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DEVELOPMENT OF BACILLUS SUBTILIS TRANSFORMATION AND MARKER RESCUE BASED MUTAGEN DETECTION ASSAYS

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DEVELOPMENT OF *BACILLUS SUBTILIS* TRANSFORMATION AND MARKER RESCUE BASED MUTAGEN DETECTION ASSAYS

by

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ABSTRACT

DEVELOPMENT OF *BACILLUS SUBTILIS* TRANSFORMATION AND MARKER RESCUE BASED MUTAGEN DETECTION ASSAYS

by

Robert Aaron Phillips

Adviser: Dr. Anthony J. Garro

The development of several new mutagen detection assays based on transformation and marker rescue in *B. subtillis* is described. In these assays isolated bacterial or phage DNA is exposed to mutagens *in vitro* and then reintroduced into competent cells; the recombinants generated by either marker rescue or transformation with this chemically treated DNA are scored for the presence of mutants.

The impetus to develop an assay in which naked DNA is used as the target for chemical modification stemmed from the failure of several highly lipophilic and/or bacteriocidal carcinogens to be detected as mutagens in microbial assays where intact bacterial cells were incubated with the compound under study. We reasoned that the highly lipophilic carcinogens were not detected as mutagens in whole cell assays because they may have been trapped within the cell envelope of bacterial cells, and therefore had little or no opportunity to react with the cell's DNA. In the case of the bacteriocidal compounds, the failure to detect mutagenesis may have been attributable to low cell survival.

The unique feature of the mutagen assays described in this thesis is that there are no membrane barriers interposed between the test chemicals and the target DNA.

Several sets of genetic markers, located on either the *B. subtilis* chromosome or on the chromosome of the *Bacillus* phages Ø105, Ø3T and *rho*11, were evaluated for their response to known base-substitution or frame-shift mutagens. It was found that the Ø105 immunity region genes and *B. subtilis* chromosomal genes involved in isoleucine-valine biosynthesis and sporulation could be used to detect mutagenic modification of isolated DNA. The mutagenic potential of hydroxylamine (HA), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), chloroacetaldehyde and propylene oxide were detected in the transformation assays, in which reversion of the *ilv*B2 base-substitution mutation was measured, and in the marker rescue assay, in which induction of forward mutations in the immunity region of replicating Ø105 DNA were scored. The marker rescue assay detected the mutagenicity of the frame-shift mutagens N-acetoxy-2-acetylaminofluorene, (±) *anti* and (±) *syn* benzo(a) pyrene diolepoxide, but reversion of the *leu*A169 frame-shift mutation was not detected in the transformation assay. Forward mutations were induced in the *spo* OH cluster of transforming DNA with chloroacetaldehyde, and the mutagenicity of a presumed metabolite of halothane, 2-bromo-2-chloro-1,1-difluoroethylene also was detected using this assay. Forward mutations in the *thy*B gene of *B. subtilis* transforming DNA were

not detected after exposure of DNA to HA, MNNG and chloroacetaldehyde. A forward mutation assay based on the mutagenesis of the *thy*P3 gene of *B. subtilis* phages Ø3T and *rho*11 was examined, but it was determined that it would not be possible to detect mutations in this gene.

Finally, preliminary work was done to ascertain the feasibility of coupling a microsomal activation system to the marker rescue and transformation assay. It was found that microsomes do not unlink genetic markers in transforming DNA nor severely diminish transforming activity, suggesting that it should be possible to develop a coupled microsome-DNA mutagenesis assay.

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CHAPTER I

INTRODUCTION

A. Rationale for the Development of New Mutagen Assays.

This thesis describes the development of rapid mutagen assay systems based on the *in vitro* induction of chemical modifications in isolated DNA. The mutagenic effects of the chemically induced lesions are scored through either transformation or marker rescue of phage infected competent cells of *Bacillus subtilis*. Three mutation detecting systems were developed. One identifies mutagens by their ability to revert point mutations in the bacterial genome. The second detects forward mutations in genes controlling lysogenization by the temperate *B. subtilis* phage Ø105. The third, whose use has been superceded by the development of the more sensitive and rapid phage assay, scores forward mutations in one of the sporulation operons of the bacterial chromosome.

There have been a number of microbial tests developed over the past 10 years for the purpose of detecting potentially carcinogenic agents. Many organisms have been used in these tests, including fungi such as *Saccharomyces cerevisiae* (Zimmerman, 1977) and *Neurspora crassa* (Malling, 1967), as well as various species of bacteria including *Salmonella typhimurium* (Ames, *et al* 1975), *Escherichia coli* (Rosenkranz, *et al.*, 1976; Mohn and Ellenberger, 1977; and Green and Muriel, 1976), and *Bacillus subtilis* (Freese and Strack, 1962; MacGregor and Sacks, 1976; and Kada, *et al.*, 1972). The end-points measured in these assays are either

the production of mutant cells or some reflection of DNA damage induced in the test organism by the compound under study. These assays are able to identify potentially carcinogenic chemicals because most, if not all, carcinogens or their metabolites are electrophiles and hence can bind to nucleophilic centers in macromolecules such as DNA (Miller, 1970, Miller and Miller, 1977). Since modifications of DNA can produce mutations either through base mispairing (Watson and Crick, 1953; Lawley and Brookes, 1961), or misrepair of the chemical modification (Witkin, 1976), a compound's carcinogenic potential can be assayed by its ability to cause heritable changes in microorganisms. In addition, DNA damage can be detected by growth inhibition of strains defective for DNA repair (Slater, *et al*.1971), as well as by monitoring other phenomena related to DNA damage, such as the induction of resident prophage (Mohn and Ellenberger, 1977).

The impetus to develop microbial-carcinogen detection systems such as those noted above derived from the realization that mutagenesis and carcinogenesis in higher organisms may be mediated through similiar mechanisms (McCann, *et al.*, 1976; Maher, *et al.*, 1976; Miller and Miller, 1977). Furthermore, as pointed out by the Council of the Environmental Mutagen Society, Committee on Environmental Mutagenic Hazards (1975), as compared to mammalian mutagen screening tests, microbial assays are inexpensive to operate, have low initial investment costs, take only a short time to yield results, and the gene mutations are relatively easy to detect.

The carcinogen assay systems which are described in this thesis are similar to other mutagen assays in the sense that they measure the ability of a compound to induce mutations in DNA. There are several general methods of detecting the various classes of chemical mutagens. Reversion assays detect the induction of point mutations by monitoring the reversal of a particular phenotype, for example, the reversion of a His^- auxotroph to a His^+ prototroph (Ames, *et al.*, 1975; Mohn and Ellenberger, 1977; and Green and Muriel, 1976). However, since base-substitution mutations tend to be reverted by base-substitution mutagens, and frame-shift mutations by frame shift mutagens, in order for a reversion assay to have broad detection capabilities, it should be able to detect both classes of mutagens. For example, tester strains TA 1530 and TA 1538, two of the revertable auxotrophs used in the Ames *Salmonella*/microsome (Ames, *et al.*, 1975) assay, are phenotypically His^- due to a base-substitution mutation, *his*G46 in TA1530 and a frame-shift mutation, *his*D3052 in TA 1538. Base-substitution mutagens generally revert TA1530 but not TA1538, and frame-shift mutagens usually revert 1538, but not 1530. Forward mutation assays can theoretically detect both base substitution and frame-shift mutagens. A forward mutation test can be designed to measure one or more of several phenomena resulting from a change in nucleotide sequence, such as the alteration or inactivation of a pre-existing gene product, or changes in operator and repressor sites leading to constitutive production of a protein (Mohn

and Ellenberger, 1977; Freese and Strack, 1962).

Point mutations arising from chemical exposure are generally considered to be the result of one of two phenomena, which may be classified as direct or indirect effects (Yamamoto, *et al.*, 1978; Caillet-Fauquet, *et al.*, 1977). Direct effects result from a modification of a base, which leads to mispairing (Watson and Crick, 1953). For example, alkylation of O^6-guanine may cause the modified base to pair with thymidine, leading to a GC-AT transition (Loveless, 1969). Indirect effects involve mutagenesis due to misrepair of modified nucleotides. Bacterial cells have several pathways which participate in the repair of chemically or physically damaged DNA sequences. Although most of these pathways accurately repair the damage, there is at least one pathway, referred to as SOS repair, which is error-prone and results in mutagenesis (Radman, 1975). The SOS repair pathway is inducible by agents which damage DNA and thus mutagens are able to induce error-prone repair in cells (Miura and Tomizawa, 1968; Radman, 1975; Meyn, *et al.*, 1977; Smith and Oishi, 1978).

If error-prone repair is increased relative to error-free repair the mutagenic response of an organism to some mutagenic compounds is actually enhanced (Sedgwick, 1975). Advantage has been taken of this phenomena to increase the sensitivity with which some bacterial assays detect certain classes of mutagens such as the monofunctional alkylating agents (Bridges, 1978). For example, in many of the Ames

Salmonella tester strains the uvrB gene, whose product is essential for error free excision repair, has been deleted and an R factor has been introduced, which by an unknown mechanism, increases the level of error prone repair (McCann, *et al.*, 1975). It is particularly relevant with respect to the transformation based assays described here that an error prone repair pathway may be induced in *B. subtilis* during the period when the cells become competent for the uptake of DNA (Yasbin, 1977a; Yasbin, 1977b).

There are many different classes of chemicals which have mutagenic activity and it is generally believed that compounds should be tested in mutagen assays which have been optimized for detection of the class to which the chemical under study belongs (Purchase, *et al.*, 1976; Ashby and Purchase, 1977). For example, the Ames test, which is currently being used in over 1000 laboratories around the world (McCann and Ames, 1977), was optimized for measuring the mutagenic activity of bulky polycyclic aromatic hydrocarbon carcinogens, as well as compounds structurally related to the multi-ring pesticide 2-acetylaminofluorene. This was accomplished by introducing mutations into the tester strains which interfered with the synthesis of the polysaccharide chains of the outer cell membrane (Ames, *et al.*, 1973), and thus increased their permeability to these bulky hydrocarbons. The Ames test has an excellent, though not flawless record in identifying as mutagens the carcinogenic members of the classes for which it was optimized (McCann and Ames, 1976;

Bartsch, *et al.*, 1977).

The stimulus that prompted us to develop a system which could detect chemically induced mutagenic lesions in isolated DNA was the inability of the Ames assay to identify as mutagens the carcinogenic pesticides Dieldrin and DDE, and the drug Diethylstilbesterol (DES), (McCann and Ames, 1976). We postulated that these bulky and highly lipophilic compounds (Leo, *et al.*, 1971) were not positive in the Ames assay because they may have failed to traverse the cell envelope of the tester strains employed. Certainly it is possible that not all carcinogens are mutagens, but DDE (Kelly-Garvert and Legator, 1973) Dieldrin (Markaryan, 1966) and DES (Blackburn, *et al*) have been shown to mutate or interact with the DNA of mammalian cells. It is obvious that in order to exert a genetotoxic effect, a compound must first penetrate an organism's cell envelope before it can interact with genetic material. *S. typhimurium*, the bacterial species used in the Ames assay, is a gram-negative organism, and its cell envelope is composed of three layers, two of which are lipid rich membranes. In fact, lipids make up about 25% of the dry weight of gram negative cells (Gunsalus and Stanier, 1960). The potential effect of this lipid rich envelope on chemical permeability was illustrated in a large study of antibacterial agents which revealed that highly lipophilic compounds were bound very strongly within the envelope of gram negative cells (Lien, *et al.*, 1968). Since lipophilic compounds only slowly diffuse from lipid rich membranes into a cell's cytoplasm (Hansch and

Clayton, 1973), their concentration may never be high at sites where they could exert genetic effects. Consequently, it seemed plausible that the false negative results obtained for Dieldrin, DDE and Diethylstilbesterol in the Ames assay was due to their being bound or trapped within the cell's membranes. We believed it worthwhile, therefore, to develop a mutagen assay in which the lipid barrier between the test chemical and the DNA was entirely removed. This could be achieved by a mutagen assay in which DNA could react directly with the chemical outside of the cell.

Reaction of chemicals with isolated DNA could offer solutions to other technical problems posed by standard tests. For example, chemicals which are bacteriocidal at very low concentrations are difficult to test in the usual bacterial assays because of poor cell survival. Out of the 300 chemicals which were chosen to test the reliability of the *Salmonella* reversion assay for distinguishing known carcinogens from noncarcinogens, ten could not be adequately tested for mutagenicity because of their toxic effects (McCann, *et al.*, 1976). Although it appears that at least one whole cell assay, the Rosenkranz *E. coli* pol A1⁻ DNA damage test, is not as susceptable to the toxic "side-effects" of mutagens as the Ames assay (Rosenkranz, *et al.*, 1976), we reasoned that use of isolated DNA may completely bypass this problem because the chemicals are bound to DNA when they enter the cell and are probably not free to exert their toxic effects. This feature may prove to be especially important

when studying mixtures which contain both bacteriocidal and mutagenic components.

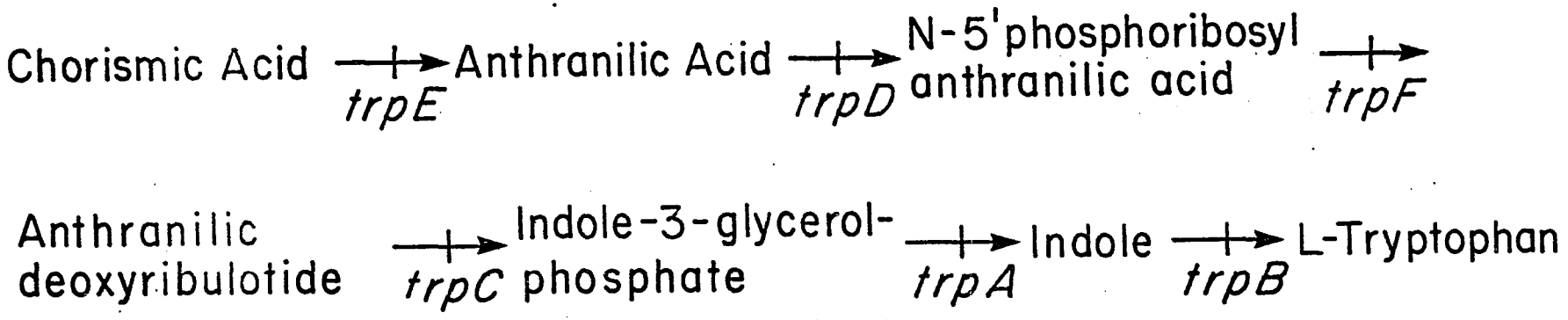
Furthermore, a DNA transformation based assay not only would circumvent the membrane lipid barrier present in whole cell assays, it would also circumvent potential trapping of the electrophilic mutagens by the nucleophilic proteins and phosphates which are present in the membrane. This feature may be particularly important when testing chemicals which are relatively more reactive with the nucleophilic sulfhydryl groups in proteins than with weakly nucleophilic sites such as ring oxygens in DNA (Osterman-Golkar, *et al.*, 1970; Veliminsky, *et al.*, 1970). The importance of being able to detect pyrimidine and purine ring oxygen alkylations is related to observations which correlated mutagenicity and carcinogenicity with this type of alkylation (Loveless, 1969; Goth and Rajewsky, 1974; Singer, 1976). Since proteins make up a significant amount of a bacterial cell, it is possible that a mutagenic compound with relatively greater reactivity toward protein sulfhydryl groups than toward purine and pyrimidine ring oxygens could be missed in a whole cell assay.

Our own preliminary experiments, as well as previous reports by other investigators, indicated that prospects were good for the successful development of a general mutagen screening assay based on exposing chemicals directly to DNA. First of all we knew that chemically modified DNA could be taken up by competent cells and that the modifications could be detected as mutations. This had been shown for a variety of

chemicals including: hydroxylamine and nitrous acid (Freese and Strack, 1962; Bresler, *et al.*, 1968), alkylating agents such as MNNG and N-methyl-N-nitrosourea (Bresler, *et al.*, 1972), N-acetoxy-2-acetylaminofluorene (Maher, *et al.*, 1968) and a derivative of benzo(a)pyrene (Maher, *et al.*,1971). Secondly, the longer range goal of coupling the transformation assay to a microsomal metabolic activation system, which is necessary to convert secondary carcinogens to their DNA reactive form (Miller, 1970), also seemed feasible. Preliminary experiments showed that DNA retained a significant amount of transforming activity even after a 30 min incubation with hepatic microsomes (Table 18).

The transformation assay utilized in the mutagenesis studies noted above was based on the induction of mutations in the tryptophan biosynthetic pathway illustrated in Fig. 1 (Freese, 1961). Anthranilic acid and N-5'-phosphoribosyl anthranilic acid are fluorescent compounds which are excreted by *trp*C, D or F mutants growing on media containing limited amounts of indole. Only cells mutated in *trp*B fail to produce colonies on indole supplemented plates. In order to test compounds for mutagenicity, DNA from wild-type cells is treated with the test chemical, and then used to transform *trp*B mutant cells. *Trp*B transformants prototrophic for growth on indole (Ind^+) are selected on media containing a limiting amount of indole. As the transformants grow and the media becomes depleted of indole, the *trp* operon is derepressed. Those clones with a defect in the *trp*C, D or F

Fig. 1. Pathway of tryptophan biosynthesis (adapted from Carlton and Whitt, 1969).


Chorismic Acid
trpE
Anthranilic Acid
trpD
N-5'phosphoribosyl anthranilic acid
trpF
Anthranilic deoxyribulotide
trpC
Indole-3-glycerol-phosphate
trpA
Indole
trpB
L-Tryptophan

genes excrete fluorescent metabolites which can be detected by viewing the plates under short-wave UV light.

The problem with the use of this assay as a general mutagen screen stems from the fact that Ind+ transformants must first be selected and then screened for clones excreting fluorescent metabolites. In order to detect such clones, the colony density can not exceed 150-200 Ind+ transformants per plate. For very potent mutagens, relatively few plates would have to be screened in order to detect mutagenesis, but for weak mutagens, it would be necessary to screen large numbers of plates in order to detect an increase in the number of fluorescent mutants generated. Two examples from the literature illustrate these points. Bresler (1968) reported that the most efficient mutagen in the fluorescent assay system was HA. Using conditions similiar to those described by Freese and Strack (1962), Bressler obtained 9 fluorescent mutants per 246 Ind+ colonies scored; however, when Maher (1971) investigated activity of benzo(a)pyrene in the fluorescent assay, approximately 15,000 Ind+ colonies had to be screened in order to establish the mutagenicity of this compound. This obviously required the screening of at least 75 plates containing Ind+ transformants. The magnitude of each experiment which would be necessary to test an unknown and weakly mutagenic compound clearly precluded the fluorescent assay from being used as a general screening procedure.

Since we believed, for the reasons described above, that a transformation mutagen assay offered several features which should be exploited, we began to seek other markers in which mutations might be more easily scored. The characterization of the mutagenic responsiveness of several gene clusters to a series of characterized mutagens thus became the principal goal of this thesis.

Since one of the major problems with the fluorescence assay was that it was not selective for the growth of only those transformants which had received a piece of mutated DNA, the initial criterion in determining if a marker would be suitable for use in a mutagenicity screen was whether or not selective conditions could be applied for the growth of the mutants induced. Since transformation frequencies of about 1% are achievable with *B. subtilis*, direct selection of transformants from cultures containing 10^8 cells/ml affords the opportunity to monitor low levels of mutagenesis. For example, following the plating of 10^6 transformants, if one in ten thousand were a mutant of the desired phenotype, then between 100 and 150 clones should grow on a selective plate. Thus an increase in the frequency of mutant clones from a background of approximately 1×10^{-5} to 4×10^{-5} could could be confirmed statistically with four replicate plates. This level of sensitivity would be 50-200 times greater than that possible in the fluorescence assay.

Of the three assays which we have used, only the reversion assay offers the advantage of direct selection of

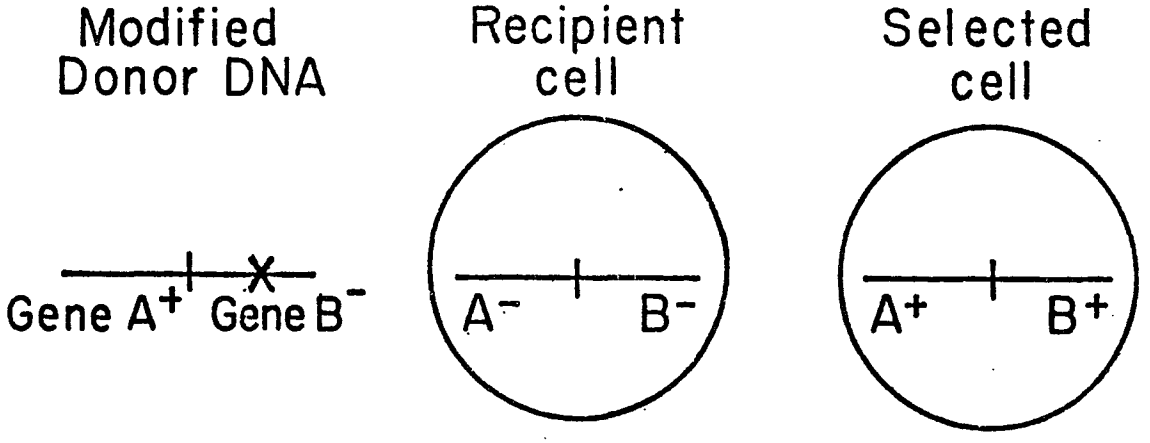
mutant clones. However, in the phage assay, which scores forward mutations in operons regulating lysogeny of Ø105 the clear plaques produced by mutant phage can be identified against a background of $>10^4$ turbid wild type plaques per plate. This offers a sensitivity 50 times greater than that possible in the fluorescent assay. The third assay, developed before the above and superceded by them, measured forward mutations in the sporulation OH cluster. In this assay Spo^- mutants could be detected against a background of 1000 Spo^+ colonies, which is 5 times more sensitive than the fluorescent assay.

To test a chemical for mutagenicity in the reversion assay, the chemical is incubated separately with DNA from a strain carrying the base substitution mutation *ilv*32, in the isoleucine-valine biosynthetic pathway, and with DNA carrying the frame shift mutation *leu*A169, in the leucine biosynthetic pathway (Ward and Zahler, 1973). These DNAs are then used to transform cells which carry both of the above closely linked mutations, and the induction of mutations is assayed by selecting for Ilv^+, Leu^+ recombinants. The reason for using a recipient with both of the above mutations stems from the fact that only 10% of the cells in a *B. subtilis* culture ever become competent for DNA uptake. Thus steps must be taken to ensure that the majority of the mutants scored, i.e. the Ilv^+, Leu^+ prototrophs are the result of transformation by a piece of mutagenized DNA, and are not spontaneous mutants arising from the bulk of the cell population that is not competent

for DNA uptake. Using a recipient with mutations in two closely linked genes solves this problem in the manner diagramatically illustrated in Fig. 2.

In the diagram, gene A and B are tightly linked, and thus can be carried on the same piece of transforming DNA. The donor DNA is isolated from a cell that has the genotype A^+B^-. The donor DNA is mutagenized and used to transform the recipient. We can then select for the A^+ phenotype and score for B^+, or plate under conditions which select for the A^+B^+ phenotype. It is unlikely that an A^+B^+ clone will arise spontaneously, since this cell would require simultaneous mutations in two genes. Therefore, almost all the cells which grow on the selective plates arise from the incorporation of the A^+ gene from the donor and the modified B^- gene that is mutated to the B^+ genotype as a result of exposure of the transforming DNA to the mutagen. A small number of A^+B^+ clones may arise from those transformants which received only the A^+ gene and spontaneously mutated from B^- to B^+. Also, transformation with completely homologous DNA is known to increase the mutation frequency (Yoshikawa, 1966). For example, in the reversion assay, when DNA carrying the *ilv*B2 mutation is mutagenized, the modified DNA is then used to transform cells carrying the same *ilv* mutation and a closely linked *leu* mutation, namely *leu*A169. After allowing time for DNA uptake, cells are plated on media lacking leucine, isoleucine and valine. Therefore, the only cells that will grow are transformants that have obtained a Leu^+

Fig. 2. Diagramatic representation of the use of linked genes in the transformation assay. The X on the B^- gene of the mutagen treated donor DNA represents a lesion which leads to a B^+ phenotype when the recipient chromosome recombines with the donor DNA.


Modified
Donor DNA
Recipient
cell
Selected
cell
Gene A+
Gene B-
A-
B-
A+
B+

gene and a mutated Ilv^+ gene from the donor DNA, as well as a small number of Leu^+ transformants that spontaneously mutated to Ilv^+.

In the sporulation assay, mutagenesis is detected by the induction of asporagenous mutants among the cells transformed with the treated DNA. The asporogenous mutants are detected by their inability to produce the dark brown pigment characteristic of Spo^+ colonies. Low numbers of colorless Spo^- mutants are detectable even against a background of more than 1000 pigmented Spo+ colonies on a plate. There are several regions of the *B. subtilis* chromosome that are involved in the regulation of spore formation. One of Spo loci, the OH locus, is tightly linked to the gene for rifampin(Rif) resistance (Piggot, 1973). In the actual test, Rif^r, Spo^+ DNA is incubated with a test chemical and used to transform a Rif^s, Spo^+ culture, and Rif^r transformants are selected. The transformants are then screened for the presence of Spo^- mutants.

As noted above, the sporulation assay has been superceded by a phage assay which detects the induction of lysogenization-defective mutants of the temperate phage Ø105. This phage has six complementation groups which regulate lysogeny. A mutation in any of these six cistrons will interfere with the phage's ability to lysogenize infected cells (Scher, *et al.*, 1978). The lysogenization-defective mutants are readily detected by their clear plaque phenotype as opposed to turbid plaques produced by wild-type phage.

The phage-mutagenesis assay is based on the phenomenon of marker rescue, a process in which competent cells are both infected with phage carrying a conditional lethal mutation and exposed to DNA carrying the corresponding wild-type gene for the mutation. Following recombination between the added wild-type DNA and the replicating phage DNA, phage are produced carrying the "rescued" wild-type gene. In the mutagenesis assay the wild-type DNA is first treated with a test chemical and the rescued phage, which can now replicate under restrictive conditions since they no longer carry the conditional lethal mutation, are scanned for clear plaque mutants.

Marker rescue is used rather than a simpler transfection assay, in which mutagen treated phage DNA would be scored directly for its ability to produce clear plaquing phage when added to competent cells, because of the relatively greater sensitivity of transfection to inactivation by mutagen treatment. The greater sensitivity to inactivation of transfection relative to marker rescue is readily understood in terms of target theory; in order to produce a plaque by transfection all the essential genes of the phage must survive the mutagen treatment whereas in marker rescue only a single gene, the one being selected in the rescue, need survive the treatment.

The marker which is used in the phage assay is *Jsus* 11. The J gene is tightly linked to the cluster of six genes whose functions are required for lysogeny (Scher, *et al.*, 1978).

Since *Jsus* 11 phage can not produce plaques in cells which lack the su^+ suppressor, only those phages which acquire a wild-type J gene introduced on the added DNA will produce plaques under the restrictive assay conditions. If the added DNA contains chemically induced lesions in any of the lysogenization specific genes, clear plaque mutants will be produced.

Attempts were made to develop a forward mutation assay in which it would be possible to directly select for *B. subtilis* mutants, which as a result of transformation with mutagen treated DNA would become defective for thymidylate synthetase. Thy^- cells with a defect in this enzyme are able to survive and replicate in the presence of dihydrofolate reductase inhibitors such as trimethoprim, as long as the growth media is supplemented with thymidine (Wilson, *et al.*, 1966). The growth of cells with an active thymidylate synthetase is inhibited in the presence of the drug. The growth inhibition of Thy^+ cells by trimethopin is based on the fact that one of the major which tetrahydrofolate is oxidized is in the conversion of dUMP to dTMP, which is the reaction catalyzed by the enzyme thymidylate synthetase. Tetrahydrofolate must be regenerated from the oxidized form by dihydrofolate reductase in order that purine, histidine and methionine synthesis can continue. When dihydrofolate reductase is blocked by trimethoprim the only cells that can continue to replicate are those which do not exhaust their supply of tetrahydrofolate,

such as Thy$^-$ cells supplemented with exogenous thymidine.

B. subtilis has two genes *thy*A and *thy*B which code for thymidylate synthetases (Neuhard, *et al.*, 1978). The *thy*B locus is linked to *ilv*A (Zahler, *et al.*, 1976). It was reasoned that a mutagen assay could be developed in which the recipient cell in the transformation was *ilv*A*thy*B$^+$ *thy*A$^-$ and the donor was chemically treated *ilv*A$^+$*thy*B$^+$ *thy*A$^-$ DNA which had been converted to *thy*B$^-$ by the exposure to a mutagen. Induction of mutations in the transforming DNA would be monitored by selecting for Ilv$^+$,Thy$^-$ transformants grown in the presence of trimethoprim.

A variation of this assay which seemed particularly attractive involved the use of a phage which was purported to carry a gene homologous to the *B. subtilis thy*A gene. Phage Ø3T and *rho*11 carry a gene, *thy*P3 which codes for a thymidylate synthetase (Tucker, 1969; Williams and Young, 1976). When these phage lysogenize a Thy$^-$ host, they integrate between the *thy*A and *thy*B genes, and convert the cell to a Thy$^+$ phenotype (Williams and Young, 1977a,b). Naked phage DNA also had been used by Williams and Young to transform Thy$^-$ cells to Thy$^+$, and in the transformation mediated conversion it had been suggested that the thymidylate synthetase gene from the phage actually recombined directly with the host *thy*A gene. Based on this information, it was thought that it would be possible to monitor mutagenesis in the phage *thy*P3 gene by transforming *thy*B$^-$, *thy*A$^+$ cells with chemically treated phage DNA and selecting for Trm^r resistant colonies.

