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MULTIPLICITY REACTIVATION AND REPAIR OF LETHAL LESIONS INDUCED BY NITROUS ACID OR ULTRAVIOLET IRRADIATION IN BACTERIOPHAGE T^4

by

Eileen Marie Nonn

A Dissertation Submitted to the Faculty of the DEPARTMENT OF MICROBIOLOGY In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY WITH A MAJOR IN MOLECULAR BIOLOGY In the Graduate College THE UNIVERSITY OF ARIZONA

1977.
I hereby recommend that this dissertation prepared under my direction by Eileen Marie Nonn entitled MULTIPLICITY REACTIVATION AND REPAIR OF LETHAL LESIONS INDUCED BY NITROUS ACID OR ULTRAVIOLET IRRADIATION IN BACTERIOPHAGE T4 be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy.

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Date

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ABSTRACT

An effort was made to define the gene functions that are involved in the multiplicity reactivation of damaged T4 phage particles. First, wild type phage were either treated with nitrous acid or exposed to ultraviolet irradiation. The treated phage were used to infect two populations of bacteria: one population was singly infected by the phage and is referred to as monocomplexes, the second population was infected with an average of two or more damaged phage and is referred to as multicomplexes. When the log of the fractional survival was plotted as a function of increasing dosage it was found that the multicomplexes were inactivated less rapidly than the monocomplexes. This increased survival ability of the multicomplexes is referred to as multiplicity reactivation. Then phage carrying mutations in genes whose products had previously been shown to be needed for normal levels of genetic recombination were used. These phage mutants were defective either in gene 47, 46, 32, px or y. Growth of host-phage monocomplexes and multicomplexes was carried out at semi-permissive or semi-restrictive conditions. At semi-restrictive growth conditions, levels of multiplicity reactivation were substantially reduced, while under semi-permissive conditions multiplicity reactivation returned to a level closer to that of wild type. When phage were used which had mutations in genes y, 30 or 44, multiplicity reactivation of nitrous acid damaged phage occurred at the wild type level. These results
indicate that the same gene functions that are required for marker exchange are also required for multiplicity reactivation which implies that both processes occur by the same or similar recombination pathways. In addition, it was found that host-phage monocomplexes formed from phage that are defective in gene 46, 47 or 32 were more sensitive to nitrous acid inactivation than monocomplexes formed from wild type phage. Quantitation of this increased sensitivity revealed that about 20% of the nitrous acid induced lethal lesions remain unrepaired in the mutant monocomplexes. These results are indicative of a post-replicative recombination repair pathway in bacteriophage T4.
INTRODUCTION

Wild type T4 bacteriophage are able to adsorb to a susceptible host and inactive it by turning off certain metabolic processes and redirecting others. During this infective cycle, they code for the production of new phage structures and functions within the cell. The resultant effect of this infection is the production of a population of phage progeny, usually numbering anywhere from fifty to three hundred per cell depending upon the conditions of infection. Phage able to carry out this process are referred to as 'active' particles (Hayes 1968; Dulbecco 1952).

'Inactive' phage particles may adsorb to a bacterial host and inactivate it by turning off or redirecting a limited number of metabolic pathways, but they are blocked at some point in the infectious cycle and do not give rise to the production of active phage progeny (Hayes 1968; Dulbecco 1952).

Populations of bacteriophage that have been exposed to a deleterious agent, such as ultraviolet (UV)\(^1\) light, sustain damages in a fraction of their chromosomes that can result in phage inactivation (Dulbecco 1952; Luria 1947; Luria and Dulbecco 1949). When the appropriate bacterial host is singly infected with these inactive particles, no progeny are produced. However, when a suitable host is multiply

1. Abbreviations used: UV, ultraviolet; MR, multiplicity reactivation; h, hershey's; ts, temperature sensitive; m.o.i., multiplicity of infection; EHA, enriched Hershey's agar.
infected with these phage, a repair process is initiated that can have the ultimate effect of allowing the production of active phage progeny. This phenomenon is referred to as multiplicity reactivation (MR) (Luria 1947; Luria and Dulbecco 1949).

Luria (1947) formulated a 'recombination theory of multiplicity reactivation.' He assumed that each phage particle had a number of genetic units that were inactivated by UV light independently of one another. He postulated that if one or more of these units in a particular phage particle was inactivated, the whole particle became inactive, but when host cells were multiply infected with these phage, the active genetic units from each of the particles would be able to replicate and recombine to produce a complete set of active units and viable phage progeny would be produced.

Luria and Dulbecco (1949) expanded Luria's work and offered further proof in support of Luria's original theory. They observed that reassembly of 'active genetic units' from damaged phage within a multiply infected bacterial cell was an extremely efficient process. To account for this efficiency, it was proposed that the active units of the individual phage particles multiplied independently forming a 'gene pool' and that genetically complete phage could be reassembled from this pool.

Dulbecco (1952) pointed out that, if such a substitutive mechanism were indeed operative, and a damaged genetic unit in one of the infecting phage chromosomes is simply replaced by a similar undamaged unit from another chromosome, then, with increasing dosage of UV
light, there would also be an increasing likelihood of inactivation of the same genetic sub-unit in any group of phage. Therefore, the ultimate slope of a multiply infected population of bacteria (multicomplexes) would approach that of the curve of singly infected bacteria (monocomplexes). He tested this theory and found that when killing was allowed to proceed so that the surviving fraction was as low as $10^{-6}$ of the total population, the ultimate slope of the multicomplexes did not decline at the same rate as that of the monocomplexes, but was actually one-fifth of that slope. Dulbecco (1952) concluded that the simple recombination theory of MR was not borne out by his data and he decided the principal phenomenon in MR is the reduction of the ultimate slope of the survival curves of multicomplexes, as compared to that of the monocomplexes. It was theorized that either there is a repair system, other than simple recombination, that is functional only in multiply infected cells; or that there is a mechanism that makes certain parts of the phage particle dispensable in multicomplexes.

Since certain phage products are needed to carry out successful recombination between damaged genomes, the question arose as to whether the genes coding for these products would have to be present in an undamaged state within the multiply infected cell to ensure efficient MR. Addressing himself to this problem, Epstein (1958) devised a selective technique which yielded a population of infected bacteria in which the genotype of the parental phage was known. This technique involved infecting *Escherichia coli* K12 ($\lambda$) with a mixture of UV damaged phage, 50% of which contained a mutation in the rIIA cistron and 50%
of which carried a mutation in the rIIIB cistron. At the low multiplicities of infection which he used, the majority of infective centers which could yield viable phage had received only one phage of each genotype. He compared levels of MR of these infective centers with infective centers formed from wild type, UV damaged phage and found that the level of MR of the infective center population made up of rIIA, rIIIB mutant phage was decreased as compared to the level of MR of the infective center population made up of wild type phage. It was concluded that the pre-early gene products coded for by the rII cistrons were needed for efficient MR, that one hit was sufficient to inactivate these genes, and that functional complementation contributes in part to MR.

In other work it has been found that lesions induced by other agents, i.e., soft X-rays, $^{32}$P decay, and nitrous acid, can also be repaired by MR (Weigle and Bertani 1956; Symonds and Ritchie 1961; Bautz-Freese and Freese 1961).

Symonds and Ritchie (1961) investigated levels of MR of $^{32}$P labeled phage adsorbed onto either host bacteria that were heavily labeled with radio-phosphorus or bacteria that were unlabeled. They found that in both types of experiments there are two populations of multicomplexes, one of which exhibits efficient MR while the other population undergoes a relatively low level of MR. When the phage and host bacteria are both labeled with $^{32}$P, about 80% of the population are efficiently rescued by MR. In cases where the phage contains radio-phosphorus and the host bacterium is not labeled, only 30% of the population exhibits MR.
Bautz-Freese and Freese (1961), in their studies on the mutagenic effects of nitrous acid, noticed that the lethal lesions caused by nitrous acid could be repaired by MR, but they did not quantitate this effect.

When soft X-rays were employed as the phage inactivating agent, MR occurred to a much greater extent in host bacteria that had been treated with radiation prior to infection than in untreated bacteria (Weigle and Bertani 1956).

Extensive work has been done investigating repair pathways leading to the restoration of active particles from phage or bacteria that have sustained damage from UV irradiation. Much of this work involves studies of the repair mechanisms operable in E. coli and the bacteriophage lambda. Huskey (1969) demonstrated that the MR of damaged lambda particles was dependent upon the recombination system of the E. coli host and that of the infecting phage. When both systems were inoperative, the phage were not able to undergo MR. Also, Blanco and Devoret (1973) demonstrated that prophage reactivation is severely depressed when UV irradiated lambda infects a lysogenic host that carries a recA mutation. They found that the lambda recombination system could compensate for host deficiency in recombination, albeit less efficiently. More importantly, by comparing the mechanisms of prophage reactivation and UV reactivation, they were able to distinguish between two distinct types of post-replicative repair. These two repair pathways have been defined in E. coli and have been shown to be effective in the elimination of DNA damages resulting from
exposure of the bacterium to UV irradiation. One pathway (prophage reactivation) has been shown to be error-proof, recA dependent and exr (lexA) independent; the alternate pathway (UV reactivation) is error-prone, dependent on a functional exr (lexA) product, and does not involve recombinational mechanisms (Witkin 1967).

In this dissertation, I will present evidence that particular gene products of T4 bacteriophage are necessary for the efficient MR of nitrous acid damaged T4 phage.

It has been assumed that MR is the result of recombination between damaged genomes, but this theory has not been experimentally demonstrated as true. In view of the fact that MR is one of the main repair pathways of damaged T4 particles, it was considered important that this pathway be carefully defined. I have shown that the same gene functions that are required for marker exchange are also required for MR and this implies that both processes occur by the same or similar recombinational pathways.

Another interesting and important aspect of the work presented here is that the gene products shown to be required for efficient MR and marker exchange are also required for a repair process operative in singly infected cells. This implies the presence of a post-replicative recombinational repair pathway in T4 phage.

Nitrous acid was used as the deleterious agent in the majority of experiments since its effect on phage survival has not been as widely investigated as that of UV. Also the kinetics of survival of the multicomplexes infected with nitrous acid damaged phage differed
from that of the multicomplexes infected with UV irradiated phage and provided interesting insights into the replicative entity formed within the multiply infected cell.

Work is also presented that demonstrates that a gene product necessary for MR of nitrous acid damaged phage is also needed for MR of UV irradiated phage. This work was done to indicate the presence of a common repair pathway of phage damaged by these two agents.
NATURE OF DELETERIOUS AGENTS EMPLOYED

Nitrous Acid Inactivation of Phage

Nitrous acid has been shown to react with DNA in vitro and in vivo, causing the deamination of adenine, guanine and cytosine converting them to hypoxanthine, xanthine and uracil respectively (Schuster 1960). It has also been shown in a number of systems that nitrous acid treatment results in either mutation or the inactivation of transforming DNA or phage particles. The lethal effects follow one-hit kinetics, meaning that a single chemical event can cause lethality or mutation (Vielmetter and Schuster 1960).

Schuster (1960) found that adenine and cytosine underwent deamination at a substantially slower rate than guanine. It was thought that the hydrogen bonds linking the amino groups of adenine and cytosine with the keto groups of thymine and guanine respectively tended to retard the deamination of these molecules.

