

STIMULATION OF RECOMBINATION IN BACTERIOPHAGE T4  
BY NITROUS ACID-INDUCED LESIONS

by

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## ABSTRACT

A number of agents are capable of stimulating recombination by phage T<sup>4</sup>. Experiments with nitrous acid show that recombination can be stimulated above the normal level by more than seven times indicating that nitrous acid induced lesions are recombinogenic. Temperature-sensitive mutants defective in an exonuclease function showed less than the normal spontaneous level of recombination even after nitrous acid treatment. A ligase mutant showed an extremely high frequency of recombination approaching the level of genetic equilibrium after treatment with nitrous acid. These results imply that HNO<sub>2</sub> induced lesions in DNA stimulate a recombinational repair process similar in some of its enzymatic steps to normally unstimulated recombination.

## INTRODUCTION

### The Role of Recombination

Recombination is ubiquitous. It occurs in bacteriophage, animal viruses and higher organisms (see reviews by Hayes 1974; Fenner et al. 1974; Haywood and Magee 1976). Despite its prevalence in biological systems, its role in nature has not yet been clarified. Recombination has been thought to be preserved by natural selection in order to generate genetic diversity but recently an alternative role for recombination as primarily a DNA repair process has been proposed (H. Bernstein 1977).

It seems reasonable to assume that when macromolecules first began to bear genetic information, mechanisms must have evolved to stabilize this information against the disruptions introduced by the environment. The rate of evolution of a species may have been directly related to the efficiency and discrimination of its DNA repair mechanisms. A number of DNA repair processes are known to exist in nature (Hanawalt and Setlow 1975). Among the most well characterized are photoreactivation, excision, and recombination repair.

Photoreactivation is the simplest of the repair mechanisms and occurs through the action of a photoreactive enzyme. This enzyme recognizes and binds to dimer-containing regions of DNA in the dark. Visible light provides the energy required to catalyze the subsequent cleavage of the dimer to generate the normal adjacent thymines.

Excision repair occurs when a segment containing the lesion is excised. This can occur in the absence of light. Incisions are made on either side of the lesion and the released segment replaced by replicative gap filling. This synthesis is directed by the intact complementary strand of DNA acting as a template.

Recombination repair can occur when a lesion is replaced by a segment of DNA from an homologous chromosome or when a gap formed opposite a lesion by replication is filled by a segment of DNA from a homologous chromosome. In the first case the altered segment may be excised by incisions on both sides of the lesion and a homologous strand of DNA used to fill in the gap, producing a repaired duplex (for review see Kornberg 1974). Evidence has been obtained that recombination is used to repair lesions produced by UV-light, x-rays,  $^{32}\text{P}$ -decay, nitrous acid and psoralen + light (Epstein 1958; Harm 1958; Symonds and Ritchie 1961; Nonn and Bernstein 1977; Cole 1973).

Several DNA repair processes are found to occur by recombination in E. coli and its phages (for review see H. Bernstein 1977). The studies reported here in the repair of nitrous acid lesions of phage T4 provide insight into the repair of damage to DNA through recombination.

The original evidence for the occurrence of genetic exchange or recombination of DNA between bacteriophage comes from the work of Delbruck, Bailey and Hershey in 1946 (Delbruck and Bailey 1946; Hershey 1946). They showed that recombination was a population phenomenon requiring more than one chromosome. Their results were consistent with a number of models for genetic recombination. These models are breakage and reunion, breakage and copying, and complete copy choice.

In 1935 Darlington developed a theory of genetic exchange which was based on the observed behavior of chromosomes at meiosis (Darlington 1935). He proposed that recombination occurred through breakage and reunion. Breakage and reunion involves the physical break in a parent chromosome with a subsequent physical exchange of genetic material with another chromosome. This model of breakage and reunion was ultimately supported by direct evidence, the most significant being a radioactive labeling experiment with bacteriophage  $\lambda$  (Meselson and Weigle 1961). They conducted two factor crosses between unlabeled  $\lambda$  and  $\lambda$  heavily labeled with isotopes  $^{13}\text{C}$  and  $^{15}\text{N}$ . Density gradient centrifugation was used to determine the distribution of labeled parental DNA among both parental and recombinant gene types of the progeny. Their results demonstrated the presence of discrete portions of parental DNA in recombinant phage. These experiments strongly indicated breakage and reunion as the mechanism for recombination in bacteriophage  $\lambda$ .

The present experiments with nitrous acid study general recombination. This is the exchange between homologous segments of DNA which can take place anywhere along the chromosome. Recombination comprises a series of enzyme mediated steps (for review see Hayes 1974). These events include the breakage of DNA by enzymes, rejoining of segments of different parental molecules to form a joint molecule, the synthesis of new DNA from complementary strands to fill gaps, and the covalent joining of the DNA strands of different parental origins. It is known that, in general, genes that affect recombination in E. coli and phage T<sup>4</sup> also affect the repair of lesions in DNA (for

review see Kornberg 1974; Radding 1973). The role of recombination in the repair of DNA in eucaryotic organisms remains unclear.