Although it would not be possible to utilize linkage to some other host marker to lower the spontaneous Trm^r background in this system, it was thought that the spontaneous Trm^r background could be controlled with a sufficiently high concentration of trimethoprim. The advantage of using the phage DNA would derive from the relative increase in *thy*P3 gene copies/mg phage DNA relative to the *thy*B gene copies/mg bacterial DNA.

In addition to measuring the induction of mutations in DNA, the extent of DNA damage induced by a chemical was routinely evaluated by following loss of transforming activity in the reversion and sporulation assay, and by following the decrease in plaque forming units in the phage assay. An example of a lesion which would decrease transforming activity is one that causes strand scissions (Morrison and Guild, 1972). The extent of this type of damage could also be measured by quantifying the ability of treated DNA to co-transform two closely linked genes, since strand scission would unlink genes carried on the same piece of transforming DNA. Ability to induce this type of damage could indicate that a chemical has the ability to break chromosomes (Savage, 1975). Chromosomal aberrations and sister chromatid exchanges have been implicated in carcinogenesis, and these phenomena probably require breakage and reunion of chromosomes (Perry and Evans, 1975). Therefore, the ability of a transformation based assay to sensitively measure events that lead to strand scission may indicate a potential of an agent to cause serious lesions in eukaryotic organisms.

B. Rationale for choice of compounds tested.

In order to assess the ability of the transformation and marker rescue systems to detect potential mutagens, the reversion and phage assays were tested with previously characterized genetically active compounds. These chemicals were chosen so that different chemical classes which produced distinct lesions in DNA, and which did not require metabolic activation for this DNA reactivity, would be represented. The specific compounds chosen were the epoxide propylene oxide; the nitrosamide, N-methyl-N'-nitro-N-nitrosoguanidine; the halogenated aldehyde, chloracetaldehyde; hydroxylamine; the aromatic amide, N-acetoxy-2-acetylaminofluorene (N-2-fluorenylacetamide); and the *syn* and *anti* forms of the polycyclic aromatic hydrocarbon 7,8-dihydrodiol,9,10-epoxy-7,8,9,10 tetrahydrobenzo(a)pyrene.

1. Hydroxylamine (HA)

HA is thought to induce GC-AT transitions by modifications of cytosine residues (Freese, *et al.* 1961). However, at least one group (Budowsky, *et al.*, 1975) reported that AT-GC transitions may be caused by modification of adenine. HA has been shown to be mutagenic in several systems, including the fluorescent transformation assay (Freese and Freese, 1962), but is negative in the Ames test (McCann, 1975).

2. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)

The mutagenic potency of MNNG has been related to the extensive degree to which it alkyates oxygens in nucleic acids.

The role of oxygen alkylation in mutagenesis has become appreciated since Loveless's work (Loveless, 1969) which suggested that mutagenic potency of alkylating agents could be directly related to the ratio of O^6-guanine/N^7-guanine modification. The most complete analysis reported for the reaction of a nitrosamide with ring nitrogens and oxygens in DNA utilized ethylnitrosoguanidine (Singer, 1976), but similar reaction patterns were also obtained with MNNG, the only difference being that there was somewhat less alkylation at ring oxygens (Singer, 1976). The salient feature of the nitrosoguanidine reaction is that there is considerable alkylation of O^6-guanine in both double and single-stranded DNA, and a three-fold increase in O^4-thymidine in single stranded DNA. Both of these lesions can lead to mispairing of bases during replication. Mispairing by RNA polymerase has been demonstrated using an O^6-methyl guanine modified template (Gerchman, 1973).

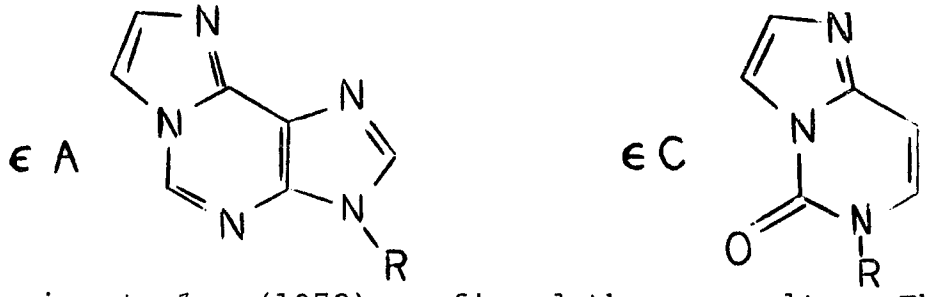
Mutagenicity of MNNG has been demonstrated in several systems (Freese, *et al.*, 1967; Singer, 1967). It is more mutagenic at the replication point of *E. coli*, Cerrudo and Olemda, 1968) where the DNA is single-stranded, than at other sites. This may be rationalized on the basis of increased binding to O^4-thymidine. Alternatively, the repair of MNNG induced damage at DNA replication points may be inhibited because of a local concentration of polymerizing enzymes which might interfere with the repair enzymes (Lawley, 1974).

3. Chloroacetaldehyde

Chloracetaldehyde is a metabolite of vinyl chloride (VC), a widely used compound which is produced at the rate of 2.5×10^9kg/year (McCann, *et al.*, 1975b). The carcinogenic potential of VC was first reported in 1971, in studies which showed that male rats exposed to 30,000 ppm VC for 4hr/day, 5 days/week for 12 months developed tumours of the skin, lungs and bones (Viola, 1971). Maltoni extended these studies by administering VC to males and females of different species over a wide range of concentrations with varying lengths of treatment, routes of exposure, and age of animals (Maltoni, 1977. Among the important findings of this work was that a dose response relationship was established with respect to overall incidence of tumours as well as to the latency period for development of tumours. Unfortunately, demonstration of the carcinogenicity of VC has not been limited to animal models. VC polymerization workers have been found to develop angiosarcomas of the liver (Creech and Johnson, 1976) and also show an increased incidence of brain tumours, lung carcinomas, leukemias and lymphomas (Waxweiler, *et al.*, 1976). Mutagenicity of VC has been demonstrated by several groups, including our laboratory (Garro, *et al.*, 1976).

Chloroacetaldehyde was identified as one of the metabolites of vinyl chloride in the rat (Hathaway, 1977), as well as in an *in vitro* rat metabolizing system (Gothe, *et al.*, 1974). Kotechov, *et al.* reported in 1971 that chloroacetal-

dehyde reacted with adenine and cytosine at low pH to form the imidazonucleoside derivatives illustrated below.



Barrio *et al.*, (1972) confirmed these results. These products have also been identified in the DNA of rats exposed for one year to VC through drinking water containing 250ppm (Green and Hathaway, 1977). When DNA was reacted with chloroacetaldehyde in 50% DMF at pH 4.5, Lee and Wetmur (1973) showed that the melting temperature of DNA was lowered, indicating that the chloroacetaldehyde lesions produced mispairing of bases. Additional work from the Wetmur group indicated that chloroacetaldehyde reacted more with single-stranded DNA. Furthermore they showed that the reaction of chloroacetaldehyde with cytosine bases in DNA had a pH optimum of 4.5, and was essentially over at pH 4.7, while the reaction with adenine had a very broad pH optimum which continued to at least 5.2 (Ruyechan, 1976). Recent work by Kohwi-Shigematsu, *et al.*, (1978) indicates that chloracetaldehyde reacts with the adenines of single-stranded DNA even at pH 6.8.

The mutagenicity of chloroacetaldehyde was demonstrated by McCann *et al.* (1975b). In that report, TA100, but not TA1535 was reverted by the compound, and no metabolic activation was required. Rannug *et al.* (1975) suggested that the imidazole derivatives formed from reaction of chloroacetalde-

hyde with DNA are probably responsible for the mutagenic action of the compound. This is based on the demonstration that chloroacetaldehyde reacts only slightly with oxygens compared to other alkylating agents, but nonetheless is very mutagenic (Hussain, S. and Osterman-Golkar, 1976).

4. Propylene oxide

Propylene oxide is an aliphatic epoxide which is used as a sterilant (Fishbein, 1969) of foodstuffs. Inhalation studies on several animal species did not reveal carcinogenic activity, (Rowe, 1956), but rats developed sarcomas after subcutaneous injection (Walpole, 1958). Only a limited number of publications exist on the nature and effects of propylene oxide alkylation of nucleic acids. Lawley and Jarman (1972) found that propylene oxide formed two principal products when reacted with DNA in aqueous solution. These are 7-(2-hydroxypropyl) guanine and 3-(2-hydroxypropyl) adenine. No O^6-guanine alkylation product was found. Walles (1974), on the basis of the strand scissions observed after propylene oxide modifications of DNA, suggested that propylene oxide also reacted with the phosphodiester backbone of the molecule.

There have been only a few reports on the mutagenicity of propylene oxide. Propylene oxide induced reversions in *Neurospera crassa* (Kølmark and Giles, 1955), and the IARC monograph, volume 11, noted two reports in the Russian literature indicating that propylene oxide induced recessive lethal mutations in *Drosophila melanogaster* (Rapoport, 1948a,b).

Fraenkel-Conrat (1961) did not find propylene oxide to be mutagenic toward TMV-RNA, but neither was the known mutagen dimethylsulfate mutagenic in that study.

5. Benzo(a)pyrenediolepoxides (BPDE'S)

In 1974 Sims *et al.* presented evidence that the metabolite of benzo(a)pyrene that bound to DNA of hamster embryo cells was 7,8-dihydrodiol, 9,10-epoxy-7,8,9,10-tetrahydrobenzo (a)pyrene (Sims, 1974). This was an extension of an earlier finding by Borgen *et al.* (1973), who established that the most extensive DNA modification resulted when benzo(a)pyrene 7,8 dihydrodiol was the substrate for syrian hamster liver microsomes. BPDE exists into two isomeric forms. In the *syn* form, the 7-hydroxyl and the 9,10-oxide groups are on the same side of the ring system, whereas in the *anti* form, they are on opposite sides. Humans appear to metabolize benzo(a) pyrene to the *syn* BPDE, whereas hamster embryo cultures produce both *syn* and *anti* BPDE (Jeffrey, *et al.*, 1977, Ivanovic, *et al.*, 1978) The mutagenicity of both BPDEs to bacterial (Wood, *et al.* 1976) and mammalian cells (Huberman, *et al.*, 1976) was quickly established following Sims' finding.

Evidence for a site at which BPDE bound to DNA was first presented by Weinstein, *et al.* (1976). Analysis of the DNA adduct formed from the metabolism of benzo(a)pyrene by cultured bronchial mucosa, as well as from binding of chemically synthesized *syn* and *anti* BPDE to denatured calf thymus DNA, enabled the Weinstein group to conclude that BPDE deriva-

tized the exocyclic 2-amino group of guanine. These results were confirmed by several groups, including Calvin's (Straub, *et al.*, 1977) who extended these findings to include binding of BPDE to the exocyclic amino group of adenine. In addition, it has been demonstrated that at least the *anti* form of BPDE binds to the N^7-position of guanine (Osborne, *et al.* 1978).

6. N-acetoxy-2-acetylaminofluorene(AAAF)

When N-2-acetylaminofluorene is ingested by rats it is converted to an electrophile, N-sulfate-2-AAF, which is capable of interacting with nucleophilic centers in macromolecules(Miller, 1970). N-acetoxy-2-acetylaminofluorene is a chemically prepared analogue of the biological metabolite and is used because of its increased stability compared to N-sulfate-2-AAF(Maher, 1968). The direct mutagenicity of AAAF for isolated DNA was first demonstrated by Maher (1968) who used the fluorescent transformation assay described above. Kriek, *et al.*, (1967) first showed that AAAF bound to guanosine *in vitro* forming N-(deoxyguanosin-8-yl)-AAF. Administration of AAF to rats has demonstrated that in addition to this C^{8-} guanine adduct, there is another guanine modification which yields 3-(deoxyguanosin-N^2-yl)-AAF. Westra, 1976). This latter product is persistant, whereas the C^{8-} derivative is readily removed from rat liver DNA *in vivo*. Yamasaki *et al.* (1977) have extended these findings by demonstrating that when AAAF modified DNA is incubated with the single strand

-specific nuclease S_1 only the C^{8-} adduct is removed. Yamasuki, *et al*. suggests that the N-(deoxyguanosin-8-yl)-AAF residue leads to local denaturation which may be recognized by repair systems, whereas the 3-(deoxyguanosin-N^2-yl)-AAF derivative is persistent because it does not disrupt the helix and hence is not removed by repair enzymes. They further argue that the persistent product may be responsible for mutagenesis and carcinogenesis.

CHAPTER II

MATERIALS AND METHODS

A. Bacterial and Phage Strains.

The bacterial strains used are listed in Table 1. All *B. subtilis* are derivatives of the transformable strain 168. The *S. typhimurium* strains are derivatives of LT-2 and were kindly provided by B. Ames.

Wild-type Ø105 and the conditional lethal mutant Ø105 *Jsus*11 were obtained from L. Rutberg. The Ø105 deletion mutant was obtained from J.I. Flock. The clear plaque mutant Ø105*cng*-2 was isolated by Garro and Law (1974) after MNNG mutagenesis of Ø105-infected cells. Mutant and wild-type Ø3T phages were obtained from D. Dean, and *rho* 11 phage from C. Orego.

B. Chemicals and Enzymes.

The following were purchased from Sigma: lysozyme; N-lauryl sarcosine salt; sodium lauryl sulfate; ribonucleic acid, tRNA type XXI from *E. coli* strain W; mitomycin-C; N,N-dimethyl formamide; 9-aminoacridine; daunomycin; and trimethoprim. Pronase was purchased from Calbiochem, and ribonuclease A and ribonuclease T_1 were purchased from Worthington Biochemical Corp. Proplylene oxide, 99% pure and N-methyl-N'-nitro-N-nitrosoguanidine were obtained from Aldrich Chemical Co. Chloroacetaldehyde, 50% in H_2O was purchased from K&K, Life Sciences Group. Hydroxylamine was from Fischer. Crystalline phenol was obtained from Mallinckrodt and distilled

Table 1. List of Strains and Their Characteristics

Strain	Synonym	Relevant Genetic Markers or Genotype	Source
A. *B. subtilis*			
GB43	MB 228	su^{3+}, *leu*8(*met*B *thr*-5)	C.P. Georgopoulis
GB1115	GB43 (Ø105J*sus*11)	GB43, lysogenic for Ø105J*sus*11	B.M. Scher
GB7018	CU229	*ilv*B2 *trp*C2	S. Zahler
GB7022	CU258	*leu*A5 *ilv*B2 *trp*C2	S. Zahler
GB7037	CU614	*leu*A169 *trp*C2	S. Zahler
GB7044		*leu*A169 *ilv*B2	R. Phillips[a]
GB7047		*leu*A169	R. Phillips[b]
GB7051	IS78	*thr*-5 *trp*C2 *rif*-1	I. Smith
GB7054	CU405	*ilv*A8 *thy*A	S. Zahler
GB7055		*thy*A	R. Phillips[c]
GB7056		*thy*A *thy*B	R. Phillips[d]
GB7057	BD204	*thy*A *thy*B *his*B2	D. Dubnau
GB7058	168(Ø3T)	168 lysogenic for Ø3T	D. Dean
GB7059	DB52(Ø3T)	BR52, lysogenic for Ø3T*thy*P3	D. Dean
GB7060	BC109	*trp*C2 *thy*A *thy*B	C. Orego
GB7061	BC109(*rho*11)	BC109 lysogenic for *rho*11	C. Orego
GB7062	BC108(*rho*11 *thy*P3531)	BC108 lysogenic for rho11 *thy*P3531	C. Orego
GB7068	SB1158	*ilv*D6 *thy*B Δ*thy*A	J. Lederberg
GB7071		Δ*thy*A	R. Phillips[e]
GB7073		*ilv*A8	R. Phillips[f]
GB7074		*ilv*A8 Δ*thy*A	R. Phillips[g]

Table 1. (Cont'd)

GB7077	Prototroph	R. Phillips
BD170 (Ø105)	*trp*C2 *thr*-5 lysogenic for Ø105	A. Garro
BD99	*his*A *trp*C2 *thr*-5	D. Dubnau
44AO Str^r	*trp*C2 *thy*A *thy*B str^r	A. Garro

B. *S. typhimurium*

TA 98	*his*D3052 *rfa* Δ*uvr*B, pKM101	B.N. Ames
TA 100	*his*C3076 *rfa* Δ*uvr*B, pKM101	B.N. Ames

Footnotes: Table 1

a
Constructed in the following steps: GB7022 was transformed to Leu$^+$ with GB7018 DNA. The *leu*A169 marker from GB7047 was introduced into the Leu$^+$ derivative of GB7018 by congression (Nester, *et al.*, 1963), initially by selecting for Trp$^+$ transformants and then screening these for the Leu$^-$,Ilv$^-$ phenotype.

b
Constructed by transforming GB7037 with prototrophic DNA and selecting for Trp$^+$ transformants.

c
Constructed by transforming GB7054 with GB7057 DNA, and selecting for Ilv$^+$,Thy$^+$ transformants which were Trm^s at 37°C, but Trm^r at 46°C on plates containing thymidine. This selection is based on the following rationale. *B. subtilis* genes *thy*A and *thy*B both code for thymidylate synthetases (Neuhard, 1978). Mutations in both of these genes, or inactivation of their gene products are required to create a thymidine requirement, and at the time enable *B. subtilis* to be resistant to trimethoprim when exogenous thymidine is provided. The *thy*B gene product is temperature sensitive, and therefore at 46°C, 7055 *thy*A *thy*B$^+$ is phenotypically Trm^r.

d
Constructed by transforming GB7054 with GB7057 DNA and selecting transformants which were Ilv$^+$,Trm^r at 37°C on thymidine supplemented plates.

e
Constructed by transforming GB7068 with GB7044 DNA and selecting for Thy$^+$ clones which were screened for Trm^s at 37°C and Trm^r at 46°C on thymidine supplemented plates. These clones were then transformed to Ilv$^+$ with GB7037 DNA, and the Trm^s phenotype at 37°C and Trm^r phenotype at 46°C was confirmed to assure retention of the Δ*thy*A marker.

f
Constructed by transforming GB7054 with BD99 DNA, and selecting for Thy$^+$ at 46°C on plates containing isoleucine and valine. These Thy$^+$ colonies were purified and tested for retention of the Ilv$^-$ phenotype.

g
GB7073 was transformed with GB7071 DNA, selecting for Trm^r at 46°C, on plates supplemented with thymidine. These transformants were purified and the Ilv$^-$ phenotype was confirmed.

[h] Str^r colonies are resistant to 5mg/ml of streptomycin sulfate. 44AO $\underline{Str^s}$ was initially obtained from K. Bott. The Str^r derivative is a spontaneous mutant.

at 181-182°C. (±)*syn* and (±)*anti* benzo(a)pyrene diolepoxide was supplied by the National Cancer Institute, and Dr. B. Weinstein, and N-acetoxy-2-acetylaminofluorene was kindly supplied by Drs. E. and J. Miller. 2-bromo-2-chloro-1,1-difluoroethylene (BCD) was obtained from Columbia Organic Chemicals Co., Columbia, S.C.

C. Media and Growth of Bacteria and Phage.

1. Media

In the reversion assay, transformants were selected on minimal media containing Spizizen's salts (Spizizen, 1958) plus 0.5% glucose, 25µg/ml of asparagine, 25µg/ml of glutamic acid, and 50µg/ml of required amino acids (Copeland and Marmur, 1968). Media were solidified with 2% Difco agar, except for top agars which contained 0.6% agar. Plates used to select for trimethoprim resistant transformants either were prepared with 50µg/ml of thymidine and 7.5µg/ml of trimethoprim, or were supplemented with trimethoprim by layering top agar containing the drug onto the plates after transformants had been spread. In the sporulation assay, Rif^r transformants were selected on sporulation medium (Bott and Davidoff-Abelson, 1966) containing 10µg/ml of rifampin. The plates used to measure total viable cells consisted of 3.3% tryptose blood agar broth, 0.5% agar, 4mM $MgSO_4$ and 1.5 x 10^{-5}M $MnCl_2$ (TBAB plates).

Plaques were assayed by the standard overlay procedure (Adams, 1959), using a medium containing 1% Bacto tryptone broth, 0.5% NaCl, 10mM $MgSO_4$, 0.2% maltose and 1 mg/ml of strep-

tomycin. This medium (MB-Str) was solidified with 1% agar while the corresponding top agar contained 0.6% agar. In order to keep the plates moist prior to use, they were wrapped tightly in plastic bags and stored at 4°C. VY medium consisted of 2.5% Veal Infusion, plus 0.5% Yeast Extract in distilled water.

2. Growth of Cells to Competence.

Cells were made competent for uptake of DNA by the method described by Rudner and Remeza (1973) which is a modification of the two-step growth procedure of Anagnostopoulos and Spizizen (1961). Because of the convenience offered by preparing large batches of cells which could be stored in small aliquots for subsequent use, following growth to competence the cells were pelleted by centrifugation and resuspended in 0.1 volume of the culture supernatant which had been made 10%, v/v glycerol. After shaking for 5 min at 37°C, the cells were distributed in 1 or 2 ml fractions to disposable plastic tubes and then frozen in a dry ice-ethyl alcohol bath before storing at -80°C.

3. Preparation of Ø105J*sus*11, Ø3T and *rho*11 lysates.

Ø105J*sus*11 phage was isolated by induction of GB43 (Ø105J*sus*11) by the addition of mitomycin C at a concentration of 0.4µg/ml to exponentially growing cells (Garro, 1973). The spontaneous reversion frequency of each Ø105J*sus*11 lysate was determined by plaque assay on the permissive and restrictive cell lines GB43su$^+$ and 44AOsu$^-$ respectively. Ø3T and

*rho*11 lysates were prepared by mitomycin C induction of 168(Ø3T) and BC109(*rho*11) respectively. Confirmation that these phages carried a thymidylate synthetase gene was obtained by transducing GB7057 *thy*A *thy*B *his*B2, to *Thy*$^+$ with each of the lysates, using the procedure described by Tucker (1969). In essence, exponentially growing GB7057 were infected with either phage Ø3T or *rho*11 in VY at a multiplicity of infection (MOI) of 5. After shaking for 30 min at 37°C the infected cells were pelleted, resuspended in Spizizen salts, and streaked on minimal media supplemented only with histidine, which only allowed growth of GB7057 that had been converted to Thy$^+$.

D. Preparation of Bacterial DNA and Replicating Ø105 DNA.

Bacterial DNA was isolated by a modification of a procedure described by Dubnau and Davidoff-Abelson (1971). Cells were grown in VY to mid-exponential phase and lysed by treatment with 1mg/ml lysozyme at 37°C for about 15 min. The crude cell lysate was first treated with 1mg/ml of pronase, and then with redistilled phenol, saturated with BPES buffer (Crothers and Zimm, 1967), pH 7.8, in order to separate nucleic acids from other macromolecules. BPES buffer consists of 0.15M NaCl, 0.001M EDTA, 0.006M Na_2HPO_4, 0.002M NaH_2PO_4, 0.1M mercaptoethanol, pH 7.8. Phenol was removed from the DNA preparations by dialysis against SSC/10 at 4°C. RNA was then digested with 50µg/ml of pancreatic RNase and 5 units of T_1 RNase for 30 min at 37°C, and then the phenol treatment

was repeated.

Replicating Ø105 DNA was isolated either from mitomycin C induced cultures of the lysogen BD170(Ø105) or from Ø105 infected 44AO cells. In the case of lysogen DNA, mitomycin C was added to BD170(Ø105) at a concentration of 0.4µg/ml and the culture was shaken for 45 min at 37°C, during which time the prophage DNA begins to replicate (Rutberg, 1973). The cells were lysed by the addition of lysozyme and Ø105 lysogen DNA was then isolated in the same manner as described above for cellular DNA. In order to isolate replicating Ø105 wild-type DNA or replicating Ø105 DII:6c DNA from infected cells, a culture of 44AO, growing exponentially in VY-0.01M NaCl, was infected at an MOI of approximately 1.5 with either Ø105 phage or DII:6c phage. Twenty min later the cells were diluted 1:10 into fresh, warmed VY, and then shaken for an additional 30 min at 37°C. At this time the DNA was isolated in the same manner as described above for cellular DNA.

E. Isolation of Mature Ø105, Ø3T and *rho*11 DNA.

Phage lysates were prepared by mitomycin C induction of the respective lysogens (Garro, 1973) and the lysates treated with ribonuclease and deoxyribonuclease in order to digest cellular nucleic acids. The phage particles were concentrated by polyethylene glycol precipitation (Yamamoto, *et al.*, 1970) and purified by banding in a CsCl step gradient (Scher, *et al.*, 1977). The purified phage were dialyzed overnight against 2,000 volumes of phage diluent consisting of 0.01M Tris (pH

7.4), 5 x 10^{-3}M $MgCl_2$, 2 x 10^{-5}M $MnCl_2$ and 0.01 mg of gelatin per ml. The DNAs were extracted by pronase digestion, followed by treatment with BPES-buffer saturated phenol, pH 7.8. Phenol was removed from the DNA preparations by dialysis against SSC/10 at 4°C.

F. Reversion of the Tester Strains.

In order to confirm the genotype of the GB7018 *ilv*B2 *trp*C2, and GB7037 *leu*A169 *trp*C2 strains, their ability to be reverted by MNNG and 9AA respectively was periodically checked. The *ilv*B2 mutation had been created by MNNG mutagenesis (Ward and Zahler, 1973), and was revertable in strain GB7018 by the same mutagen. Our initial characterization of the GB7037 strain indicated that the *leu*A169 marker could be reverted by 9AA, indicating that it was a frame-shift mutation.

To test reversion of the *leu*A169 marker in strain 7037 2.5 ml of molten top agar, containing 0.5% glucose, 5µg/ml of leucine and 50µg/ml of tryptophan was seeded with 0.1 ml of an overnight culture and layered on minimal Spizizen plates supplemented with 50µg/ml of tryptophan. The minimal amount of leucine (3.5µM final) was sufficient to allow a few rounds of GB7037 multiplication, but unless the cells were exposed to an appropriate mutagen, only a small number of spontaneous Leu^+ revertants which could form colonies would arise. However, when a disc containing a mutagen such 9AA (25µg/ml) dissolved in EtOH or DMF, was placed in the

center of the plates, several hundred Leu^+ colonies could be observed immediately adjacent to a zone of inhibition around the disc after incubation at 37°C for 48 hours. When more quantitative analysis was desired, the mutagen was incorporated in the top agar at various concentrations so that a dose response could be obtained. Similar procedures were used to test reversion of the *ilv*B2 marker in GB7018, except that trace amounts of isoleucine and valine were not required in this case. When MNNG was used as the test mutagen, discs were impregnated with 10µg/ml of the mutagen dissolved in DMSO or DMF.

Reversion tests with the Ames *Salmonella* strains were carried out by the procedure described by Ames, *et al.* (1975). 2.5 ml of molten top agar, consisting of 0.6% agar, 0.09M NaCl, and 0.05mM of histidine and biotin, was seeded with overnight cultures of either TA98, TA100 or TA1537, and the mutagen was then added. The top agar was layered on minimal plates to yield a final histidine and biotin concentration of 5uM. After 48 hours of growth at 37°C the number of revertant colonies per plate were scored.

G. Transformation.

Transformations were carried out in medium optimized for maximum uptake of either single or double-stranded DNA. The medium for double-stranded DNA was that described by Rudner *et al.* (1967) and consisted of the standard Spizizen's salts supplemented with 0.5% glucose, 0.05% yeast extract,

0.01% casamino acids, 5 x 10^{-4}M spermine tetrahydrochloride, 10^{-3}M $CaCl_2$, 2.5 x 10^{-3}M $MgCl_2$ and 50µg/ml of required amino acids. In this medium single-stranded DNA transforms only 1% as efficiently as double-stranded DNA for any given marker. Tevethia and Mandel (1970, 1971) reported that by withholding $CaCl_2$ from the transformation medium and either adding EDTA to 10^{-3}M or simply lowering the pH to 6.0, transformation with single-stranded DNA became 40% as efficient as that with double-stranded DNA. Furthermore, they showed that a single stranded nuclease was produced by competent cells, and they conjectured that this endonuclease used Ca^{++} as a cofactor, had a pH optimum near 7.0, and rapidly lost activity in more acidic medium. Dubnau (personal communication) found that a combination of the lower pH and 10^{-3}M EDTA yielded the most efficient transformation with single-stranded DNA, and therefore our transformation medium for single-stranded transformation consisted of Spizizen salts supplemented with 0.5% glucose, 0.05% yeast extract, 0.01% casamino acids, 2.5 x 10^{-3}M $MgCl_2$, 10^{-3}M EDTA, 50µg/ml required amino acids, and a final pH of 6.0. Single-stranded transformation medium was prepared immediately before use.

For transformation, competent cells were thawed and diluted 1:5 into the transformation medium. DNA was added to a final concentration of 10µg/ml, which is below saturation, and the transformation mixture was shaken at 37°C for 45 min. When the donor DNA was chemically modified GB7018 DNA *ilv*B2 *trp*C2 and the recipient competent cell was

GB7044 *ilv*B2 *leu*A169, Leu$^+$ single marker transformants were selected by streaking the transformation mixture on minimal plates supplemented with 50 µg/ml of tryptophan, 50µg/ml of isoleucine and 50µg/ml of valine. Ilv$^+$, Leu$^+$ double marker transformants were selected by streaking 0.1ml of a 10 fold concentrate of the transformation mixture on minimal plates supplemented only with 50µg/ml of tryptophan. After incubation at 37°C for 48 hours, Leu$^+$ and Ilv$^+$, Leu$^+$ clones were scored. When the donor DNA was chemically modified GB7037 *leu*A169 *trp*C2 and the recipient was GB7044 *ilv*B2 *leu*A169, Ilv$^+$ single marker transformants were selected by streaking the transformation mixture on minimal plates supplemented with 50µg/ml of tryptophan and 50µg/ml of leucine. Ilv$^+$, Leu$^+$ double marker transformants were selected by seeding top agar supplemented with 0.5% glucose, 5µg/ml of leucine, and 50µg/ml of tryptophan with 0.1ml of a 10-fold concentration of the transformation mix, and layering this mixture on minimal plates supplemented with 50ug/ml of tryptophan. After incubation at 37°C for 48 hours, Ilv$^+$ and Ilv$^+$,Leu$^+$clones were scored.