Vielmetter and Schuster (1960) treated phage T2 with nitrous acid under conditions in which the pH was systematically increased. Comparing decreases in the deamination rates of the above mentioned bases with corresponding decreases in rates of mutation and inactivation, they found that the deamination of adenine or cytosine or both was likely to be responsible for the induction of mutations. They also concluded that inactivation of the phage could possibly be due to the deamination of either adenine, cytosine or guanine but this
deamination was not conclusively shown to be the primary inactivating factor.

Becker, Zimmerman and Geiduschek (1964) found that nitrous acid treatment, in addition to causing deamination, also causes cross-linking of complementary DNA strands. Various DNA's were treated with nitrous acid. The DNA was then heat-denatured, quenched, and its density determined on a CsCl gradient. Two classes of DNA were found, one class was identical to that of single stranded DNA and the other class sedimented at the density of double stranded DNA. With continuing nitrous acid treatment, the amount of DNA sedimenting as double stranded molecules increased, until the entire population was composed of this class of molecules. The chemical event causing this was proposed to be the intra-molecular cross-linking of complementary DNA strands. This cross-linked DNA was shown to retain its transforming activity at high temperatures. They were not able to define the chemical nature of the cross-links, but they did develop a quantitative analysis that enabled them to determine the average number of cross-links per molecule. The calculated ratio of cross-links to deamination was found to be one cross-linking event for every four deaminations. Vielmetter and Schuster (1960), working with phage T2, found that four deaminations resulted in one inactivation of phage infectivity. Because of the similarity in frequency of cross-linking events to frequency of lethality, Becker, Zimmerman and Geiduschek (1964) hypothesized that a cross-link is the most frequent inactivating event caused by nitrous acid treatment.
Alterations of DNA Caused by UV Irradiation

Much work has been done in order to define the type of lesions produced in the DNA of UV irradiated organisms. Setlow (1966) summarized this work, placing particular emphasis on the role of pyrimidine dimers in UV treated biological systems. He cites previous work in which it was demonstrated that, at low doses of UV irradiation, 90% of the biological damage sustained by transforming DNA is subject to enzymatic photoreactivation. From this evidence, he concludes that about 90% of the inactivation at low doses arise from pyrimidine dimers. It has been reported that the UV sensitivity of *E. coli* strains that carry a mutation in both the *recA* and *uvrA* genes is such that a single pyrimidine dimer per DNA strand results in lethality (Howard-Flanders and Boyce 1966).

Dimerization of pyrimidines can result in five types of molecules: TT, CC, UU, TC and/or CT (Setlow 1966). Setlow cites evidence to indicate that the ratio of AT/GC sequences in DNA determines which of these molecules will predominate in a particular species. Thus, UV irradiated *E. coli*, whose DNA contains 50% AT sequences, forms dimers of which 59% are from thymine-thymine interactions and UV irradiated *Micrococcus lysodeikiticus*, whose DNA contains only 30% AT sequences, forms only 19% of its dimers from purely thymine-thymine interactions. *T4* would presumably form mainly thymine-thymine pyrimidine dimers since, as shown by Marmur and Doty (1962), its AT content comprises 65% of its DNA.
It has been demonstrated that dimerization of pyrimidines is not the only result of UV irradiation (Marmur and Grossman 1961; Setlow and Setlow 1962). Since Becker et al. (1964) related cross-linking to lethality in nitrous acid treated Bacillus subtilis transforming DNA, the question was raised as to whether cross-linking events are involved in the inactivation of UV irradiated DNA. Becker et al. (1964) cite work in which it is shown that the inactivation rate due to UV irradiation is twenty times greater than the cross-linking rate. Thus DNA cross-links are not a significant UV lethal lesion.
THE MULTIPLICITY REACTIVATION REPAIR PATHWAY

Possible Gene Involvement

In order to define the nature of the MR repair pathway various mutant strains of T4 phage were examined for their effect on levels of rescue by MR. The mutants tested are described below. They include mutants that have been shown to either decrease or increase levels of genetic recombination in T4 phage. Px and y strains were tested since it has been shown that mutations in these genes have a small effect on levels of recombination (Harm 1963) and also on levels of MR of UV-damaged phage (Boyle and Symonds 1969). The v genes was included since Symonds, Heindl and White (1973) demonstrated that when phage have a mutation in the v gene, lethal lesions induced by UV irradiation cannot be efficiently repaired by means of MR.

The v Gene

This gene has been shown to code for an endonuclease that catalyzes the formation of single stranded breaks in UV irradiated double stranded DNA (Friedberg and King 1969, 1971; Yasuda and Sekiguchi 1970). The enzyme has been purified and characterized by Friedberg and King (1971) and Yasuda and Sekiguchi (1970). The product of this gene has been shown by Minton et al. (1975) to specifically recognize thymine dimers. Isolated T7 DNA was exposed in vitro, first to photoreactivating enzyme, and then to the v gene endonuclease. It was found that
the number of incisions made by the \textit{v} gene product decreased dramatically when the DNA was first treated with photoreactivating enzyme.

This enzyme also recognizes other conformational distortions in addition to the thymine dimer. Benz and Berger (1973) prepared deletion-wild type heteroduplex DNA \textit{in vitro} and, with a transformation assay, measured selective allele loss following treatment with cell free extracts of phage infected cells. Treatment of these heteroduplex complexes with extracts from \textit{v}+ infected cells markedly reduced the frequency of transformation of the wild type segment opposite the deletion. This effect was not observed following treatment with extracts of \textit{v}− infected cells. This indicates that the \textit{v}+ gene product is needed in a repair process that is capable of removing 'loops' in heteroduplex DNA molecules.

The \textit{v} gene endonuclease has been shown to initiate a repair pathway in \textit{T}4 phage infection of \textit{Escherichia coli} that results in the excision of pyrimidine dimers (Friedberg and King 1971), reinsertion of the appropriate nucleotides, and the rejoining of these newly formed fragments to the parental strand. Sato and Sekiguchi (1976), working with temperature sensitive mutants, have firmly demonstrated that the formation of the UV endonuclease, endonuclease \textit{V}, is controlled by the \textit{v} gene of \textit{T}4. They also demonstrated that the repair of DNA that is initiated by this enzyme occurs early in the infectious cycle of \textit{T}4.

\textbf{The \textit{x} Gene}

Harm (1963) isolated the \textit{x} mutant after treatment of wild type phage with nitrous acid. He found that it produced a sensitivity level
to UV irradiation that was intermediate between wild type and the $v$ mutant. He also demonstrated that the $x$ allele is not closely linked to the $v$ allele and double mutants with the $vx$ genotype show an additive UV sensitivity demonstrating that the $x$ gene does not code for a product involved in the $v$-mediated excision repair pathway. This T$^4x$ mutant was subsequently shown to carry mutations in two distinct genes: $px$ (for pure $x$) and $hm$ (for high mutability) of which only $px$ determines the UV repair characteristics (Drake 1973).

The exact function of the $x$ gene is not known, but it has been implicated in recombination in experiments reported by Harm (1964). He performed marker rescue experiments in which bacterial hosts were multiply infected with irradiated phage carrying the genetic markers $r145$ (A cistron) or $r102$ (B cistron) to be rescued and either the $x^+$ or $x^-$ allele and unirradiated phage that were wild type with respect to the rII genes and either genotypically wild type or mutated in the $x$ gene. The extent of marker rescue was shown to be dependent upon the allelic state of the $x$ gene of the unirradiated parent.

The $x$ allele was more directly shown to be influential in recombination in experiments testing its effect on the recombination of rII markers. Recombination was shown to be three-fold higher in all crosses in which at least one parent had the $x^+$ genotype (Harm 1964). Symonds et al. (1973) demonstrated that the gene products specified by the $x$, $y$ and 1206 genes also are involved in the MR pathway. Phage carrying mutations in any of these genes did not produce the characteristic shoulder present in typical survival curves of multicomplexes.
formed from UV damaged phage. On the other hand, a \textit{v} mutation reduced the final slope of the MR survival curve. They interpreted their results as showing that UV-induced lesions in T\textit{4} phage are repaired in multicomplexes by means of two separate processes. One process, mediated by the \textit{x}, \textit{y} and 1206 genes, is referred to as replication repair. The other process, mediated by the \textit{v} gene, is the excision repair pathway.

**The \textit{v} Gene**

\textit{T\textit{4v}} is an amber mutant in that its UV sensitivity can only be demonstrated on a \textit{su} host (Boyle and Symonds 1969). Maynard-Smith and Symonds (1973) mapped \textit{T\textit{4y}} and demonstrated that it was between genes \textit{24} and \textit{25} and not near \textit{x} or \textit{y}. The UV survival curve of this mutant phage is intermediate between \textit{T\textit{4x}} and \textit{T\textit{4y}}. Compared to wild type it is also more sensitive to gamma irradiation, has slightly reduced multiplicity reactivation of UV damage, a slower rate of intracellular phage development, and a burst size reduced by about one-half. In crosses homozygous for \textit{v}, the recombination frequency between two standard markers is reduced by about one-half (Boyle and Symonds 1969).

Since the double mutants \textit{vx} and \textit{vy} show greater additive sensitivity than the individual mutants and the \textit{xy} mutant does not show additive UV sensitivity over that of \textit{x} and \textit{y} single mutants, it has been proposed that \textit{x} and \textit{y} are defective in a common repair pathway. In view of the fact that \textit{x} and \textit{y} are similar to one another but qualitatively different from \textit{v} in regards to gamma ray survival, growth and recombination, it was concluded that there are two classes of radiation
sensitive mutants of T4: those represented by T4<sup>y</sup>, and those represented by T4<sup>x</sup> and T4<sup>y</sup> (Boyle and Symonds 1969).

T4<sup>y</sup>, T4<sup>px</sup>, and T4<sup>x</sup> have been shown to have increased sensitivity to nitrous acid whereas v has the same sensitivity as wild type phage. This characteristic, plus the fact that the xy system repairs damages caused by UV irradiation and also gamma irradiation whereas v only affects repair of UV damage suggests that the xy repair pathway is less specific regarding the type of damage that it is able to repair than that of the v-mediated excision repair pathway (Harm 1974).

**Genes 46 and 47**

The products of genes 46 and 47 are necessary for normal phage replication. Mutants defective in either gene stop DNA synthesis prematurely (Epstein et al. 1963). This is probably due to the fact that the DNA becomes detached from the membrane as no concatenate formation results when the phage DNA loses its ability to attach to the host cell membrane (Shah and Berger 1971; Shalitin and Naot 1971). The 46-47 genes are thought to produce or control the production of an exonuclease that degrades single stranded nicks to gaps. In the intracellular DNA formed by 46-47 phage, the strand interruptions produced by endonucleases remain as nicks (Prashad and Hosoda 1972).

Tomizawa and Anraku (1964) and Anraku and Tomizawa (1965) demonstrated two steps in the interaction of parental chromosomes under conditions in which phage DNA replication was inhibited. The first step consisted of the formation of 'joint DNA molecules' containing DNA segments derived from different phage particles joined in heteroduplex
form over a portion of the length. In the second step, joint molecules were converted to recombinant molecules in which the components are covalently linked. Broker and Lehman (1971) examined infecting phage DNA under conditions in which DNA replication was inhibited using polymerase ligase mutations using electron microscopy and showed that up to 25% of the DNA fragments had one or more branches. These branched molecules were interpreted as intermediates of molecular recombination between parental DNA's. They further examined the effects of mutations in other genes on branched molecule formation by constructing lig-pol-X mutants and found that branched molecules were not formed if X was either gene 32, 46, or 47. Both Bernstein (1968) and Berger, Warren and Fry (1969) found that mutations in genes 46 and 47 limited recombination and Davis and Symonds (1974) found a decrease in recombination within tandem duplications in 46-47 phage.