### Recombination in Phage T<sup>4</sup>

#### General Recombination

Molecular and genetic recombination in phage T<sup>4</sup> has been reviewed by Broker and Doerman (1975). Broker and Lehman (1971) demonstrated the existence of branched DNA molecules that are intermediate in the process of recombination. These intermediates were found by electron microscopy in extracts of Escherichia coli infected by wild-type phage T<sup>4</sup> but were absent in infections with mutants defective in recombination. These results led to the formulation of a model for recombination in bacteriophage T<sup>4</sup> (Broker 1973).

Their idealized pathway for recombination is shown in Figure 1. The first step involves the injection of a number of phage T<sup>4</sup> chromosomes which have base pair sequences that are circular permutations of each other. This DNA is acted upon by an endonuclease creating nicks, an example of which is indicated by the 'V' in the figure. These nicks can be sealed by the phage T<sup>4</sup> DNA ligase which is coded for by gene 30. Thus the ligase counteracts the effect of the endonuclease.

The next step involves the enlargement of nicks to gaps and the exposure of stranded regions by a DNA exonuclease coded for by genes 46 and 47. The action of genes 46 and 47 are counteracted by a DNA polymerase coded for by gene 43.

The single stranded regions created by exonuclease bind the product of gene 32, a single stranded DNA binding protein (Alberts and

# T4 RECOMBINATION

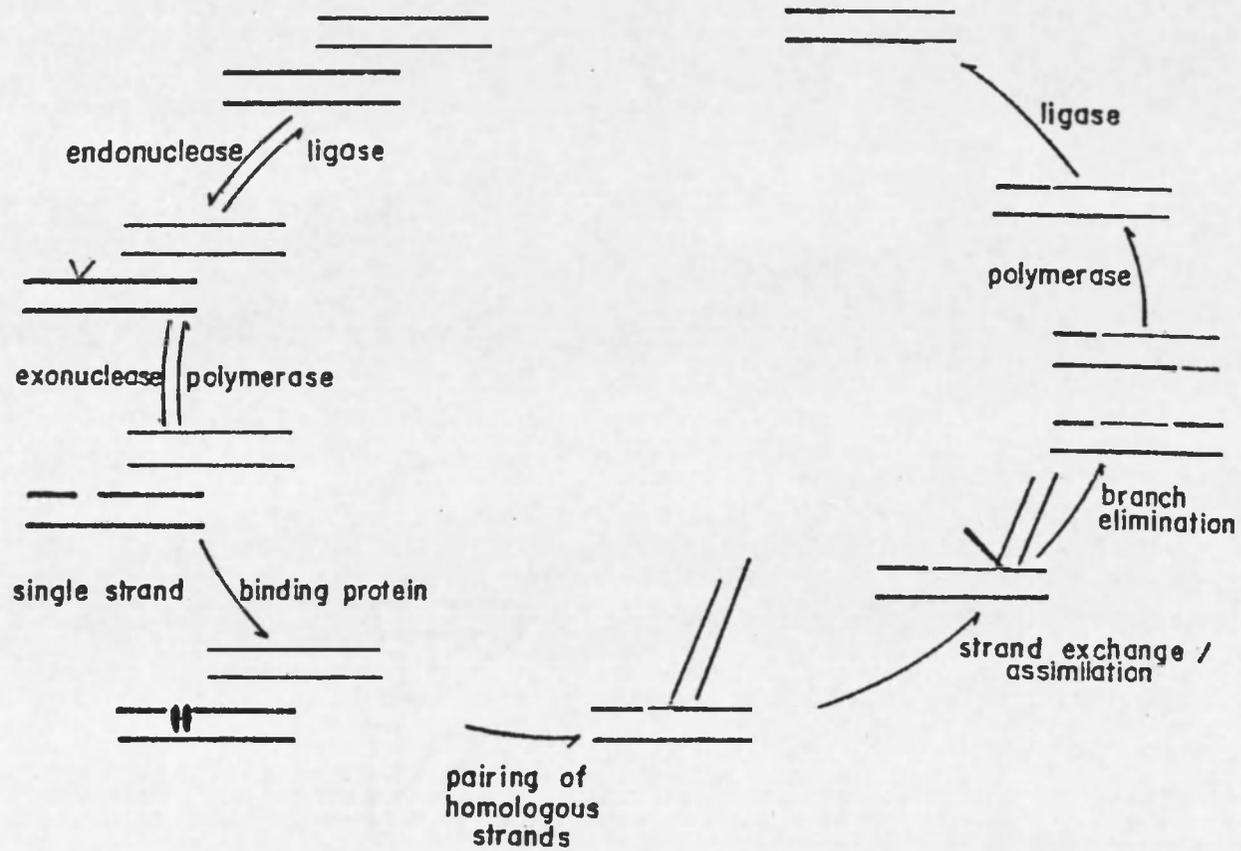


Figure 1. Broker and Doerman model for recombination in phage T<sup>4</sup>.

Frey 1970). It is thought that the gene 32 protein protects the single stranded regions from nucleases and promotes the pairing of complementary sequences of DNA. The gene 32 protein is indicated by the solid ovoid shapes in Figure 1.

The next step involves the pairing of homologous strands of DNA. The mechanism for this phenomenon is not known. The pairing leads to a heteroduplex region with duplex branches. Paired regions are extended either by a diffusion controlled strand exchange or a nuclease directed strand assimilation.