The number of Ilv$^+$, Leu$^+$ transformants per plate was expressed directly, or as Ilv$^+$,Leu$^+$/Leu$^+$ when the transforming DNA was GB7018, and as Ilv$^+$,Leu$^+$/Ilv$^+$ when the transforming DNA was GB7037. Dividing the double marker transformants by the number of single marker transformants corrected for variations in double marker transformants due to differences in single marker transforming activity of each particular

DNA sample.

In the sporulation assay, competent cells first were exposed to mutagen treated DNA for 45 min and then were transferred to VY and allowed to grow for 3 hours before streaking on sporulation plates containing rifampin. The growth in VY allowed time for expression of the Rif^r phenotype, which is due to an altered RNA polymerase subunit (Heil and Zillig, 1970).

When transforming cells to Trm^r, competent cells were exposed to DNA for 45 min at 37°C, and then either directly streaked on plates containing trimethoprim, or were first grown for 16 hours at room temperature on minimal plates, and then exposed to trimethoprim. This later procedure was used in the event that an expression time was needed for turnover of the wild-type thymidylate synthetase.

H. Measurement of Gene Linkage.

Gene linkage was monitored in some experiments to determine if the test compound was capable of inducing lesions in DNA which produced strand scission. Since the appearance of Ilv^+, Leu^+ colonies is principally dependent upon the recipient cells acquiring the linked *ilv*B and *leu*A markers from the donor DNA, and the marker rescue assay is dependent upon simultaneous acquisition of the J^+ gene and the immunity region, it was important to know if mutagenesis was being missed because of decreases in gene linkage.

Gene linkage can be monitored by comparing the frequency of simultaneous acquisition of two known, linked genetic markers to the independent acquisition of each of the two genetic markers. If donor markers are designated "1" and recipient markers as "0", then the cotransformation index, r, is described as (Nester and Lederberg, 1961):

$$r = \frac{11}{10 + 01 + 11}$$

Typically after transformation, colonies were streaked on media which selected for "10" and "11", and no selection was made for "01" because it was equal to "10" in the linkage groups used. The selection method did not distinguish double transformants among the singles, and hence a sum of the single transformants would count the number of double transformants twice. To correct for this, r was calculated in the following manner:

$$r = \frac{11}{2(10) - 11}$$

where $11 = Ilv^{+}Thy^{+}$
$10 = Thy^{+}$

Disruption of linkage between genes in mutagen treated GB7018 *ilv*B2 *trp*C2 and GB7037 *leu*A169 *trp*C2 DNAs was determined by transforming GB7068 *ilv*D6 *thy*B Δ*thy*A with the treated DNAs and selecting for Ilv^{+}, Thy^{+} and Thy^{+} transformants.

The *ilv*D and the *thy*B genes are known to be linked, and exhibit a typical r value of 0.25.

Disruption of linkage in the marker rescue assay was determined in a similar manner. Ø105DII:6c carries a deletion in the immunity region which gives this phage a clear-plaquing phenotype. When this DNA is used in a marker rescue assay with *Jsus*11, approximately 45% of the plaques will be clear. However, as single strand nicks are introduced into this DNA, the percentage of clear plaques among the rescued phage decreases because of the disruption of linkage between the J gene and the immunity region. After reacting test chemicals with replicating Ø105DII:6c DNA the DNA was used in a marker rescue. The effect of treatment was measured by determining the frequency of clear plaquing phage among the total PFU's.

I. Marker Rescue.

The production of mutations in the immunity region was scored by a modification of the marker rescue procedure described by Armentrout and Rutberg (1970). Competent BD99su$^-$ (0.5ml) suspended in double-stranded transformation media were infected at a multiplicity of approximately 5 with Ø105 *Jsus*11 phage. After shaking for 15 min at 37°C the cells were centrifuged and resuspended in either double or single-stranded transformation media depending on the conformation of the rescuing DNA. Infected cells were exposed to treated DNA at a final concentration of 1-5μg/ml and shaken for 30 min at

37°C. After the addition of 5ml of VY,the cells were pelleted, resuspended in 2ml of VY, and then shaken for an additional 210 min to allow for phage replication. The phage produced were then diluted and mixed with 44AO $Str^r su^-$ in molten MB-str top agar. This mixture was then layered on MB-str plates. After 18 hours of growth at 37°C turbid and clear plaques were scored.

J. Treatment of DNA with Mutagens.

1. Reactions in DMF.

Immediately prior to the addition of the test chemical, DMF was added at room temperature to 200µg/ml of DNA in SSC/10, to a final concentration of 46-50%DMF. To avoid precipitation of the DNA during addition of the DMF, the solvent was added in 0.1ml aliquots accompanied by shaking. The final pH of this DMF-SSC/10 solution was 6.1. If the DNA were to be reacted in single-stranded form, the DNA-DMF mixture was warmed in a screw-capped tube for ten minutes at 50°C, and then placed on ice. For time course experiments, the test compound, dissolved in DMF, was added to the DMF-DNA mixture and then incubated at the reaction temperature. For dose response experiments, aliquots of the DMF-DNA mixture were distributed to sterile screw-capped glass vials or to sterile, capped, polyethylene tubes which were resistant to DMF. The pH of the reactions was monitored and consistently remained at pH 6.1.

Reactions were stopped by mixing 0.1-0.5ml of the DNA reaction mixture with 0.5ml of a 200µg/ml solution of *E. coli*

tRNA in 2X SSC. The nucleic acids were precipitated by the addition of 2 volumes of ice-cold ethanol. The precipitate was chilled on ice for 10 min, and then pelleted by centrifugation at 7,000 rpm in a Sorvall SS34 rotor. The DNA-RNA pellet was washed with 5 ml of cold ethanol. The nucleic acids were redissolved in 0.5ml of SSC/10 to give a final concentration of 100µg/ml for GB7018, GB7037, and GB7054 DNA, and 20µg/ml for replicating Ø105 DNA.

2. Reactions in Aqueous Buffer.

The only reaction carried out in aqueous buffer was between DNA and hydroxylamine. The procedure was a modification of that described by Freese and Strack (1962). A 2M solution of HA was prepared by mixing 278mg of HA.HCl with 1.1ml of H_2O, 0.3ml of 5N NaOH, and 0.4ml of 0.2M sodium phosphate buffer, pH 6.2. This solution was sterile filtered and kept warm to avoid formation of a precipitate. A control solution consisted of 5.84 g of NaCl mixed with 40.0ml of H_2O and 10.0ml of 0.2M sodium phosphate buffer, pH 6.2. To start the reaction the HA solution was added to DNA in equal volumes to achieve a 1M solution HA. Samples were removed at various time points and the reaction was stopped either by diluting the reaction mixture into a solution consisting of 10% acetone, 1M NaCl and 0.05M Tris buffer, pH 7.4 or by precipitating the DNA with ethanol. When the reactions were stopped by the acetone mixture, an additional control was

included in which DNA was first added to the acetone mixture at 4°C and then an amount of hydroxylamine equivalent to that used in the experiment was added in the cold.

Base-denatured DNA (single-stranded) was prepared by the addition of 0.1 volume of 1N NaOH to double stranded DNA, which was kept on ice for 10 min, and then neutralized by the addition of 1.0M NaH_2PO_4.

K. Determination of DNA Melting Temperatures.

The absorbance-temperature profile of DNA dissolved in dimethylformamide was determined with a Gilford 2400 spectrophotomer. The solution temperature was raised at the rate of 0.8°C per minute.

L. Preparation of Rat Liver Microsomes.

Rat liver microsomes were prepared from Aroclor 1254 pretreated Sprague Dawley rats by the procedure described by Czygan, *et al.* (1973). Following isolation of the rat livers a homogenate was prepared in 1mM EDTA, 0.1 M potassium phosphate buffer, pH 7.4. Undamaged cells, nuclei and large plasma membrane fragments were pelleted by centrifugation for 10 min at 800 x g, and mitochondria were pelleted by centrifugation for 10 min at 20,000 g. The resulting supernatant was centrifuged at 105,000 g for one hour. The pellet was washed with and resuspended in 0.1M potassium phosphate buffer, pH 7.4, and then centrifuged again at 105,000 x g for one hour. The pellet from this last centrifugation was the microsomal fraction. The protein content of this fraction was measured by the biuret reaction (Layne, 1957).

M. Statistical Analysis.

Three types of statistical analysis were used to evaluate the significance of the results obtained with the reversion assay. The first, analysis of variance (Armitage, 1971) was used to determine whether an increase in the number of Ilv^+, Leu^+ transformants generated by the treated DNA samples was significantly different from the number of Ilv^+, Leu^+ transformants generated by the control DNA. If it was established by analysis of variance that the treated DNA samples generated more Ilv^+, Leu^+ transformants than the control DNA samples, then a second test was performed to determine if the numbers of mutants produced by DNA samples treated either with different doses of mutagen or length of mutagen exposure differed significantly from each other. In this latter test, called Duncan's multiple range test (Duncan, 1955), the mean number of Ilv^+,Leu^+ transformants obtained with each DNA sample, including the control was compared to every other group, to determine whether or not two groups were statistically different from each other. The level of significance of this test is set at the $p=0.05$ level. One of the underlying assumptions of both analysis of variance and Duncan's multiple range test is that the standard deviations in the various groups are approximately equal. When the standard deviations between various groups in an experiment were grossly different, the data were converted to $\log_e$, a procedure which tends to reduce the range in the standard deviations. In the data obtained in the reversion assay, however, the only time that

major differences in standard deviations between groups occurred was when there was such a large increase in the number of Ilv^+,Leu^+ colonies that logarithmic conversion did not effect the statistical significance of the tests. The third test, the paired t-test (Armitage, 1971) tests whether differences observed between two means is significant. It is applicable only when the samples being compared are matchable member for member.

The data generated in the marker rescue mutagenesis assay was analyzed by the Poisson heterogeneity test ("Student", 1907). This procedure is suited for testing the significance of data which measures increases or decreases in rarely occurring events, for which means of particular treatment groups are not established. In the case of the marker rescue assay, the analysis determined whether the different frequencies of clear-plaquing phage, obtained with the mutagen treated DNAs, differed significantly from the control samples.

N. Use of DMSO as a Solvent

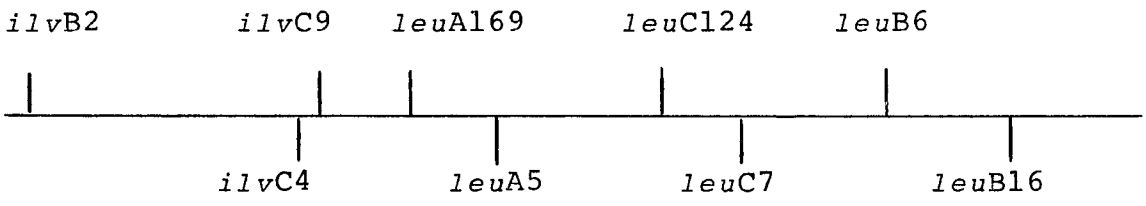
Since DMSO was known to interact with several compounds, including 2-bromo-2-chloro-1,1-difluoroethylene, it was not used either as a solvent for DNA nor as a diluent for compounds used in these studies.

CHAPTER III

RESULTS

A. Construction of a Recipient Strain for the Reversion Assay.

As indicated in the introduction, reversion assays require indicator organisms capable of detecting base substitution and frame shift mutagens. With respect to the proposed *in vitro* mutagenesis assay it therefore was desirable to have a strain carrying closely linked base substitution and frame-shift mutations, which could be used as the recipient for all transformations with the chemically tested DNAs. Ward and Zahler (1973) had generated a collection of mutants with lesions in several different genes of the isoleucine-valine and leucine biosynthetic pathways by using a variety of mutagens. Their mapping data indicated that there was tight linkage between the *ilv*B, *ilv*C, *leu*A, *leu*C, and *leu*B genes as shown below.



Since many of these mutations were uncharacterized with regard to the type of lesion they carried, *i.e.* base-substitution mutation or frame-shift mutation, the ability of the base-substitution mutagen MNNG and the frame-shift mutagen 9AA to revert strains carrying either *ilv*B2, *ilv*B3,

*ilv*B6, *ilv*C4, *ilv*C9, *leu*A5, *leu*A169, *leu*C7, *leu*C124, *leu*B6, or *leu*B16 was tested. Note that not all the mutations have been ordered within each gene, and that therefore some do not appear on the above map. The two strains which gave a high frequency of reversion were GB7018, which carries the *ilv*B2 mutation, and was reverted by MNNG, and GB7037, which carries the *leu*A169 mutation, and was reverted by 9AA. The reported cotransformation index for *leu*A5 and *ilv*B2 was r=0.4, and reference to the map above indicates that *ilv*B2 and *leu*A169 would be even more tightly linked. Therefore it was decided to construct a strain carrying both the *ilv*B2 and *leu*A169 mutations.

Since preliminary tests indicated that 7018 *ilv*B2 *trp*C2 was highly transformable, it was advantageous to introduce the *leu*A169 marker into GB7018. It is difficult, however, to directly select for a transformant that has obtained a marker such as *leu*A169 which renders it auxotrophic for an amino acid. This problem was circumvented in the following manner. First, a Trp^+ derivative of GB7037 *leu*A169 *trp*C2, designated GB7047, was prepared by transformation of GB7037 with DNA from the prototrophic strain GB7077. Reversion tests confirmed that 7047 retained the *leu*A169 mutation. Strain GB7018 *ilv*B2 *trp*C2 was then transformed with 40ug/ml of GB7047 *leu*A169 DNA, and Trp^+ transformants were selected. The use of this saturating concentration of DNA in the transformation increased the probability that a single competent

cell would be transformed simultaneously for unlinked genetic markers, such as *trp*C2 and *leu*A169, a process referred to as congression (Nester, *et al.*, 1963). The Trp^+ GB7018 transformants were screened for conversion to a Leu^- phenotype by replicating the Trp^+ colonies onto plates which lacked leucine. Of 688 Trp^+ colonies tested, one was found which failed to grow without leucine supplementation. This isolate was tested to confirm that it carried the *ilv*B2 and *leu*A169 markers by assaying its revertability to Ilv^+ with MNNG and to Leu^+ with 9AA, and by measuring the cotransformation index of the *ilv* and *leu* markers. A typical r value for the contransformation of the isolate to an Ilv^+, Leu^+ phenotype was 0.6, and approximately 1% of the cells became competent for the uptake of exogenous DNA.

B. Development of the Ø105 Marker Rescue Forward Mutation Assay.

1. Efficiency of Marker Rescue vs. Transfection with UV-Irradiated Replicative Form Ø105 DNA.

Theoretically, a forward mutation assay which measured as an end-point the production of clear plaque mutants following the *in vitro* exposure of Ø105 DNA to mutagenic compounds could have been based either on transfection or on marker rescue. It was known that the transfecting activity of undamaged replicative form Ø105 DNA was nearly as high as the marker rescue activity of that DNA (Rutberg and Rutberg, 1970). Therefore, it was possible that either transfection

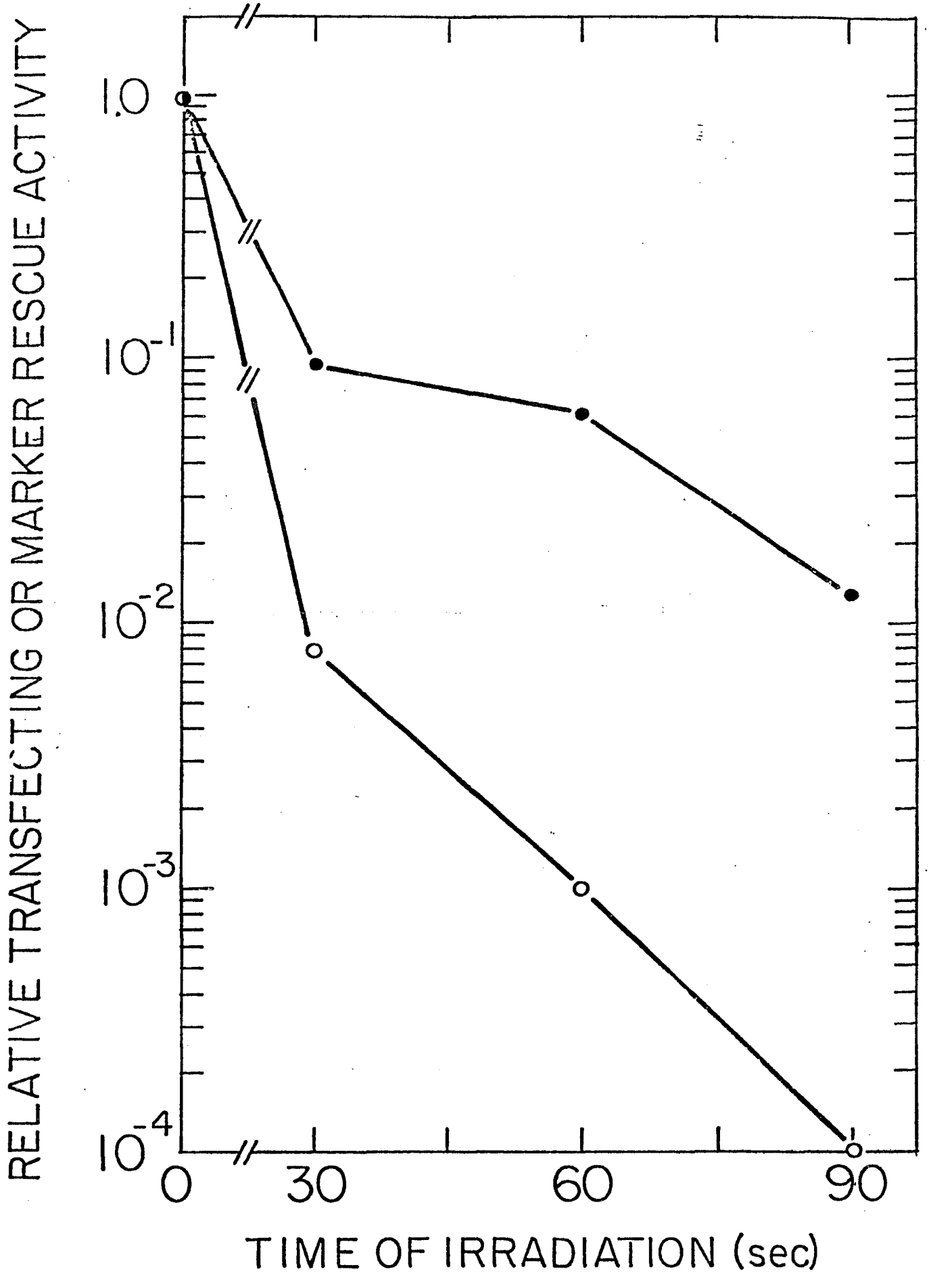
or marker rescue would be able to generate the large number of phage which would be necessary to detect a rarely occurring mutant. However, since all the essential genes of Ø105 must survive chemical treatment in order for DNA to be active in transfection, we were concerned that as the test DNA became damaged by reaction with a mutagen, its transfecting activity would be rapidly lost. Marker rescue, on the other hand, requires that only a single gene, the one being selected for, remain intact. It seemed reasonable therefore that marker rescue would be less susceptible to inactivation by mutagenic compounds than transfecting activity.

In Fig. 3 are shown the results of an experiment in which the transfecting and marker rescue activities of UV-irradiated replicating Ø105 DNA was measured. Transfecting activity at various doses of UV is 10-100 times lower than the marker rescue activity with the same DNA. This experiment suggested that marker rescue would provide a more efficient means to detect mutations in replicating Ø105 DNA.

2. Determination of the Latent Period for Marker Rescue.

In the marker rescue procedure described by Armentrout and Rutberg (1970), 15 min post addition of phage and rescuing DNA the competent cells were plated with cells which form the lawn for plaque development. Since 15 min is shorter than Ø105's latent period, even if a rescue event generating a clear plaque mutant did occur in the assay, both mutant and wild-type phages would be liberated within the same burst and

Fig. 3. Relative transfecting activity (o) and marker rescue activity (●) of UV-irradiated replicating Ø105 lysogen DNA. DNA in SSC/10 at 10μg/ml was placed in sterile glass petri dishes containing a magnetic stirrer. The DNA was irradiated with approximately 10 erg/mm^2/sec UV light generated from a General Electric Short Wave UV lamp. Samples of DNA were removed at the times shown and used either to transfect BD99 or diluted tenfold and used to rescue the *Jsus*11 marker. Plaque forming units were assayed on 44A0 Str^r. In the marker rescue and transfection assays, the PFU/ml of the 0 sec samples were 3.1×10^7 and 1.1×10^7 respectively.


RELATIVE TRANSFECTING OR MARKER RESCUE ACTIVITY
1.0
10-1
10-2
10-3
10-4
0
30
60
90
TIME OF IRRADIATION (sec)

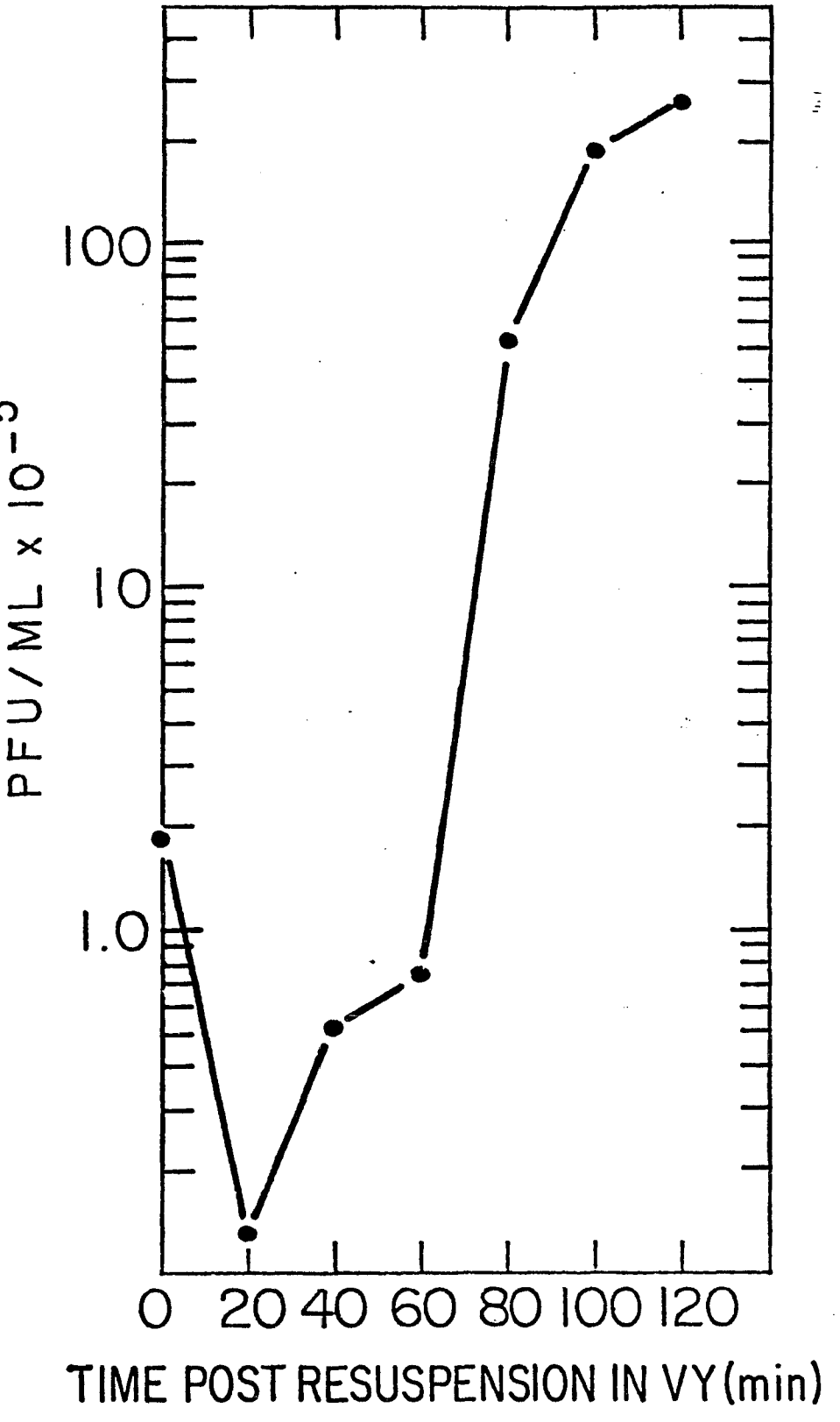
be within the same developing plaque. This was of serious concern since Scher *et al*. (1978) had shown that wild-type phage were able to complement clear-plaquing phage, thus generating lysogenized cells. If such complementation occurred within a developing clear plaque, the plaque would contain lysogenized cells and thus appear turbid. This would obscure the detection of clear-plaquing phage in the total population, and decrease the sensitivity of the assay. It was decided, therefore, to determine when the phage burst occurred from the competent cells so that the plaques obtained would be the result of a single free phage particle.

In the experiment shown in Fig. 4, host cells were infected with double-stranded Ø105 DNA, shaken for 15 min at 37°C, superinfected with J*sus*11 phage, and shaken for an additional 15 min. The cells were then pelleted, washed with Spizizin salts, and resuspended in warm VY, and shaken at 37°C. At the time intervals indicated, aliquots were removed and sampled for PFU. A steep rise in PFU was observed starting at about 60 min and ending at about 120 min. Based on these data it was decided to resuspend infected cells in VY and shake them for an additional 120 min before assaying for PFUs.

3. Determination of Optimum Conditions for Marker Rescue with Single-Stranded DNA.

During marker rescue the competent cells normally are first exposed to the DNA and then after 15 min are infected with phage in the same media. As discussed in Methods, com-

Fig. 4. Kinetics of phage release during marker rescue. Replicating Ø105 lysogen DNA was added at a concentration of 7.5 µg/ml to competent GB7044 cells and incubated with shaking at 37°C for 15 min. Ø105 *Jsus*11 was added to a multiplicity of infection (MOI) of approximately 5 and the incubation continued for another 15 min. The cells were then pelleted, washed with Spizizen salts, and then resuspended in warm VY. The cells were then shaken at 37°C, and at the times indicated in the figure, samples were removed and assayed for PFU activity on 44AO Str^r.


100
10
1.0
PFU/ML x 10⁻⁵
0 20 40 60 80 100 120
TIME POST RESUSPENSION IN VY (min)

petent cells efficiently take up single-stranded DNA in transformation media which is 10^{-3}M EDTA and pH 6.0. However, these are not the optimal conditions for phage infection. A series of experiments therefore were undertaken to develop conditions for marker rescue with single-stranded DNA which would yield sufficiently high numbers of plaques to monitor the single stranded DNA for induced forward mutations. The first approach taken was to determine the effect of the single-stranded medium on marker rescue. DNA was added to cells in a single-stranded transformation medium, and shaken for 15 min at 37°C. The cell suspension was then divided in half, and phage added directly to one portion. The second portion was centrifuged and resuspended in VY-0.01M NaCl before phage addition. PFU were assayed 120 min later. PFU arising from each sample are given in Table 2, and also shown are the effects of the centrifugation and resuspension steps on the efficiency of marker rescue. The results showed that while centrifugation and resuspension adversely affected the efficiency of double-stranded marker rescue, the modification improved single-stranded marker rescue 7 fold. However, the efficiency of single-stranded marker rescue was still several fold lower than the double-stranded marker rescue.

It thus appeared that prior exposure of cells to single-stranded transformation medium was adversely affecting either phage adsorption or replication. The effect of reversing the order of addition of phage and DNA was then examined so that the phage could adsorb and begin to replicate prior to exposure

Table 2. Effect of Resuspension of BD99 in VY-0.01M NaCl Prior to Phage Infection on the Efficiency of Marker Rescue.

DNA Conformation	Cell Treatment Following Addition of DNA	PFU/ml x 10^{-6}
Double-stranded	Centrifuged and resuspended	2.7
Double-stranded	Not treated	9.3
Single-stranded	Centrifuged and resuspended	0.7
Single-stranded	Not treated	0.1

Double and single-stranded replicating Ø105 DNA was added to competent BD99 cells in single and double-stranded transformation medium, respectively, and shaken for 15 min at 37°C. Each cell suspension was then divided in half. Phage were added directly to one portion, while the second portion received phage after the cell suspension was pelleted and resuspended in VY-0.01M NaCl. The cell suspension were shaken for an additional 2 hours and PFU assayed on 44AO.

to single-stranded transformation medium. The results of this experiment are shown in Fig. 5. It can be seen that at 120 min post resuspension in VY the number of PFU generated from the rescue with single-stranded DNA was about 25 fold lower than that from double-stranded DNA. However, if sampling was extended to 210 min, there was only about a three-fold difference between the number of PFU produced using the two types of DNA. In addition, by 210 min there were over 7.3 x 10^8 PFU/ml generated in the single-stranded rescue. There also appear to be two bursts of phage during the 210 min incubation period. Since only 1% of the cells are competent to take up the DNA which contains the wild type allele for *Jsus*11, it is only these cells which contribute to the first burst of wild type phage. These phage, however, are capable of replicating in the total cell population and it is probably the burst from this population which accounts for the second rise in PFU.

4. Detection of Clear PFU.

In the marker rescue assay, any clear plaque mutants generated by rescue of *Jsus*11 with mutagenized DNA would be present within a large population of wild-type turbid-plaquing phage. In order to determine the sensitivity with which the mutant PFU could be detected against a high background of wild type PFU, two types of mixing experiments were conducted. In the first experiment (Table 3), a constant amount of Ø105c4 phage, which has a clear plaque phenotype, was mixed with

Fig. 5. Marker rescue of *Jsus*11 phage with double (o) and single (●) stranded replicating Ø105 DNA. When single-stranded DNA was used, competent BD99 was infected with *Jsus*11 in double-stranded transformation media, shaken for 15 min at 37°C, and then centrifuged and resuspended in single stranded transformation media before exposure to DNA. Thirty min after DNA addition, the cells were centrifuged and resuspended in VY. Incubation was continued at 37°C, and aliquots were removed at the times indicated and assayed for PFU on 44AO Str^r cells. Marker rescue with double-stranded DNA was similiar except that the DNA was added to the cells in double stranded transformation media.