Gene 32

The product of this gene was first shown by Tomizawa and Anraku (1964) to be necessary for the hydrogen bonded joint DNA molecules believed to be the initial products of genetic recombination. Alberts and Frey (1970) then isolated the protein product of gene 32. It was found to specifically bind to single stranded DNA and to enhance DNA denaturation and renaturation in vitro. The finding that genetic recombination is decreased under conditions in which the gene 32 function is limiting gave support to the hypothesis that the gene 32 product stabilized free single strands of DNA allowing recombination to take place (Berger et al. 1969; Carroll, Neet and Goldthwait 1972). In
regards to its role in recombination, gene 32 protein is thought to cause local denaturation of the strands in a DNA molecule and so induce pairing over a short region with a complementary single strand from another molecule (Carrollet al. 1972). Broker and Lehman (1971) found the protein to be essential for the formation in vivo of branched molecules interpreted to be recombination intermediates and Davis and Symonds (1974) found that, in its absence, the recombination within tandem duplications of T4 was depressed.

**Gene 30**

This gene codes for the protein, polynucleotide ligase. The enzyme catalyzes the covalent joining of nicks in double stranded DNA (Cozzarelli et al. 1967; Becker et al. 1967; Weiss and Richardson 1967) and is also needed for normal DNA replication (Okazaki et al. 1968; Newman and Hanawalt 1968). Baldy (1970) has demonstrated that the product of gene 30 is necessary for the repair of UV damage. H. Bernstein (1968), working with temperature sensitive mutants and Berger et al. (1969), working with amber mutants have shown that a defect in this gene leads to an increase in recombination.

**Gene 44**

The gene 44 mutant strain was first tested by Epstein et al. (1963). They found that under restrictive conditions, this strain did not synthesize DNA. Its product has been purified and has been isolated tightly complexed with the product of gene 62 (Barry and Alberts 1972). Evidence has been presented showing that it is able to interact
with the products of genes 32, 41, 43, 45 and 62 to catalyze DNA synthesis in vitro (Morris, Sinha and Alberts 1975).

Table 1 summarizes the properties of the various mutant strains of T4 used in this study.
Table 1. Properties of mutant strains of bacteriophage T4.

<table>
<thead>
<tr>
<th>Phage Strains</th>
<th>Functional Pathway</th>
<th>UV Sensitivity</th>
<th>Recombination Frequency</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>tsB110(44)</td>
<td>Replication</td>
<td>Increased</td>
<td>No effect</td>
<td>Epstein et al. 1963, Barry and Alberts 1972, Baldy 1970</td>
</tr>
<tr>
<td>X</td>
<td>Replication-Repair</td>
<td>Increased</td>
<td>Decreased</td>
<td>Harm 1963</td>
</tr>
<tr>
<td>Y</td>
<td>Replication-Repair</td>
<td>Increased</td>
<td>Decreased</td>
<td>Boyle and Symonds 1969</td>
</tr>
<tr>
<td>v</td>
<td>Excision Repair</td>
<td>Increased</td>
<td>No effect</td>
<td>Sato and Sekiguchi 1976</td>
</tr>
</tbody>
</table>
POSSIBLE PATHWAYS FOR REPAIR OF DAMAGED PHAGE PARTICLES

Genetic Recombination

A model of genetic recombination in phage T4 has been described by Broker (1973) and is diagramed in Figure 1. This model necessitates the formation of heteroduplex molecules and reciprocal recombination does not necessarily occur in certain mutant infections. Branched molecules, as shown in Structure 4 of Figure 1, are not formed in lig-pol-32-, lig-pol-46-, or lig-pol-47- mutants. The 46 and 47 gene products seem to be necessary for gap formation (Structures, 3) and, in addition, lig-pol-46- mutants do not produce hybrid density recombinant intermediates in cells that are multiply infected with phage of two different densities. Branched recombinant intermediates (Structure 4) are reduced ten-fold in T4 32- or T4 lig-pol-32- mutant strains.

Known Model for Repair of Cross-links

Cross-linking of DNA has been demonstrated after nitrous acid treatment of transforming DNA (E. F. Becker et al. 1964). These authors argue that since cross-linking frequency coincides with the frequency of phage inactivation, a cross-linking event may indeed precipitate the inactivation of the phage particle.

Marmur and Grossman (1961), working with bacterial DNA, have demonstrated the formation of interstrand DNA cross-links after UV irradiation. In the case of UV inactivation of phage particles,
Figure 1. Pathway for T4 recombination in the absence of DNA replication. -- Broker and Doerrmann 1975.
cross-linkage of DNA strands cannot be the main inactivating event since the inactivation rate is considerably greater than the cross-linking rate (Glisin and Doty 1962).

In any event, repair of these cross-links presents special problems since both DNA strands sustain damages at either the same sites or very closely neighboring sites. An excision-type repair, such as that mediated by the \( y \) gene, does not seem feasible, as gaps left after dimer excision are repaired by insertion of nucleotides complementary to those of the intact opposite strand.

Addressing themselves to this problem, Cole, Levitan and Sinden (1976) have proposed a model for the repair of cross-links. They reasoned that if the cross-link was excised from one strand leaving a gap, the resulting structure would be more or less stable and could be corrected by a recombinational event. In support of this hypothesis, they found that \( E. \) coli strains that are mutated in the \( uvrA, uvrB, uvrC \) and \( polA \) (coding for the pol 1 \( (5'-3') \) exonuclease) are unable to remove cross-links (Cole et al. 1976) and Cole (1971) also found that strains which are deficient in recombination \( (\text{rec}A^-) \) are much more sensitive to cross-linking agents.

Figure 2 illustrates the proposed model of Cole et al. (1976) for the removal of cross-links. The UV endonuclease nicks the DNA strand containing a cross-link (Structure a) and the exonuclease of \( polA \) nicks the DNA strand on the opposite side of the cross-link creating a gap (Structure b) so that a recombinational event becomes possible.
1. Homologous duplexes

2. UV endonuclease
   \( uvrA, uvrB \)

3. 5'-3' exonuclease
   \( polA \)

4. Strand exchange (recA) and repair synthesis

5. Duplex separation

Figure 2. Pathway for repair of inter-strand cross-links. -- (Cole et al. 1976)
Post-replicative Repair

The present model for the repair of UV lesions by the error-proof post-replicative recombinational pathway had its beginnings with a discovery reported by Rupp and Howard-Flanders (1968). They found that DNA synthesized in uvr^- cells after irradiation contained many single stranded breaks. Howard-Flanders et al. (1968) clearly demonstrated that these breaks were opposite thymine dimers and Iyer and Rupp (1971) showed that the breaks in the DNA were not short gaps, but excluded approximately 800 nucleotides from the defective strand. Rupp et al. (1971) presented evidence that these gaps were filled, via a recombinational process, by an homologous length of the parental DNA. The number of insertions of parental DNA into daughter strands corresponded to the number of daughter strand gaps initially produced.

Ganesan (1974) demonstrated that pyrimidine dimers in the parental strands are found in daughter strands and proposed that recovery from UV damage by means of the post-replicative repair process was a result of dilution away from pyrimidine dimers rather than dimer excision.

The alternate, error-prone, post-replicative repair pathway is inducible and has been carefully defined by Radman (1975) who refers to it as SOS Repair. Sedgwick (1975) proposed that the SOS-inducing lesion may be a daughter strand gap that partially overlaps another daughter strand gap in the sister DNA molecule. These molecules could not be repaired by recombination, since this would involve the donation of DNA of both parental strands and would result in a discontinuity of the parental molecule. Therefore it was proposed that either one or both strands were repaired by a polymerase that used the dimer
containing parental strand as template and sometimes insertion of an incorrect base was made opposite the thymine dimer or that an error-prone polymerase that did not require a template for gap repair was employed.

Genes 32, 46, 47, x and y, all of which are necessary for recombination, would be needed for each of the recombinational processes diagrammed in Figures 1, 2 and 3. Processes of genetic recombination (Fig. 1) and cross-link repair (Fig. 2) would depend on multiple infection. Post-replicative recombination repair (Fig. 3) could occur with single infection. Thus these genes were tested for their involvement in repair, under conditions of both single and multiple infection.
1. Homologous duplexes containing thymine dimers

a. Replication of these molecules

b. Strand exchange and repair synthesis

c. Duplex separation

Figure 3. A model for post-replicative recombinational repair. -- (Ganesan 1974)
MATERIALS AND METHODS

Media

Bacterial cultures were grown in Hershey's (H) broth with the exception of the cultures used for the growth of phage stocks. These were grown in M9 media (Adams 1959) supplemented with 2.5 mg/ml of vitamin-free casamino acids and 2.7 μg/ml of FeCl₃·H₂O. An M9 salt solution was used for suspension of host bacterial cultures (per liter of distilled deionized water: 1.0 gm NH₄Cl, 6.0 gms Na₂HPO₄, 3.0 gms KH₂PO₄). Two hundred μg/ml of tryptophan was added to this salt solution to enhance adsorption in experiments measuring degrees of multiplicity reactivation of T₄B mutant strains (Harm 1956).

Chemicals

Crystalline sodium nitrite and anhydrous sodium acetate were purchased from the J. T. Baker Chemical Company, Phillipsburg, New Jersey. A stock solution of 1M sodium nitrite was made fresh each week and stored in a screwcap tube under nitrogen.

Acetate buffer solution: a 1M stock solution of acetic acid and a 1M stock solution of sodium acetate was prepared. Ten mls of 1M sodium acetate was added to approximately 55 mls of 1M acetic acid to obtain a 1M acetate buffer with a pH of 4.0. Twenty-five mls of this solution was mixed with 225 mls of distilled, deionized water to obtain a 0.1M acetate buffer solution of pH 4.0 (Harm 1974). The pH of the diluted solution was verified to be as stated on a standard pH meter.
Bacteria

*Escherichia coli* S/6/5, a $Su^{-}$ derivative of strain B (Doermann and Hill 1953) was used throughout both as host bacterium and as a plating indicator, with the exception of experiments in which an amber suppressor strain was needed. In these instances *E. coli* CR63, which contains the amber suppressor $SuI$, was used both as the host bacterium and as a plating indicator.

Every few months, cultures of *E. coli* S/6/5 and *E. coli* CR63 were individually grown from a slant inoculum in H broth at 30°C with aeration for twelve hours. These cultures were subsequently used as the source of inocula for overnight broth cultures. Fresh overnight cultures were made weekly by dilution (1:1000) into H broth. These cultures were incubated for twelve hours, with aeration, at 30°C and subsequently stored at 4°C. To prepare fresh plating indicator cultures, a portion of an overnight culture was diluted 1:100 into fresh H broth and incubated with aeration for 2-1/2 hours at 30°C. These cultures were then centrifuged at 3,800 x g for 30 minutes and the resulting pellet was resuspended in 15 mls of H broth.

Bacterial hosts that were to be used for high multiplicity of infection (m.o.i.) experiments were grown as described for plating indicators with the following modifications. After centrifugation, the pellet was washed once with approximately 100 mls of M9 salt solution, centrifuged, and resuspended in 10 mls of M9 salt solution. This culture was supplemented with 200 μg/ml tryptophan when the bacteria were to be infected with a T^4B phage strain. Cell density was
determined by Petroff Hausser counts and confirmed by plating for colony formation. All platings for both bacteria and bacteriophage were carried out with enriched Hershey agar plates (EHA) and overlays (Steinberg and Edgar 1962).

