ronen (1971) found great variation in the frequencies with which different rII ochre mutants are converted to amber or opal by 2-AP mutagenesis. Since all three codons are nonsense, the variation cannot be dismissed as being due to differential tolerance of the protein to the 'wrong' amino acid which could well be a factor if the frequencies of reversion were measured. These workers classified the mutational derivatives from ochre on the

basis of their behaviour on different restrictive E.coli strains carrying known nonsense suppressors. However they failed to note that it is known that the efficiency of nonsense suppression itself can vary considerably from site to site (Salser 1969; Yahata et al. 1970). The reason for this differential suppressability cannot be put down to variation in the restoration of protein activity by the insertion of the wrong amino acid at different sites. Therefore the results of Salts and Ronen may be alternatively interpreted as being due to differential suppressability rather than mutability.

Related to the phenomenon of differences in suppression at different sites is the fact that the AUG triplet acts as a signal for f-methionine when it occurs at the initiation site of a cistron but that it codes for methionine when it occurs elsewhere. The base sequences of the initiation sites of several cistrons of some RNA phages have been elucidated (Steitz 1969 and 1972; Hindley and Staples 1970; Staples et al. 1971; Staples and Hindley 1971). However no consistent sequence has been found common to all initiation sites and it would seem that if the signal which causes f-methionine to be inserted resides in the nucleotide sequence, then it is the gross stereochemistry of a large region of nucleic acid rather than the immediate neighbours of the AUG site which is responsible (Golini and Thach 1972).

Still on the subject of suppression but at a rather different level, Levisohn (1970), as has been mentioned, found that HA could cause phenotypic suppression of certain T4rII mutants. The mechanism of the suppression is not known but it may involve the conversion of C $\rightarrow$ U in mRNA opposite the mutant base. The fact

that different rII's responded differently to HA suppression though they contained the same mutant base pairs was interpreted as showing the influence of neighbouring bases on the action of HA on different cytosines at different sites in RNA.

Reversion studies which did not completely exclude the possibility that observed differences in mutation frequencies were due to differential tolerance of the protein to the wrong amino acid at different sites have been done in T4 using HA as mutagen (Brenner, Stretton and Kaplan 1965) and in yeast with UV (Sherman et al. 1969). Both studies demonstrated considerable variation in induced frequencies at different sites.

Very interestingly it has been shown recently that a mutator allele of E.coli (mutS1 of Siegel and Bryson (1967)) exhibits considerable variation in the frequencies with which it induces transitions at different sites at the trpA locus (Cox, Degnen and Scheppe 1972). The authors surmised that this variation was due to fairly local influences of neighbouring base sequence.

de Vries et al. (1971 and 1972) found, on close examination, that base pairs at certain sites were quite refractory to the mutagenic action of T4 mutator DNA polymerase, suggesting that the specificity of action of this enzyme is subject to modification by local base sequence.

Though not strictly apposite in a consideration pertaining to mutagenesis, reports on the effect of neighbouring bases on the reactivity of cytosine bases in tRNA bear mention here.

Bisulphite can only convert C $\rightarrow$ U when C is in the single-stranded state (Shapiro et al. 1973). When tRNA is treated,

bisulphite converts cytosine residues which are not engaged in base pairing to uracil with high efficiency. However in E.coli f-met tRNA there is one cytosine residue which, although in a single-strand configuration is immune from attack. This particular residue is adjacent to the unusual base pseudouridine. The significance of this is a matter of conjecture since the refractory cytosine is the only such base in the entire tRNA loop. It may be that the stereochemistry of the loop rather than the neighbouring pseudouridine base is responsible for rendering the cytosine unreactive.

Similarly, methoxyamine, which is closely related to HA can also convert C → U in single-stranded DNA. However the homologous cytosine to that mentioned above also failed to react with this agent in E.coli tyr-tRNA (Cashmore, Brown and Smith 1971). These workers showed that in an isolated sequence of TψCG (ψ = pseudouridine) the cytosine would react with methoxyamine, indicating that it is the gross stereochemistry of the loop which is the cause of the immunity of the cytosine and not the pseudouridine neighbour. Cashmore (1970) noted that methoxyamine treatment of tRNA did not change the specificity of aminoacylation.