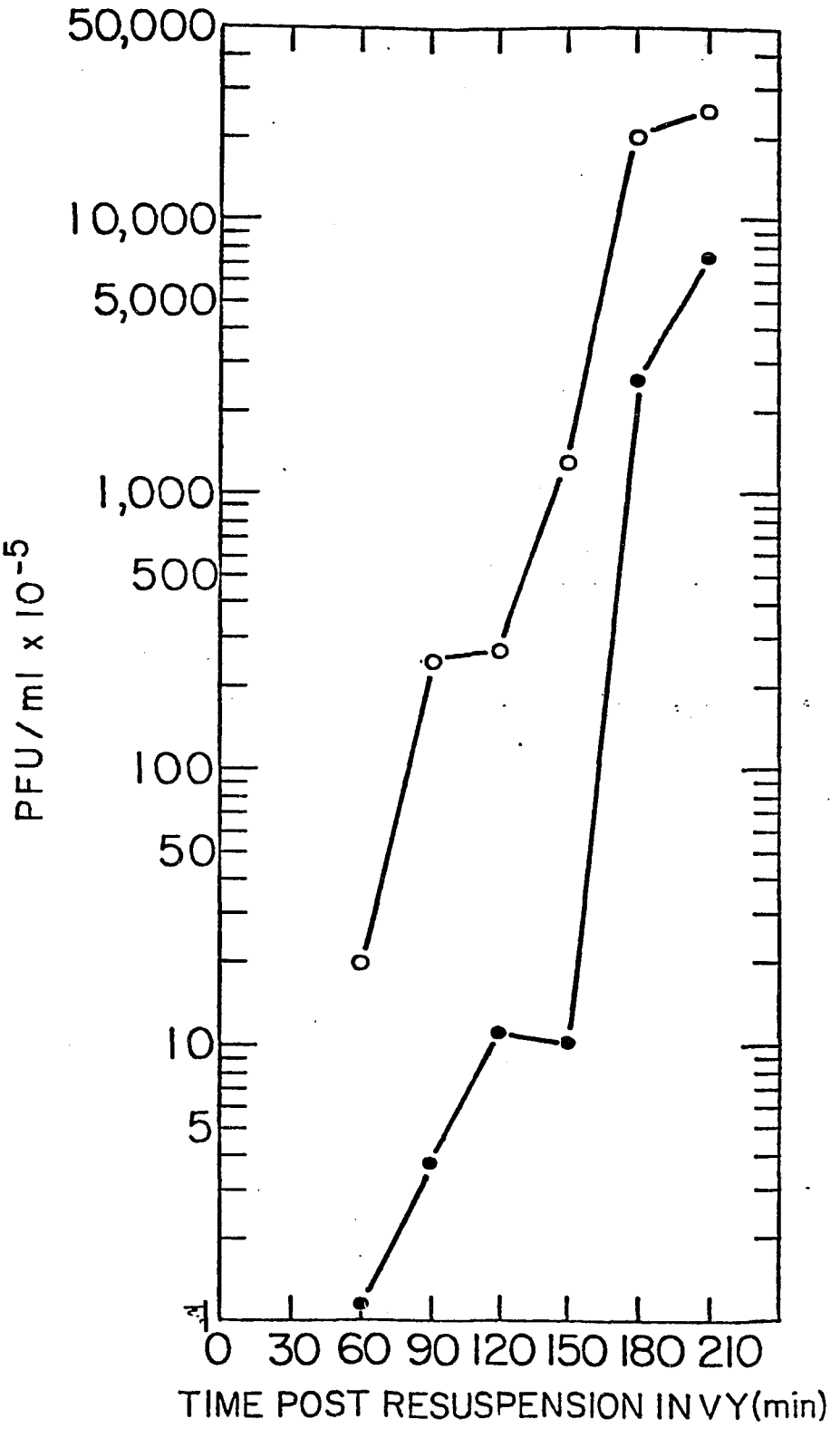

50,000
10,000
5,000
1,000
500
100
50
10
5
1
PFU/ml x 10-5
0 30 60 90 120 150 180 210
TIME POST RESUSPENSION IN VY(min)

Table 3. Detection of Mutant Clear Plaque PFU in the Presence of Increasing Concentrations of Wild-Type Turbid PFU.

Input PFU, c4 Phage	Input PFU, Wild-type Phage	Clear PFU Observed
450	0	442
450	2×10^3	103
450	2×10^4	42
450	2×10^5	3

A constant amount of Ø105c4 phage which has a clear plaquing phenotype, was mixed with increasing concentrations of wild-type phage. The PFU of each of these mixtures was determined on 44AO.

increasing concentrations of wild-type phage. This mixture was then added to top agar previously seeded with 44AO. In the second experiment, (Table 4), a marker rescue was performed with a mixture of wild-type DNA and varying amounts of DNA from the clear plaque mutant Ø105*cng*-2. This type of experiment closely simulates a marker rescue in which DNA exposed to a test chemical includes some mutagenized molecules.

In both of these experiments it can be seen that as the level of wild-type PFU's increased, the ability to detect clear plaques diminished. These experiments demonstrated that the most accurate reflection of the frequency of clear plaques in a population is determined at the highest dilution of phage at which clear plaques are detectable. For example, in Table 3, when no wild-type phage were mixed with the Ø105c4 phage, 442 clear plaque forming units were observed. However, when approximately this same number of clear plaque phage were mixed with increasingly greater numbers of wild-type phage, fewer clears could be detected among the turbids. Similarly, it can be seen in Table 4 that when 2% of the DNA in the marker rescue was *cng*-2, and 98% wild-type DNA, the expected number of clear-plaquing phage were visible at the 10^{-4} dilution of infected cells, but only 20% of the expected number of clears were visible at the 10^{-3} dilution, and only 10% of the expected number were visible at the 10^{-2} dilution. Similar effects were seen when 0.4% of the DNA in the marker rescue was *cng*-2.

Table 4. Detection of Clear PFU Generated in a Marker Rescue of *Jsus*11 Phage by a Mixture of Ø105 Wild-type DNA and Ø105*cng*-2 DNA.

%Wild-type DNA	%*cng*-2 DNA	%Clear PFU at Lysate Dilution of		
		10^{-2}	10^{-3}	10^{-4}
0	100	94	94	94
98	2	0.2	0.4	2.5
99.6	0.4	0.02	0.2	0.8

Competent GB7044 cells were infected with a mixture of *cng*-2 and wild-type Ø105 DNA. After 15 min of shaking at 37°C, the cells were exposed to Ø105*Jsus*11 Phage, and shaken for an additional 15 min. After the addition of anti-Ø105 serum the cells were pelleted by centrifugation, and then resuspended in warm VY. After shaking at 37°C for 140 min, serial tenfold dilutions of the resulting lysates were scored for PFU on 44AO.

C. Development of a Trimethoprim Assay for the Induction of of Thy$^-$ Mutants.

1. Determination of the Optimum Concentration of Trimethoprin for the Selection of Thy$^-$ Clones.

Although it had been reported that *B. subtilis* Thy$^-$ clones could be selected by plating cells in the presence of 10ug/ml of trimethoprim (Neuhard, *et al.*, 1978), the possibility existed that this concentration might be somewhat toxic to Thy$^-$ cells, and thereby reduce the sensitivity of the assay. An experiment was therefore conducted to determine a dose of trimethoprim which would have minimal toxicity for Thy$^-$ colonies, but which would inhibit the growth of Thy$^+$ cells when they were present at 10^7 to 10^8 CFU per plate. This was important because in order to select for rarely occurring transformants that had acquired a Thy$^-$ phenotype following transformation of Thy$^+$ cells with chemically treated DNA, 10^7 to 10^8 Thy$^+$ cells would have to be streaked on the selective plates. If a concentration of trimethoprim allowed a significant number of Thy$^+$ cells to form colonies when these cells were plated at high density,then that trimethoprim concentration could not be used, because the colonies of Thy$^+$ cells would obscure the detection of the rarely occurring Thy$^-$ mutants.

Competent Thy$^+$ GB7055 *thy*A and Thy$^-$ GB7056 *thy*A *thy*B, which are isogenic except for the *thy*B marker, were streaked on plates containing trimethoprim at concentrations ranging from 0.2µg/ml to 10µg/ml,with 50µg/ml of thymidine in each

plate. It should be noted that in order for a cell to be phenotypically Thy^-, both the *thy*A and *thy*B gene products must be inactive. Toxicity to the Thy^- and Thy^+ cells was determined by comparing the colony forming units (CFU) at each particular dose to CFU in the absence of the drug. As can be seen in Table 5, 10µg/ml of trimethoprim completely inhibited growth of the Thy^+ cells, but also reduced the number of Thy^- CFU by 90%. Trimethoprim at 5µg/ml reduced the CFU of the Thy^+ cultures by a factor of 3×10^7, and was not significantly toxic to the Thy^- cells. 1µg/ml of trimethoprim was not toxic to the Thy^- cells, but it only reduced the CFU of the Thy^+ cultures by a factor of 10^3. Further testing, which is not shown here, indicated that 7.5µg/ml reduced the CFU of the Thy^+ cultures by a factor of 4×10^7, and was not toxic to Thy^- cells. This latter concentration was used in all further experiments.

2. Determination of Expression Time for the Appearance of Trm^r Following Transformation of Trm^s Cells.

Ability to grow in the presence of trimethoprim requires inactive *thy*A and *thy*B gene products, which in both cases is a thymidylate synthetase. If Trm^s cells were exposed to trimethoprim immediately after transformation with Thy^- DNA they therefore might be inhibited from growing because of a reservoir of wild-type thymidylate synthetase that could make these cells Trm^s. In order to test this possibility competent GB7055 *thy*A cells were exposed to GB7057 *thy*A *thy*B DNA for 45 min and then either were plated immediately on trimethoprim

Table 5. Colony Production by Thy^+ GB7055 *thy*A and Thy^- GB7056 *thy*A *thy*B, on Plates Containing Various Concentrations of Trimethoprim.[a]

Trimethoprim Concentration µg/ml	Cells	CFU at Culture Dilutions of:		
		10^0	10^{-3}	10^{-5}
0		n.c.[b]	n.c.	580
0.2		n.c.	n.c.	200
1.0	GB7055	$>10^4$	0	0
5.0		2	0	0
10.0		0	0	0
0		n.c.	n.c.	470
0.2		n.c.	n.c.	390
1.0	GB7056	n.c	n.c.	360
5.0		n.c.	n.c.	400
10.0		n.c.	n.c.	43

a GB7055 *thy*A and GB7056 *thy*A *thy*B were grown to competence. Ten fold serial dilutions of the competent cultures were streaked on minimal plates containing 50ug/ml of thymidine and either 0, 0.2µg/ml, 1.0µg/ml, 5.0µg/ml or 10µg/ml of trimethoprim.

b n.c. indicates that the colonies were confluent on the plate and too numerous to count.

containing medium or were first grown for 3 or 6 additional hours in thymidine supplemented VY before plating. Essentially no differences were observed between plating protocols, with respect to either the total viable cells or levels of Trm^r transformants (Table 6).

3. Construction of a Δ*thy*A Strain for Use as a Recipient in the Trm^r Assay.

One of the problems frequently encountered after growth of GB7054 *ilv*A8 *thy*A to competence was that a large fraction of the population would consist of Thy^+ cells. This presumably reflects a selective growth advantage of the *thy*A^+ revertants present in cultures of GB7054 cells. In order to be able to consistently obtain a competent population of *ilv*A8 *thy*A cells, the *thy*A point mutation in 7054 *ilv*A8*thy*A was replaced with a *thy*A deletion. To accomplish this, competent 7054 cells first were transformed with 7037 Thy^+ DNA and *thy*A^+ clones were selected by growing transformants at 46°C without thymidine supplementation. Since the *thy*B gene product is temperature sensitive (Neuhard, 1978), the only clones which will grow without thymidine supplementation are those made up of *thy*A^+ cells. After purification of the Ilv^- *thy*B^+ *thy*A^+ colonies, a single isolate was grown to competence and transformed with DNA prepared from 7071 Δ*thy*A. Clones carrying the Δ*thy*A marker were selected by growth at 46°C in the presence of trimethoprim and thymidine. Growth of *thy*A^+ cells is inhibited under these conditions, whereas cells carrying the

Table 6. Determination of the Expression Time for Trm^r Following Transformation of Trm^s Cells[a]

Expression Period[b] (hours)	Trm^r Transformants/ml	Total Viable cells/ml
1	2.77×10^4	2.48×10^7
4	2.56×10^4	2.13×10^7
7	3.00×10^4	2.59×10^7

[a] Competent GB7055*thy*A cells were exposed for one hour to 1.5 µg/ml of GB7057*thy*A *thy*B *his*B2 DNA in transformation medium at 37°C. The cells were then either plated directly to determine Trm^r and total CFU or were diluted five-fold into VY supplemented with 50 µg/ml of thymidine and incubated for 3 or 6 additional hours at 37°C before plating. Trm^r CFU were assayed on a minimal medium containing 50 µg/ml thymidine and 7.5 µg/ml trimethoprim. The number of total CFU was determined by plating on TBAB.

[b] Expression period is the time elapsed between addition of DNA to competent cells and the plating of those cells on trimethoprim containing medium or TBAB.

*thy*A deletion are phenotypically Thy$^-$ at 46°C and hence Trm^r.

4. Attempts to Utilize the Ø3T *thy*P3 gene in the Trm^r Assay.

Theoretically, since the *thy*P3 gene carried by Ø3T or *rho*11 was thought to be homologous to the cell's *thy*A gene it should have been possible to inactivate the *thy*A gene through the integration of a mutagenized *thy*P3. Inactivation of *thy*A in the absence of a functional *thy*B gene product would produce a Trm^r phenotype. Since the extent of homology between *thy*P3 and *thy*A was not known, and to avoid the possibility of reduced transformation efficiencies in the mutagenesis assays because of possible heterology between *thy*P3 and *thy*A, it was decided to construct a recipient strain which was Thy$^+$ because it carried the wild type *thy*P3 gene. Although such a strain was constructed, other features of this strain which are detailed below made it unsuitable for use in the proposed assay.

The *thy*P3 gene was introduced into Thy$^-$ cells by transforming GB7057 *thy*A *thy*B *his*B2 with either *rho*11 or Ø3T DNA and selecting Thy$^+$ recombinants by plating on medium supplemented only with histidine. Eight Thy$^+$ transformants, four from the Ø3T DNA and four from the *rho*11 DNA, were purified by restreaking on the same medium used in the original selection. However, when these colonies were tested for the trimethoprim sensitivity expected on the basis of the Thy$^+$ phenotype, all appeared Trm^r. This result suggested either that the purification process did not adequately separate Thy$^-$ from Thy$^+$

cells or that the Thy^+ cells were reverting to their original Thy^- phenotype. The same eight clones were repurified by single colony isolation on the original selective medium, grown overnight in a liquid medium supplemented with thymidine, and then streaked at dilutions that yielded single colonies on a medium supplemented only with histidine. This extensive purification should have allowed the isolation of Thy^+ clones which arose from single cells.

In order to characterize the phenotypes of these Thy^+ isolates, they were grown in liquid medium and plated on several different media, which selected either for a) Trm^r, b) Thy^+ or c) viable cells. A representative result of this procedure is shown in Table 7. Approximately 5% of the total viable cells in the culture were trimethoprim resistant, and about 95% were Thy^+. Furthermore, all of the Trm^r colonies that were tested were Thy^-.

For reasons described in detail in the Discussion, it is doubtful that any assay for Trm^r based on the introduction of a mutated *thy*P3 gene into a *thy*B *thy*A^+ background would have been successful.

D. Use of DMF as a Solvent in the Mutagenesis Assays.

A procedure for reacting chemicals with DNA in solutions of 50%DMF-50%SSC/10 was developed for two reasons. First of

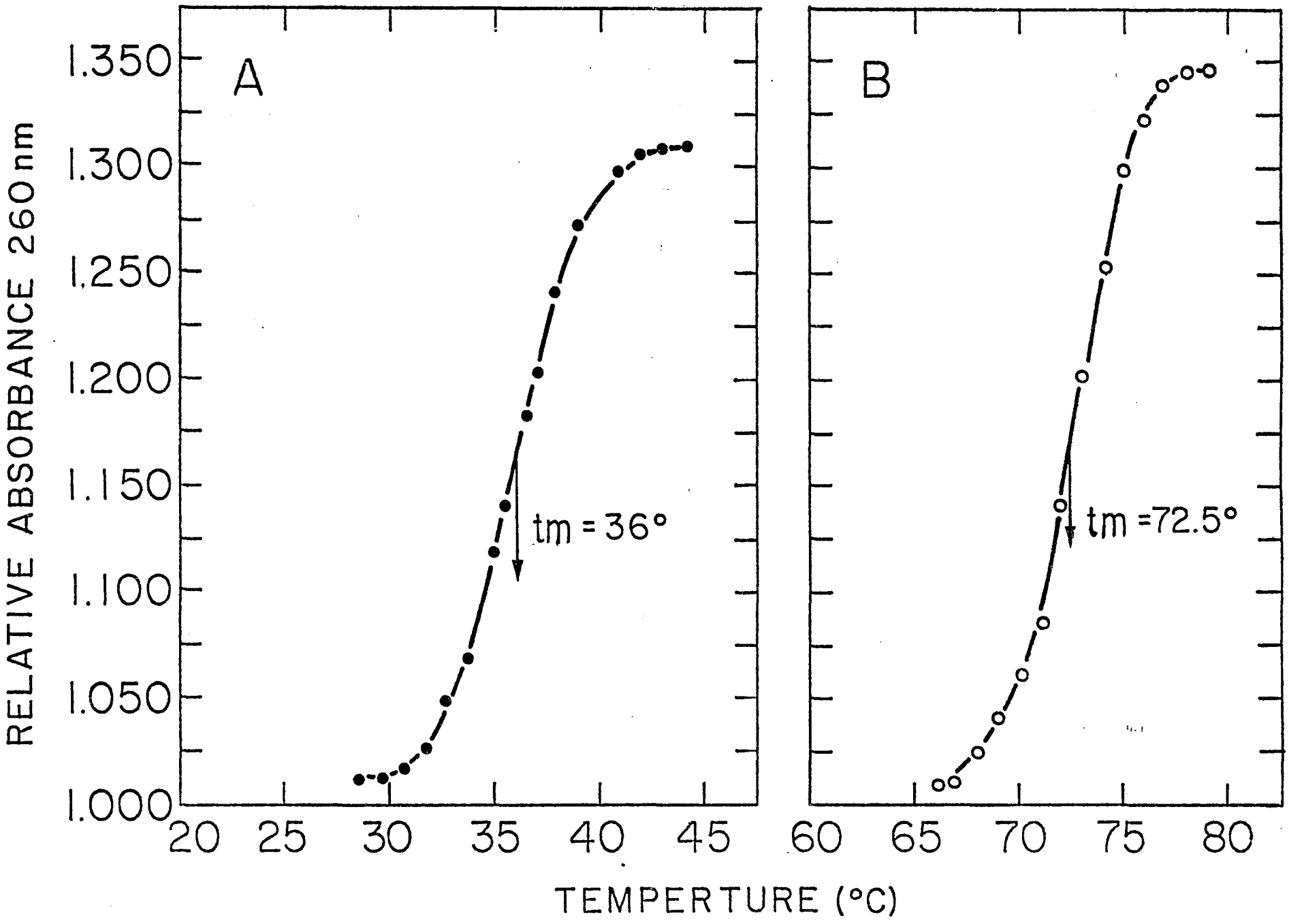
Table 7. Phenotypes of Cells in a Culture of a Thy^+ Isolate Generated by the Introduction of the *thy*P3 gene of *rho*11 into GB7057 *thy*A *thy*B *his*B2.

Viable cells/ml $\times 10^{-8}$	Thy^+ cells/ml $\times 10^{-8}$	Trm^r cells/ml $\times 10^{-7}$
3.3	3.1	1.7

A Thy^+ clone resulting from transformation of GB7057 *thy*A *thy*B *his*B2 by *rho*11 DNA was extensively purified as described in the text and grown to stationary phase in competence medium supplemented with histidine. The number of viable cells in the culture were assayed on TBAB plates, the number of Thy^+ cells on minimal medium lacking thymidine, and the number of Trm^r cells on plates containing 7.5μg/ml of trimethoprim, and 50μg/ml of thymidine.

all, some compounds such as chloroacetaldehyde (Ruyechan, 1976) and alkylating agents such as ethylnitrosourea react preferentially with DNA bases when the DNA is single-stranded (Singer, 1970). As shown in Fig. 6, B. Subtilis DNA in solvent SSC/10 is only completely single stranded above 80°C. If this DNA were cooled, some intrastrand base-pairing would occur, making some of the regions double-stranded and potentially less reactive with certain compounds. Although reactions between a chemical and DNA in an aqeuous solvent could take place at 80°C, the compound might decompose much more rapidly than if the reaction were conducted in the 40-60°C range. As shown in Fig. 6, DNA in 47%DMF-53%SSC/10 is completely single stranded at temperatures above 40°C, and therefore the solvent provides a means of reacting chemicals with single stranded DNA at relatively low temperatures. Furthermore, Bresler, *et al.*, (1972a) had shown that when transforming DNA was dissolved in 90% formamide and reacted with MNNG, N,N'-dimethyl-N-nitrosourea, and N-methyl-N-nitrosourea, the levels of fluorescent mutants was greatly increased as compared to reaction of these compounds with single stranded DNA in phosphate buffer. Bresler suggested that the hydrophobic environment in formamide produced more mutagenic breakdown products of MNNG, but another explanation is that the DNA is completely single-stranded in 90% formamide, with no fold-back regions, and bases were thus more accessible to the MNNG.

Fig. 6. Thermal denaturation curves of GB7018 DNA dissolved in either 47%DMF-53%SSC/10 (A,●)or in SSC/10 (B,o). Both samples contain ~ 38μg/ml of DNA. The midpoint of each transition (t_m) is indicated by the arrow.


A
B
tm = 36°
tm =72.5°
RELATIVE ABSORBANCE 260 nm
1.350
1.300
1.250
1.200
1.150
1.100
1.050
1.000
20
25
30
35
40
45
60
65
70
75
80
TEMPERTURE (°C)

The second reason for using DMF stems from the fact that many compounds are not conveniently tested in aqueous systems because they are not water soluble. DMF rather than formamide was picked as the solvent of choice because it is considered to be a "universal solvent" (Merck), and therefore had the possibility of being more versatile than formamide.

Before DNA that had been dissolved in dimethylformamide could be used in a transformation or marker rescue, the solvent had to be removed because it is toxic to cells. At the same time it was necessary to remove the unbound test chemical, since as stated in the Introduction, one of the advantages of a transformation based assay is that cells are not exposed to potentially toxic chemicals that might interfere with cell survival. When testing a water-soluble compound, such as chloroacetaldehyde, DMF as well as the test chemical could be removed by dialysis against a sodium phosphate buffer. Water-insoluble compounds, however, are not readily removed by dialysis against aqueous buffer. It was decided, therefore, to develop a standard procedure for recovering DNA from reactions with all types of compounds.

Bresler had used a Sephadex G25 column to remove formamide and mutagens from the DNA (Bresler, *et al.*, 1972a). However, rather than set up a column for each sample, it was apparent that ethanol precipitation of the DNA would be simpler, provided that the recoveries were adequate. Recovery of both single and double-stranded DNA which had been alcohol preci-

pitated from a 50%DMF-50%SSC/10 solution was determined by comparing the transforming activity of this DNA to that of DNA which had not been exposed to the DMF solvent. In addition, recovery of radioactively labeled single-stranded DNA from the solvent was also studied. In the transformation studies, single and double-stranded GB7077 DNA in SSC/10 was mixed with an equal volume of DMF for a final DNA concentration of 100μg/ml. After sitting at room temperature for 10 min, the DNA was precipitated and redissolved in SSC/10 as described in the Methods, and then used to transform GB7044 *ilv*B2 *leu*A169. The ability of this DNA to produce Ilv^+ and Ilv^+,Leu^+ transformants was determined in order to see if the exposure to and precipitation from the DMF decreased the transforming activity of the DNA, and to see if linkage was disrupted between the *ilv*B2 and *leu*A169 markers. As is shown in Table 8, the treatment did not decrease the transforming activity of either single or double-stranded DNA, and did not significantly alter the linkage between the *leu*A169 and *ilv*B2 markers. This result also suggested that all of the DNA was recovered from the DMF solvent, and this conclusion was supported by the experiment in which physical recovery of the DNA was studied. In this latter experiment, single-stranded ^{14}C-labeled T7 DNA was mixed with single-stranded GB7077 DNA. A portion of this mixture was exposed to and precipitated from the 50%DMF-50%SSC/10 solvent, and then redissolved in SSC/10. The efficiency of recovery was determined by measuring the CPM of the treated sample and

Table 8. Recovery of DNA from DMF Reaction Mixtures and the Effect of DMF Exposure on Transforming Activity and Gene Linkage.

DNA Conformation	DMF Treatment	Ilv^+ Transformants/ml[a]	r Value	CPM[b]
Single stranded[c]	-	1.32×10^5	0.47	2.2×10^3
	+	1.90×10^5	0.45	2.3×10^3
Double stranded	-	1.38×10^6	0.50	-
	+	1.65×10^6	0.48	-

a
Double-stranded and single-stranded GB7077 DNA was dissolved in 50%DMF-50%SSC/10, to yield a final DNA concentration of 100µg/ml. After standing at room temperature for 10 min, the samples were ethanol precipitated and redissolved in SSC/10 as described in Methods. GB7044 was transformed with 20µg/ml of the DMF treated or control DNA which had not been exposed to DMF nor alcohol precipitated. Ilv^+ and Ilv^+,Leu^+ transformants were selected on appropriate media. R values were determined as described in Methods.

b
40µg/ml of single-stranded ^{14}C-labelled T7 DNA, (1333 cpm/µg) was mixed with 300µg of single-stranded GB7077 DNA in a final volume of 1.5ml SSC/10. 0.5ml of this mixture was added to 0.5ml of DMF. After 10 min at room temperature the DNA was ethanol precipitated as described in the Methods, and redissolved in 0.5ml of SSC/10. 0.1ml of the untreated DNA sample, and 0.1ml of the DMF treated and alcohol precipitated DNA were placed into separate scintillation vials containing 10ml of Aquasol, and CPM were determined in a Packard Tri-Carb scintillation counter.

c
Alkali denatured single-stranded DNA was prepared as described in Methods. After denaturation the DNA solution was dialyzed extensively against SSC/10 in order to reduce the NaCl concentration so that the DNA would dissolve in DMF without precipitating the salt.

the CPM of the DNA which had not been exposed to DMF nor alcohol precipitated. As can be seen in Table 8, the CPM of both samples was equivalent, indicating that no DNA was lost as a result of exposure to the solvent.

E. Results of Standard Mutagen Testing with the Reversion Assay.

1. Hydroxylamine (HA).

Double-stranded GB7018 *ilv*B2 *trp*C2 DNA was incubated in the presence of 1M HA at 75°C and 53°C in sodium phosphate buffer, pH 6.1, and the samples were taken at the times indicated in Table 9 and Table 10, respectively. The choice of the HA concentrations was based on work of Freese and Freese (1965), who showed that at HA concentrations less than 10^{-1}M, the compound had a predominantly inactivating effect on *B. subtilis* transforming activity, whereas at concentrations greater than 1M, the effect was predominantly a mutagenic one. The temperatures chosen were similar to those used by Freese and Strack (1962). Both reaction temperatures were used because of Singer and Fraenkel-Conrat's (1969) suggestion that HA might decompose at 75°C. A pH of 6.1 was chosen on the basis of Freese and Strack's (1962) finding that this was the optimal pH for reaction between HA and DNA.

The number of Ilv$^+$,Leu$^+$ transformants obtained at all of the time points in the 75°C experiment is shown in Table 9. Analysis of variance indicated that the Ilv+,Leu$^+$ transformants obtained in the treated samples were satistically

Table 9. Ilv^+,Leu^+ Transformants Produced after Transformation of GB7044 *ilv*B2 *leu*A169 by Double Stranded HA Treated (75°C) GB7018 *ilv*B2 *trp*C2 DNA.[a]

Treatment Time (min)	Mean Ilv^+,Leu^+ Clones Per Plate[b]
0	5.3 ± 3.2
60	70.0 ± 1.7
120	124.0 ± 14.1
180	300.6 ± 54.0

a
Double stranded GB7018 DNA was treated with 1M HA at 75°C under the conditions described in Methods.

b
The means and standard deviations were calculated on the basis of three replicate platings of GB7044 cells that had been transformed with HA-treated GB7018 DNA.

Table 10. Ilv^+,Leu^+ Transformants Produced After Transformation of GB7044 *ilv*B2 *leu*A169 by Double Stranded HA-Treated (53°C) GB7018 *ilv*B2 *trp*C2 DNA.

Treatment Time (min)	Mean Ilv^+,Leu^+ Clones Per Plate[b]
0	49.8 ± 4.0
10	76.8 ± 15.2
20	62.5 ± 6.7
40	140.8 ± 15.7
90	54.5 ± 5.6

[a] Double stranded GB7018 DNA was treated with 1M HA at 75°C under the conditions described in Methods.

[b] The means and standard deviations were calculated on the basis of four replicate platings of GB7044 cells that had been transformed with HA-treated GB7018 DNA.

different from the control ($p<0.001$), and Duncans multiple range test indicated that all of the treatment groups were significantly different from each other ($p<0.05$). Analysis of the numbers Ilv^+Leu^+ transformants obtained with DNA samples treated at 53°C (Table 10) indicated that only the 40 min group could be distinguished from the control and the other HA treatment groups at a $p<.05$. When the level of transforming activity of the control and treated samples was taken into account, however, and the data plotted as $Ilv^+,Leu^+/Leu^+$, significant difference between all groups existed ($p<0.05$), (Fig. 7). Therefore the apparent dose responses observed in both the 75°C and 53°C experiment was statistically confirmed.