The branches of DNA are then eliminated by the action of nucleases creating two possible recombinants. The lower recombinant in the drawing is termed an insertion heteroduplex and the upper recombinant is termed a staggered heteroduplex. The gaps between strands are then filled by either the host or the phage T<sub>4</sub> polymerase. The strands are finally sealed by either the host or phage T<sub>4</sub> ligase.

The experiments reported here on the effects of phage T<sub>4</sub> mutations on nitrous acid induced recombination imply that the Broker and Doerman recombinational pathway applies to nitrous acid-induced stimulated recombination, particularly with respect to the first few steps of the pathway in Figure 1.

#### The Function of Genes 46 and 47

Phage T<sub>4</sub> gene 46 and gene 47 functions have been shown to be required for recombination (H. Bernstein 1968; Berger, Warren and Fry 1969; Broker and Lehman 1971). Amber and temperature-sensitive mutations in genes 46 and 47 are also defective in DNA synthesis in the

late period of a phage T<sub>4</sub> infection (Hosoda, Mathews and Jensen 1971). Electron microscopic examination of intracellular phage DNA intermediates formed by genes 46 and 47 mutants show no branched molecules in DNA. Such mutants fail to produce single stranded gaps or termini (Broker 1973). These branched structures are thought to be intermediates in phage T<sub>4</sub> recombination in wild-type phage infections.

Genes 46 and 47 were found to have an exonucleolytic activity on host and phage DNA (Kutter and Wiberg 1968). The time course of bacterial DNA degradation after infection of E. coli B with phage T<sub>4</sub> was followed with neutral and alkaline sucrose density gradient centrifugation. It was found that the degradation of both E. coli and cytosine containing phage DNA (instead of normal hydroxymethylcytosine containing DNA) occurred as a two step process. The first step involves limited endonucleolytic cleavage. This was found to occur in gene 46 and 47 mutants. The next step involves a second nuclease sequence of reactions for the degradation of large DNA fragments to an acid soluble form. It was found that mutations in either genes 46 or 47 completely blocked this second stage in degradation leaving all of the bacterial DNA as double stranded fragments (Kutter and Wiberg 1968).

Digestion of hydroxymethylated and glucosylated wild-type phage T<sub>4</sub> DNA (Wyatt and Cohen 1953) has been found to be carried out by the products of genes 46 and 47. Parental DNA did not become acid soluble when mutations in genes 46 and 47 were present (Hosoda and Mathews 1971; Prashad and Hosoda 1972).

As mentioned above gene 46 and 47 mutants have been shown to be deficient in recombination. Recombination between pairs of rII

markers was drastically reduced in infections by gene 46 and 47 temperature-sensitive mutants (H. Bernstein 1968). Other experiments using amber mutations in genes 46 and 47 have also shown a great reduction in recombination (Berger, Warren and Fry 1969).

Evidence has been presented (Prashad and Hosoda 1972) that single strand breaks in DNA were repaired with ligase alone when 46 and 47 mutations were present. This result suggests that genes 46 and 47 control either directly or indirectly an exonuclease activity which can attack T<sup>4</sup> DNA at nicks to create gaps.

In summary, genes 46 and 47 code for an exonuclease that can degrade either host or phage DNA. When mutations in genes 46 and 47 are defective the level of genetic recombination as well as the level of observable branched intermediates is greatly reduced.

### The Function of Gene 30

It has been found using temperature-sensitive and amber mutations that DNA ligase is coded for by gene 30 of phage T<sup>4</sup> (Fareed and Richardson 1967). The ligase catalyzes the repair of single strand nicks in a DNA duplex by the formation of a phosphodiester bond. In the reaction adenosine 5' triphosphate (ATP) is cleaved to adenosine 5' phosphate (AMP) and inorganic phosphate (PPi) (Becker, Gefter and Hurwitz 1967).

Mutants defective in ligase show an increase in recombination (Berger et al. 1969; H. Bernstein 1968). Cesium sulfate and sucrose density-gradient analysis of mutants defective in both the T<sup>4</sup> induced ligase and DNA polymerase result almost exclusively in the production

of joint structures. These joint structures are hydrogen bonded hybrids of parental DNA as opposed to recombinant (covalently linked) hybrids. Joint structures contain components from different parental chromosomes and are thought to require ligase for final sealing (Fig. 1). These joint structures were interpreted as being intermediates in recombination (Anraku, Anraku and Lehman 1969). Temperature-sensitive mutations in gene 30 were found to increase recombination between rII markers of T4 (H. Bernstein 1968). Amber mutations in gene 30 were also found to increase recombination (Berger, Warren and Fry 1969).

Understanding the effects of ligase mutants in the presence of an rII mutant (such as used in the nitrous acid experiments) is complicated by the suppressive effect of rII mutations on ligase mutations. It has been reported that under conditions when  $\text{lig}^-$  amber mutations are not viable (Fareed and Richardson 1967),  $\text{rII}^- \text{lig}^-$  mutations may be viable (Berger and Kozinski 1969; Karam 1969). The mechanism of this suppression has been investigated (Berger and Kozinski 1969; Karam 1969). The conferring of an rII mutation and a gene 30 amber mutation partially restored phage growth in the  $\text{Su}^-$  host E. coli B. The presence of the rII mutation also resulted in nearly complete restoration of phage DNA synthesis preventing the extensive degradation of parental phage DNA observed after infection with  $\text{lig}^-$  single mutants (Berger and Kozinski 1969).