**Bacteriophage**

Phage strains used in these experiments are listed in Table 2. The spontaneous revertant of _tsL86_ (gene 47) was selected by plating high concentrations of the _ts_ mutant at 45°C.

High titer phage stocks were prepared as follows. A log phase culture of the appropriate bacterial strains, which had been grown in M9 media (supplemented with vitamin-free casamino acids and FeCl₃·H₂O), was diluted 1:1000 into fresh M9 media. Phage were added such that the m.o.i. was 0.01. Temperature sensitive phage strains were grown with aeration at 25°C for twelve hours. All other phage strains were grown at 30°C for twelve hours. Chloroform was then added to the resulting stock and the solution was centrifuged at 3,800 x g for ten minutes to remove cellular debris. The phage were then sedimented by centrifugation at 23,000 x g for two hours and resuspended by being allowed to stand overnight in 5 mls of M9 salt solution. Remaining debris was removed by centrifugation at 3,800 x g for ten minutes. The resulting phage stocks usually contained between 2 to 4 x 10¹¹ plaque forming units (PFU)/ml. These stocks were stored at 4°C until used.

**Nitrous Acid Treatment**

Phage were diluted 1:10 into pre-warmed acetate buffer at pH 4.0 and kept in a waterbath at 27°C for ten minutes. An aliquot was
Table 2. Phage mutants used in the present study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutant Class</th>
<th>Source</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>tsL86(47)</td>
<td>DA</td>
<td>Collection of the California Institute of Technology</td>
<td>Exonuclease (Prashad and Hosoda 1972; Shalitin and Naot 1971; Shah and Berger 1971)</td>
</tr>
<tr>
<td>tsL86R</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(revertant)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tsL109(46)</td>
<td>DA</td>
<td>Collection of the California Institute of Technology</td>
<td>Exonuclease (Prashad and Hosoda 1972; Shalitin and Naot, 1971; Shah and Berger 1971)</td>
</tr>
<tr>
<td>tsL67(32)</td>
<td>DS</td>
<td>Collection of the California Institute of Technology</td>
<td>DNA Unwinding Protein (Alberts and Frey 1970)</td>
</tr>
<tr>
<td>tsB20(30)</td>
<td>DS</td>
<td>Collection of the California Institute of Technology</td>
<td>Ligase (Weiss and Richardson 1967)</td>
</tr>
<tr>
<td>tsB110(44)</td>
<td>DO</td>
<td>Collection of the California Institute of Technology</td>
<td>Unidentified</td>
</tr>
<tr>
<td>v</td>
<td></td>
<td>Marion W. Baldy</td>
<td>Endonuclease V (Sato and Sekiguchi 1976)</td>
</tr>
<tr>
<td>pX</td>
<td></td>
<td>J. W. Drake</td>
<td>Unidentified (Harm 1963; 1964; Drake 1973)</td>
</tr>
<tr>
<td>x10</td>
<td></td>
<td>S. Maynard Smith</td>
<td>Unidentified (Boyle and Symonds 1969)</td>
</tr>
</tbody>
</table>

The 'mutant class' refers to the classification of Warner and Hobbs (1967) which contains the following abbreviations: DO (no DNA synthesis), DS (some DNA synthesis) and DA (arrested DNA synthesis).
removed at this time and diluted 1:10 into an M9 salt solution which had been cooled to 4°C. At this time, 1M sodium nitrate was added to the phage suspended in acetate buffer at a dilution of 1:10 to give a final concentration of 0.1M nitrous acid (Harm 1974). Nitrous acid treatment was terminated at specified time intervals by diluting samples 1:10 into aliquots of an M9 salt solution which had been cooled to 4°C. Aliquots from each time period were appropriately diluted and immediately plated to test for survival of mono- and multi-complexes as described below.

**Ultraviolet Irradiation**

Phage stocks, which had been diluted into an M9 salt solution to obtain a final titer of approximately $2 \times 10^9$ phage/ml were irradiated in DISPOSO clear plastic trays with 3 ml wells (Linbro Chemical Co., Inc.). The trays, as obtained from the manufacturer, were cut to yield four smaller trays made up of six rows with four wells per row. A 1 ml aliquot of the phage suspension was placed in the appropriate number of wells. A stopwatch was used to time the dose. As each successive time period elapsed, the wells for that particular period were covered with a blackened glass plate. Two wells containing the phage suspension were irradiated for each time period and subsequently mixed in screwcap tubes. Aliquots were diluted and immediately plated to test for survival. The treated phage were also tested for the degree of rescue by MR as described below.

A 15W germicidal lamp (G15T8) was used for irradiation, the phage suspension being placed 40 cm beneath the lamp. Irradiation was
carried out at room temperature. Under these conditions the incident radiation at the phage suspension is about 11 ergs/mm²/sec.

**Plating**

To determine the degree of survival, either from nitrous acid treatment or ultraviolet irradiation, treated aliquots were diluted to yield between 10 and 1000 plaques per plate and to have a multiplicity of total phage (viable plus inviable) to bacteria of less than 0.01. 0.1 ml of the phage suspension was added to 2.5 ml soft agar. The appropriate strain of _E. coli_ was added as plating bacteria, and the mixture was poured onto a bottom layer of EHA. These plates, that had been prewarmed, were incubated overnight in a controlled heat room at designated temperatures. Surviving plaques were then counted.

**Multiplicity Reactivation**

Damaged phage were inactivated at a significantly faster rate in monocomplexes as compared to multicomplexes. Methods of determining the fractional survival of these complexes, their respective inactivation rates, and the relationship of these two values is described below.

**Measurement of Monocomplexes**

A control phage suspension was titered to determine the 100% survival value. The control for nitrous acid inactivation experiments was obtained by removing an aliquot from the acetate buffer phage suspension immediately prior to the addition of sodium nitrite. This aliquot was diluted 1:10 into a chilled M9 salt solution and titered. The control for the UV-treated phage was simply the untreated phage
mixture. This was titered for the 100% survival value. Treated phage from each successive time period were titered. Fractional survival at each time period was computed as the ratio of survivors at a particular time period to that of the control.

**Measurement of Multicomplexes**

Host bacteria were diluted into an M9 salt solution to give a final concentration of 1 to $4 \times 10^8$ cells/ml. An aliquot of 0.5 ml of these bacteria were placed in an adsorption tube. An equal volume of phage at approximately 1 to $4 \times 10^9$ particles/ml was then added. This bacterial/phage suspension was placed in a controlled temperature Dri Block (36°C) for 10 minutes to obtain good adsorption. An aliquot of this suspension was then diluted, mixed with plating indicator, and plated for infectious centers. Aliquots were also diluted and placed directly to measure uninfected bacteria. Immediately after these dilutions were made, chloroform was added to the adsorption tube and this phage-bacterial suspension was later titered for unadsorbed phage. Levels of unadsorbed phage were always found to be less than 5% and usually ranged from 0.5 to 1%. A control phage suspension was allowed to enter multicomplexes as described above to determine the 100% survival value. Treated phage from each time period were allowed to enter multicomplexes and assayed as described above. Fractional survival at each time period was computed as the ratio of infectious centers at a particular time period to that of the control. At time intervals in which the extent of killing of the monocomplexes was 90% or less, the experimentally determined number of infectious centers were corrected,
whenever possible, for unadsorbed phage and monocomplexes. The unadsorbed phage were measured directly. The fraction of monocomplexes was determined by assuming a Poisson distribution of phage particles among the bacteria. The second term of this distribution \( m e^{-m} \) describes the fraction of input bacteria that will be infected with only one phage particle (monocomplexes). \( m \) is the m.o.i. which was determined in two ways. First, the ratio of adsorbed phage to the number of input bacteria was measured. In addition, the number of uninfected bacteria was determined. From the uninfected bacteria, the m.o.i. was computed using the first term of the Poisson distribution \( e^{-m} \).

The average m.o.i. calculated from the two measurements was considered to be nearest to the correct multiplicity and it is this value that is given in the legend of the figures. At doses of nitrous acid that gave more than 90% killing of the monocomplexes, the contribution of unadsorbed phage and monocomplexes to the infectious center counts were ignored.

**Normalization of Data**

When phage are damaged by nitrous acid or UV irradiation, the rate of inactivation can vary due to a number of causes. Inactivation of phage by nitrous acid is dependent upon the pH and the molarity of the nitrous acid solution and the temperature of this solution. The change in inactivation rate that was solely due to the presence of multicomplexes was most clearly shown when the inactivation curves of the mono- and multicomplexes were plotted as a function of phage lethal
hits delivered to monoclonal antibodies. Thus all curves were normalized in this way.
RESULTS

Inactivation of Extracellular T4 Bacteriophage

It has been proposed that MR is the result of recombination between damaged genomes or their replicative products, either of these processes producing an undamaged phage genome which is then able to replicate normally and produce phage progeny (Epstein 1958; Kozinski, Doermann and Kozinski 1976). Also, it has been shown that mutations in genes whose functions are involved in known repair pathways affect the level of reactivation of UV damaged genomes (Symonds, Heindl and White 1973).

In order to determine the nature of the MR pathway, and particularly whether it is a recombinational and/or repair process, wild type and mutant strains of T4D were inactivated with nitrous acid or UV irradiation and the kinetics of inactivation of monocomplexes and multicomplexes formed from these damaged phage particles were examined.

Relative Survival of Wild Type Phage Monocomplexes and Multicomplexes after Nitrous Acid Treatment

Figure 4 summarizes the results of six experiments. Host bacteria were infected with damaged phage and the survival of monocomplexes and multicomplexes were measured. The survival of these complexes are plotted as a function of lethal hits delivered to the monocomplexes. Experimental temperatures ranged from 25 to 40°C. As can be seen, the survival curve of the multicomplexes decreases
Figure 4. Survival of wild type phage monocomplexes and multicomplexes after nitrous acid treatment.

Survival of monocomplexes as a function of average phage lethal hits.

In each experiment the surviving fraction was first plotted as a function of time of nitrous acid treatment of the free phage on semi-logarithmic coordinates. A straight line was drawn to fit the points. The time at which the straight line passed through a surviving fraction value of 0.37 was used as the time taken to deliver an average of one phage lethal hit. The surviving fraction values for all six experiments were then plotted on this graph, using as a unit of time the time taken to deliver one phage lethal hit in each experiment.

For each experiment the growth temperature and the symbol are:
25° (●) (●); 35° (▼); 37° (▲); 38° (▲); 40° (□).

Survival of multicomplexes as a function of monocomplex phage lethal hits.

For each experiment the growth temperature, m.o.i. and the symbol are:
25°, m.o.i. 6.6, (●); 25°, m.o.i. 2.5 (▲); 35°, m.o.i. 2.5, (▼); 37°, m.o.i. 7.8, (▲); 38°, m.o.i. 2.5, (▲); 40°, m.o.i. 5.1, (□).
Figure 4. Survival of wild type phage monocomplexes and multicomplexes after nitrous acid treatment.
exponentially with increasing dosage, indicating that a single chemical change in the DNA of one of the infecting particles is able to inactivate the complex. The repair of nitrous acid lesions is very efficient in multiply infected cells. Wild type phage T4 which have been treated extracellularly with nitrous acid and subsequently allowed to form multicomplexes required five times the dose as that delivered to monocomplexes to reach the same level of survival. The m.o.i. does not seem to have a significant effect on the rate of inactivation of the multicomplexes under the conditions of these experiments. In the six curves that are represented by Figure 1, the m.o.i. values ranged from 2.5 to 7.8.