The reactivity of methoxyamine towards cytosine in synthetic oligonucleotides has been shown to vary according to the sequence of closely neighbouring bases (Steinschneider and Lesham 1972).

Lastly in this catalogue there has been one report on the influence of neighbouring DNA structure on the frequency of spontaneous deletions in E.coli (Coukell and Yanofsky 1971). The frequency of trp-tonB deletions varies considerably between dif-

ferent E.coli strains (Spudich et al. 1970). Coukell and Yanofsky (1971) found that the strain-specific differences were determined by the DNA structure at, or very close to the trp locus. Not only was the frequency of deletions affected by the local chromosome structure, but also the positions of the termini were affected. Mutants of E.coli deficient in DNA polymerase (pol^-) generate higher deletion frequencies (Coukell and Yanofsky 1970) but the possibility was ruled out that the differences in the deletion frequencies between strains were due to differences in the DNA polymerases.

All these studies have been concerned with the influence of neighbouring bases on the frequency of fully expressed changes is nucleic acid sequence. There is a recent report which demonstrates that the frequency of premutational lesions is also subject to modification by neighbouring DNA structure (Brunk 1973). He found that the number of thymine dimers induced by in vitro UV-irradiation of Tetrahymena-pyriformis DNA was greater the longer the run of pyrimidines in a tract, to a much greater extent than would be due simply to the fact that more thymines will be adjacent in the longer tracts .

Several comparative studies have been carried out on the differences between AT-rich and GC-rich DNA. Studies on the dependence of physical structure of DNA on its base composition have been at a gross level and hence may not be relevant to the fine-scale influences of neighbouring bases which would be required for mutational site specificities. It is clear nevertheless that the secondary stnucture of DNA does depend upon the base composition

(Bram 1971; Bram and Tougard 1972).

Other workers have looked at the dependence of base composition upon the specificity of interaction between DNA and various proteins which act on it.

For instance, E.coli RNA polymerase has been shown to bind preferentially to AT-rich regions of bacteriophages λ (Le Talier and Jeanteur 1971) and f1 (Shishedo and Ikeda 1971 and 1972). E.coli RNA polymerase also transcribes the T4 genome (e.g. Sokolova et al. 1970) but the comparative binding studies at different T4 regions have not been done.

In contrast to the RNA polymerase, calf thymus histone binds much more strongly to GC-rich DNA (Clark and Felsenfield 1972).

Three other examples will be given to illustrate the diversity of ways in which DNA base sequence determines protein specificity.

There is in E.coli an endonuclease which cleaves the DNA of Simian virus at a highly specific point suggesting recognition by this enzyme of a particular base sequence (Morrow and Berg 1972).

In phage T2, the extent of methylation of cytosine and adenine was dependent upon neighbouring base sequence (Vanyushin et al 1971). If the methylated and non-methylated purines differ in their response to mutagenic attack, here is a novel way for the generation of non-random spectra due to the influence of neighbouring bases on physiological factors.

The most obvious example of specificity of protein-DNA interactions is of course that found between the operator of bacterial operons and repressor proteins. Presumably the specificity is dictated by the base sequence of the DNA in the operator.

From all the examples discussed above it can be deduced that the influence of neighbouring bases on the frequency with which several classes of mutants arise is considerable. Is it sufficient to explain the hotspot phenomenon, and if so is it necessary to postulate any role for physiological processes in deciding which particular sequences will be most prone to error?

The most striking hotspots of all are the spontaneous frameshifts, r131 and r117. Without knowledge of the base sequence at these sites it is impossible to be categorical, but in the light of the findings of Okada et al (1972) it would not be too surprising if they contained long tracts of the same base pair. A comparatively simple model for the generation of spontaneous frameshift mutants does exist, (Streisinger et al 1966) but it is not clear whether the error-proneness of runs of the same base pair is a necessary direct corollary of the physical stereochemistry of the DNA at such regions or whether it is mediated by inaccuracies of an enzyme system when it encounters such tracts.