E. coli host ligase has also been shown to have a role in substituting for the defective phage ligase in  $\text{rII}^- \text{lig}^-$  infections (Krisch, Shah and Berger 1971). Increased recombination in ligase

deficient  $r_{II}^-$  infections is reduced in bacterial strains which produce a greater than normal level of host ligase (Karam 1969).

In summary these results show that mutations in the phage T4 gene 30 ligase decreased viability and increased recombination. Double mutants  $r_{II}^- \text{lig}^-$  show increases in viability compared to single  $\text{lig}^-$  mutations. The E. coli host ligase may play a role in  $r_{II}^- \text{lig}^-$  infections.

### Multiplicity Reactivation

When multiplicity reactivation was first discovered by Luria - in 1947 he suggested the possible involvement of recombination (Luria 1947). Luria found that after irradiation of phage and infection of cells at either high or low multiplicity, the cells infected at high multiplicity of infection, moi, showed a much higher plaque forming ability than cells infected at low moi.

These experiments point to a relationship between recombination and survival after UV-irradiation but they give no insight into the mechanism at the molecular level.

In 1958 Harm studied the survival of multicomplexes at different moi after irradiation with X-rays (Harm 1958). It was found that multiplicity reactivation of intracellular X-rayed phage occurred, indicating that multiplicity reactivation was not limited to UV-induced lesions.

Recent work by Nonn and Bernstein (1977) has implicated a number of T4 gene functions in multiplicity reactivation. These experiments involved the multiplicity reactivation of phage T4 damaged

by nitrous acid. The survival of monocomplexes and multicomplexes of wild-type phage after  $\text{HNO}_2$  treatment was determined. Multicomplexes showed a much greater survival than monocomplexes after treatment suggesting that the DNA lesions produced by nitrous acid were effectively repaired by multiplicity reactivation.

The survival of mutants defective in gene 46 (tsL109) and gene 47 (tsL86) was determined and compared to wild-type infections. The level of multiplicity reactivation was far less in 46<sup>-</sup> and 47<sup>-</sup> multicomplexes than in wild-type multicomplexes. This indicates that genes 46 and 47 have a role in multiplicity reactivation.

The series of experiments with UV-light, X-rays and nitrous acid as inactivating agents suggests that multiplicity reactivation is a recombinational process useful in the repair of a variety of DNA lesions. Both recombination of genetic markers and multiplicity reactivation requires at least two homologous chromosomes. Also the experiments with mutants defective in genes 46, 47 and 30 show that multiplicity reactivation and recombination as measured by marker exchange require common gene functions.

#### Stimulated Recombination

It has been shown in phage T4 that the frequency of exchange between genetic markers can be stimulated by a variety of agent. These agents include X-rays, UV-light,  $^{32}\text{P}$ -decay, and 9-aminoacridine (Harm 1958; Epstein 1958; Symonds and Ritchie 1961; Mattson 1970). All of these experiments were carried out with phage that did not carry mutations deficient in unstimulated recombination. Thus it was not

clear whether the same enzymatic pathway was used for stimulated and unstimulated recombination.

It has been shown that recombination increases after treatment with X-rays when the percentage of wild-type recombinants was plotted as a function of X-ray dose (Harm 1958). This stimulation of recombination was greater than four times the level in untreated phage. UV-light has also been shown to stimulate recombination in phage T<sub>4</sub> by over three times (Epstein 1958). Symonds and Ritchie (1961) reported stimulation of recombination by the incorporation of <sup>32</sup>P in phage infected E. coli cells. This increase was two times that of untreated T<sub>4</sub>/E. coli complexes. In addition it has been shown that 9-aminoacridine is capable of stimulating recombination in phage T<sub>4</sub> (Mattson 1970). An increase of genetic exchange between rII markers of over three times was reported. These experiments also showed that this increase in recombination was not attributable to a delay in maturation of the phage particle.

X-rays, UV-light, <sup>32</sup>P-decay, and 9-aminoacridine are known to have a variety of effects on DNA. UV-light introduces pyrimidine dimers (Bedkers and Berends 1960) and it is thought that 9-aminoacridine intercalates into the DNA double helix (Lerman 1964). X-rays and <sup>32</sup>P-decay (see review by Dertinger and Jung 1970) have been shown to cause both single strand and double strand breaks in DNA. The following experiments will show the effects of nitrous acid-induced lesions on recombination in phage T<sub>4</sub>. It is known that nitrous acid causes oxidative deaminations (Schuster 1960) and crosslinks in DNA (Becker,

Zimmerman and Geiduschek 1964). It is proposed that the damage caused by nitrous acid may be responsible for the effects on recombination.

## MATERIALS AND METHODS

### Phage and Bacterial Strains

T4 phage and E. coli bacterial strains were obtained from the California Institute of Technology. Double mutant phage were constructed in this laboratory. Phage stocks were prepared by inoculating a slightly turbid culture of S/6/5 with approximately  $10^6$  phage/ml. These stocks were grown at 37° overnight. The cells were lysed with chloroform and the debris removed by centrifugation in the cold. The phage were then concentrated by ultracentrifugation in a Sorvall SS-34 rotor at 17,000 r.p.m. for 2.5 hours to a titer of greater than  $2.25 \times 10^8$  phage/ml.