Relative Survival of Host-Phage Complexes of a Gene 47 Mutant and its Revertant After Nitrous Acid Treatment

Figure 5 compares inactivation of the multicomplexes of a temperature sensitive strain of T4 phage with those of the wild type strain. The mutant strain tested is tsL86, which is defective in gene 47. The product of this gene is thought to be an exonuclease Prashad and Hosoda 1972) and the recombination frequency between markers is progressively reduced as growth temperatures approach restrictive conditions (Bernstein 1968).

Figure 5a summarizes the results of five experiments in which growth occurred at the semi-restrictive temperatures designated in the legend. All of the experimental data for multicomplex inactivation fall close to a straight line consistent with single hit inactivation kinetics. It is immediately apparent that when growth of complexes
Figure 5. Survival of a gene 47 mutant in monocomplexes and multicomplexes after nitrous acid treatment.

(a) Semi-restrictive temperatures.
(b) Semi-permissive temperatures.

Survival of monocomplexes as a function of phage lethal hits.
For each experiment the mutant strain, growth temperature, and the symbol are:
(a) tsL86 (47): 35.5°, (●); 36°, (▲); 37°, (■); 38°, (▼).
    tsL86+ (47): 36°, (●).
(b) tsL86 (47): 25°, (●, ▲, ■).

Survival of multicomplexes as a function of monocomplex phage lethal hits.
For each experiment the mutant strain, growth temperature, m.o.i., and the symbol are:
(a) tsL86 (47): 35.5°, m.o.i. 6.8, (●); 36°, m.o.i. 5.8 (▲);
    37°, m.o.i., 5.8 (■); 38°, m.o.i. 2.5 (▼).
    tsL86+ (47): 36°, m.o.i. 6.8 (+).
(b) tsL86 (47): 25°, m.o.i. 6.8 (●); 25°, m.o.i. 6.4 (▲);
     25°, m.o.i. 6.6 (■).

The dashed line in both panels indicates the relative multicomplex survival expected for wild type, taken from Figure 4.
Figure 5. Survival of a gene 47 mutant in monocomplexes and multicomplexes after nitrous acid treatment.
occurs at semi-restrictive temperatures, levels of MR are severely depressed. When the revertant of tsL86 was tested for multicomplex survival, a level of MR was found that approximated that of wild type. There seems to be no correlation of MR with m.o.i.

Figure 5b shows the levels of survival of tsL86 phage-host complexes in three experiments performed at a semi-permissive temperature of 25°C. Under these conditions, the level of MR approaches that of wild type.

Survival of Monocomplexes and Multicomplexes of a Gene 46 Mutant after Nitrous Acid Treatment

Figure 6 compares inactivation of the multicomplexes of a temperature sensitive mutant of gene 46, tsL109, with those of the wild type strain. The phenotype of strains defective in gene 46 is similar to those strains that are defective in gene 47 in that mutants in gene 46 are defective in recombination (Bernstein 1968; Berger et al. 1969).

Figure 6a summarizes the results of three experiments in which growth occurred at semi-restrictive temperatures. The level of MR in this mutant strain at semi-restrictive temperatures is comparable to that of tsL86 at semi-restrictive temperatures and is well below wild type reactivation.

Figure 6b summarizes the results of three separate experiments with tsL109 where the complexes were grown at semi-permissive temperatures. Under these conditions, the level of MR approaches that of wild type.
Figure 6. Survival of a gene 46 mutant in monocomplexes and multicomplexes after nitrous acid treatment.

(a) Semi-restrictive temperatures.
(b) Semi-permissive temperatures.

Survival of monocomplexes as a function of phage lethal hits.
For each experiment the mutant strain, growth temperature, and the symbol are:
(a) tsL109 (46): 40.5°, (●); 40°, (▲, ■).
(b) tsL109 (46): 25°, (●); 31°, (▲).

Survival of multicomplexes as a function of phage lethal hits.
For each experiment the mutant strain, growth temperature, and the symbol are:
(a) tsL109 (46): 40.5°, m.o.i. 6.6, (○); 40°, m.o.i. 4.1 (▲); 40°, m.o.i. 4.9, (□).
(b) tsL109 (46): 25°, m.o.i. 4.9, (○); 31°, m.o.i. 5.3, (▲).

The dashed line in both panels indicates the relative multicomplex survival of wild type.
Figure 6. Survival of a gene 46 mutant in monocomplexes and multicomplexes after nitrous acid treatment.
Relative Survival of Host-Phage Complexes of a Gene 32 Mutant after Nitrous Acid Treatment

Figure 7 indicates that another gene that has been shown to be defective in recombination is also defective in MR. The strain tested, tsL67, carries a temperature sensitive mutation in gene 32. The protein product of this gene is commonly referred to as the 'unwinding protein.' This enzyme has been shown to aid DNA denaturation and renaturation \textit{in vitro} (Alberts and Frey 1970) and is necessary for the recombination of complementary DNA strands \textit{in vivo} (Broker and Lehman 1971).

Figure 7a summarizes the results of two experiments. Survival at semi-restrictive temperatures follows single hit kinetics and the level of MR is somewhat closer to the wild type MR level than observed with the gene 46 and 47 mutants at semi-restrictive temperatures. At semi-permissive conditions of growth (Fig. 7b) the wild type level of MR is attained.

Survival of Host-Phage Mono-complexes and Multicomplexes of T4px after Nitrous Acid Treatment

Strains of T4 phage that are defective in the px allele have increased sensitivity to UV irradiation (Harm 1963); Symonds et al. 1973; Boyle and Symonds 1969) and nitrous acid inactivation (Harm 1963). Recombination is also decreased 3 to 4 fold compared to that of the wild type strain (Harm 1963). Multicomplexes, formed by host infection with phage defective in the px allele, were grown at various temperatures. Figure 8 shows that levels of MR of these complexes are
Figure 7. Survival of monocomplexes and multicomplexes of a gene 32 mutant after nitrous acid treatment.

(a) Semi-restrictive temperatures
(b) Semi-permissive temperatures

Survival of monocomplexes as a function of phage lethal hits.

For each experiment the mutant strain, growth temperature, and the symbol are:
(a) tsL67(32): 40\(^\circ\), (○, ▲).
(b) tsL67(32): 25\(^\circ\), (▼); 35\(^\circ\), (▲); 36\(^\circ\), (●).

Survival of multicomplexes as a function of phage lethal hits.

For each experiment the mutant strain, growth temperature, the m.o.i., and the symbol are:
(a) tsL67(32): 40\(^\circ\), m.o.i. 5.8, (▲); 40\(^\circ\), m.o.i. 3.0, (○).
(b) tsL67(32): 25\(^\circ\), m.o.i. 3.0, (▼); 35\(^\circ\), m.o.i. 3.0 (▲); 36\(^\circ\), m.o.i. 3.9, (○).

The dashed line in both panels indicates the relative multicomplex survival curve of wild type.
Figure 7. Survival of monocomplexes and multicomplexes of a gene 32 mutant after nitrous acid treatment.
Figure 8. Survival of T4px monocomplexes and multicomplexes after nitrous acid treatment.

Survival of monocomplexes as a function of average phage lethal hits.

For each experiment the growth temperature, and the symbols are:
25°, (●,●); 31°, ( ■ ); 36.5°, ( ▼ )

Survival of multicomplexes as a function of phage lethal hits.

For each experiment the growth temperature, the m.o.i. and the symbol are:
25°, m.o.i. 4.3, ( ○ ); 25°, m.o.i. 3.3, ( △ ); 31° m.o.i. 3.3, ( □ ); 36.5°, m.o.i. 4.3, ( ▽ ).

The dashed line indicates the relative multicomplex survival of wild type.
Figure 8. Survival of T4px monocomplexes and multicomplexes after nitrous acid treatment.
significantly decreased as compared to the wild type level of multiplicity reactivation. No effect was observed due to the temperature of growth of these multiply infected cells as is expected, since px is not a temperature sensitive mutation.

To check the phenotype of the x mutant used in this study, as well as that of y and y, the UV inactivation constant of these strains were determined and compared to previously measured inactivation constants from the literature. These data are presented in Table 3. The rate constants, (k), in column 2 are computed from the equation \( N/N_o = e^{-kt} \). The units of k are lethal lesions per minute. Average relative sensitivities of phage strains used in this work (column 3) correlate well with previously published data (columns 4 through 7). T4px is approximately 1.7 times as sensitive to UV as the wild type strain (see column 3). This strain, compared to wild type, also shows 1.2 times as much sensitivity to nitrous acid damage which is in agreement with the observation of Harm (1963).

**Survival of Host-Phage Monocomplexes and Multicomplexes of T4y after Nitrous Acid Treatment**

Mutant strains that are defective in the y allele also show increased sensitivity to UV irradiation (Boyle and Symonds 1969) and nitrous acid treatment (Harm 1974). Recombination between markers is reduced by about one-half (Boyle and Symonds 1969). Figure 9, panel a, shows that levels of MR of y are decreased to about the same extent as the gene 32 (as grown at semi-restrictive temperatures) and px mutant strains. Since T4y is an amber mutant, it is possible to grow
Table 3. U.V. sensitivities of T4 strains in monocomplexes.

<table>
<thead>
<tr>
<th>Phage Strain</th>
<th>Inactivation Constant (k) in Lethal Hits per minute</th>
<th>Average relative sensitivities&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Boyle and Symonds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>This Work</td>
<td>Harm (1963)</td>
</tr>
<tr>
<td>wild type</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.7</td>
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<td></td>
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<td>2.5</td>
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<td>5.9</td>
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</tr>
<tr>
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<td>4.5</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y</td>
<td></td>
<td>4.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> $k_{mut}/k_{wt}$
Figure 9. Survival of monocomplexes and multicomplexes of $T^4_{\text{Y}}$ after nitrous acid treatment.

Survival of monocomplexes as a function of phage lethal hits.

For each experiment the phage strain, host strain, growth temperature and the symbol are:
(a) $T^4_{\text{Y}}$, S/6/5, 25°, (●); 31°, (▲).
(b) $T^4_{\text{Y}}$ CR63, 36°, (●).
(c) Wild type, CR63, 36°, (●).

Survival of multicomplexes as a function of phage lethal hits.

For each experiment the phage strain, host strain, growth temperature, and the symbol are:
(a) $T^4_{\text{Y}}$, S/6/5, 25°, (○), 31°, (△).
(b) $T^4_{\text{Y}}$, CR63, 36°, (○).
(c) Wild type, CR63, 36°, (○).

The dashed line in all panels indicates the relative multicomplex survival of wild type when grown on the host strain, S/6/5.

The dot and dash line in panel (b) indicates the relative multicomplex survival of wild type when grown on the host strain, CR63. This is taken from panel c.
Figure 9. Survival of monocomplexes and multicomplexes of T₄ after nitrous acid treatment.
this strain on a semi-permissive host, CR63. Levels of MR of the wild type strain, when grown on CR63 are slightly increased relative to growth on S/6 (panel c). When T^y is grown in CR63, levels of MR approximate those of wild type grown on S/6 (panel b).

Comparison of the relative UV sensitivity of the y strain used in these experiments to the relative sensitivities from the data in the literature are shown in Table 3. There was good agreement among the observations.