When GB7018 DNA was incubated in the control solution consisting of 1.0M NaCl, 0.1M sodium phosphate buffer, pH 6.1, there was no increase in mutants over a three hour period (data not shown). HA was also tested for its ability to revert the *leu*A169 frame-shift mutation. When GB7037 *leu*A169 *trp*C2 DNA was reacted with 1M HA at 75°C and used to transform GB7044 cells, there was no detectable mutagenesis (data not shown).

2. MNNG.

MNNG, at a final concentration of 100mM was reacted with single-stranded GB7018 *ilv*B2 *leu*A169 DNA in 50%DMF-50% SSC/10, pH 6.1 at 45°C. Samples were taken at the times shown in Table 11. Analysis of variance indicated that the numbers of Ilv^+,Leu^+ transformants produced with the MNNG

Fig. 7. Mutagenesis of GB7018 *ilv*B2 *trp*C2 DNA with HA. Double-stranded GB7018 DNA was treated with HA at 53°C under conditions described in Methods. GB7044 *ilv*B2 *leu*A169 competent cells were transformed with the DNA samples taken at the times indicated and Leu^+,Ilv^+ transformants (0) and Leu^+ transformants were selected by plating on appropriate media. The transforming activity of each sample relative to the 0 min sample (●) is also shown.

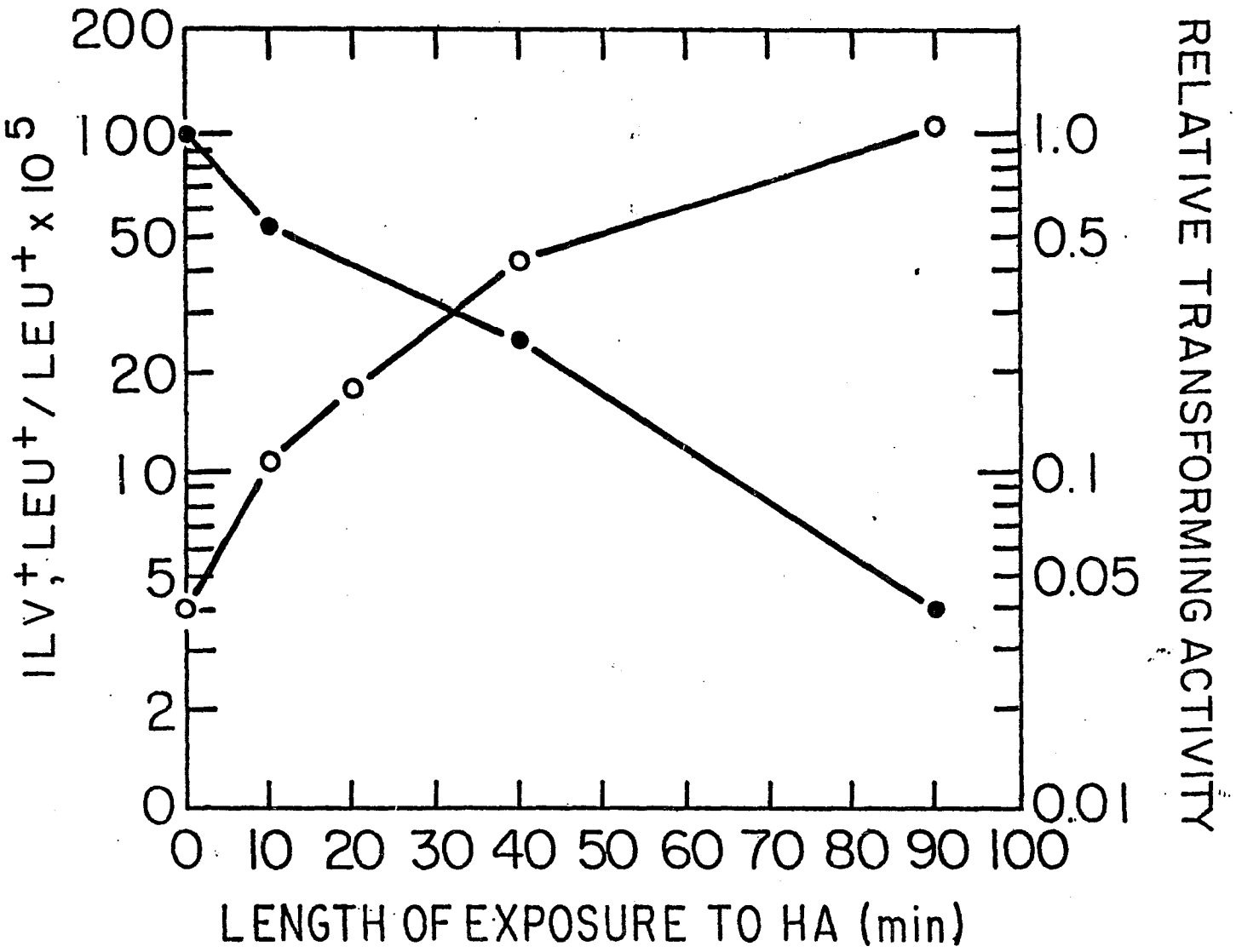

200
100
50
20
10
5
2
0
ILV+, LEU+ / LEU+ x 10^5
1.0
0.5
0.1
0.05
0.01
RELATIVE TRANSFORMING ACTIVITY
0 10 20 30 40 50 60 70 80 90 100
LENGTH OF EXPOSURE TO HA (min)

Table 11. Ilv$^+$,Leu$^+$ Transformants Obtained After Exposure of Competent GB7044 *ilv*B2 *leu*A169 to MNNG-Treated GB7018 *ilv*B2 *trp*C2 DNA.[a]

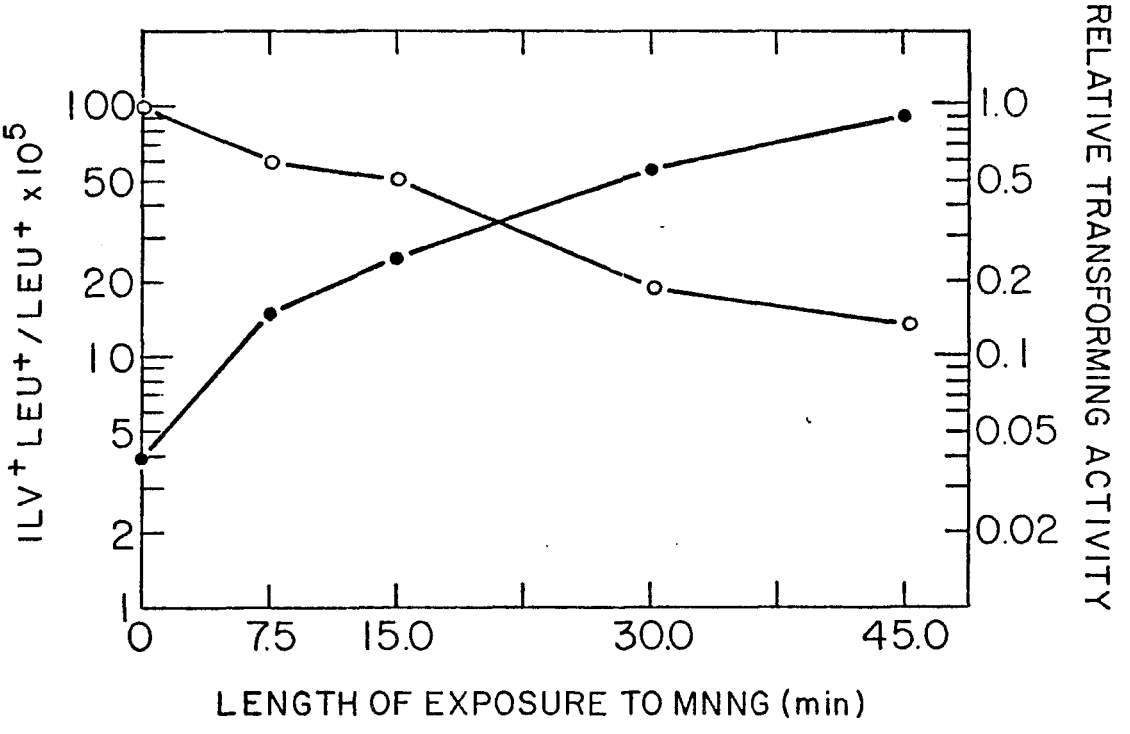
Treatment Time (min)	Relative Transforming Activity	Mean Ilv$^+$,Leu$^+$ Clones[b] Per Plate
0	1.0	18.8 ± 8.8
7.5	0.60	42.8 ± 8.2
15	0.53	63.2 ± 10.9
30	0.19	67.0 ± 13.8
45	0.14	61.0 ± 8.5

a
MNNG at 100mM was reacted with single-stranded GB7018 DNA in 50%DMF-SSC/10, pH 6.1, at 45°C for the times indicated.

b
The means and standard deviations were computed on the basis of four replicate platings of GB7044 cells that had been transformed with MNNG-treated GB7018 DNA.

treated samples differed significantly from the control ($p<0.001$). Duncan's multiple range test indicated that the number of Ilv^+,Leu^+ transformants generated with the 0 min DNA sample and the 7.5 min DNA sample were statistically different ($p<0.05$) suggesting that mutagenesis was detectable even at the earliest time point. As can be seen in Table 11, the number of Ilv^+,Leu^+ transformants at the 15 min, 30 min and 45 min samples were very similiar, and these could not be statistically distinguished from each other. Over the course of the reaction, however, there was a seven-fold drop in Leu^+ transforming activity, and a plot of the data as Ilv^+Leu^+/Leu^+ transformants is shown in Fig. 8. An apparent dose response is seen, and statistical analysis indicated that all groups were statistically distinguishable, except for the 30 min and 45 min treatments. In addition, although a two-fold drop in the r value was noted in the MNNG treated samples (data not shown), control experiments indicated that this drop also occurred in 50%DMF in the absence of MNNG. Furthermore, paried t-tests analysis indicated that the decrease in r values observed over time was not statistically significant. Finally, the ability of MNNG to revert the *leu*A169 frame-shift mutation was also tested. When GB7037 *leu*A169 *trp*C2 DNA was reacted with MNNG, under the conditions described above, and used to transform GB7044, there was no detectable mutagenesis.

Fig. 8. Mutagenesis of GB7018 *ilv*B2 *trp*C2 DNA with MNNG. MNNG at a final concentration of 100mM was reacted with single-stranded GB7018 DNA in 50%DMF-50%SSC/10, pH 6.1, at 45°C for the times indicated. GB7044 *ilv*B2 *leu*A169 competent cells were transformed with the MNNG treated DNA samples and Ilv^+,Leu^+ (0) and Leu^+ transformants were selected by plating on appropriate media. The transforming activity of each sample relative to the 0 min sample (●) is also shown.


ILV+ LEU+ / LEU+ x10^5
100
50
20
10
5
2
1
0
7.5
15.0
30.0
45.0
LENGTH OF EXPOSURE TO MNNG (min)
RELATIVE TRANSFORMING ACTIVITY
1.0
0.5
0.2
0.1
0.05
0.02

3. Chloroacetaldehyde.

Single-stranded GB7018 *ilv*B2 *trp*C2 DNA was exposed to various concentrations of chloroacetaldehyde in 45%DMF, 0.03N sodium acetate buffer, pH 4.5 for 15 min at 53°C. The Ilv^+,Leu^+ transformants obtained when GB7044 *ilv*B2 *leu*A169 competent cells were transformed with the various samples is shown in Table 12. Analysis of variance indicated that the number of Ilv^+,Leu^+ transformants produced with the chloroacetaldehyde treated samples differed significantly from the control ($p<0.001$). The Duncan's multiple range test indicated that the 5.0mM and 1.67mM treatment groups could not be distinguished from each other, but all other treatments were significantly different from each other with $p<0.05$. Relative transforming activity decreased in a dose-dependent fashion but no decrease in r values was noted (data not shown). The ability of chloroacetaldehyde to revert the *leu*A169 frameshift mutation was also tested. When GB7037 *leu*A169 *trp*C2 DNA was reacted with chloroacetaldehyde, under the conditions described above, and used to transform GB7044 cells, there was no detectable mutagenesis.

4. Propylene Oxide (PO)

Single-stranded GB7018 *ilv*B2 *leu*A169 DNA was treated with a wide range of PO concentrations at 45°C for 30 min in 50%DMF-50%SSC/10, pH 6.1. The number of Ilv^+,Leu^+ transformants are shown in Table 13. Analysis of variance treatment of this data indicates that there is a significant difference

Table 12. Ilv^+,Leu^+ Transformants Produced After Exposure of Competent GB7044 *ilv*B2 *leu*A169 to Chloroacetaldehyde-Treated GB7018 *ilv*B2 *trp*C2 DNA.[a]

Chloroacetaldehyde mM	Relative Transforming Activity	Mean Ilv^+,Leu^+ Clones Per Plate[b]
0.00	1.0	7.3 ± 1.5
0.19	1.0	61.3 ± 9.3
0.57	0.78	194.0 ± 40.2
1.67	0.67	330.0 ± 22.2
5.00	0.49	346.0 ± 45.8

a
Single-stranded GB7018 DNA was exposed to various concentrations of chloroacetaldehyde in 45%DMF-55° SSC/10, 0.03N sodium acetate buffer, pH 4.5 for 15 min at 53°C.

b
The means and standard deviations were calculated on the basis of three replicate platings of GB7044 cells that had been transformed with chloroacetaldehyde-treated GB7018 DNA.

$p<0.01$, between the treated groups and the untreated group, but there is no difference between the treated groups themselves. However, when decreases in transforming activity are taken into account, and the data is computed as Ilv^+Leu^+/Leu^+, analysis of variance treatment indicates that significant differences among samples existed with a $p<0.005$. Furthermore, PO disrupted the linkage between the *thy*B and *ilv*D genes, and the r values for each treated sample are listed in Table 13. When there is a decrease in the r value of a treated DNA, some of the Ilv^+,Leu^+ revertants will be missed because of disruption of linkage between the *ilv*B2 and the *leu*A169 genes. In fact, a 50% decrease in the r value will approximately halve the number of Ilv^+,Leu^+ transformants. The degree to which linkage disruption affected the appearance of Ilv^+,Leu^+ transformants was determined by first calculating the expected number of Ilv^+,Leu^+ transformants for each sample based on the r value of the OmM sample, which was 0.27. The ratio of the expected Ilv^+,Leu^+ transformants to the observed Ilv^+,Leu^+ transformants for each sample was then multiplied by the respective $Ilv^+,Leu^+/Leu^+$ values to give the corrected number. Plots of the data, corrected and uncorrected for the effect of PO on linkage number, are shown in Fig. 9. Paired t-tests, performed on the observed and corrected $Ilv^+,Leu^+/Leu^+$ values were significant at a $p<0.05$. Of the eight treatment groups, Duncan's multiple range analysis of the corrected data identified five statistically different groups ($p<0.05$): OmM, 25 and 50mM, 100 and 150mM, 200 and 250mM, and the 300mM group. Finally, the

Table 13. The Effect of PO on Mutagenesis, Linkage and Transforming Activity of GB7018 *ilv*B2 *trp*C2 DNA.[a]

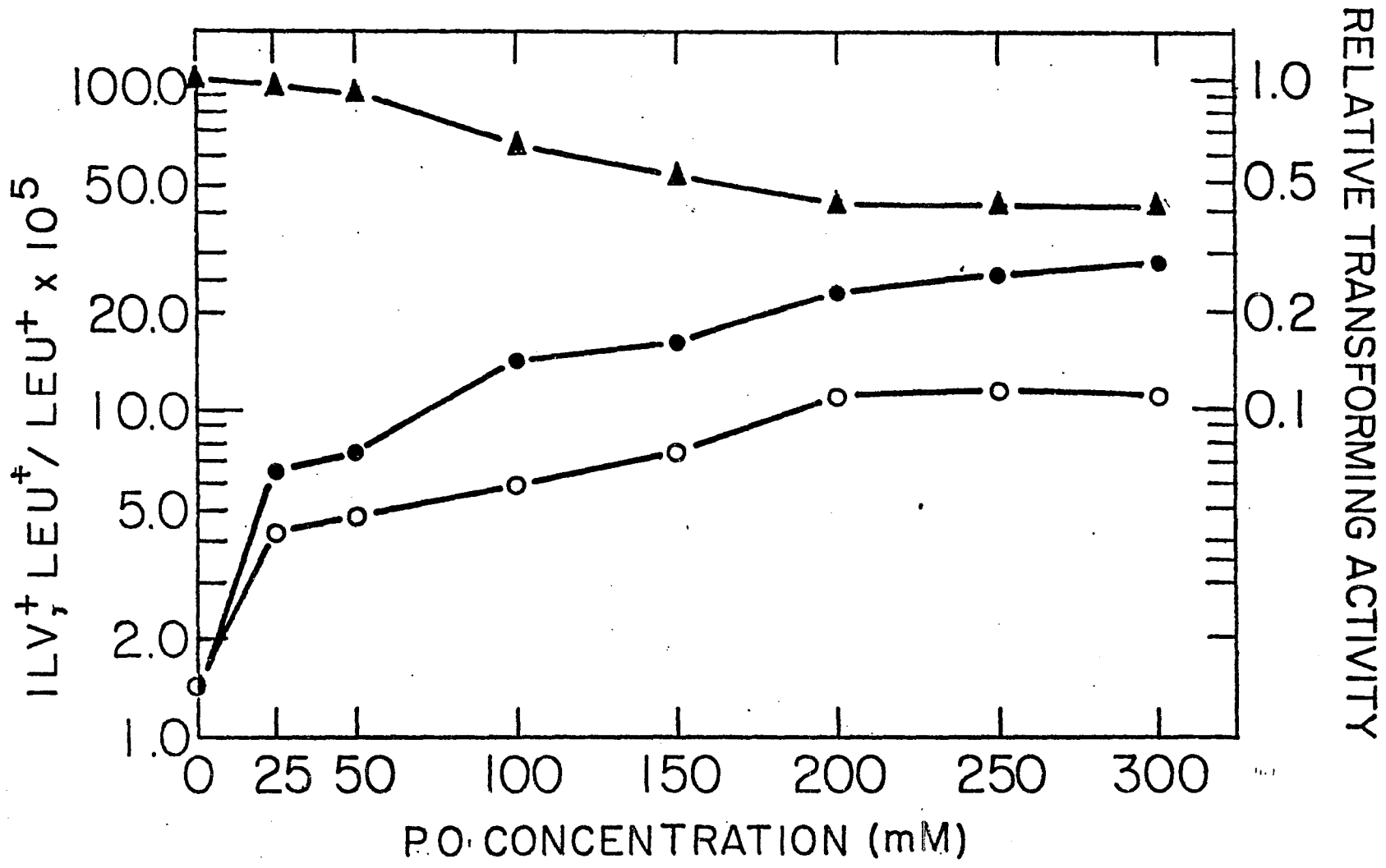
PO mM	Relative Transforming Activity	Mean Ilv^+,Leu^+ Clones Per Plate[b]	r value[c]
0	1.00	6.8 ± 1.0	0.27
25	0.96	19.3 ± 5.0	0.20
50	0.92	21.3 ± 5.4	0.15
100	0.62	28.0 ± 5.0	0.17
150	0.50	17.0 ± 2.1	0.11
200	0.42	21.0 ± 1.7	0.11
250	0.42	18.0 ± 4.2	0.09
300	0.42	21.5 ± 5.0	0.09

a
Single stranded GB7018 DNA was treated with various concentrations of PO at 45°C for 30 min in 50%DMF-50%SSC/10, pH 6.1 in screw-capped vials.

b
Means and standard deviations were calculated on the basis of four replicate platings of GB7044 cells which had been transformed with PC-treated GB7018 DNA.

c
The r value calculation was performed as described in Methods. The donor DNA was PO^- treated GB7018 DNA, the recipient was GB7068 *ilv*D6 *thy*B Δ*thy*A, and Ilv^+, Thy^+ and Thy^+ transformants were selected on appropriate media.

Fig. 9. Mutagenesis of GB7018 *ilv*B2 *leu*A169 DNA by PO. Single-stranded GB7018 DNA was treated with the indicated concentrations of PO at 45°C for 30 min in screw capped vials because of the volatility of PO. GB7044 *ilv*B2 *leu*A169 competent cells were transformed with the PO treated DNA samples and then assayed for Ilv^+,Leu^+ and Leu^+ transformants on appropriate media. In addition, the same DNA sample were used to transform GB7068 *ilv*D6 *thy*A *thy*B, and Ilv^+,Thy^+ and Thy^+ transformants were selected on appropriate media in order to calculate an r value for each sample. Ilv^+Leu^+/Leu^+ transformants, corrected (●) and uncorrected (0) for disruption of gene linkage are plotted in the figure. The transforming activity of each sample relative to the 0 min sample is also shown (▲).


ILV$_7^+$ LEU$^+$/ LEU$^+$ x 10^5
100.0
50.0
20.0
10.0
5.0
2.0
1.0
0
25
50
100
150
200
250
300
P.O. CONCENTRATION (mM)
RELATIVE TRANSFORMING ACTIVITY
1.0
0.5
0.2
0.1

ability of PO to revert the *leu*A169 marker was not tested.

5. N-acetoxy-2-acetylaminofluorene (AAAF)

Levine, *et al.* (1974) had reported that AAAF modified denatured DNA 3x more extensively than native DNA. It is possible that this difference was a result of either a shift in the relative proportions of N-(deoxyguanosin-8-yl)-AAF and 3-(deoxyguanosin-N^2-yl)-AAF, or that minor and as of yet unidentified products are formed in the reaction with the denatured DNA. Since the AAAF modifications of DNA which lead to mutagenesis have not been unequivocally identified, the AAAF was reacted with GB7037 *leu*A169 *trp*C2 DNA under conditions in which the DNA was either double or single-stranded, so that if mutagenesis preferentially occurred in either physical state of the DNA, it might be detected.

When AAAF was reacted with GB7037 DNA at a ratio of 1.7moles of AAAF:1.0 moles of DNA nucleotide under conditions where the DNA was single-stranded, there was a rapid drop in the transforming activity of the DNA, but no increase in the number of mutants (Fig. 10). When various concentrations of AAAF were reacted with GB7037 DNA under conditions where the DNA was double-stranded (Table 14), there was a decrease in transforming activity, but again no increase in mutants was observed.

6. BPDEs.

Double-stranded GB7037 *leu*A169 *trp*C2 DNA was incubated with (±)7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-

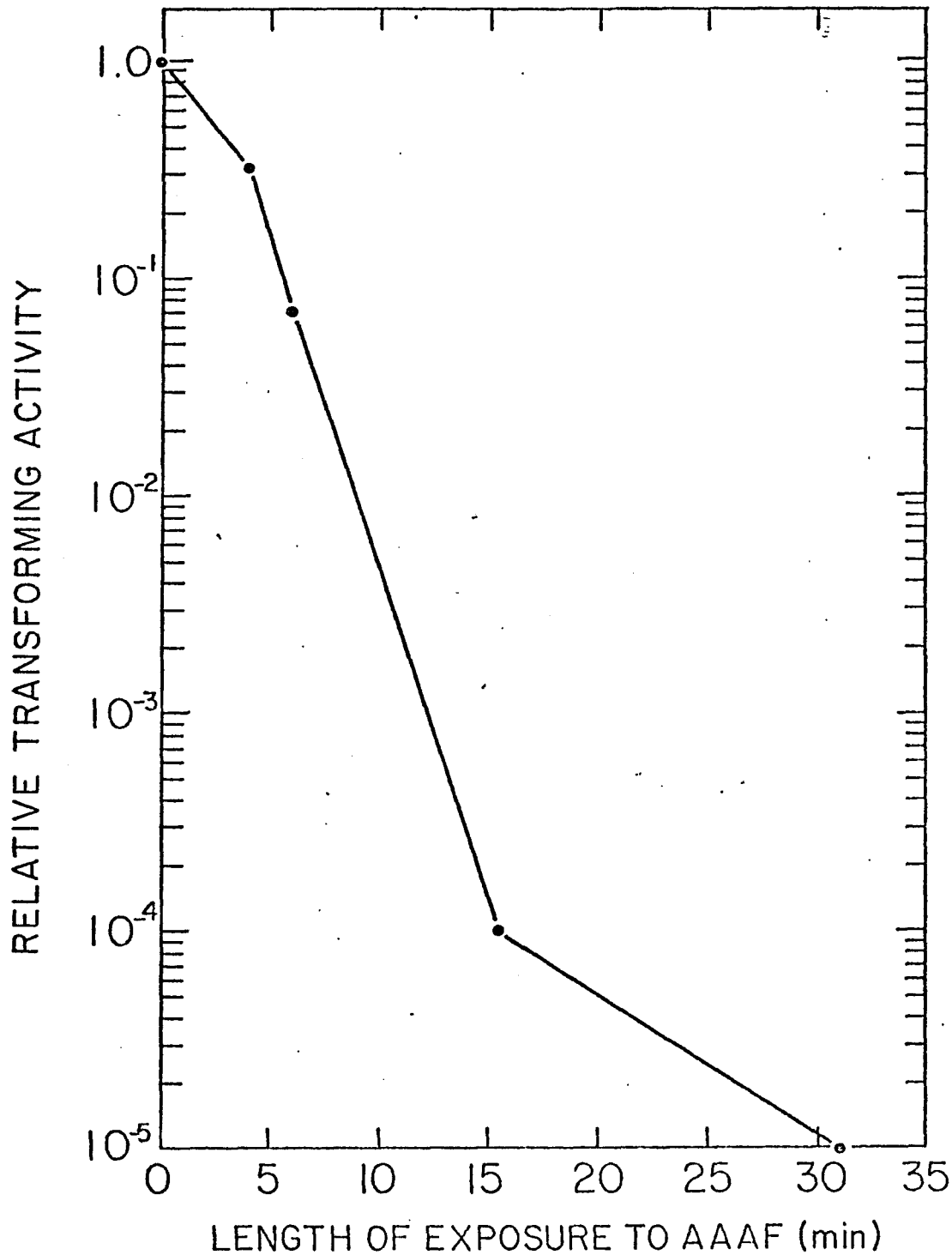
Table 14. The Effect of Increasing Doses of AAAF on the Transforming Activity of Double Stranded GB7037 DNA.[a]

Ratio of mM AAAF to mM DNA Nucleotide[b]	Relative Transforming Activity
0.0	1.0
0.7	0.9
1.4	0.75
2.8	0.75
5.5	0.45
11.0	0.45

a
Double stranded GB7037 *leu*A169 *trp*C2 DNA was reacted with various concentrations of AAAF in 50%DMF-50%SSC/10 pH 6.1 at 20°C for 15 min. Competent GB7044 *ilv*B2 *leu*A169 cells were transformed with the different samples, and Ilv$^+$ transformants were selected on appropriate medium.

b
A ratio of 1:1 corresponds to an AAAF concentration of 0.35mM and a DNA concentration of 100µg/ml. The DNA concentration was held constant in all experiments and the AAAF concentration was varied.

Fig. 10. The effect of AAAF on the transforming activity of single-stranded GB7037 *leu*A169 *trp*C2 DNA. GB7037 DNA was reacted with AAAF at a ratio of 1.7moles of the compound to 1.0 moles of DNA nucleotide in 50%DMF-50%SSC/10, pH 6.1, at 45°C for the times indicated above. Competent GB7044 *ilv*B2 *leu*A169 cells were transformed with the different DNA samples, and Ilv$^+$ transformants were selected on appropriate medium.


RELATIVE TRANSFORMING ACTIVITY
1.0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0
5
10
15
20
25
30
35
LENGTH OF EXPOSURE TO AAAF (min)

tetrahydrobenzo(a)pyrene (*anti*BPDE)at a concentration of 0.6 hydrocarbons per DNA nucleotide in 50%DMF-50%SSC/10, pH 6.1 at 30°C for 90 min. When this BPDE-treated DNA was used to transform GB7044, there was no increase in the number of mutants, no decrease in transforming activity,and no apparent decrease in the r values (Fig. 11). When different concentrations of the (±)*anti* BPDE, ranging from a ratio of BPDE:DNA nucleotide of 12:1 to 0.05:1, were reacted at 30°C for 15 min in 50%DMF-50%SSC/10, there was no dose dependent decrease in transforming activity, and no increase in the number of mutants (date not shown). R values were not determined in this latter experiment.

In contrast to the reaction with GB7037 DNA with the (±) *anti* BPDE,when GB7037 was incubated with the (±)*syn* BPDE at a concentration of 0.6 hydrocarbons per DNA nucleotide under the conditions described above, there was a decrease in transforming activity with increasing times of treatment (Fig. 11). However, there was no increase in the number of mutants. R values were not determined in this experiment.

In order to rule out the possibility that AAAF and the (±)*syn* and (±)*anti* forms of BPDE were not mutagenic in the transformation assay because they simply were not mutagenic for *B. subtilis*, reversion studies of the 7037 *leu*A169 *trp*C2 tester strain were conducted with these mutagens. The response of the *leu*A169 marker in 7037 to AAAF and the *syn* and *anti* forms of BPDE are shown in Figs. 12,13,14. The controls for these experiments included the Ames *Salmonella* tester

Fig. 11. Transforming activity of double-stranded GB7037 *leu*A169 *trp*C2 DNA following reaction with (±)*anti* (0) or (±) *syn* (●) BPDE. GB7037 DNA was reacted with either compound at a ratio of 0.6 moles of BPDE:1.0 moles of DNA nucleotide in 50%DMF-50%SSC/10, pH 6.1 at 30°C over 90 min. Competent GB7044 *ilv*B2 *leu*A169 cells were transformed with samples which had been treated with BPDE for the times indicated, and Ilv^+ transformants were selected on appropriate medium.