The phage strains used were rED144(rIIA), r71(rIIA), tsL109(46) rED144(rIIA), tsL109(46)r71(rIIA), tsL86(47)rED144(rIIA), tsL86(47)rIIA, tsB20(30)rED144(rIIA), tsB20(30)r71(rIIA), and T4D wild-type. The ts symbol indicates that the mutant was sensitive to increases in temperature. The brackets indicate which gene was defective.

### Media

Hershey's Broth (Steinberg and Edgar 1962) were used for the growth of phage lysates and growth media for the phage crosses. Phage to be treated with nitrous acid were suspended in modified M-9 media containing  $\text{NaHPO}_4$  (6.0g/L),  $\text{KH}_2\text{PO}_2$  (3.0g/L),  $\text{NH}_4\text{Cl}$  (1.0g/L) (Adams 1959). The agar overlay method on agar plates (Adams 1959) was used for measuring phage plaque formation.

### Nitrous Acid Treatment

Phage concentrated at approximately  $2.5 \times 10^{11}$ /ml were diluted 10-fold into 4.5 ml of sodium acetate buffer (0.1 M and pH 4.0). Then 0.45 ml of 1.0M  $\text{NaNO}_2$  was added to the phage in acetate buffer to give a final concentration of 0.1M  $\text{NaNO}_2$  (Bernstein et al. 1976). At short intervals after adding  $\text{NaNO}_2$  samples of phage were removed and diluted 10-fold into modified M-9 solution to stop the reaction.

### Plating

Indicator bacteria were prepared by inoculating 250 ml of Hershey's broth with 0.4 ml of a saturated E. coli broth culture that had been incubated overnight (Steinberg and Edgar 1962). Plating cultures were incubated at 37°C until they reached a titer of approximately  $1 \times 10^8$  cells/ml. The cells were then harvested by centrifugation for 30 minutes at 3,000 rpm and resuspended in 15 ml of Hershey's medium for use as plating indicators. The permissive plating host for the rII mutant was E. coli S/6/5. The restrictive plating host was E. coli K594 $\lambda$ . Plates were incubated overnight at 25°C.

### Cross Protocol

Log phase E. coli were prepared by inoculating 250 ml of Hershey's Broth with 0.4 ml of a fresh saturated culture of bacteria and growing the cells at 37°C until a titer of  $1 \times 10^8$  cells/ml was achieved. Cells were concentrated by centrifugation and adjusted to  $4 \times 10^8$  viable cells/ml with modified M-9 solution. A klett meter was used to estimate the titer and a viable bacterial count was obtained by diluting an aliquot of the adjusted suspension and plating at 25°C to measure colony formation.

Aliquots of log phage bacteria were suspended in modified M-9 salts solution for 30 minutes and prewarmed to 37°C in absorption tubes. Aliquots of phage mixtures for crosses were added at concentrations giving 5-25 phage per bacteria. After 10 minutes of incubation infected cells were diluted  $4 \times 10^4$  into prewarmed growth tubes at 37°C. Unabsorbed phage were sampled within 23 minutes after infecting the host cells. After 90 minutes the growth cycle was terminated by the addition of chloroform. Total progeny was determined by diluting and plating on E. coli S/6/5 and rII<sup>+</sup> progeny were determined by diluting and plating on E. coli K594λ. The recombination frequency was twice the rII<sup>+</sup> progeny divided by total progeny. This fraction was multiplied by 100 so that it could be expressed as a percentage.

## RESULTS

### Genes 46 and 47

Treatment with nitrous acid resulted in survival curves that followed single hit kinetics for both wild-type and mutant strains of phage T4. Nitrous acid treatment was found to produce approximately one lethal hit for 2.2 minutes of exposure at the concentration used (0.1M).

The assay for recombination could detect two or more  $r^+$  recombinants out of 10,000 phage progeny. The spontaneous  $r^+$  revertant frequency for all of the ts rII mutants used was less than  $10^{-7}$ . Figure 2a and 2b each show the results of two crosses. In all crosses the frequency of recombination was measured between the markers rED144(rIIA) and r71(rIIA). When these strains are untreated, crosses give recombination frequencies of 1-2%. In Figure 2a the percent recombinants obtained in the control cross ts<sup>+</sup> rED144 x ts<sup>+</sup> r71 is represented by closed circles and labeled (ts<sup>+</sup>). It shows a frequency of recombination at zero minutes of treatment of 1.5%. After treatment with nitrous acid the frequency of recombination rose to 10%. The open circles in Figure 2a represent the cross between mutants tsL109rED144 (gene 46) and tsL86r71 (gene 46). The initial frequency of recombination was 0.012% and the final frequency after treatment with nitrous acid was 0.017%.