Bernstein (1971) in an exhaustive study of the effects of mutations at many T4 loci on the frequency of reversion of rII frameshifts found that some alleles of gene 43 (DNA polymerase), gene 32 (Alberts' protein), gene 44 (DNA synthesis) and gene 47 (deoxyribonuclease) enhanced the frameshifts' reversion frequencies. Mutants at four other loci involved in DNA metabolism had no mutator effect. Drake and Allen (1968) found that an antimutator allele of gene 43 did not depress the frequencies of frameshift mutations. Nevertheless it is clear that a large array of enzymes do have a role in the production of frameshift mutations, but in

the absence of forward mutation studies involving the mapping of the rII mutants in the T4 strains carrying mutations in any of the loci studied by Bernstein (1971), one does not know which, if any of the loci are important in determining the siting of frameshift mutations.

More relevant to the work in this thesis is a consideration of the generation of hotspots by mutagens which induce base pair substitutions.

In the catalogue of reports on the influence of neighbouring bases on mutagen site specificity, some of the examples came from work done *in vitro*. These were on the modification of tRNA sequence by bisulphite and by methoxyamine, and on the induction of pyrimidine dimers in *T.pyriformis* DNA by UV. Clearly in such experiments the role of physiological factors can be discounted.

Paradoxically, the fact that Brunk (1973) showed the influence of neighbouring bases on the distribution of premutational lesions *in vitro* may well substantiate, rather than negate the role of physiological factors in dictating the siting of mutations. Certainly it is a big jump from bacteriophages to ciliates but in T4, although thymine dimers are induced exclusively *in vitro* by UV, the majority of the base substitution mutations have AT at the mutant site. Obviously, so fine an analysis does not exist for *Tetrahymena*. Nevertheless, one can speculate that, although neighbouring bases may well influence the siting of premutational lesions, the positioning of the fixed mutant is a matter for the jurisdiction of physiological mechanisms.

No direct attack on the possible influences of neighbouring

bases on the site specificities of either of the two mutagens, NA and 5-BU, which were used in this work, comparable to the examples mentioned above with other mutagens has been made.

In the case of NA, if neighbouring bases have a role in determining the positioning of premutational lesions, it must be of a purely physico-chemical nature since the treatment was done extracellularly. In fact the nature of the gross steric shape of the DNA has been implicated in determining the reactivities of NA on DNA bases in polynucleotides (Shapiro and Yamiguchi 1972) and on RNA in viruses (Sehgal and Soong 1972).

Physiological factors are clearly implicated in the determination of the frequency with which the lesions induced in the T4 DNA are converted into full mutations as witnessed by the antimutator effect on NA-induced mutation frequencies of some gene 43 alleles (Drake and Greening 1970) and the comutagenic effects of the hm mutation and the presence of PFPA found in this work.

The fact that in these last two cases, the increases in frequencies were general and did not involve a change in the rII spectra does not say one way or the other whether cellular physiology determines the nature of the spectrum, just that no factor involved in generating site specificity is affected by the hm mutation or by PFPA. Given the precedent set by UV, of the ability of a mutagen to induce one class of lesion but to generate mutations which do not occur at the site of the lesion, it may be possible that such a situation holds for other mutagens. The fact that the classes of mutation induced by NA can apparently be readily explained on the basis of the types of lesion it can induce in vitro, perhaps argues

against this possibility. Nevertheless in T4, the mutagenicity of NA has been shown to be amenable to physiological modification in three distinct ways, all of which differ slightly from each other in their particular effects.

(a) T4 antimutator DNA polymerase was shown to specifically depress the frequency of NA-induced AT → GC transitions (Drake and Greening 1970).

(b) The T4hm strain exhibited nonspecific synergism with NA, coupled to an increased resistance to its lethal action.

(c) PFPA-treatment resulted also in a nonspecific increase in NA-induced mutation frequency of a similar order to that found for hm^+ but the sensitivity to NA-killing was not affected.