Survival of Host-Phage Monocomplexes and Multicomplexes of a Gene 30 Mutant, a Gene 44 Mutant and the y Mutant after Nitrous Acid Treatment

Figure 10 shows that wild type levels of MR occur in three mutant strains none of which have been shown to decrease levels of recombination between markers. Panel a gives the results of an experiment with a strain carrying a temperature sensitive defect in gene 30. This gene codes for the enzyme polynucleotide ligase (Cozzarelli et al. 1967; Becker et al. 1967; Weiss and Richardson 1967), which has been shown to seal nicks in DNA. Growth of multicomplexes occurred at a semi-restrictive temperature. Panel b shows the results of an experiment with a strain carrying a temperature sensitive defect in gene 44. The function of this gene product is not known, but phage carrying a defect in this gene are not able to synthesize any DNA (Warner and Hobbs 1967). Growth of multicomplexes was at an intermediate temperature. Panel c shows the results of an experiment with a strain carrying a defect in the y gene. This gene codes for the enzyme,
Figure 10. Survival of monocomplexes and multicomplexes of three mutant strains of T4D after nitrous acid treatment.

Survival of monocomplexes as a function of phage lethal hits.

For each experiment the mutant strain, growth temperature, and the symbol are:
(a) tsB20(30), 32°, (●).
(b) tsB110(44), 34°, (●).
(c) T4v, 25°, (●).

Survival of multicomplexes as a function of phage lethal hits.

For each experiment the mutant strain, growth temperature, and the symbol are:
(a) tsB20(30), 32°, (○).
(b) tsB110(44), 34°, (○).
(c) T4v, 25°, (○).

The dashed line in all panels indicates the relative multi-complex survival of wild type.
Figure 10. Survival of monoclonal and multicomplexes of three mutant strains of T4D after nitrous acid treatment.
endonuclease V (Sato and Sekiguchi 1976), which has been shown to initiate the dark repair pathway that excises pyrimidine dimers from DNA. The relative sensitivity to UV irradiation of this strain is shown in Table 3. It has not been shown to have increased sensitivity to nitrous acid treatment.

**Survival of Host-Phage Monocomplexes and Multicomplexes of a UV Irradiated Gene 47 Mutant**

Figure 11 summarizes the results of four experiments. In the first experiment, host bacteria were infected with UV irradiated wild type phage particles. The kinetics of inactivation of monocomplexes and multicomplexes correlated very well with previously published work (Symonds et al. 1973). As shown, there is an initially high rate of survival of multicomplexes, inactivation becoming exponential at a time when the phage entering these complexes were receiving a mean number of nine lethal hits. UV irradiated gene 47 (tsL86) mutants, when grown at the fairly permissive temperature of 25°C, have the same inactivation kinetics as the wild type phage multicomplexes. However, when growth occurs at an intermediate temperature of 31.5°C, the survival curve is strictly exponential, indicating that one hit is sufficient to cause lethality to these multicomplexes. The exponential inactivation of multicomplexes, when growth occurs at this temperature, is equivalent to the latter portion of the inactivation curve of wild type multicomplexes. When growth of tsL86 occurs at a higher temperature, there is no significant MR of multicomplexes, the kinetics of inactivation being comparable to that of the monocomplexes.
Figure 11. Survival of monocomplexes and multicomplexes of a UV irradiated gene 47 mutant.

Survival of monocomplexes as a function of phage lethal hits.

For each experiment the phage strain, growth temperature, and the symbol are:

- tsL86(47); 25°, (▼), 31.5°, (▲), 34.5°, (●).
- Wild type, 32°, (●).

Survival of multicomplexes as a function of phage lethal hits.

For each experiment the phage strain, growth temperature, and the symbol are:

- tsL86(47); 25°, (▼), 31.5°, (▲), 34.5°, (○).
- Wild type, 32°, (＋).

The dashed line indicates the multicomplex survival curve of wild type.
Figure 11. Survival of monocomplexes and multicomplexes of a UV irradiated gene 47 mutant.
Correlation of Fraction of Average Wild Type MR Factor Retained by Strains of Phage T4 with Fraction of Average Wild Type Recombination Frequency Retained by these Strains

Nitrous acid treated phage have been shown to be more efficiently repaired when allowed to form multicomplexes than when the host bacteria are singly infected with these particles. When a host is infected with more than one damaged phage and progeny are ultimately produced; a repaired, genetically complete, replicative entity is most likely formed by interaction between the infecting phage. Possibly, damages are not 'repaired' as such, but damaged portions of each genome could be selected against during a recombinational event and simply not be incorporated into the proposed replicative entity. By this means the replicative entity of the multicomplexes would receive fewer unrepaired hits than the replicative entity of the monocomplexes. One way of expressing the decrease in lethality is to determine the factor by which the monocomplex dosage must be increased in the multicomplexes to yield equal survival levels in both populations. This factor is referred to as the MR Factor and can be shown to be equal to the ratio of the rate constant of the monocomplexes to the rate constant of the multicomplexes. The demonstration that this factor is \( \frac{k_{\text{mono}}}{k_{\text{multi}}} \) is as follows:

Inactivation of monocomplexes and multicomplexes both follow one hit kinetics. Therefore their inactivation can be described by the equation
\[ \frac{N}{N_0} = e^{-kt} \]

where \( \frac{N}{N_0} = \) fractional survival

\( k = \) rate constant

\( t = \) dosage expressed in units of time

Taking the natural log of both sides of the above equation for a given fraction of survivors, \( n \), we have:

\[ \ln \left( \frac{N}{N_0} \right) = -kt \]

and rearranging we get:

\[ t_{\text{mono}} = -\ln \left( \frac{N}{N_0} \right) \frac{1}{k_{\text{mono}}} \]

when we are describing the inactivation of the monocomplexes or:

\[ t_{\text{multi}} = -\ln \left( \frac{N}{N_0} \right) \frac{1}{k_{\text{multi}}} \]

when we are describing the inactivation of the multicomplexes and, for any given fraction of survivors:

\[ \text{MR Factor} = \frac{t_{\text{multi}}}{t_{\text{mono}}} = \frac{-\ln \left( \frac{N}{N_0} \right) \frac{1}{k_{\text{multi}}}}{-\ln \left( \frac{N}{N_0} \right) \frac{1}{k_{\text{mono}}}} = \frac{k_{\text{mono}}}{k_{\text{multi}}} \]

When wild type levels of MR do not occur in mutant phage, it is reflected by decreases in the MR Factor. These decreases, plotted as the MR Factor of the mutant/average MR Factor of wild type, are
presented in Figure 12. The data used to generate these values are included in the experiments shown in Figures 4 through 7. However, now, a straight line is fit to the points for each separate experiment, and a separate MR Factor is calculated for each experiment. Panel a shows the wild type MR Factor/average wild type MR Factor at different temperatures. In comparison, wild type recombination frequency/average wild type recombination frequency obtained with two rII markers at different temperatures is shown. Panel b shows there is a decrease in the fraction of MR Factor retained by tsL86(V?) mutants with increase in temperature. The progressive decrease in MR as shown by the decrease in the MR Factor correlates with the progressive decrease in the fraction of average wild type recombination retained by this mutant. Panel c shows there is a decrease in the fraction of MR Factor retained by tsL109(46) mutants with increase in temperature. Again, this decrease in the MR Factor correlates with the progressive decrease in the fraction of average wild type recombination retained. Panel d shows there is a decrease in the fraction of MR Factor retained by tsL67(32) mutants with increase in temperature. This decrease coincides with the progressive decrease in the fraction of average wild type recombination retained.

Multiplicity Reactivation of Phage
with UV-Induced Lesions

Table 4 presents data obtained in MR experiments in which ultraviolet light was used to inactivate phage populations. The normalized data from these experiments is presented graphically in Figure 8.
Figure 12. Fraction of average wild type MR Factor retained by strains of phage T4 and fraction of average wild type recombination retained by these strains as a function of growth temperature.

(a) —: MR Factor of wild type phage/average MR Factor of wild type. Each point represents a single determination except the 25°C point which is the average of 2 values.

—: Wild type recombination frequency/average wild type recombination frequency for crosses between rED144 and r71. Data taken from Bernstein (1968) with the addition of four subsequently obtained points (H. Bernstein, personal communication 1976).

(b) —: MR Factor of tsL86(47)/average MR Factor of wild type. Each point represents a single determination except the 25°C point which is the average of 3 values.

—: Recombination frequency in the presence of tsL86(47)/average wild type recombination frequency for crosses between rED144 and r71. Data taken from Bernstein (1968).

(c) —: MR Factor of tsL109(46)/average MR Factor of wild type. Each point represents a single determination except the 40°C point which is the average of 2 values.

—: Recombination frequency in the presence of tsL109(46)/average wild type recombination frequency for crosses between rED144 and r71. Data taken from Bernstein (1968).

(d) —: MR Factor of tsL67(32)/average MR Factor of wild type. Each point represents a single determination except the 40°C point which is the average of 2 values.

—: Recombination frequency in the presence of tsL67(32)/average wild type recombination frequency for crosses between rED144 and r71. Previously unpublished data of H. Bernstein (personal communication 1976).
Figure 12. Fraction of average wild type MR Factor retained by strains of phage T4 and fraction of average wild type recombination retained by these strains as a function of growth temperature.
Table 4. Multiplicity reactivation of phage with UV-induced lesions.

<table>
<thead>
<tr>
<th>Phage Strain</th>
<th>Temperature °C</th>
<th>Inactivation Constant (k) of Straight Line Portion of Curve in Lethal Hits per Minute</th>
<th>MR Factor ( \frac{k_{\text{mono}}}{k_{\text{multi}}} )</th>
<th>Total Lethal Hits to Phage before Exponential Multicomplex Curve becomes Exponential</th>
<th>Intercept of Exponential Part of Multicomplex Curves with the Ordinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>32</td>
<td>2.2 Monocomplexes 1.0 Multicomplexes</td>
<td>2.2</td>
<td>9.0</td>
<td>37</td>
</tr>
<tr>
<td>tsL86(47)</td>
<td>25</td>
<td>2.6 Monocomplexes 1.0 Multicomplexes</td>
<td>2.6</td>
<td>9.0</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>31.5</td>
<td>2.3 Monocomplexes 0.94 Multicomplexes</td>
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<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>34.5</td>
<td>3.3 Monocomplexes 3.3 Multicomplexes</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Phage strains are designated in column 1 and growth temperature of the mono- and multicomplexes are indicated in column 2. The inactivation constants, expressed in terms of phage lethal hits per minute, for monocomplex inactivation are presented in column 3. Inspection of these data reveal that the sensitivity of the monocomplexes is increased in the tsL86(47) mutant when growth occurs at the semi-restrictive temperature of 34.5°C. This is in agreement with previously published data (Baldy 1970). Inactivation of multicomplexes formed from wild type phage is similar to previously published work (Symonds et al. 1973). The inactivation curve, as plotted on semi-logarithmic paper, has a broad shoulder and then a linear portion (ultimate slope) which, in these experiments, extends over approximately four decades. The rate constants of the ultimate slopes of the multicomplex inactivation curves are presented in column 4. At these higher dosages, sensitivity of multicomplexes formed from wild type and mutant (tsL86) phage are similar when growth of the mutant complexes occurs at 25° or 31.5°C. However, when growth of these mutant complexes occurs at 34.5°C, sensitivity of the multicomplexes is similar to that of the monocomplexes. Column 5 presents the MR Factors for the various experiments. The MR Factors are calculated from the straight line portions of the curves. The factor of 1.0 at 34.5°C for tsL86(47) reflects the fact that the mono- and multicomplex curves are similar. Column 6 reflects the extent of the shoulder of the inactivation curve before inactivation of multicomplexes becomes exponential. Complexes formed from wild type phage and tsL86(47) phage in which the growth of
the multicomplexes occurred at the fairly permissive temperature of 25°C sustained 9 lethal hits before the curve became exponential. When growth of the \textit{tsL86} complexes occurred at 31.5 or 34.5°C, inactivation of these complexes was strictly exponential. Column 7 presents the extrapolation point of the ultimate slope of the wild type multicomplexes and the \textit{tsL86(47)} multicomplexes grown at the three listed temperatures. The inactivation curves of the wild type complexes and \textit{tsL86(47)} multicomplexes, grown at 25°C, extrapolate to 37. The inactivation curves of multicomplexes formed from \textit{tsL86(47)} when grown at 31.5 or 34.5°C both extrapolate to 1.0.