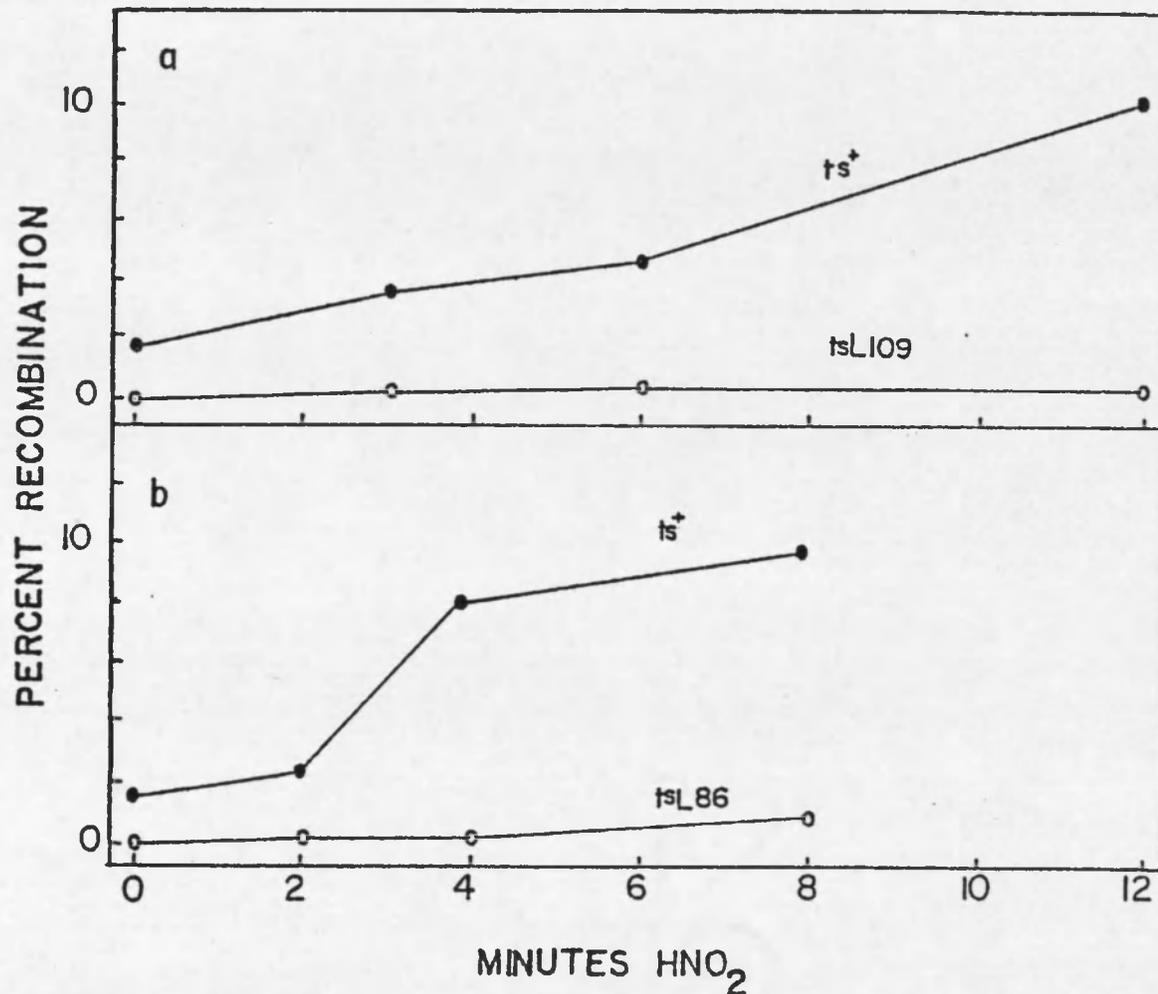


Figure 2. Percent recombination in mutant tsL109 (gene 46) and tsL86 (gene 47) and ts as a function dose of HNO<sub>2</sub>. -- (a) ts<sup>+</sup> r71 x ts<sup>+</sup>rED144 ●; tsL109r71 x tsL109rED144 ○. (b) ts<sup>+</sup> r71 x ts<sup>+</sup>rED144 ●; tsL86r71 x tsL86rED144 ○.

In Figure 2b the control cross again is represented by closed circles and labeled ts<sup>+</sup>. It shows a frequency of recombination at zero minutes of treatment with nitrous acid of 1.5%. After treatment with nitrous acid the frequency of recombination rose to 9.5%. The open circles in Figure 2b represent the cross between mutants tsL86rED144 (gene 47) and tsL86r71 (gene 47). The initial frequency of recombination was 0.047% and the final frequency after treatment with nitrous acid was 0.069%. The results shown in Figure 2a and 2b demonstrate an appreciable increase in recombination of the control crosses from 1.5% to 9.5-10% after treatment with nitrous acid. Mutants tsL109 (gene 46) and tsL86 (gene 47) showed a marked reduction in the frequency of recombination without treatment. Even after treatment with nitrous acid the frequency of recombination was well below that of ts<sup>+</sup> control frequencies of recombination. These results indicate that genes 46 and 47 are essential for both unstimulated recombination and the added recombination induced by nitrous acid lesions.