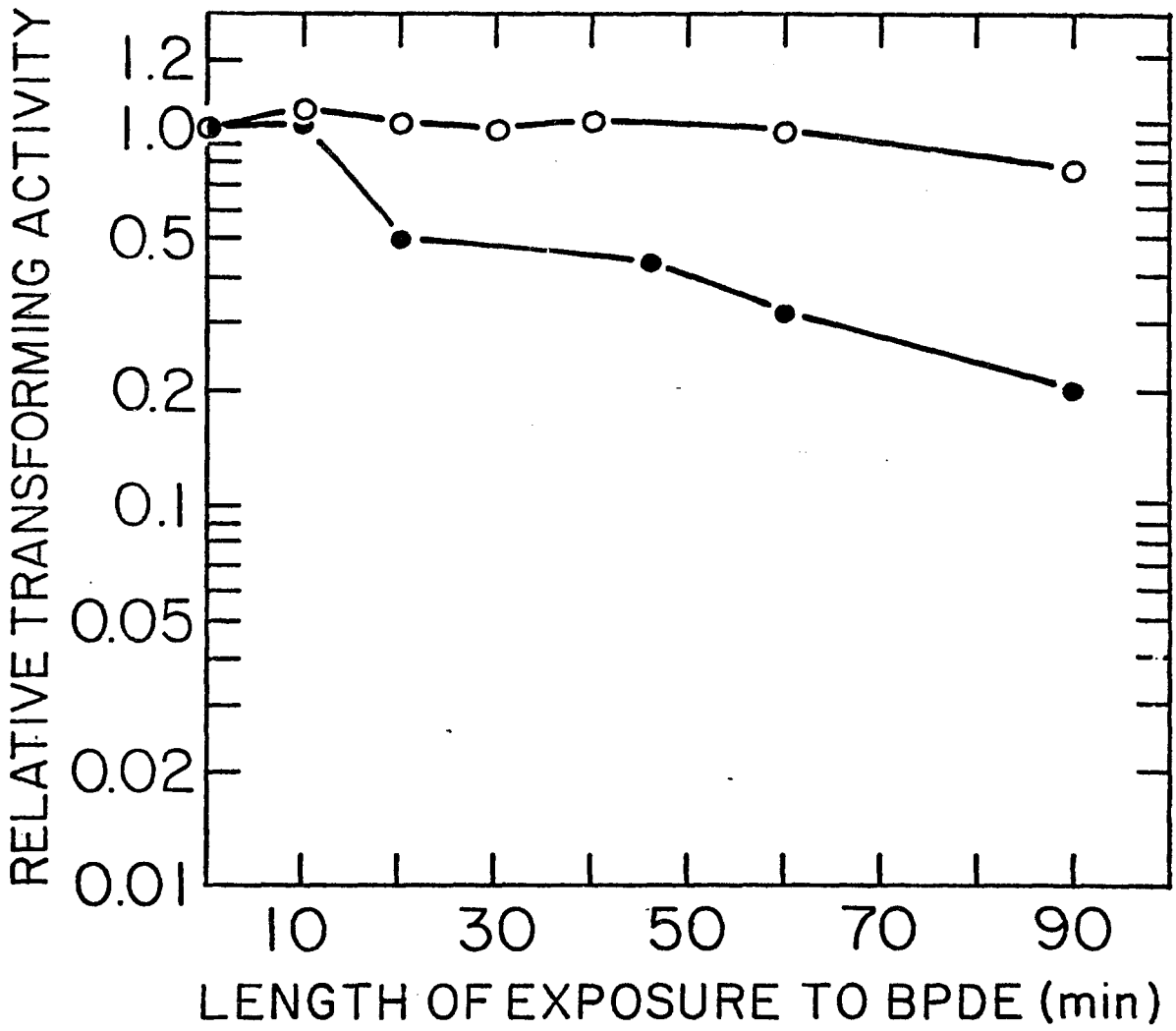

RELATIVE TRANSFORMING ACTIVITY
1.2
1.0
0.5
0.2
0.1
0.05
0.02
0.01
10
30
50
70
90
LENGTH OF EXPOSURE TO BPDE (min)

Fig. 12. Dose dependent mutagenesis of GB7037 *leu*A169 (●) and TA98 *his*D3052 (0) by AAAF. Reversion testing was carried out as described in Methods. The mutagen was incorporated into the molten top agar along with the cells.

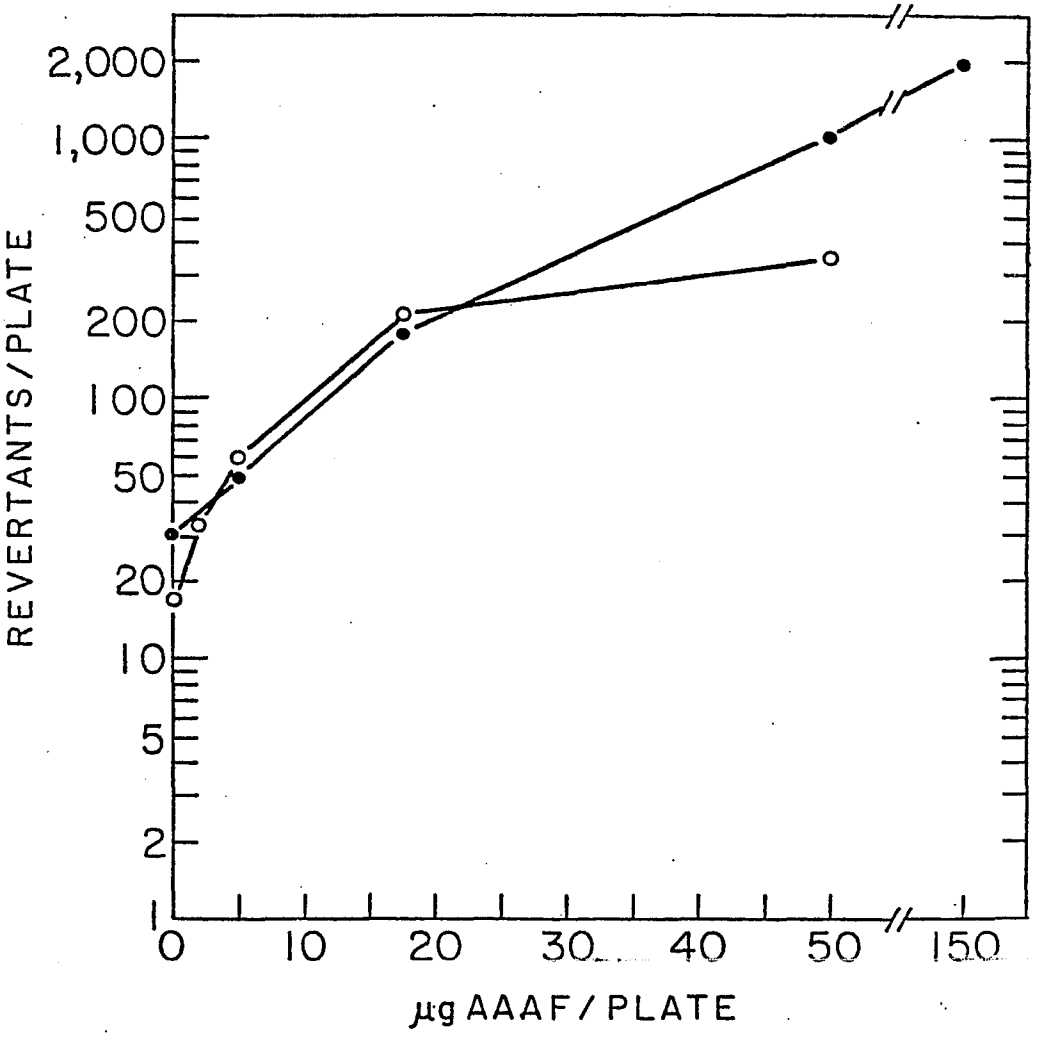

2,000
1,000
500
200
100
50
20
10
5
2
1
REVERTANTS/PLATE
0
10
20
30
40
50
150
µg AAAF/PLATE

Fig. 13. Reversion of GB7037 *leu*A169 (●), TA98 *his*D3052 (○), and TA100 *his*G46 (□) by (±)*syn* BPDE. The reversion assay was conducted as described in Methods. The mutagen was incorporated into the molten top agar along with the cells.

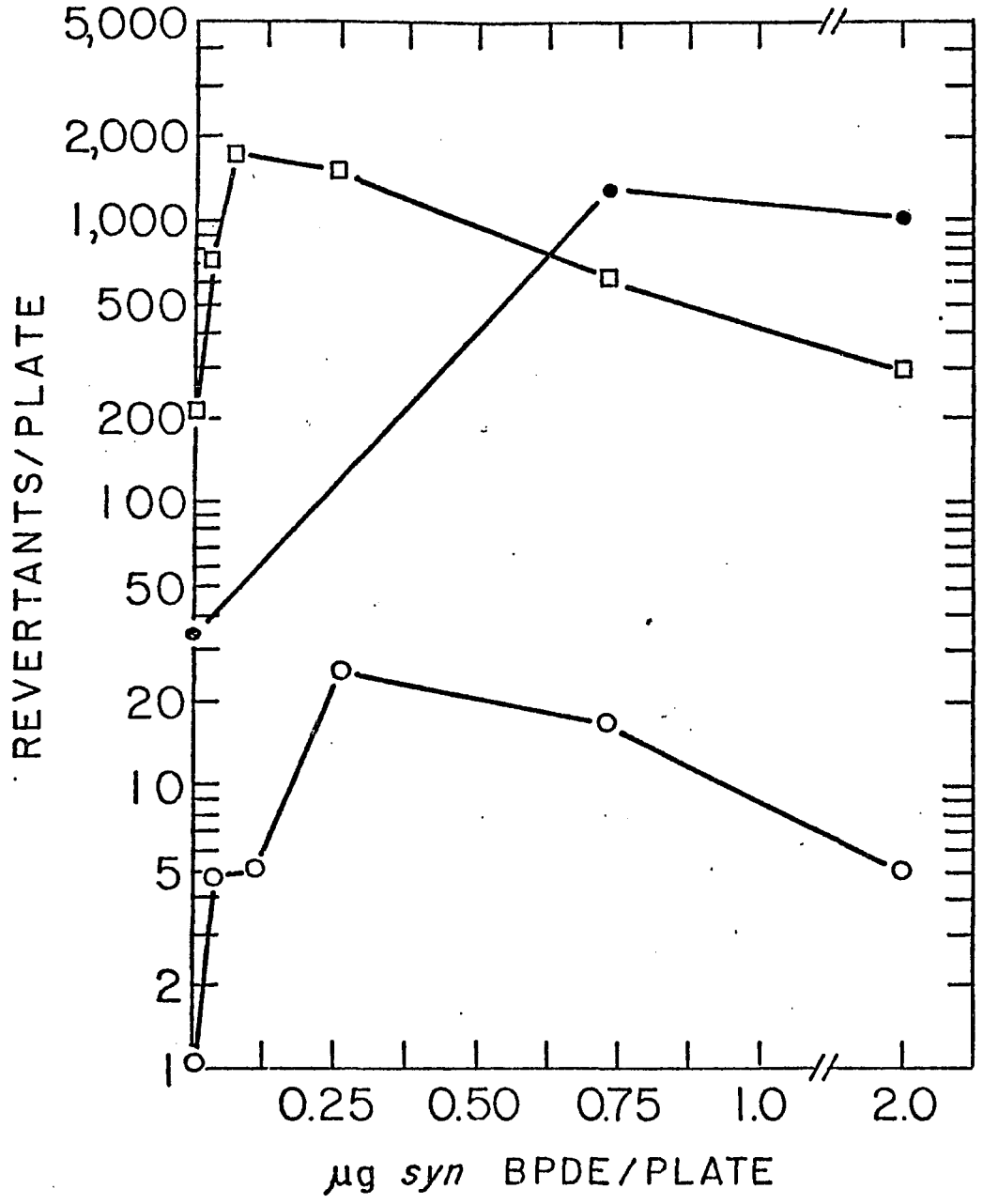
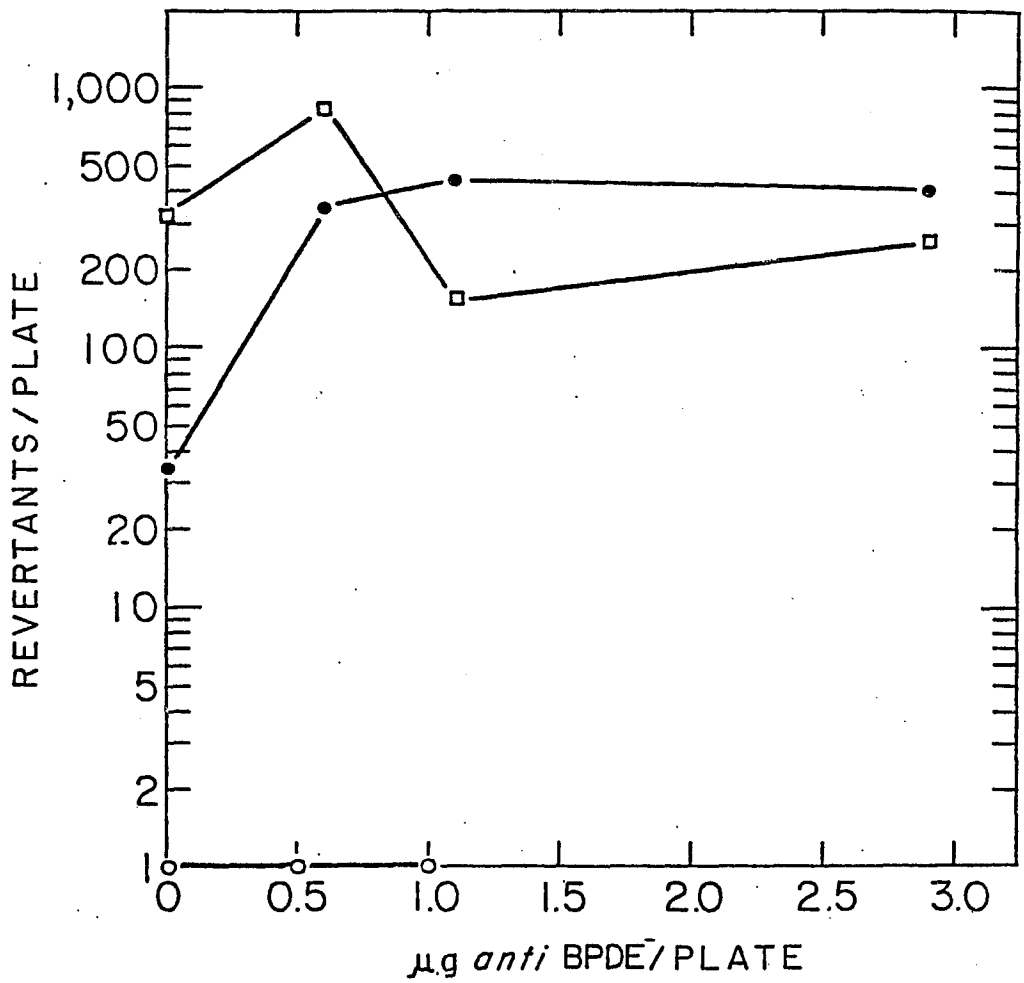

5,000
2,000
1,000
500
200
100
50
20
10
5
2
1
REVERTANTS/PLATE
0.25
0.50
0.75
1.0
2.0
μg syn BPDE/PLATE

Fig. 14. Reversion of GB7037 *leu*A169 (●), TA98 *his*D3052 (0), and TA100 *his*G46 (□) by (±)*anti* BPDE. The reversion assay was conducted as described in Methods. The mutagen was incorporated into the molten top agar along with the cells.


1,000
500
200
100
50
20
10
5
2
1
REVERTANTS/PLATE
0
0.5
1.0
1.5
2.0
2.5
3.0
μg anti BPDE/PLATE

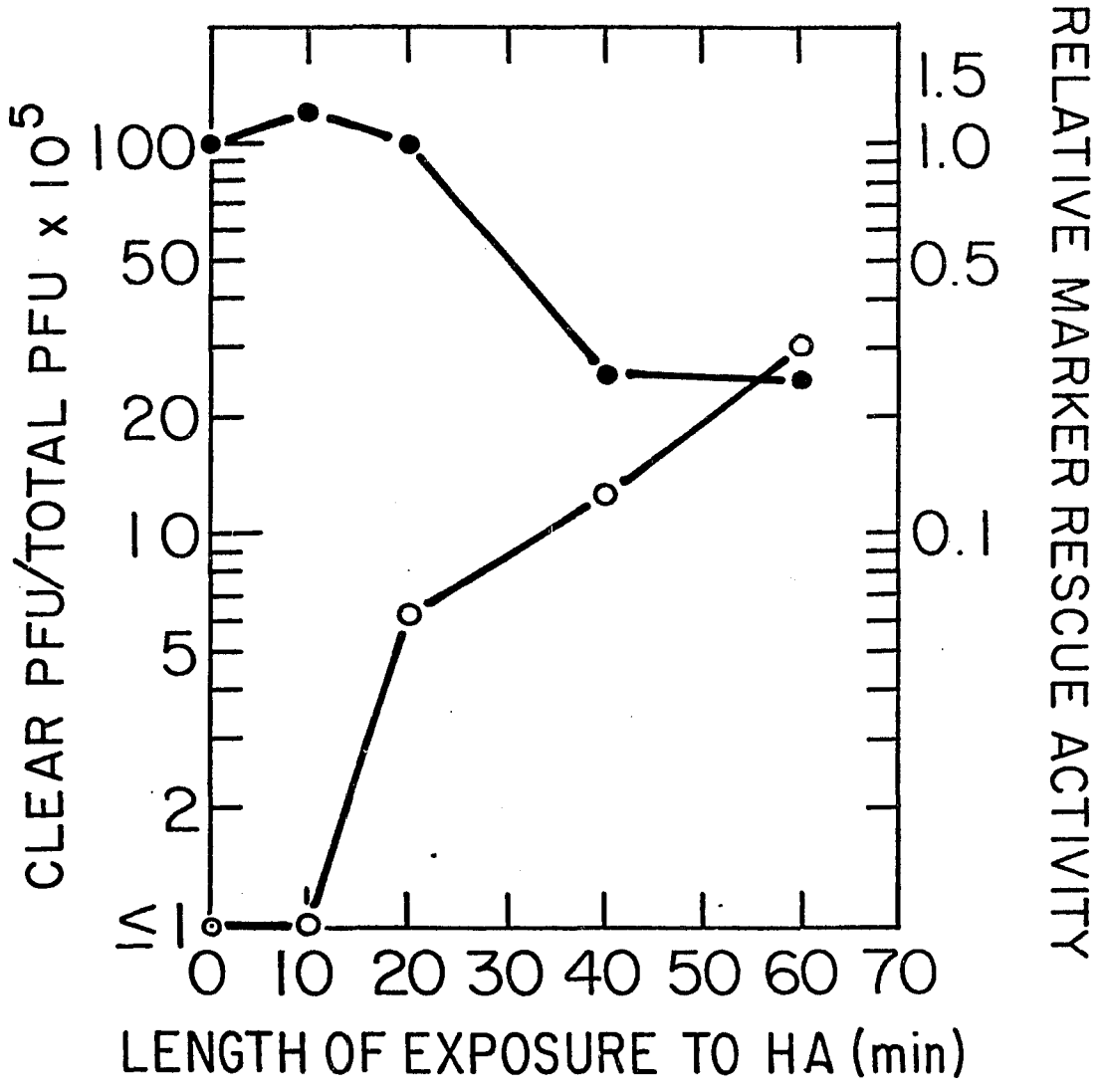
strains TA98 and TA100, which carry a frame-shift and base substitution mutation, respectively. It can be seen that the *leu*A169 marker in GB7037 was reverted by these mutagens in a dose dependent fashion. No statistical analysis was performed on these data since each dose of a given compound was incorporated into only one plate. This was sufficient for the purposes of the experiment, since only a qualitative response was of interest. The reason that there is no apparent response of TA98 to the *anti* BPDE is that the strain was so sensitive to the toxic effects of the compound that its mutagen responsiveness could not be determined.

F. Results of Standard Mutagen Testing with the Ø105 Marker Rescue Assay.

1. Hydroxylamine (HA)

HA at a final concentration of 1M was reacted with double-stranded replicating Ø105 DNA at 53°C and single-stranded DNA at 45°C in the same pH 6.1 buffer noted in the reversion assay. The results of marker rescue performed with the double stranded DNA are shown in Fig. 15. With this DNA there was at least a 30 fold increase over the background mutation frequency produced by the reaction with HA. The increase in mutation frequency during a 60 min incubation is significant at a $p<0.001$. It was not possible to determine the true background mutation frequency because no clear PFU were detected among the phage rescued by the 0 min DNA sample. A drop in marker rescue activity of the double-stranded DNA

Fig. 15. Mutagenesis of double stranded replicating Ø105 DNA by HA. The reaction mixture contained 100µg/ml DNA, 1M HA, in 0.1M sodium phosphate buffer, pH 6.1, and the temperature was 53°C. Marker rescue with each sample was performed as described in Methods, using competent BD99 as the recipient. Since marker rescue with the 0 min sample generated no clear plaques among >100,000 PFU screened, the background frequency of clear PFU/total PFU(0) is described as $\leq 10^{-5}$. The relative marker rescue activity is also shown (●).


CLEAR PFU/TOTAL PFU x 10^5
100
50
20
10
5
2
1
0 10 20 30 40 50 60 70
LENGTH OF EXPOSURE TO HA (min)
1.5
1.0
0.5
0.1
RELATIVE MARKER RESCUE ACTIVITY

occurred between 20 and 40 min of incubation.

Mutagenesis occurred very rapidly with single-stranded DNA (Fig. 16). Within 3 min of treatment the frequency of clear PFU was greater than that obtained in 60 min with double stranded DNA. The results of the single stranded mutagenesis are significant with a $p<0.001$. In addition, the marker rescue activity of the single stranded DNA dropped 56% within the first min of treatment, and after 10 min had dropped 96%.

2. MNNG.

MNNG at a final concentration of 100mM was incubated with single-stranded replicating Ø105 DNA in 50%DMF-50%SSC/10, pH 6.1 at 42°C for the times shown in Fig. 17. Mutations were induced under these conditions, with the increase in frequency of clear PFU/total PFU being significant at $p<0.001$. There was no significant decrease in marker rescue activity over the time course of the experiment.

3. Chloroacetaldehyde.

Several concentrations of chloroacetaldehyde were reacted with single stranded replicating Ø105 DNA in 50%DMF-50%SSC/10, pH 6.1, at 50°C. Marker rescue with each treated sample was performed both with BD99 and with GB7075 *hcr*-9 *trp*C2 as the recipient. This latter strain is defective in one of the DNA repair pathways (Okubo and Romig, 1965) and was included here to determine if this repair defect increased the sensitivity of the assay. The results of these experiments are shown in Fig. 18. The mutagenic activity of chloroacetal-

Fig. 16. Mutagenesis of single stranded replicating Ø105 DNA by HA. The reaction conditions were as described in Fig. 15, except for the reaction temperature, which was 45°C. Samples were taken at the times indicated, and marker rescue conducted as described in Methods, using BD99 as the recipient. Since marker rescue with the 0 min sample generated no clear plaques among >100,000 PFU screened, the background frequency of clear PFU/total PFU (0) is described as $\leq 10^{5}$. The relative marker rescue activity (●) is also shown.

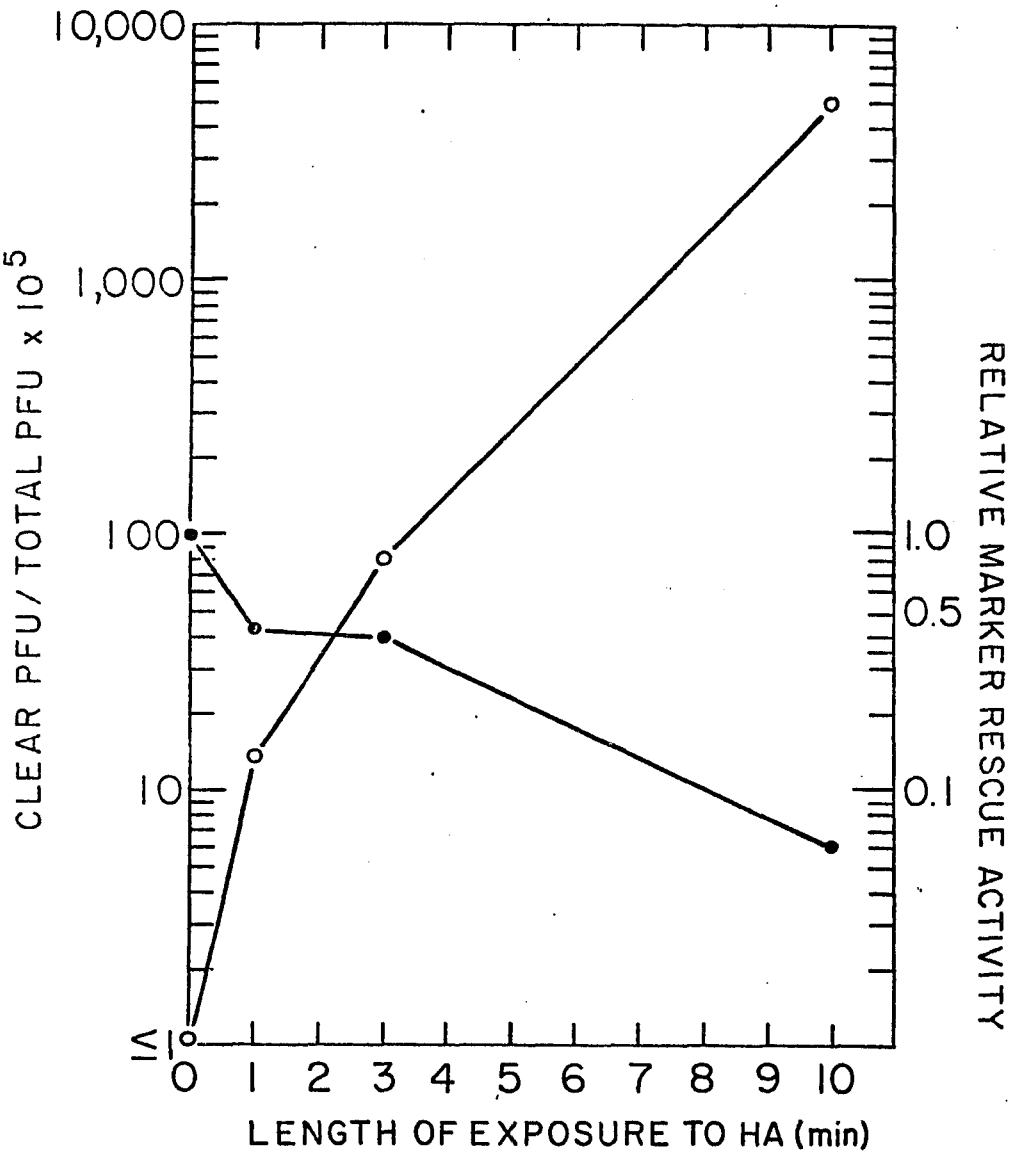

10,000
1,000
100
10
1
CLEAR PFU / TOTAL PFU x 10^5
1.0
0.5
0.1
RELATIVE MARKER RESCUE ACTIVITY
0 1 2 3 4 5 6 7 8 9 10
LENGTH OF EXPOSURE TO HA (min)

Fig. 17. MNNG mutagenesis of single-stranded replicating Ø105 DNA at 42°C. The mutagenesis reaction mixture contained 100ug/ml DNA and 100mM MNNG in 50%DMF-SSC/10, pH 6.1. Marker rescue with each treated DNA sample was performed as described in Methods, with competent BD99 as the recipient. Since with the 0 min sample there were no clear plaques observed among >100,000 PFU screened, the background frequency of Clear PFU/Total PFU is described as $\leq 10^{-5}$.