\textbf{MR of Phage with Nitrous Acid Induced Lesions}

Table 5 summarizes the actual data obtained in MR experiments in which nitrous acid was used to inactivate phage populations. The normalized data from these experiments are presented graphically in Figures 1-7. Phage strains are designated in column 1. One of the phage strains used in this study (\textit{T4y}) carries an amber mutation in the \textit{y} gene and therefore exhibits its mutant phenotype on the restrictive host (S/6/5) and has a normal phenotype on the permissive host (CR63). The temperature sensitive strains can be grown on S/6/5 since their mutant phenotype is, by definition, a function of temperature. The \textit{y} and \textit{x} strains exhibit their mutant phenotype on S/6/5 and this phenotype does not vary with changes in temperature. The bacterial strains used for host-phage infections and titrations are listed in column 2. Columns 3 and 4 present the m.o.i.'s of the multicomplexes.
Table 5. MR of phage with nitrous acid induced lesions.

<table>
<thead>
<tr>
<th>Phage Strain</th>
<th>Host Strain</th>
<th>m.o.i.</th>
<th>Growth Temp. °C</th>
<th>Inactivation Constant ( k ) Lethal Hits per minute</th>
<th>( k_{\text{mono-complexes}} )</th>
<th>( k_{\text{multi-complexes}} )</th>
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Table 5—Continued. MR of phage with nitrous acid induced lesions.

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<th>Inactivation Constant in k Lethal Hits per minute</th>
<th>k mono- Complexes</th>
<th>k multicomplexes</th>
<th>k mono- Complexes/ k multicomplexes</th>
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a. Abbreviation used: m.o.i., multiplicity of infection.
b. The m.o.i., determined from surviving bacteria, using the zero term of the Poisson distribution.
c. The m.o.i. determined from phage and host titers before infection.
d. It is not known whether this growth temperature is permissive or semi-restrictive.
e. The ratio k monocomplexes/k multicomplexes is equivalent to the ratios of the dose to multicomplexes to the dose to monocomplexes required to give the same level of survival.
for the designated MR experiments. These values were determined by two methods. The m.o.i.'s in column 3 were determined from the titer of the surviving bacteria, using the zero term of the Poisson distribution and the m.o.i.'s in column 4 were determined from phage and host titers before infection. There is a reasonably good correlation between these values; in 37 out of 42 instances, the m.o.i.'s agree within a factor of 2. Column 5 gives the experimental growth temperatures for the monocomplexes and multicomplexes. The rate constants, expressed in terms of phage lethal hits per minute, for monocomplex inactivation are presented in column 6. As pointed out in Materials and Methods, inactivation of phage by nitrous acid is dependent upon the pH and the molarity of the nitrous acid solution and the temperature of this solution. It is also dependent upon the age and conditions of storage of the phage stock being tested (Harm 1974). These conditions varied in the experiments summarized here. The rate constants for multicomplex inactivation are presented in column 7. Increased sensitivity of these multicomplexes grown under semi-restrictive or restrictive conditions can most clearly be seen by examining the changes in the MR Factor presented in columns 8 and 9. As previously stated, this is the factor by which the monocomplex dosage must be increased in the multicomplexes to yield equal survival levels in both populations. In the wild type strain, the average value of the MR Factor, for experiments in which S/6/5 was used as the bacterial host, is 5.7. When tsL86(47) multicomplexes were grown at 25°C, this value was somewhat decreased, the average value being 3.6. At semi-restrictive temperatures the decrease
is more marked, the average MR Factor being 1.5, demonstrating the extreme sensitivity of these complexes. \textit{tsL109(46)} shows almost comparable levels of multicomplex sensitivity at semi-restrictive temperatures, having an MR Factor of 2.3 or less. \textit{tsL67(32)} shows a definite increase in sensitivity of the multicomplexes when grown at semi-restrictive temperatures. Strains x and y demonstrate levels of sensitivity comparable to the \textit{tsL67(32)} strain grown under semi-restrictive conditions. The MR Factor of the \( y \) strain reverts to approximately that of wild type phage on \textit{E. coli} S/6/5 when \textit{E. coli} CR63, the permissive host, is infected with high multiplicities of nitrous acid damaged T4\textit{y} phage. The MR Factor of strains \( x, \textit{tsB20(30)}, \) and \textit{tsB110 (44)} under semi-restrictive growth conditions of the multicomplexes, is comparable to the average MR Factor of the wild type strain (5.7).

\textbf{Inactivation of Monocomplexes of Phage Treated Extracellularly with Nitrous Acid}

A carefully controlled set of experiments was done to determine if mutations in genes 32, 46 and 47 confer increased sensitivity to nitrous acid. Mutations in \( x \) and \( y \) but not \( y \) had previously been shown to increase nitrous acid sensitivity (Harm 1974) while the \textit{tsB20(30)} mutation did not (C. Bernstein et al.1976). To avoid the effects of differential aging of phage lysates on nitrous acid sensitivity (Harm 1974), lysates of wild type, \textit{tsL67(32)}, \textit{tsL109(46)} and \textit{tsL86(47)} were grown at the same time. One set of lysates was grown for 11 hours and a second set was grown for 14 hours. Each mutant lysate was then
treated in parallel with the wild type lysate from the same set, using the same reagents and plating cultures.

Figure 13 shows representative relative survival kinetics for each of these mutants grown at semi-permissive and semi-restrictive temperatures for comparison. Extracellular phage were exposed to nitrous acid and survival of monocomplexes was measured as a function of increasing dosage. In each experiment, the first sample was always taken at 24 seconds after the addition of the sodium nitrate solution, following the protocol of Harm (1974). He used this procedure so that each sample tested could be taken from the same solution and the inaccuracy inherent in an attempt to take the first sample immediately after the addition of the sodium nitrite would be avoided.

It is clear that phage that are mutated in genes 32, 46 or 47 are more sensitive to nitrous acid treatment than wild type phage when growth occurs at semi-restrictive temperatures. When growth of these mutant strains occurs at semi-permissive temperatures, the sensitivity of the phage to nitrous acid treatment is decreased, but remains more sensitive than that of the wild type phage.

Table 6 presents the data illustrated graphically in Figure 13 and also includes additional experiments with these mutant strains. Sensitivities of strains previously tested by other investigators are also presented. Column 1 lists the phage strains tested and growth temperatures are indicated in column 2. The wild type phage lethal hits per minute (k), was normalized to 1.0 and column 3 presents the relative phage lethal hits per minute for each strain tested in this
Figure 13. Inactivation of T4D wild type phage and various mutants by nitrous acid as a function of time.

Survival of monocomplexes as a function of time of treatment of the phage with nitrous acid.

For each experiment the phage strain, growth temperature, and the symbol are:

(a) \text{tsL86(47)}; 36°C (\circ), 25°C (\triangle).
   Wild type; 36°C (\bullet), 25°C (\blacktriangle).

(b) \text{tsL109(46)}; 40°C (\circ), 25°C (\triangle).
   Wild type; 40°C (\bullet), 25°C (\blacktriangle).

(c) \text{tsL67(32)}; 40°C (\circ), 25°C (\triangle).
   Wild type; 40°C (\bullet), 25°C (\blacktriangle).
Figure 13. Inactivation of T4D wild type phage and various mutants by nitrous acid as a function of time.
Table 6. Relative inactivation of T4D type and various mutants by nitrous acid.

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<th>Relative Number of Lethal Lesions per minute (k)</th>
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<td>25°C</td>
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</table>

*Values taken from Bernstein et al. 1976.
**Values taken from Harm 1974.
Columns 4 and 5 present the average relative k for strains tested in previously published work and in this work. Subsequent to nitrous acid treatment, wild type, tsB20(30) and \( v \) strains are shown to have received approximately the same number of lethal lesions per minute. On the other hand, \( x, y, tsL86(47), tsL109(46) \) and \( tsL67(32) \) show increased sensitivity to nitrous acid treatment, sustaining about 20% more lethal lesions per minute. This latter group of mutants is distinct from the wild type, \( v \) and \( tsB20(30) \) strains in that each of the strains has been shown to have decreased levels of genetic recombination.
DISCUSSION

The purpose of this work was to define the gene products needed for the efficient MR of phage that had been lethally damaged through exposure to nitrous acid. It was found that genes 32, 46, 47, x and y, which have been shown to decrease genetic recombination (Bernstein 1968; Berger et al., 1969; Boyle and Symonds 1969; Harm 1963) also decrease MR (Figs. 5 through 9) while genes 30 and v (Fig. 10) which do not decrease genetic recombination (Bernstein 1968; Berger et al. 1969) do not decrease MR. In particular, in the cases of genes 32, 46 and 47 (Fig. 12) decreases in the MR factor are similar to decreases in levels of genetic recombination. Thus, genes 32, 46, 47, x and y define a pathway for the repair of nitrous acid induced lethal lesions in T4 bacteriophage.

The possibility that MR of phage that had sustained lethal lesions from exposure to UV irradiation occurred through a similar repair pathway was also explored. The mutant strain that is defective in gene 47, which has been shown to greatly decrease the level of MR of nitrous acid damaged phage, was tested as to its effect on the MR of UV damaged phage. Figure 11 demonstrates that the MR of these phage is also dependent upon the gene 47 product.

In addition, it has been shown that the gene products needed for the efficient MR of nitrous acid damaged phage are also necessary for the repair of phage particles that are allowed to form
monocomplexes. Figure 13 shows that phage deficient in genes 32, 46 or 47 have increased sensitivity to nitrous acid treatment. Strains deficient in x and y have previously been shown to be sensitive to nitrous acid (Harm 1974).

### MR and Repair of Phage with Nitrous Acid Induced Lethal Lesions

#### The Replicative Entity in Multiply Infected Cells

Experimental data presented by Krisch, Hamlett and Berger (1972) demonstrates that much of the genetic recombination between undamaged infecting phage occurs early in infection. Krisch et al. (1972) performed two factor crosses and looked for recombinant frequency at the earliest detectable times in the infectious cycle by prematurely lysing the infected cells with chloroform. They found that at a time when only 0.07% of the final phage yield was present intracellularly, 20.5% of the final recombination values were observed. When 6.0% of the final phage yield was present, 60% of the final recombination values were observed. Also, it has been demonstrated that recombinational events occur repeatedly. Hershey and Rotman (1948) noted that when a cell is infected with three types of phage, a++, +b+, and ++c, the yield includes recombinants of the abc genotype. Hershey and Chase (1951) showed that triparental recombinants were not a rare occurrence, since their proportion approaches genetic equilibrium.