### Gene 30

Figure 3 shows the effect of the gene 30 ligase allele tsB20 on recombination. The control cross rED144 x r71 is shown by the closed circles and labeled ts<sup>+</sup>. The frequency of recombination at zero minutes of treatment with nitrous acid was 1.8%. After treatment with nitrous acid the frequency of recombination rose to 8.5%. The cross tsB20r71 x tsB20rED144 was represented by open circles. The frequency of recombination at zero minutes of treatment was 8.5% as opposed to 1.8% for the ts<sup>+</sup> wild-type control. After treatment for

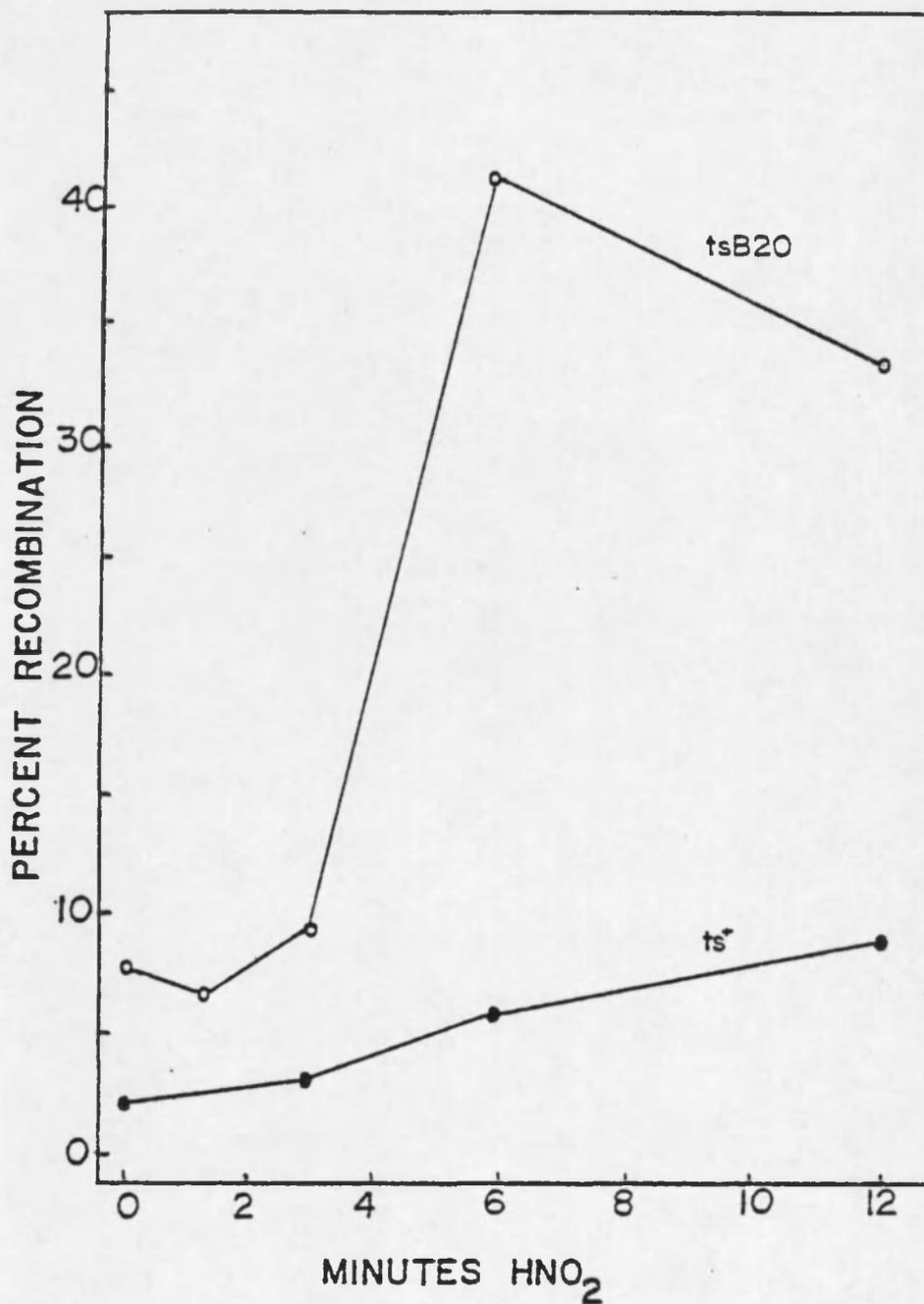


Figure 3. Percent recombination in mutant *ts*<sub>B20</sub> (gene 30) and *ts*<sup>+</sup> as a function of dose of HNO<sub>2</sub>. -- *r*<sub>71</sub> x *r*<sub>ED144</sub> ●; *ts*<sub>B20</sub>*r*<sub>71</sub> x *ts*<sub>B20</sub>*r*<sub>ED144</sub> ○.

6 minutes the level of recombination rose to 41%. At 12 minutes of treatment the level of recombination was 33%. Thus in the presence of a defective ligase the frequency of recombination both before and after treatment with nitrous acid is greatly enhanced.

### Summary of Results

A summary of the results is shown in Table 1. The results of three wild-type crosses were averaged, and they show an increase in recombination frequency of nearly six times. The results of 8 experiments (including the three shown here and five additional experiments) show average increases in recombination frequency of over seven times.

The mutant cross with a gene 30 ligase deficiency shows a substantial increase in the frequency of recombination after treatment with nitrous acid (from 8.5% to as high as 41%) whereas the crosses in the presence of genes 46 and 47 controls show little if any increase in recombination after treatment with nitrous acid.