This suggests that at least three independent mechanisms are involved in NA mutagenesis and perhaps belies the apparently simple models of its mutagenic properties.

The situation with 5-BU is somewhat more complex than that for NA since it is given intracellularly and the potential exists for physiological mechanisms to act in determining the frequency with which premutational lesions are induced. Recent work has shown that *in vitro*, purified T4 antimutator DNA polymerase incorporates less 2-AP into newly synthesized DNA than does the wild-type enzyme, and that mutator polymerase incorporates more analogue than does the wild-type (Schnaar et al. 1973). Such an analysis has not been done with 5-BU. DNA polymerase is then a good candidate for a system which decides the frequency with which incorporation of base analogues occurs. If the fidelity of the enzyme were to vary according to the shape of the replication fork (dictated perhaps

by the sequence of bases in its vicinity) then differential incorporation of the mutagen at different sites would ensue.

After the premutational lesion has been induced, there is still scope for other enzyme systems to determine site specificity. The fact that lesions induced by both NA and 5-BU are susceptible to excision repair has already been pointed out. If certain lesions are refractory to such repair then they will have an increased probability of generating full mutations. One might postulate a role for neighbouring base sequence by saying that the degree of perturbation in the helix caused by a lesion may vary according to the sequence of the neighbours and that those lesions which result in small distortions are not recognized by the enzyme and hence persist.

Although lesions induced either by NA or 5-BU are not amenable to photoreactivation repair, a finding concerning this class of repair bears on the above point. Kilbey (1967) found that in Neurospora the extent of the photoreactivability of different UV-induced lesions along the genome varied. Whether the variation was due to the lesions being of a heterogeneous nature in which some are susceptible and some are refractory to photoreactivation, or whether the actual lesions are of a single type, with the differential response being due to their position in the genome, is not known.

The possible contribution of error-prone recombination repair in the mutagenic pathways of 5-BU and NA has been mentioned. As yet there is no model for the way in which such deficiencies in the accuracy of such repair cause mutations induced by either agent.

Whatever the mechanism, enzymes are almost certainly involved.

One more factor which might be involved in the production of non-random site specificity involves the probability with which a lesion persisting in the DNA is transcribed or is used as a template for DNA replication as if it were the original unmutated base. This idea may be best illustrated by reference to base analogue mutagenesis.

If 5-BU is incorporated into DNA in place of cytosine in the sense strand at different sites, the probability with which RNA polymerase reads it as cytosine or thymine may depend on the neighbouring base sequence. If in certain environments it tends to be read as cytosine the initial contribution of mutant mRNA compared to wild-type mRNA will be reduced, and although the lesion will be resolved and segregate) a mutation on replication, it is possible that the reduced mutant contribution means that certain mutations are missed on scoring.

Analogously and perhaps more importantly, if at different sites there are differences in the frequencies with which 5-BU is recognized as cytosine or thymine by DNA polymerase during DNA replication, site specificities will again be generated.

Two reports are of some relevance to these models. Saurbier et al. (1970) have found that although the transcription of UV-irradiated T4 DNA is hindered, due to the production of shorter molecules of mRNA, and the rate of initiation is decreased, there was no change in the fidelity of transcription.

Turning to DNA replication, the fidelity of polymerization of DNA using X-irradiated template was decreased (Saffhill and Weiss

1973). Here then is an example where the primary lesion stimulates further errors by inducing error-proneness of an enzyme.

Both conjectural possibilities concerning differential recognition by polymerases implicated premutational lesions as the substrates for such recognition. There is a report from a system quite unrelated to mutagenesis in T4 in which it was found that the extent of gene conversion of various alleles in the fungus Ascobolus depended on their mutagenic origin (Leblon 1972). This finding can be interpreted as showing that the extent to which mispairing of fully established mutants (as opposed to premutational lesions) in heteroduplices are resolved by some enzyme system may also depend on neighbouring base sequence.