Epstein (1958) performed a series of premature lysis experiments with UV irradiated parental phage. There is stimulated recombination under these conditions and we can see that when only 0.07% of
the final phage yield was present intracellularly, 100% of the final recombination values were observed. He also performed single burst experiments in which clonality of recombinants were observed. His results imply that most of the recombinant particles in some bursts are derived from a single, early formed genome which supports the idea of early mating.

Other evidence relevant to the formation of the genetic structure has been presented by Rayssiguier and Vigier (1972). They studied MR using two strains of T4D that differed in twenty-six genetic markers. These strains were irradiated and the MR repaired progeny of multicomplexes formed from these phage were analyzed in a series of single burst experiments. They found that twenty-three bursts out of thirty-four contained one or more phage of a parental genotype. This could be accounted for if lesions in the parental configuration triggered a recombinational event that consisted mainly of short, single stranded regions that left the parental configuration intact.

Blanco and Devoret (1973) also have hypothesized that prophage reactivation in phage lambda, which is dependent upon the recA gene of the host, is a result of a pre-replicative recombinational process.

The inactivation of multicomplexes formed from phage that have been previously treated with nitrous acid has been shown to follow single hit kinetics. That is, when the log of the surviving fraction is plotted as a function of dosage, the experimental points delineate a straight line whose extrapolate is 1.0. This means that there is only one phage replicative entity within the bacterial host cell, and
a single chemical change in the DNA of the infecting particles is able to inactivate the complex. It would seem that formation of this replicative entity occurs very early in infection and is produced by recombination between damaged genomes.

Role of Gene Products Involved in the MR Repair Pathway

Due to the common effects of strains deficient in genes 32, 46, 47, x or y on recombination and MR, it is logical to assume that these two processes occur by the same or similar pathways. It has been hypothesized (Becker et al. 1964) that the formation of an interstrand cross-link is the most frequent inactivating event caused by nitrous acid treatment. Presumably, DNA cross-links caused by the exposure of phage to nitrous acid stimulate recombinational events in multiply infected cells, resulting in the elimination of those lesions. In reference to Figures 1 and 2, in which current models for genetic recombination and the repair of cross-links are illustrated, certain common steps can be delineated. They are as follows:

1. Nick insertion
   a. Step a of Figure 1
   b. Steps a and b of Figure 2

2. Gap formation
   a. Step b of Figure 1
   b. Step c of Figure 2

3. Synapse and branch migration
   a. Steps c and d of Figure 1
   b. Steps c and d of Figure 2

4. Formation of intact duplexes
   a. Through branch elimination, gap repair, and nick sealing. Steps e, f and g of Figure 1.
   b. Through repair synthesis and duplex separation. Steps d and e of Figure 2.
Occurrence of these common steps most probably necessitates the action of common enzymes. Recombination is dependent upon the introduction of nicks into the DNA by T4-induced endonucleases. Repair of cross-links also seems to necessitate endonucleolytic action. Altman and Meselson (1970) identified the presence of a T4-induced endonucleolytic activity that produces nicks in double stranded DNA. Kemper and Hurwitz (1973) isolated an endonuclease from T4 infected cells which attacks single or double stranded DNA. Broker and Doermann (1975) proposed that genes x or y could possibly code for the production of one of these endonucleases. It has been shown that x- or y- phage are deficient in recombination and MR (Harm 1963; Boyle and Symonds 1969; this work).

Genes 46 and 47 have been shown to be necessary for the conversion of single stranded nicks to gaps (Prashad and Hosoda 1972) and branched recombinational intermediates are not produced in these mutant strains (Broker 1973). Mutants in these genes are also deficient in the MR of nitrous acid damaged phage (this work). Perhaps the gaps left after endonucleolytic scission of cross-links (Steps a and b, Fig. 2) require further degradation in order to promote efficient synapse of complementary DNA strands. Also, branches produced by recombinational events are possibly degraded by the action of these genes (Step e, Fig. 1; Step d, Fig. 2).

The product of gene 32 has been shown to be necessary for genetic recombination (Berger et al. 1969) and for efficient MR of nitrous acid damaged phage (this work). Binding of the gene 32 protein to single stranded DNA inhibits nuclease activity (Huang and
Lehman 1972) and catalyzes the formation of recombinant duplexes (Tomizawa and Anraku 1964). Thus, it is needed to protect the single stranded region illustrated in Structure 3, Figure 1 (Broker 1973) and is possibly needed in the formation of the recombinant molecule formed in Step c, Figure 2.

**Post-Replicative Recombinational Repair**

Luria and Latarjet (1947) tested the effect of UV irradiation on intracellular vegetative T2 phage at various stages of the latent period. They were surprised to find that the UV sensitivity of these vegetative phage was greatly reduced during the early stages of the eclipse period. In the light of our present knowledge, it could be theorized that this decreased sensitivity is due to recombination between daughter molecules. In regards to T4 phage, Symonds et al. (1973) alluded to the fact that there was a post-replicative type of repair in phage T4 that was dependent upon the $x$, $y$ and $1206$ gene products. Since they did not have sufficient evidence to justify calling it post-replicative repair, they tentatively referred to it as replication repair.

Figure 13 clearly demonstrates that mutant strains defective in genes 32, 46 and 47 have increased sensitivity to nitrous acid treatment. Table 6 summarizes experiments in which the sensitivity of these strains to nitrous acid treatment was determined. The table also includes previously published data in which the sensitivity of various other mutant strains were tested. It is clear that mutants defective in genes 32, 46, 47, $x$ and $y$, which have been shown to have
decreased genetic recombination and decreased MR, are also more sensitive to nitrous acid treatment. This increased sensitivity to nitrous acid indicates the presence of a repair pathway that is operative in singly infected cells. Since all of the gene products that have been shown to be necessary for the normal functioning of this repair pathway are also necessary for normal recombination and MR, it is reasonable to assume that this pathway would involve the by-passing of lethal lesions by recombination between progeny molecules. This is an important finding since it is the first strong indication of post-replicative recombinational repair in phage T4.

Directed Recombination

It is important to speculate as to the type of recombination that results in the formation of an intact replicating entity from two or more damaged genomes. Random recombination would not be an effective mechanism as it would only result in the exchange of damages between the infecting phage particles. Also, Luria (1947) performed experiments in which bacterial cells were multiply infected with severely damaged phage (phage that had sustained up to 50 lethal hits) and undamaged phage particles. In a series of such experiments, he maintained that every infection was successful, i.e., in no instance were the lethal lesions recombined into the untreated phage. Therefore, it is reasonable to conclude that recombination is directed in such a way as to by-pass lethal lesions. As to the nature of this directional signal, a clue is presented in the repair of nitrous acid induced lethal lesions by MR. Cross-links have been implicated as the major
inactivating lesion of nitrous acid damaged phage (Becker et al. 1964). Since replication obviously cannot proceed past a cross-link, this type of damage may be the signal for a cross-over event. Cole et al. (1976) investigated the repair of cross-links caused by psoralen plus light. In his model of the repair of these cross-links (see Fig. 2 and accompanying discussion) he found that the cross-link did indeed initiate a cross-over event.

**Repair Mechanisms Operative in Multiplicity Reactivation**

A model is proposed for the MR of nitrous acid damaged phage, in which the pre-replicative repair of cross-links occurs in a manner similar to that described by Cole et al. (1976). This model is diagrammed in Figure 14. DNA duplexes containing nitrous acid induced cross-links are illustrated in the first structure (Part 1, Fig. 14) and the partial excision of these cross-links and the formation of recombinant, repaired molecules are shown in steps a through d. Steps a and b would require nucleolytic activity (perhaps the products of genes x and y) and steps c and d may require nucleases for gap formation and branch elimination (the gene 46 and 47 products), the unwinding enzyme of gene 32 to maintain single stranded intermediate recombinational structures, and the action of polymerase for repair replication. A mutation in the Kornberg polymerase has been shown to decrease MR of UV irradiated phage (Maynard-Smith, Symonds and White 1970). It is suggested that, during the replication of molecules containing partially excised cross-links (Part 2, Fig. 14), gaps are left opposite these structures (step a). These gaps would facilitate a cross-over event.
1. Pre-replicative repair of cross links.
   a. endonucleolytic scission
   b. exonucleolytic scission
   c. strand exchange and repair synthesis
   d. duplex separation

   a. replication of molecules
   b. strand exchange and repair synthesis

Figure 14. Proposed model for MR repair of nitrous acid induced lethal lesions.
as illustrated in step b leading to the formation of the replicative entity that is discussed on pages 69-71.

**MR of Phage with UV-Induced Lethal Lesions**

Much of the research pertaining to the phenomena of MR has been performed using phage carrying UV-induced lethal lesions. Symonds et al. (1973) investigated the effects of mutations in genes x, y, 1206 or v on levels of MR and identified two distinct types of repair contributing to the efficient MR of UV inactivated phage. When the log of the surviving fraction of wild type complexes are plotted as a function of time (dosage), (see Fig. 11) there is a broad shoulder formed prior to the time when the curve becomes exponential. The extent of this shoulder is significantly decreased when multicomplexes are formed from strains x, y or 1206. If the multicomplexes are formed from phage carrying a mutation in the v allele, the ultimate slope of the MR curve is decreased and becomes similar to that of the monocomplexes. Their interpretation of these data was that the products of the x, y and 1206 mutant strains, which have been shown to be deficient in genetic recombination (Harm 1963), participate in a type of recombinational repair that determines the extent of the shoulder of the MR curve, while an excision repair pathway, as mediated by the v gene, determines the final slope of these curves. They found that the double mutants, vx and vy have an additive effect on MR of UV damaged phage which also suggests that two repair pathways are involved in this process.

The greatest decreases in MR of nitrous acid damaged phage were shown to occur when the phage being tested were deficient in the
product coded for by either gene 47 or gene 46. Therefore, a gene 47 mutant strain was tested in order to see if MR of UV irradiated phage particles was dependent upon this gene product. Since, as is shown in Figure 11, all UV-MR can be removed by a defect in the gene 47 product, it can be tentatively concluded that both the $x$, $y$ and 1206 process and the $v$ process leading to MR must lead into a final step dependent on gene 47 function. This is presumably a process of recombination between defective genomes.

Referring again to Figure 11, it is clear that as the growth temperature of multicomplexes formed from $tsl86(47)$ becomes more restrictive, first, the shoulder of the MR curve is eliminated and second, the final slope becomes similar to that of the monocomplexes. Therefore, the 47 product is required for the type of recombination repair that is characterized both by the $xy$ repair pathway and the repair pathway mediated by the $v$ gene product.

The inactivation kinetics of UV irradiated phage are complex and the factors contributing to the shoulders and final slope of UV-MR curves are not completely understood. However, we can propose that, in multiply infected cells, the gaps left by the excision of pyrimidine dimers in the $v$ pathway are more efficiently repaired by recombination of single stranded sections of undamaged homologous chromosomes and consequently the damages involving only one strand of the DNA duplex may also serve as signals for cross-over events in which the products of genes $x$, $y$ and 47 are necessary. Therefore, it may be that there is only one MR pathway for repair of UV lesions and an intermediate in
excision repair may be a signal for entering this pathway. Gaps formed by dimer excision would allow pre-replicative recombination to occur in a somewhat similar manner to that illustrated in Figure 14 (Part 1, steps c and d). Replication of a molecule in which all of the dimers had not been excised would proceed in a manner similar to that diagrammed in Part 2 leading to the formation of the replicative entity.
LIST OF REFERENCES


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