Table 1. Effect of gene 30, 46 and 47 mutations on recombination of  $\text{HNO}_2$  treated phage.

Mutation	% Recombination without $\text{HNO}_2$	Maximum % Recombination with $\text{HNO}_2$	Stimulation
Wild Type*	1.6	9.3	yes
46 <sup>-</sup>	0.012	0.017	no
47 <sup>-</sup>	0.047	0.069	no
30 <sup>-</sup>	8.5	33-41	yes

\*Average of three experiments.

## DISCUSSION

A number of agents have been shown to stimulate recombination in phage T4. X-rays, UV-light,  $^{32}\text{P}$ -decay, and 9-aminoacridine have been found to increase the level of recombination by up to four times (Epstein 1958; Harm 1958; Symonds and Ritchie 1961; Mattson 1970). In the three experiments with nitrous acid reported here the average increase in recombination was six times. The average increase in recombination in eight experiments (including an additional five not reported here) was over seven times. It is postulated that the high stimulation of recombination in contrast to that found with other agents may reflect some particular characteristic of  $\text{HNO}_2$  lesions in DNA.

Phage T4 inactivation by nitrous acid follows first order kinetics. This indicates that lethal lesions are caused by single chemical alterations (Vielmetter and Schuster 1960). Nitrous acid causes the oxidative deamination of bases (Schuster 1960) and the cross-linking of DNA (Becker, Zimmerman and Geiduschek 1964). Oxidative deamination converts guanine, adenine, and cytosine respectively to xanthine, hypoxanthine, and uracil. It is not known to what degree oxidative deaminations contribute to lethality. The formation of cross-links relative to deaminations is thought to be frequent (Becker, Zimmerman and Geiduschek 1964). The existence of only a single unrepaired cross-link in DNA is thought to result in lethality.

It is postulated that a cross-link prevents the denaturation of double-stranded DNA. Strand separation must occur for the accurate replication and transcription of DNA (Becker, Zimmerman and Geiduschek 1964). Recombination is the only known repair process which can accurately repair cross-linked DNA (Cole 1973; Hanawalt and Setlow 1975, pp. 421-429, 487-500; Howard-Flanders and Lin 1973). Since nitrous acid is known to produce cross-links in DNA this may present one explanation for the increase in the level of recombination observed in wild-type crosses.

The inhibition of recombination by mutations in genes 46 and 47 strengthens current concepts of how their gene products function in phage T4. Genes 46 and 47 probably code for a DNA exonuclease (Kutter and Wiberg 1968). These gene functions act in recombination to widen the nick formed previously by an endonuclease (see Fig. 1) (Broker and Doerman 1975). The action of exonuclease results in the creation of single-strand gaps. If this exonuclease is not present, gaps will not be formed and the level of recombination will be reduced. The advancement of the recombinational pathway will then be blocked. The results obtained here with mutants tsL109 (gene 46) and tsL86 (gene 47) indicate greatly reduced frequencies of recombination even after treatment with nitrous acid. These results show that natural and  $\text{HNO}_2$  stimulated recombination require common gene functions and thus repair recombination may occur by a pathway similar to natural recombination. The inhibition of recombination obtained with mutations in genes 46 and 47 also correlates with results obtained by multiplicity reactivation. It was demonstrated that multiplicity reactivation could effectively

repair lesions produced by nitrous acid (Harm 1974; Nonn and Bernstein 1977). Mutants defective in genes 46 and 47 had levels of multiplicity reactivation appreciably lower than that of wild-type. This correlates well with the lower level of recombination in gene 46 and 47 mutants. The observations of Nonn and Bernstein support the hypothesis that multiplicity reactivation is a recombinational process requiring the same gene functions as recombination.

Gene 30 of phage T<sub>4</sub> codes for a polynucleotide ligase. It is thought to catalyze the esterification of 3'-hydroxy 5'-phosphate single stranded scissions in double stranded DNA (Becker, Geftter and Hurwitz 1967). If ligase is completely deficient the sealing of nicks in the final stages of recombination does not occur (Fig. 1) (Broker and Lehman 1971). Viability and recombination are decreased in these infections. Bernstein has shown that a mutation in gene 30 (tsB20) results in an increased level of recombination. In the present study the level of recombination at 37°C with a tsB20 ligase mutant was increased three to four times. The additional increase in recombination observed here in response to HNO<sub>2</sub> treatment can be accounted for by examining the model shown in Figure 1. If ligase is deficient the nicks created by endonuclease which initiate the pathway will accumulate. This creates a quantity of substrate for action by exonuclease. The ligase deficiency thus advances the pathway.

Ligase is also required for the final step of recombination (Fig. 1) (Broker and Lehman 1971). Either the phage T<sub>4</sub> ligase or the host ligase is thought to be available to complete the final stage in the pathway. The great stimulation of recombination by nitrous acid

in the presence of a ligase mutation could be explained by assuming that the available pool of ligase is depleted by the high number of nicks caused by the many  $\text{HNO}_2$  lesions. It is difficult to explain why this deficient level of ligase stimulates recombination while at the same time it is sufficient for the final step of recombination. Perhaps the latter time of the final ligase step or a greater contribution by the host ligase at the later stage of the maturation cycle could account for this sufficiency.

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