Given the fact that site specificity has not been yet shown to be under the control of enzyme systems it may seemaa little audacious to have listed a catalogue of candidates which might be responsible. Nevertheless, it provides a framework of thinking for possible lines of future experimental attack on the problem. Some such approaches willbbe discussed.

It seems that at long last the identities of those reluctant heroes, the rIIA and rIIB proteins may be yielding to the prolonged assault which has been made on them. If their isolation and purification could be achieved such that amino-acid sequencing could be done, and hence the rII nucleotide sequence deduced, then it should be possible to determine if there is a simple relationship between site specificity of the hotspots and their neighbouring base sequences.

However, even if such an analysis did show what type of local

base sequence was likely to favour the generation of a hotspot by a specific mutagen, this alone would not indicate which (if any) enzyme system was the culprit in deciding which sequences cause a propensity to generate hotspots. There are two basic ways by which an evaluation of the contributions of different enzymes might be done.

The first is essentially an extension of the type of approach used in this work. In other words rII spectra, both induced and spontaneous would be obtained under conditions where the cellular physiology had been modified, either by experimental manipulation or by alteration of the genetic background. The latter would be better since the modifications would be of a more specific nature.

Various conjectural mechanisms each mediated by an enzymic system were discussed above as potential condidates for generating hotspots. These included various DNA repair systems, a number of other enzymes involved in T4 DNA metabolism such as the polymerase, endonuclease, methylase, ligase and Albert's protein and the host RNA^{s} polymerase. Mutants exist at all these loci and although it would be laborious, the analyses of the spectra obtained in such genetic backgrounds might indicate which specific systems are responsible for the generation of hotspots.

Increasingly, the role of enzymes in the determination of mutation frequencies is being studied in <u>in vitro</u> experiments and herein lies the second approach. So far most of this type of work has been focussed on DNA polymerase. From the studies on this enzyme in T4, we are beginning to understand the differences in enzyme activities associated with mutator and antimutator poly-

merases (Muzyczka et al. 1972; Hershfield 1973; Hershfield and Nossal 1973; Schnaar et al. 1973).

Of some importance is the recent finding of Springgate and Loeb (1973) that DNA polymerase extracted from human leukaemic cells polymerises DNA with greatly reduced fidelity when presented with synthetic templates than does the enzyme from normal cells. Also using synthetic templates, Hall and Lehman (1968) found that T4 mutator DNA polymerase was also less accurate than the wild-type enzyme.

By using synthetic templates of known, but varied sequence, an exact analysis of the role of neighbouring bases in the determination of error-proneness of the polymerase could be made. Schnaar et al. (1973) have already looked at the incorporation of 2-AP into newly synthesized DNA by different T4 polymerases. Again by using synthetic polynucleotide templates, one could see if differential incorporation of base analogue occurred depending upon base sequence. Using such a system it should be possible to distinguish between differential incorporation of base analogue, from differential generation of errors in DNA replication caused by misreading of base analogue-substituted DNA as template. Such an approach need not of course be restricted to base analogue mutagens.

If such studies could be extended to <u>in vitro</u> work on the influence of neighbouring bases on such parameters as the siting of premutational lesions, the specificity and fidelity of action of other enzymes involved in DNA metabolism and repair, we could have a full understanding of the forces involved in the generation of intragenic mutagen specificity.

At the start of the Introduction a brief summary of the evidence which illustrates the role of cellular processes in the generation of intergenic mutagen specificities was made. Can anything be said about the relationship between inter- and intragenic specificities?