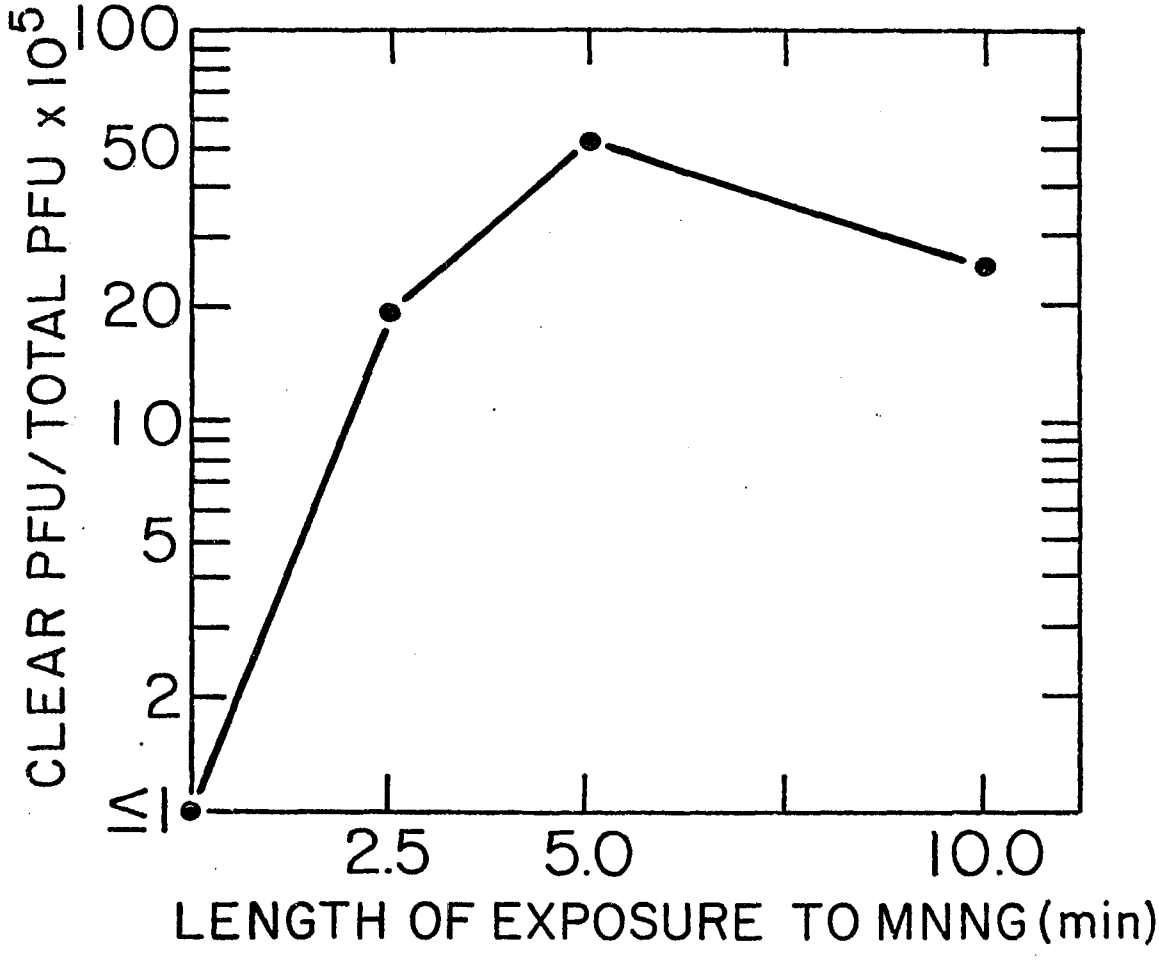
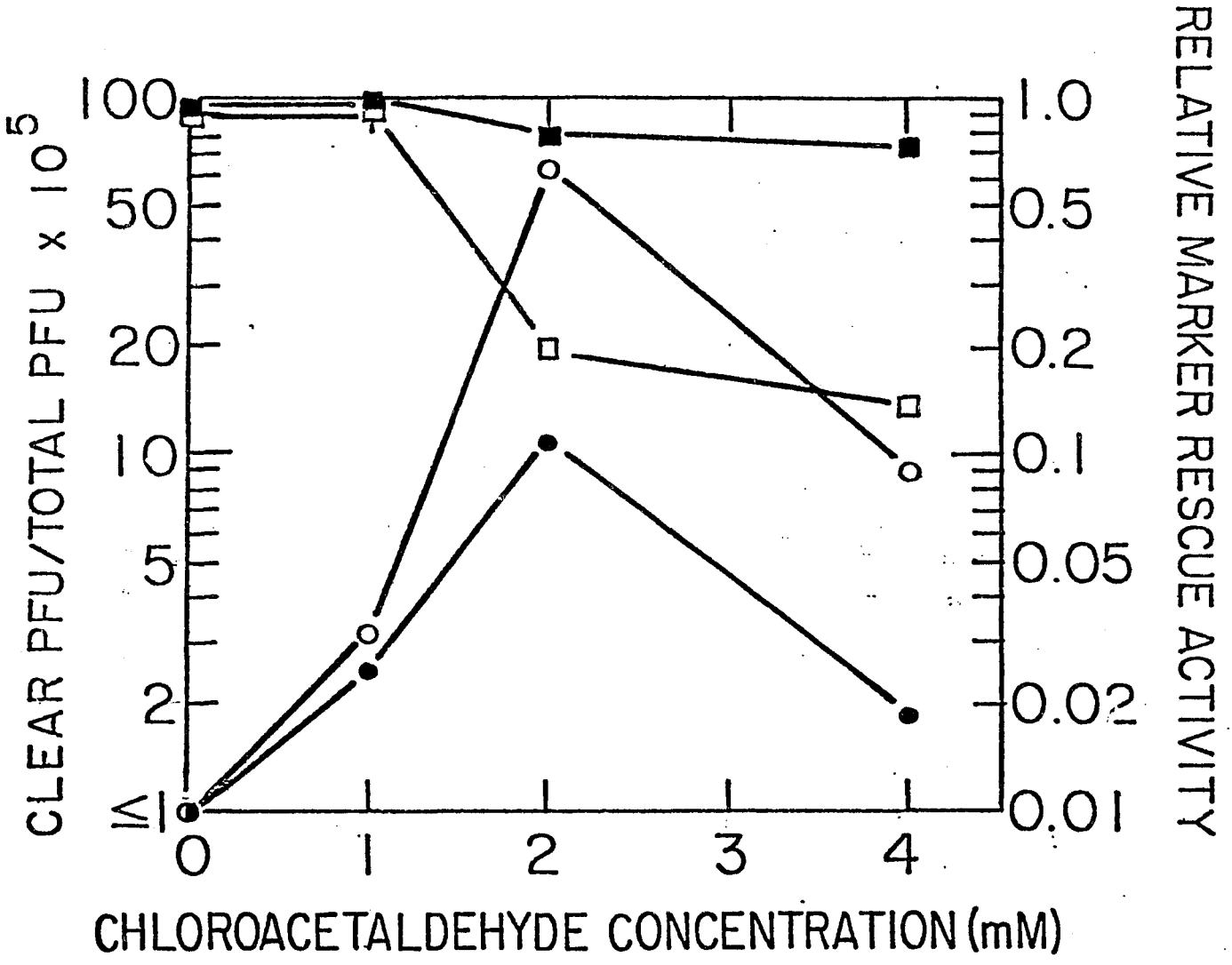

CLEAR PFU/TOTAL PFU x 10^5
100
50
20
10
5
2
1
2.5
5.0
10.0
LENGTH OF EXPOSURE TO MNNG (min)

Fig. 18. Chloroacetaldehyde mutagenesis of replicating Ø105 DNA. Chloroacetaldehyde, at the concentrations indicated, was reacted with 100ug/ml of single-stranded replicating Ø105 DNA in 50%DMF-50%SSC/10, pH 6.1 at 50°C for 15 min. The samples were used for marker rescue with either BD99 or GB7075 *hcr*-9 *trp*C2 as the recipient cells. Since when the 0 min sample was used in a marker rescue with each of the recipient strains there were no clear plaques observed among >100,000 plaques screened, the background frequency of Clear PFU/Total PFU is described as $\leq 10^{-5}$. Clear PFU/Total PFU, BD99 (●), GB7075 (○); Relative Marker Rescue Activity, BD99 (■), GB7075 (□).


CLEAR PFU/TOTAL PFU x 10^5
100
50
20
10
5
2
≤1
0
1
2
3
4
CHLOROACETALDEHYDE CONCENTRATION (mM)
RELATIVE MARKER RESCUE ACTIVITY
1.0
0.5
0.2
0.1
0.05
0.02
0.01

dehyde is detected in the marker rescue assay, with the increase in the frequency of clear PFU/total PFU being significant at $p<0.001$. It can be seen that a higher mutation frequency was produced when GB7075 was used as the recipient for the rescue. In addition, the relative marker rescue activity decreased more rapidly in GB7075 than in BD99. An unexplained event, which occurred with both strains, was the decrease in mutation frequency observed with the 4.0mM sample.

4. Propylene Oxide (PO).

PO at concentrations of 25 and 100mM was reacted with single-stranded replicating Ø105 DNA in 50%DMF-50%SSC/10, pH 6.1, at 42°C for 30 min. Marker rescue was performed with GB7075 *hcr*-9 *trp*C2 as the recipient, and the results are shown in Fig. 19. The increase in the frequency of clear PFU/total PFU was significant at $p<0.001$. No consistent decrease in marker rescue activity was observed. Since PO had disrupted linkage in GB7018 DNA, an experiment was performed to see if it had the same effect on replicating Ø105 DNA. To accomplish this, replicating DNA from Ø105D11:6c, a phage which carries a clear plaque mutation linked to *Jsus*11 (Scher, *et al.*, 1978) was exposed to various concentrations of PO. These DNA samples were then used to rescue *Jsus*11 phage, with BD99 as the recipient, and the frequency of clear plaquing phage produced was scored (Table 15). Interestingly, over the concentration range in which mutagenesis was examined, there was no disruption of linkage observed between the clear plaque mutation and the *Jsus*11 marker.

Fig. 19. Propylene oxide mutagenesis of single-stranded replicating Ø105 DNA. Propylene oxide, at a concentration of 25mM and 100mM was reacted with Ø105 replicating DNA at 42°C for 30 min in 50%DMF, pH 6.1. Marker rescue with each treated sample was performed as described in Methods. Since when the 0 min sample was used in a marker rescue there were no clear plaques observed among >100,000 screened, the background frequency of Clear PFU/Total PFU is described as $\leq 10^{-5}$.

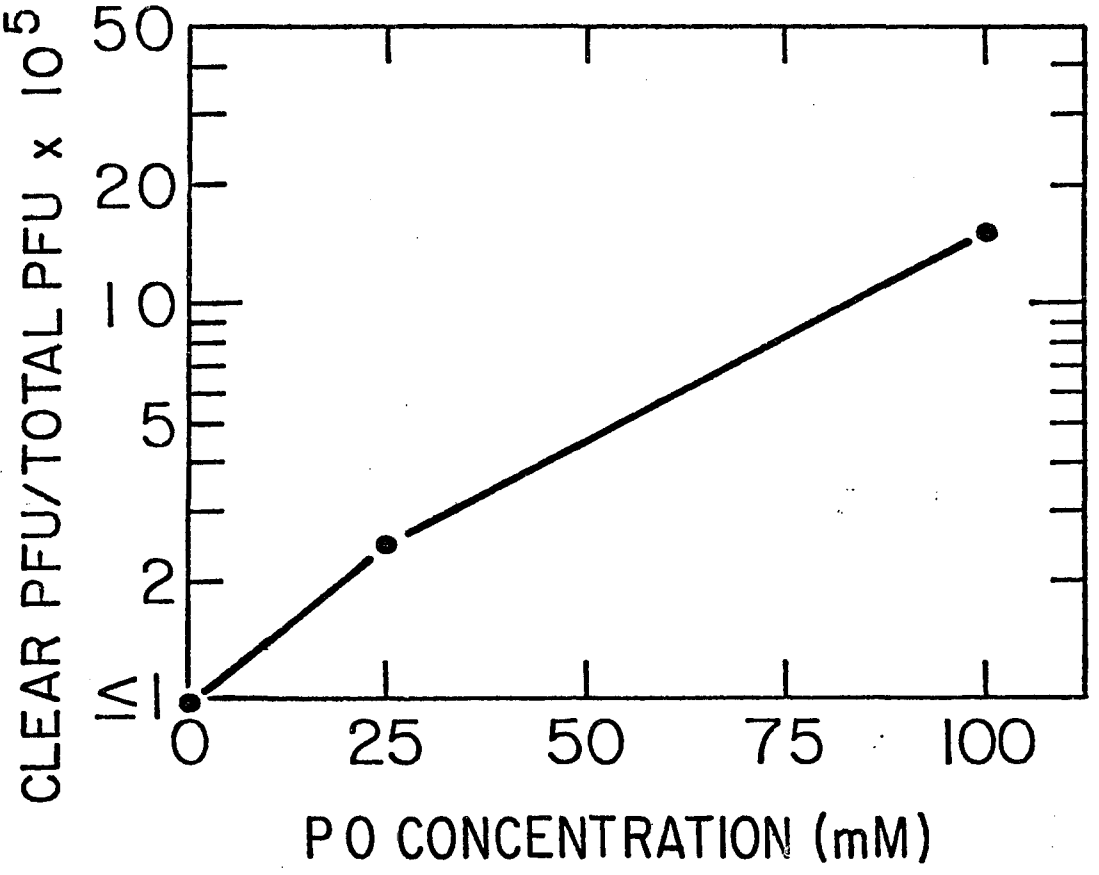

CLEAR PFU/TOTAL PFU x 10^5
50
20
10
5
2
1/
0
25
50
75
100
PO CONCENTRATION (mM)

Table 15. The Effect of PO on Linkage of Replicating Ø105 D11:6c DNA

PO Concentration	Clear PFU/Total PFU
0	0.56
25	0.66
50	0.62
100	0.54

Single-stranded replicating Ø105 D11:6c DNA at a concentration of 100µg/ml in 50%DMF-50%SSC/10 was incubated at 42°C for 30 min with PO at the concentrations listed. Marker rescue with each treated sample was performed with BD99 as the recipient, and the number of Clear PFU/Total PFU was scored.

5. AAAF.

AAAF was reacted with single-stranded replicating Ø105 DNA in 50%DMF-50%SSC/10, pH 6.1 at 37°C for a 9 min period. The ratio of AAAF to DNA nucleotide was 1.3:1.0. The AAAF treated DNA samples were examined for their ability to generate clear plaque mutants during rescue of *Jsus*11 phage, using both BD99 and the repair deficient strain GB7075 *hcr*-9 *trp*C2 as recipients. The results obtained are shown in Fig. 20. AAAF produced a significant increase in the frequency of clear PFU/total PFU with both recipients, at a $p<0.001$ with BD99 and $p<0.025$ with GB7075, and as can be seen, the drop in marker rescue activity was greater in the repair deficient strain.

6. BPDEs.

Both the (±)*anti* and (±)*syn* forms of BPDE were reacted with single-stranded replicating Ø105 DNA in 50%DMF-50%SSC/10, pH 6.1, at 42°C, over a 15 min period. The ratio of hydrocarbon to DNA nucleotide was 1.2:1.0, and marker rescue with the treated samples was performed with BD99. The mutagenic activity of the (±)*anti* BPDE was detected in the 11 min and 15 min treated DNA samples, but only in the 2 min sample of the (±)*syn* BPDE treated DNA (Table 16). Analysis by the Poisson heterogeneity test indicated that with both forms of the BPDE the increase in the frequency of clear PFU/total PFU was significant at a $p\leq 0.05$. The marker rescue activity was decreased 90% over 15 min treatment with the (±)*anti* BPDE, and 70% in 2 min treatment with the (±)*syn* BPDE.

Fig. 20. AAAF mutagenesis of replicating Ø105 DNA. AAAF was reacted with single-stranded replicating Ø105 DNA at a ratio of AAAF to DNA nucleotide of 1.3:1.0. The reaction was carried out in 50%DMF-50%SSC/10, pH 6.1 at 37°C over a 9 min period, and marker rescue with the treated samples was performed both with BD99 and the repair deficient strain GB7075 *hcr*-9 *trp*C2. Since when the 0 min sample was used in a marker rescue with BD99 as the recipient there was one clear plaque among approximately 120,000 screened, and no clear plaques observed among >100,000 PFU screened when the 0 min sample was used in a marker rescue with GB7075 as the recipient, the background frequency is described as $\leq 10^{-5}$. Clear PFU/Total PFU, BD99 (●), GB7075 (○); Relative Marker Rescue Activity, BD99 (■), GB7075 (□).

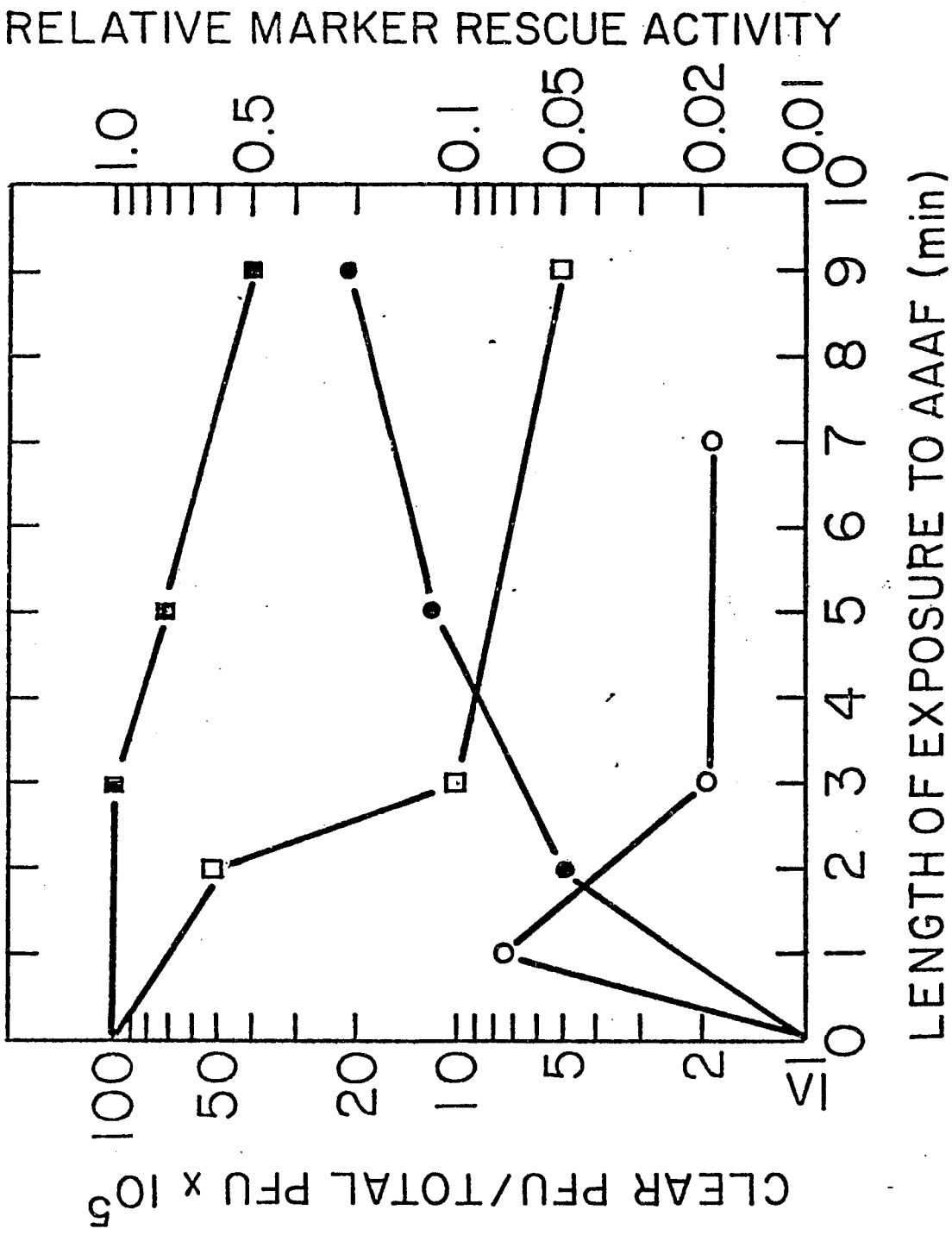

RELATIVE MARKER RESCUE ACTIVITY
1.0
0.5
0.1
0.05
0.02
0.01
0 1 2 3 4 5 6 7 8 9 10
LENGTH OF EXPOSURE TO AAAF (min)
100
50
20
10
5
2
VI
CLEAR PFU/TOTAL PFU x 10^5

Table 16. BPDE-Mediated Mutagenesis and Inactivation of Marker Rescue Activity of Replicating Ø105 DNA.[a]

BPDE (±)isomer	Treatment Time (min)	Clear PFU/Total PFU x 10^5
	0	≤1[b]
(±)*anti*	11	63
	15	1.8
	0	≤1
(±)*syn*	2	19

[a] Single stranded replicating Ø105 DNA was reacted with (±) *anti* and (±) *syn* BPDE at a ratio of hydrocarbon to DNA nucleotide of 1.2:1.0. A ratio of 1.2:1.0 corresponds to a BPDE concentration of 0.36mM and a DNA concentration of 100 µg/ml. The reaction was carried out in 50%DMF-50%SSC/10, pH 6.1 at 42°C over a 15 min period.

[b] The background frequency of Clear PFU/Total PFU is listed as $\leq 10^{-5}$ since there were no clear plaques observed among 100,000 PFU screened.

G. Sporulation Assay.

1. Chloroacetaldehyde.

The only standard mutagen which was tested in the sporulation assay was chloroacetaldehyde. GB7051 *thr*-5 *trp*C2 *rif*-1 was treated with several doses of the compound in 45% DMF, 0.03N sodium acetate buffer, pH 6.0, at 53°C for 15 min. The results obtained are illustrated in Fig. 21, where it can be seen that mutagenic activity of chloroacetaldehyde was detected with this assay. The increase in the Spo^-/Rif^r clones in the chloroacetaldehyde treated samples compared to the control was significant in the Poisson heterogeneity test at a $p<0.001$. There was an absolute increase compared to the control, in the number of Spo^- clones obtained with each of the mutagen treated DNA samples, and the transforming activity decreased 44% over the concentration range.

2. 2-bromo-2-chloro-1,1-difluoroethylene (BCD).

Halothane (2-bromo-2-chloro-1,1,1-trifluorethane) is one of the most widely used inhalation anesthetics. At least 25% of the halothane absorbed during the course of anesthesia is metabolized (Cascorbi, *et al.*, 1970) and one of the proposed intermediates (Cohen, *et al.*, 1975) is the dehydrofluorination product 2-bromo-2-chloro-1,1-difluorethylene. This compound was particularly interesting because of its structural similarity to many carcinogenic haloalkenes. When this compound was tested in the sporulation transformation assay, it was shown to be mutagenic (Table 17). In this

Fig. 21. Chloroacetaldehyde mutagenesis of GB7051 DNA. GB7071 *thr*-5 *trp*C2 *rif*-1 was reacted with several different concentrations of chloroacetaldehyde in 45%DMF, 0.03N sodium acetate buffer pH 6.0 at 53°C for 15 min. In order to remove chloracetaldehyde and DMF the treated samples were dialyzed extensively at 4°C against a buffer consisting of 0.15M NaCl, 0.005M sodium phosphate pH 7.4. Transformation with GB7044 as a recipient was carried out as described in Methods. Differentiation between Rif^r Spo^+ and Rif^r Spo^- colonies was possible after 96 hours of incubation at 37°C on rifampin supplemented plates.

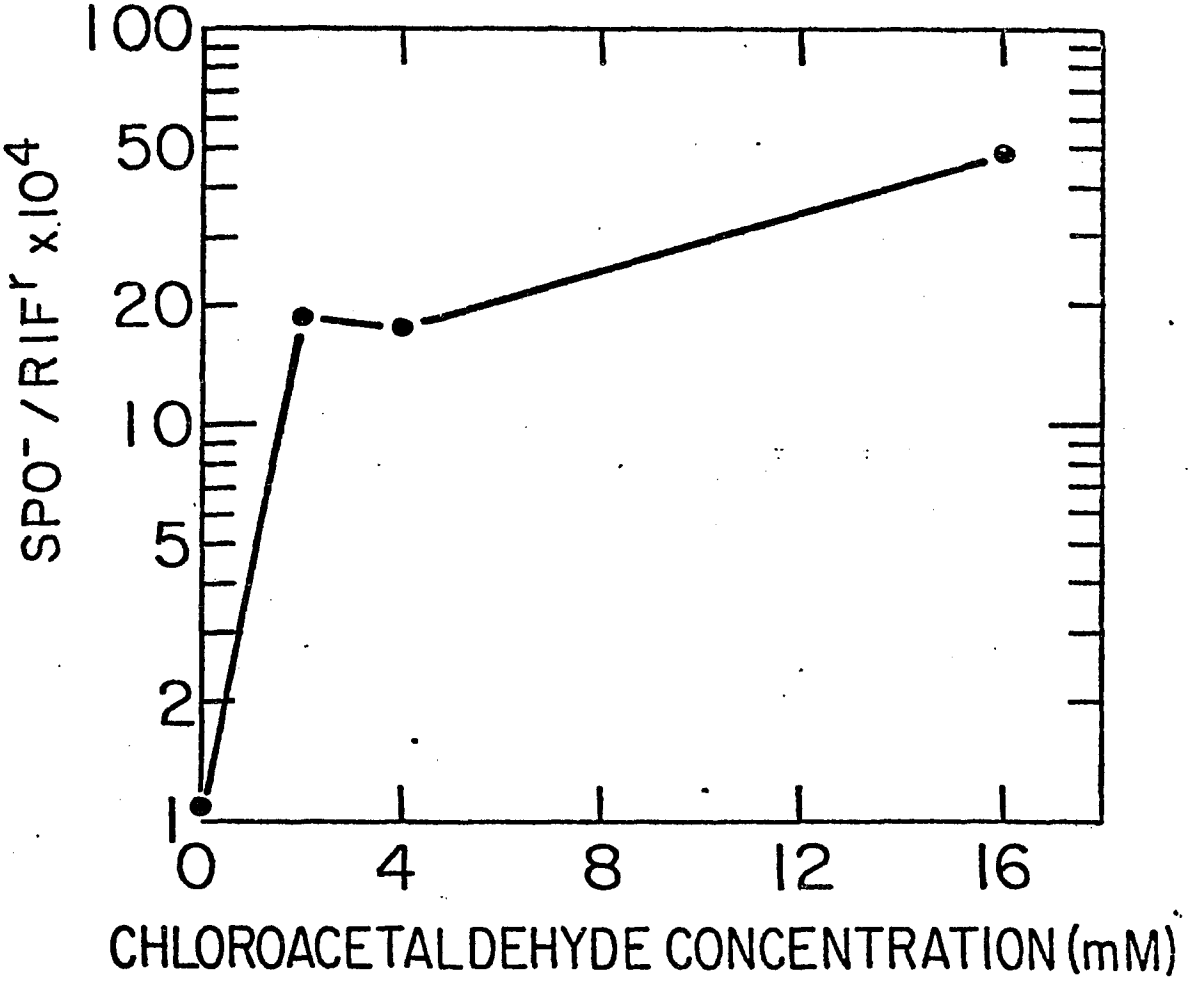

100
50
20
10
5
2
1
SPO⁻/RIF^r x10^4
0
4
8
12
16
CHLOROACETALDEHYDE CONCENTRATION (mM)

Table 17. BCD-Mediated Mutagenesis and Inactivation of Transforming Activity of GB7051 Rif^r Spo^+ DNA.

Incubation Time (min)	mmoles BCD Added x 10^2	Rif^r Transformants/ml x 10^{-3}	Spo^-/Rif^r Colonies x 10^4
0	0	104	5
15	4.8	26	33
30	9.6	8	112
45	14.4	1	344

GB7051 DNA was alkali-denatured and treated at a final concentration of 74μg/ml with BCD in the presence of 45%DMF, 0.14M phosphate, pH 7 at 53°C. The initial reaction volume was 3.6ml. At each time point a 0.5ml sample was removed and 0.05ml of a 0.96M solution of BCD in ethyl ether was added. The DNA was dialyzed and added at a final concentration of 5.5μg/ml to competent cultures of *B. subtilis* Spo^+,Rif^s. The transformation mixtures were incubated at 37°C for 1 hour and then diluted 5-fold into nutrient broth, and the incubation continued for 3 hours prior to plating to allow expression of the Rif^r phenotype. Differentiation between Rif^r,Spo^+ and Rif^r,Spo^- colonies was possible after 96 hours of incubation at 37°C on rifampin supplemented plates.

experiment there was an increase in the absolute number of Spo^- clones obtained with the treated DNA samples (Data not shown) and the increase in the Spo^-/Rif^r transformants was significant at a $p<0.001$.

H. Trimethoprim Assay.

GB7055 *thy*A DNA was treated with HA, MNNG and chloracetaldehyde under the same conditions as described for the reversion assay using GB7018 *ilv*B2 *trp*C2 DNA. The treated GB7055 DNA was used to transform GB7074 *ilv*A8 Δ*thy*A. Two procedures were used to assay for Trm^r transformants. In one after exposure to DNA competent cells were plated directly onto medium containing 7.5ug/ml of trimethoprim and 50ug/ml of thymidine and incubated at 37°C for 48 hours. In the second procedure, after a 60 min exposure to DNA the cells were mixed with thymidine supplemented top agar, layered onto thymidine supplemented plates, and incubated at room temperature for 16 hours. After this time, top agar supplemented with trimethoprim and 50ug/ml of thymidine was layered onto the plates to yield a final trimethoprim concentration of 7.5µg/ml. The cells were then incubated at 37°C for 48 hours.

No Trm^r colonies were detected above the background frequency in any of the experiments using mutagen treated DNA (data not shown). However, when DNA from a Trm^r strain, GB7057 *thy*A *thy*B was used to transform GB7074, Ilv^+ Trm^r colonies were obtained with a transformation efficiency of 0.1%. Therefore, if any mutations had been induced in the *thy*B gene of GB7055 DNA, they should have been readily detected.

I. The Effect of Microsomes on *B. Subtilis* DNA.

In order for the transformation or marker rescue based mutagen detection assays to have the ability to detect the mutagenic potential of carcinogens such as benzo(a)pyrene and dimethylnitrosamine, which are inactive until metabolized by microsomal enzymes, the assays must be coupled with a microsomal activation system. One of the potential problems is that the microsomal fraction might contain nucleases which could destroy the transforming and marker rescue activity of DNA, or unlink the genetic markers. In order to test this possibility, transformation of linked genes of the tryptophan biosynthetic pathway (Fig. 1) was evaluated after *B. subtilis* DNA had been exposed to microsomes and an NADPH generating system (Table 18). The donor DNA used carried a wild-type *trp*B gene (Ind+), and a closely linked mutated *trp*D gene. The recipient cell was Ind$^-$ and carried a wild-type *trp*D gene. As described in the Introduction, when Ind$^+$,*trp*D colonies are grown on limited amounts of indole, they excrete a fluorescent metabolite after the indole is exhausted. In order to test the effect of the microsomes on transforming activity and gene linkage, Ind$^-$ cells were transformed with Ind$^+$,*trp*D DNA which had been exposed to microsomes. Ind$^+$ transformants were selected and these transformants were then screened for co-transformation of the mutant *trp*D gene by viewing the clones under UV light, to identify those excreting fluorescent metabolites (Anagnostopoulous, and Crawford, 1961). The % linkage was determined by dividing the number of Ind$^+$*trp*D transformants

by the total number of Ind^+ transformants.

As can be seen in Table 18, during the course of a 30 min incubation of DNA with microsomes there was an 86% decrease in the transforming activity and a 7% decrease in linkage of double-stranded DNA, whereas with single-stranded DNA there was a 72% decrease in transforming activity and a 6% decrease in linkage. The decrease in linkage was therefore negligible, and would not affect the sensitivity of the assays. Because of the high initial transforming and marker rescue activity in the reversion and forward mutation assays respectively, it is unlikely that the decrease in transforming activity observed over 30 min would be severe enough to obscure the detection of mutants. It should be noted that the high transforming activity of the single-stranded DNA relative to the double-stranded DNA seen in Table 18 can be accounted for by the fact that different preparations of competent cells were used for the single-stranded and double-stranded transformations. Finally, in order to determine if the decrease in transforming activity was due to the presence of microsomes in the transforming mixture, after exposure of double-stranded DNA to the microsomes the DNA was isolated by phenol extraction before exposure to competent cells. In this latter experiment (data not shown) there was still a 50-60% decrease in transforming activity, indicating that the presence of the microsomes in the transformation mixture was not the cause for the decrease in transforming activity.

Table 18. Effect of Incubation of *B. subtilis* DNA With Rat Liver Microsomes on DNA Transforming Activity and Genetic Linkage.

DNA Conformation	Incubation Time(min)	Ind^+ Transformants[a,c] per ml x 10^{-3}	%Linkage of Ind^+[b] Marker and *trpD*
Double-stranded	0	462	0.70
	15	210	0.62
	30	66	0.65
Single-stranded	0	900	0.63
	15	436	0.60
	30	248	0.59

[a] 80µg/ml of double stranded or base denatured *B. subtilis* Ind^+,*trpD* DNA was incubated with 3mg/ml of hepatic microsomes at 37°C, with an NADPH generating system consisting of 32mM trisodium DL-isocitrate, 1.3mM $NADP^+$, 10mM $MgCl_2$ and 40µl isocitrate dehydrogenase, in a 0.1M phosphate buffer, pH 7.4. At the times indicated, samples were removed, heated at 65°C for 10 min to inactivate microsomal enzymes, and then used to transform a competent Ind^-, $trpD^+$ strain of *B. subtilis*.

[b] Cotransformation of *trpD* with Ind^+ was assayed by screening the transformants for the excretion of fluorescent metabolites.

[c] Transformation with single and double-stranded DNA was carried out with different preparations of competent cells, which accounts for the higher transforming activity obtained with the single-stranded DNA.

CHAPTER IV

DISCUSSION

The results presented above establish the validity of the use of two new mutagen detection assays in which isolated DNA is used as the target for chemical modification. Both the transformation based assay, in which the reversion of the *ilv*B2 base-substitution mutation was scored, and the marker rescue assay, in which the induction of forward mutations in the immunity region of replicating Ø105 DNA were scored, detected the mutagenic potential of the base-substitution mutagens HA, MNNG, chloroacetaldehyde and PO. In addition, the marker rescue assay detected the mutagenicity of the frame-shift mutagens AAAF, (±)*syn*BPDE and (±)*anti*BPDE. In all experiments in which the assays detected a chemical as being mutagenic, the difference between the control groups and the treated groups was significant with p values ranging from <0.05 to <0.001 depending on the experiment. Although attempts were made to revert the *leu*A169 marker in transforming DNA with AAAF and the BPDEs, no mutagenic effect was apparent. Therefore at the moment only the forward mutation system can be used to detect both base-substitution and frame-shift mutagens.

A. Gene Linkage Effects.

In addition to the difference in their capacity to detect frame shift mutagenesis the reversion and marker rescue assays also appeared to respond differently to alkylations which led

to DNA strand scissions. This was reflected in the fact that cotransformation of linked genes was decreased after PO treatment of GB7018 *ilv*B2 *leu*A169 DNA (Table 13) while there was no detectable effect of PO treatment on gene linkage in replicating Ø105 DNA (Table 15). It was expected that PO would disrupt gene linkage in single-stranded DNA since there is physical evidence suggesting that PO induces single strand breaks in DNA (Walles, 1974). It had been observed (Walles, 1974) that PO treatment decreased the temperature at which double-stranded DNA was irreversibly denatured. The induction of single strand breaks would explain this observation since they would increase the probability that DNA segments would totally dissociate at temperatures where intact molecules would still be held together by nucleation sites of high GC content. Walles (1974) proposed two mechanisms by which PO could induce single-strand breaks in DNA, First he suggested that the N^7-guanine modification reported by Lawley and Brookes (1972) could cause strand scissons since alkylation at this site is known to destabilize glycosydic linkages. Secondly, he argued that PO alkylation of DNA phosphates, which had been reported to occur (Lett, 1962), could lead to single-strand breaks since the phosphotriester produced would have a hydroxyl group vicinal to the phosphate and such modified nucleic acids are labile in neutral solutions (Kriek and Emmelot, 1963).

Since PO probably introduces the same number of single-strand breaks into both replicating Ø105 and *B. subtilis* genome DNA, it is possible that the observed difference in

sensitivity to linkage disruption is due to structural differences between the bacterial and replicating phage DNAs The replicating form of Ø105 DNA is a concatameric molecule. Flock (1978) had shown that when double-stranded replicating Ø105 DNA was phenol extracted from infected cells, it had a molecular weight 2 to 4 times greater than the mature Ø105 genome. This molecule therefore contains 2 to 4 full length Ø105 genomes which are linked together. This means that there are 2 to 4 copies of each linkage group (e.g. *Jsus*11 and the clear region) on a single piece of DNA. Although it is not known whether the double-stranded concatamers contain many single strand nicks, it is quite possible that single-stranded molecules of greater than one genome's length exist . It is known, for example, that during replication of concatameric bacteriophage λ DNA, the cellular *rec*BC nuclease is inhibited (Greenstein, M. and Skalka, 1975). If a similar situation occurred during Ø105 replication,then host nucleases which could introduce nicks during the isolation of *B. subtilis* DNA might be inhibited,thus allowing the isolation of replicating Ø105 DNA which contained relatively few nicks.

The relationship of the structure of single-stranded replicating Ø105 DNA to its apparent resistance to linkage disruption is based on the fact that cotransformation of two linked markers is essentially dependent upon the binding by competent cells of DNA fragments carrying the two genes. If the replicating DNA existed as a concatemer, it might be more difficult to observe disruption of linkage between its genes

because even if linkage was disrupted in one part of the Ø105 DNA concatemer, there would still be 1 to 3 copies of each linkage group which remained intact. This situation is quite different, however, than would exist with *B. subtilis* DNA. When this DNA is phenol extracted, each isolated fragment carries only about 0.4% of the total genome (Morrison and Guild, 1972). Therefore each fragment carries only one copy of two linked markers, and an event leading to strand scission between these two genes would effectively exclude the possibility of these two genes entering a cell and participating in a linked transformation event.

The unlinking effect that PO had on GB7018 DNA was taken into account when determining the mutagenic potential of this chemical. As can be seen in Fig. 9, when a correction was made for a decrease in linkage, the calculated number of Ilv^+, Leu^+/Leu^+ increased 2 to 3 fold. This indicates that when using this assay to determine the mutagenicity of a chemical it may be advantageous to determine the effect that the compound has on linkage. In the extreme case, a compound which is mutagenic but also induces extensive single strand breaks could be missed in this assay.

B. Frame-Shift Mutagens

The reason for the failure to detect mutagenesis by the frame shift mutagens AAAF and the BPDEs in the reversion assay is not known. The possibility that the *leu*A169 marker was not revertible by these compounds was excluded by the results presented in Figs. 12, 13 and 14. These experiments clearly

show that cells carrying the *leu*A169 marker are reverted to Leu prototrophy by exposure to AAAF and the BPDEs.

The *in vitro* DNA mutagenesis reversion assay differs, however, from the whole cell reversion assay in a fundamental way, since in order for a mutant to be generated in this assay a piece of modified DNA must be taken up by a cell and recombined into its chromosome. If either uptake or recombination are blocked by the chemical modification then the mutant will not be generated. For example, Kubinski (1978) recently found that exposure to β-propriolactone or 1,3-propane sultone decreased the capacity of *B. subtilis* DNA to attach to and penetrate the cell envelope of competent cells. Furthermore, if the chemical exposure produced lesions in essential genes and if DNA fragments carrying such damaged genes were incorporated into the same cells as the mutagenized fragement which was being selected for, the mutant would not be detected because of cell death (Bresler, *et al.*, 1968b). It is unlikely, however, that these events were the cause for the failure to detect mutagenesis of the *leu*A169 marker since AAAF and the BPDE modified replicating Ø105 DNA successfully participated in marker rescue and produced mutants. In addition, Maher (1968,1971) had reported that AAAF and a derivative of benzo (a)pyrene were mutagenic for transforming DNA when assayed with the Freese forward mutation test (Freese and Strack, 1962). Thus DNA modified with these compounds can be taken up by cells, recombine with either chromosomal or phage DNA, and the mutations they carry can be expressed.

Although the AAAF and the BPDEs reverted the *leu*A169 when whole cells were exposed to these compounds in aqueous buffer, it is possible that different lesions which would not revert the *leu*A169 marker were produced when DNA was treated with these compounds in the DMF solvent system. In this context, Osborne, *et al.*, (1978) have reported that solvent conditions do affect the type of lesion produced when (+)*anti*-BPDE is reacted with DNA and purine bases. They found that the reaction of (+)*anti*-BPDE with *E. coli* DNA in sodium cacodylate-methanol buffer produced both an N^2-guanine and an N^7-guanine adduct. When the same compound was reacted with guanine in DMF, however, at least three derivatives separable by Sephadex LH-20 chromatography were produced, one of which was the N^7-guanine adduct. The N^2-guanine adduct was not formed. It is possible therefore that the N^2-guanine adduct is what causes reversion of the *leu*A169 marker when the whole cells are exposed in aqueous buffer, and this product may not have formed when the DNA was incubated in DMF with the BPDE's. On the other hand, N^7-guanine adducts, or other possible adducts formed in DMF could account for the forward mutations observed in replicating Ø105 DNA.

Although there is no evidence to support the possibility that AAAF modifications of DNA are altered by solvent conditions, it is known that when AAAF is reacted with denatured DNA in phosphate-ethanol buffer, or when cells are treated with AAAF (Yamasaki, *et al.*, 1977, Westra, *et al.*, 1976), a major and a minor product are formed. The major product is N-(deoxy-

guanosin-8-yl)-N-acetyl-2-aminofluorene, and the minor product is 3-(deoxyguanosin-N-yl)-N-acetyl-2-aminofluorene. It is possible that only one of these lesions can revert the *leu*A169 marker, and that this lesion is not formed to the same extent in the DMF solvent.

The importance of the specific adducts formed by frame-shift mutagens can be related to the fact that in order to revert a preexisting frame-shift mutation, several relatively strict requirements must be met. First of all, a chemical modification of DNA, resulting in either an addition or deletion of base pairs must occur close to the original mutation, so that the proper reading frame can be restored. For example, it has been reported that reversion of the *his*D3052 frame-shift mutation in Ames strain 1534 is probably the result of a single base-pair deletion in a particular sequence of nucleotides (Ames, 1972). Furthermore, it appears that a particular modification might be necessary in order for the frame-shift to occur. For example, it has been suggested that binding of the activated metabolite of aflatoxin B_1 to a specific N^7-guanine is responsible for reversion of the *his*D3052 mutation (D'Andrea and Hazeltine, 1978). It is possible that if the aflatoxin derivative bound to another site on guanine, it might not revert the *his*D3052 mutation, even though it might damage the DNA. On the other hand, a change in any part of the nucleotide sequence of a gene could lead to a forward mutation. Therefore, if a change in solvent altered the type of products which were formed, this might

more readily decrease the mutagenicity of a compound in a reversion assay than in a forward mutation assay. In light of these arguments, even though DMF has several advantages as a solvent system, it may be prudent when examining unknown compounds for mutagenicity to conduct such tests in several different solvent systems.

A final point with respect to the differences between the assays' ability to detect mutagenesis by the BPDEs is that the compounds were reacted with single-stranded replicating Ø105 DNA, whereas they were reacted with double-stranded GB7037 *leu*A169 DNA. This difference in DNA conformation also could have affected the types of lesions which were formed. For example, Meehan and Straub (1979) reported that when racemic (±)*anti* BPDE was reacted with double-stranded calf-thymus DNA in phosphate buffer, pH 7.5, 86% of the products resulted from the binding of the (+) enantiomer to N^2-guanine, 4% were from binding of (-)*anti* BPDE to N^2-guanine, and 10% resulted from the binding of the (+) and (-) enantiomers to N^6-adenine. When the DNA was reacted in single-stranded form, however, both the (+) and (-) enantiomers of *anti* BPDE reacted equally with N^2-guanine, contributing 60% of the total products formed. The remaining 40% of the adducts produced with the single-stranded DNA were deoxyadenosine derivatives.

It should be noted that the only reason that BPDE was not tested with single-stranded GB7037 DNA was because of limited quantities of the compound available. Since it was

known that BPDEs bound to double-stranded DNA (Weinstein *et al.*, 1976), and because Maher (1971) had found that benzo-(a)pyrene was mutagenic to double stranded transforming DNA in the Freese assay, it was originally decided to test the ability to revert the *leu*A169 marker in GB7037 DNA by reacting the DNA in its double-stranded form. When this procedure failed, the decision was made to test the remaining BPDE in the Ø105 clear plaque marker rescue assay, using single-stranded DNA.

C. DNA Repair and Mutagenesis.

In the marker rescue assay the effect of using a recipient cell which carried the *hcr*-9 mutation was studied in order to see if the use of this strain would enhance the ability to detect the mutagenicity of AAAF and chloroacetaldehyde. The *hcr*-9 mutation results in an increased sensitivity to UV light and a decreased ability to support multiplication of UV damaged phage, but does not have a significant affect on recombination (Okubo and Romig, 1965). The *hcr*-9 mutation probably affects an excision repair pathway. Mutants of *E. coli* such as *uvr*A and B,which have a similar phenotype to *hcr*-9, are defective for excision repair (Howard-Flander, 1966), and in fact *hcr*-9 has been mapped in the *B. subtilis* *uvr*A locus (Munakata, 1977). We reasoned that the *hcr*-9 mutation might enhance mutagenesis by increasing the probability that a mutagen induced DNA lesion would be repaired by an error-prone pathway. Error prone repair, commonly called SOS repair (Radman, 1975), is induced in cells in

response to DNA damage. It has been postulated that SOS repair produces mutations by decreasing the fidelity of DNA synthesis (Caillet-Fauquet, *et al.*, 1977).

As pointed out in the Introduction, there is evidence that SOS related functions are spontaneously induced when *B. subtilis* cells are grown to competence. Among the first observations to support this possibility was Garro and Law's (1974) finding that resident *B. subtilis* prophages were induced as cells became competent for transformation. Since prophage induction is one of the functions coordinately derepressed with SOS repair when *E. coli* cells are exposed to certain DNA damaging agents (Witkin, 1976), it seems reasonable that prophage induction in competent *B. subtilis* represents one aspect of an SOS-like system. Recently, Yasbin (1977a) has shown that there is inducible repair in *B. subtilis* which is activated when cells are exposed to UV irradiation. Since all other known inducible repair systems are error-prone (Witkin, 1976), it is likely that *B. subtilis*, like *E. coli*, carries out error-prone repair. The induction of SOS functions during growth to competence is probably due to the decreased rate of DNA synthesis associated with this physiological state (Dooley, *et al.*, 1971); inducers of SOS related functions in *E. coli* also inhibit DNA synthesis (Witkin, 1976). Therefore, in competent GB7075 *hcr*-9 the state of the repair system is not only excision defective, but probably error prone as well. On the other hand, competent BD99

would be induced for error prone repair, but they would also have a greater level of error-free repair relative to GB7075.

With respect to the sensitivity with which the Hcr^+ and the Hcr^- recipients detected mutations in chloroacetaldehyde treated DNA, it can be seen in Fig. 18 that with 1mM chloroacetaldehyde, mutagenesis was detected equally well with either recipient. However, at higher doses of chloroacetaldehyde, at which marker rescue activity was falling off more rapidly with the Hcr^- recipient relative to the Hcr^+ recipient, a higher frequency of mutants was generated in the Hcr^- host. This could be explained by the *hcr*-9 strain having a low level of error-free repair which can act on chloroacetaldehyde lesions. The mutagenesis seen at low doses of chloroacetaldehyde would then be due to occasional error-prone repair that may take place in any competent cell. The hypothetical low level of error-free repair in the *hcr*-9 strain would be easily saturable, resulting in higher mutation frequencies with more heavily modified DNA.

In the AAAF experiments DNA was exposed to a single concentration of AAAF for varying lengths of time. It appeared that both the Hcr^- and Hcr^+ strains were equally capable of expressing the AAAF induced lesions as mutations with the DNA exposed for the shorter periods of time. With more extensively modified DNA the marker rescue activity was rapidly lost with the Hcr^- recipient as was the ability to detect mutations with the host. The marker rescue activity of this same DNA was less drastically affected with the Hcr^+

host in which the frequency of clear plaque mutants generated by the AAAF treated DNA increased in a dose dependent fashion. As was previously noted, AAAF is known to produce two types of lesions in DNA, a C^8-guanine adduct and an N^2-guanine adduct. The C^8-adduct is thought to produce a major distortion in double-stranded DNA and is believed to be rapidly repaired by the excision pathway (Yamasaki, *et al.*, 1977). The N^2 adduct, on the other hand is not thought to distort the helix, and is more persistent in the DNA; it also is believed to be the principle mutagenic and carcinogenic lesion of AAAF (Westra, *et al.*, 1976, Yamasaki, *et al.*, 1977). Assuming that the AAAF-treated DNA contained both adducts, in the Hcr^- strain the C^8-adduct presumably would not be readily repaired in the initial donor-recipient DNA complexes, thereby possibly interfering with the completion of the recombination process. In heavily modified DNA this might limit the incorporation of the mutagenic N^2-adduct with concommitant loss in the ability to detect AAAF induced mutations. Finally, with regard to the use of the Hcr^+ and Hcr^- strains as recipients in mutagenesis assays the results presented here suggest that both should be used with unknown compounds as the sensitivity with which they detect mutagenesis appears compound dependent.

D. Sporulation Assay.

The sporulation assay was tested with only one of the standards mutagens, chloroacetaldehyde, which it detected with a sensitivity equal to that of the marker rescue assay.

The sporulation assay also detected the mutagenic activity of the presumed halothane metabolite BCD. The mutagenicity of BCD toward *Salmonella* strains TA92, TA98 and TA100 was demonstrated in this laboratory (Garro and Phillips, 1978). In addition, investigators at Stanford recently found that a second halothane metabolite, 1,1-difluoro-2-chloroethylene is mutagenic in the Ames test (Edmunds, 1979). The Stanford group also observed that BCD can be generated non-metabolically from halothane in anesthesia machines equipped with soda-lime canisters (Sharp, 1979).

These results are important in light of epidemiological studies which suggest that operating room personnel exposed to low doses of inhalation anesthetics may experience an increased risk of cancer, spontaneous miscarriages and congenital anomalies among their offspring (Cohen, 1974,1975). In addition, the possibility has been raised in a report submitted by W.B. Coate, *et al.*, (1978) of Hazelton Laboratories to the National Institute of Occupational Safety and Health (NIOSH) that halothane administered with nitrous oxide causes genetotoxic effects. In the Hazelton study, halothane was administered to rats at a concentration of 1 part per million (ppm) with 50ppm of nitrous oxide, which is twice the recommended exposure limits (NIOSH, 1977) and at 10ppm with 500ppm of nitrous oxide, which is the average of unscavenged operating rooms. Dose-dependent increases in cytogenetic aberrations in both bone marrow and spermatogonial cell populations, as well as retardation in fetal development

were observed. In addition, decreases in ovulation and implantation were found at the higher level of exposure. These findings, in conjunction with the mutagenicity data, suggest that exposure to halothane should be kept to an absolute minimum through use of proper scavenging equipment (Garro, *et al., 1979)*.

Although both the sporulation assay and the marker rescue assay detect the induction of forward mutations, the marker rescue assay has significant advantages for use as a mutagen screening test. First, after exposure of DNA to a compound, it only takes 16 additional hours to determine if the chemical is mutagenic in the marker rescue assay as compared to 96 hours in the sporulation assay. Second, the marker rescue assay has the potential to more readily detect weak mutagens relative to the sporulation assay. This is because only a small fraction of organisms, generated from DNA exposed to a weak mutagen, would be mutants. While mutants can be scored in the marker rescue assay against a background of approximately 10^5 wild-type phage per plate, in the sporulation assay mutants can not be scored on plates containing more than about 10^3 Rif^r, Spo^+ transformants. Therefore, many more plates would have to be screened in order to detect a mutant in the sporulation assay as compared to the marker rescue assay.

F. Trimethoprim Assay: *Thy*B Mutagenesis.

In order for a *B. subtilis* cell to exhibit a Trm^r phenotype, both the *thy*A and *thy*B gene products must be inactive.

In the trimethoprim assay, DNA from a cell carrying a mutant *thy*A gene was exposed to chemicals in an attempt to mutagenize the *thy*B gene. Attempts were made to score mutagenic events by transforming *thy*A cells with the treated DNA, and assaying for the incorporation of a mutated *thy*B gene by selecting Trm^r transformants. This assay, however, was unable to detect the mutagenicity of HA, MNNG or chloroacetaldehyde. Since DNA from GB7057 *thy*A *thy*B *his*B2 could transform GB7074 *ilv*A-8 *thy*A to a Trm^r phenotype, it was apparent that the inability to detect *thy*B mutations in GB7055 *thy*A DNA was not due to some methodological problem such as toxic trimethoprim concentrations. One possible explanation is that the *thy*B gene exists as a merodiploid, so that more than one copy of the gene is present in the chromosome. If this were the case, in order to transform a *thy*A cell to Trm^r, the transforming DNA would have to carry mutations in all of the *thy*B genes. It is possible that GB7057 DNA carries mutations in all of these postulated *thy*B genes, thus accounting for the ability of this DNA to transform a *thy*A cell to Trm^r. On the other hand, the probability of inducing mutations in all *thy*B genes of GB7055 *thy*A transforming DNA would be low, which could account for the failure of this chemically treated DNA to transform cells to Trm^r.

G. Trimethoprim Assay: *Thy*P3 Gene Mutagenesis.

The genetic basis for the use of *rho*11 or Ø3T DNA in a mutagenesis assay was Williams and Young's (1977a,b) report that transformation of Thy^- cells with DNA from these phages

converted the cells to Thy$^+$. It was implied that the phage *thy*P3 gene (thymidylate synthetase), which was responsible for the transformation, physically replaced the *thy*A gene of the host chromosome. We postulated that if cells with a mutation in *thy*B were transformed with mutagenized Ø3T or *rho*11 DNA, trimethoprim resistant cells defective for both the *thy*A and *thy*B gene products should be produced via mutations introduced into *thy*A by recombination with a mutated *thy*P3 gene.

Development of this assay was abandoned after it was found that the Thy$^+$ transformants generated by transformation of GB7057 *thy*A *thy*B with Ø3T and *rho*11 DNAs were unstable. In routine testing of these Thy$^+$ transformants, it was found that they reverted to a Thy$^-$, Trm^r phenotype at a relatively high frequency (Table 7). It is not surprising that this phenomena had been overlooked by other workers, since previously only the conversion to Thy$^+$ by Ø3T and *rho*11 had been of interest. A mechanism explaining these results is that rather than simply replacing the *thy*A gene of the host chromosome, the *thy*P3 gene may also or exclusively integrate adjacent to the *thy*A gene. This would yield a cell with a tandem arrangement of two closely related genes. Anderson and Roth (1977), have suggested that mutants and recombinants with such a genetic structure have a great tendency to revert to the original state through intrastrand recombination between homologous DNA sequences. Such recombinations lead to the loss of the DNA segment situated between the homologous, re-

combining sequences. In the present case, recombination between the wild-type *thy*P3 gene and a mutant *thy*A gene on a chromosome carrying both of these genes could cause the cell to revert to a Thy^-, Trm^r phenotype because of a loss of the wild-type *thy*P3 gene.

The implication of the results in Table 7, with respect to the use of the *thy*P3 gene was that even if *thy*P3 was mutagenically inactivated, this event might never be detected, since the mutated *thy*P3 gene might integrate adjacent to the *thy*A gene, rather than within it. The transformed cell would therefore be genetically *thy*B *thy*P3, but retain a Trm^s phenotype because of the presence of a wild-type *thy*A gene.

There is evidence which suggests a mechanism whereby the *thy*P3 gene could integrate adjacent to *thy*A. pCD1 is a chimeric plasmid which was formed by cloning the wild-type *thy*P3 gene of Ø3T in plasmid pMB9 (Duncan, *et al.*, 1978). Although pMB9 can not integrate into the *B. subtilis* chromosome, pCD1 can, and thereby convert a Thy^- cell to Thy^+. More relevantly, however, Duncan,*et al.* (1978) showed that when the *thy*P3 segment and the usual phage integration site were removed from pCD1, which left only segements of Ø3T DNA with no known genetic activity, the new plasmid pCD2 could still integrate into the *B. subtilis* chromosome. It is reasonable to assume that the fragments of Ø3T on the chimeric plasmid were responsible for the integration of the plasmid since as noted above, no homology between the *B. subtilis* chromosome and the pMB9 plasmid could be demonstrated.

Thus, a *thy*P3 gene, which is linked to this integrating Ø3T segment may integrate near *thy*A through regions of phage DNA responsible for integration of the chimeric pCD2 plasmid. Finally, an additional mechanism for integration of the *thy*P3 gene into the host chromosome has been suggested by Dean (personal communication), who has shown that extensive homology exists between phages SPβ, Ø3T and *rho*11. Since phage SPβ is carried by all but a single cured *B. subtilis* strain, the possibility exists that fragments of incoming DNA from Ø3T and *rho*11 could recombine with segments of the SPβ prophage, thereby providing another way for *thy*P3 to integrate into the chromosome without replacing the *thy*A gene.

I. Microsomal Coupling.

As the present time the *in vitro* DNA mutagenesis assays described here have only been used to detect the mutagenic activity of compounds capable of direct reaction with DNA. It will be important to broaden the scope of the assays so that they will be able to detect the mutagenic activity of compounds requiring metabolic activation. This will be necessary because the reactive metabolites of many suspected carcinogens will not be known *a priori*. The results shown in Table 18 indicate that coupling with a microsomal activation system should be feasible. After a 30 min incubation of both single and double-stranded transforming DNA with rat liver microsomes, there was only a 6% and 7% disruption of gene linkage, respectively. Although contact with microsomes may decrease transforming activity 72%-86%, there should still be

enough residual transforming activity to detect a chemical's mutagenic potential. It therefore appears that standard microsome preparations are not heavily contaminated with nucleases which would limit the ability to couple microsomal activation to the marker rescue and transformation based assays. Our observation that purified microsomes are relatively free of nucleases is consistent with the results recently presented by D'Andrea and Hazelton (1978) who utilized a DNA sequencing technique to determine the sites at which Aflatoxin B_1 bound to DNA after microsomal activation. If endonucleases in the microsomes had severely damaged the DNA, then the sequencing technique would not have been feasible, since the method which they used required that the molecules of DNA be of uniform length. These authors do point out, however, that some degradation occasionally occurred, but it could be minimized by the use of fresh preparations of microsomes.

The potential applicability of a coupled microsome marker rescue and/or transformation assay will be determined by the same type of characterization as was done above with the direct acting mutagens. Compounds such as dimethylnitrosamine, benzo(a)pyrene and aflatoxin B_1 are likely candidates for characterizing the coupled system. After the system was characterized, one of the more interesting compounds to test would be the carcinogen diethylstilbesterol (DES). As mentioned in the Introduction, DES is highly lipophilic and was not detected as a mutagen in the Ames assay. DES has

been shown, however, to bind to calf thymus DNA after metabolic activation by microsomes (Blackburn, 1976). Therefore, the reason for failure in the Ames assay was probably not due to a problem such as inability of microsomes to activate the compound to a reactive form. This suggests that the compounds inability to revert the Ames strains was because it may have been trapped in the bacterial membranes and did not reach the DNA. Since much of the theoretical basis for the use of the transformation and/or marker rescue based assays is that no membranes are present to interfere with uptake of compounds, testing of DES would provide a model situation for the use of these assays.

APPENDIX

ABBREVIATIONS

AAAF	N-acetoxy-2-acetylaminofluorene
2AAF	2-acetylaminofluorene
9AA	9-aminoacridine
BCD	2-bromo-2-chloro-1,1-difluoroethylene
BPDE	benzo(a) pyrenediolepoxide
CPM	counts per minute
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDTA	ethylenediamine tetraacetic acid
*his*A	requires histidine; probably blocked before imidazoleglycerol phosphate
*his*B	requires histidine, some mutants respond to histidinol
His	histidine
*Ilv*B	requires isoleucine and valine; lacks condensing enzyme
*ilv*C	requires isoleucine and valine; lacks reducto-isomerase
Ilv	isoleucine, valine
HA	hydroxylamine
ICR-191	2-methoxy-6-chloro-9-[3-(2-chloroethyl)amino-propylamino]acridine
Ind	indole
*leu*A	requires leucine; lacks α-IPM synthetase
*leu*C	requires leucine; lacks β-IPM isomerase
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MOI	multiplicity of infection
ppm	parts per million

Rif	rifampin
RNase	ribonuclease
Spo	sporulation
SSC	0.15M NaCl, 0.015M sodium citrate
Str	streptomycin
Thy	thymidine
*thy*A	defective for the major thymidylate synthetase of *B. subtilis*
*thy*B	defective for the secondary thymidylate synthetase of *B. subtilis;* the *thy*B gene product is inactivated at 46°C
t_m	melting temperature
dTMP	deoxythymidylic acid
Trm	trimethoprim
Trp	trypthopan
*trp*B	requires tryptophan; lacks tryptophan synthetase B
*trp*C	requires tryptophan; lacks indole-3-glycerol phosphate synthetase
*trp*D	requires tryptophan; lacks phosphoribosyl transferase
dUMP	deoxyuridylic acid
UV	ultraviolet
Val	valine

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