It is rare enough to find reports of intergenic forward mutagen specificities. As far as I know there is no case where, after the demonstration of such specificity, the work has been extended into an analysis of the mapping spectra of the mutants within the appropriate loci. To date the best example of such an analysis comes from the r system itself, though even here only the rII region can be finely mapped. It has been mentioned earlier and it has been shown in this work that in a collection of spontaneous r mutants the majority are rII's. In contrast, after treatment by 5-BU or NA the rI mutants are the majority class. So here is a nice example of intergenic mutagen specificity. If however the two giant hotspots in the rII spontaneous spectrum did not exist, the ratio of spontaneous rI's to rII's would be close to unity. This then demonstrates that hotspots can be responsible at least for enhancing differences in intergenic mutagen specificity. One feels that it is largely a matter of chance that the two giant hotspots, r131 and r117 are in different cistrons. If however they happened to be in the same cistron then the ratio of rIIA to rIIB mutants would be different depending upon whether the rII's were of spontaneous origin or were induced with NA or 5-BU. In other words it may not be impossible for the differential siting of hotspots within and between cistrons to be responsible for intergenic mutagen specificities.

If the type of non-random spectra found in the rII region are

typical of all loci, it would be interesting to see if a change in forward mutagen specificities by some ancillary treatment could be interpreted as being due to the effect of the treatment on the relative intensities of hotspotting within the relevant loci.

Lastly, in the light of the current interest in the evolutionary aspects of mutation rates (as witnessed by the recent issue of a supplement broadly devoted to this topic:- "The Genetic Control of Mutation" Genetics 73, 1-205 (1973)) let us look at hotspots from this point of view.

Most of the thinking about the ways in which organisms determine their own mutations frequencies revolves around the fact that spontaneous mutation rates can be modified by genetic alteration of various enzymes. The fact that T4 DNA polymerase alleles and E.coli exrA(W) alleles whose only phenotypic manifestation is a decrease in mutation frequency can be isolated, suggests that the wild-type alleles at at least two loci have been selected during the course of evolution to be less accurate than they could be. It is not unreasonable to suppose that the wild-type alleles at other loci which have been shown to be implicated in the control of mutation rates have also been selected so as to maintain optimum mutation rates.

It takes two to tango and this role of cellular systems in the determination of mutation frequencies might be complemented by the role of local base sequences in the DNA in dictating the positions and the rates at which such cell-mediated mutations arise. After all if the two spontaneous rII hotspots, r131 and r117, were absent the spontaneous mutation at this region would fall by some 50%.

In fact it is very hard to imagine if and how any selective forces could act to maintain error-prone sequences in order to maintain optimum mutation rates. With regard to these two particular hot-spots, the fact that the mutants at both these sites comprise frameshifts argues against their being present in order to provide a source of new mutants upon which evolutionary selection can act since frameshifts cannot be considered to be the best raw material for selection, since such mutations cause a great distortion in amino acid sequence.

We can ask the complementary question. If the hotspots contribute nothing to natural selection and if there is no apparent mechanism for maintaining highly mutable sites by selection, why has natural selection not got rid of them?

If in fact the two giant hotspots do occur in runs as was suggested, the runs would code for a consective sequence of one of the following amino acids:- phe (UUU), pro (CCC), lys (AAA) or gly (GGG). Now each of these amino acids has a choice of bases in third position of their codons:- U,C,A or G for pro and gly, U or C for phe, and A or G for lys. One might think simplistically that the postulated run could be broken up by the insertion of heterologous bases in the third position of each triplet and by this means the site specific mutation frequency could be reduced.

It is possible that other selective forces override the advantages gained by so punctuating the runs. T4 is an AT-rich organism and in 14 out of 15 codons unambiguously typed in the e gene, the last position in the triplet was filled by A or U although the choice of C or G was available for the third position of these

triplets. (Okada et al. 1970).

In a comparative study of variant proteins (human haemoglobin and cytochrome c) no evidence of evolutionary hotspotting (i.e. certain sites of the protein which were peculiarly prone to mutational change) could be found (Zuckerhandl et al. 1971; Vogel 1972). Fitch (1973 a and b) found that the intracodon distribution of many different mutations in the same two proteins was highly non-random, the first position of the codon being the most susceptible to mutation. However in this study also, no hotspots were detected.

Whether all the hotspots are an inevitable consequence and are a cost which must be borne, due to selective forces not primarily concerned with the determination of mutation rates, or whether their siting and intensities are parameters amenable to natural selection is a knotty problem and is perhaps best left to the theologians of evolutionary genetics.

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