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GENETIC CONSEQUENCES OF TRANSFORMING *BACILLUS SUBTILIS* WITH NITROGEN MUSTARD CROSSLINKED DNA

by

Saul Scheinbach

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1977

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October 13, 1976
Date

Chairman of Examining Committee
Professor R. Rudner

October 22, 1976
Date

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Abstract

GENETIC CONSEQUENCES OF TRANSFORMING
BACILLUS SUBTILIS WITH NITROGEN
MUSTARD CROSSLINKED DNA

by

Saul Scheinbach

Advisor: Professor Rivka Rudner

Bacillus subtilis DNA was treated with nitrogen mustard (HN_2) and alkali denatured. The resulting crosslinked molecules (HN_2-D-DNA) were used in transformation assays to determine:

1. The ability of crosslinked DNA to cotransform a segment containing the *trpC2* and *hisB2* markers.
2. The effect of crosslinks on the production of mutations during transformation.

The crosslinked molecules were detected by their ability to remain double stranded and their activity in transformation assays following exposure to denaturing conditions. These molecules were separated from single strands by hydroxyapatite chromatography. The single marker transforming activity of HN_2-D-DNA was less than that of native but higher than that of renatured DNA. When tested for cotransforming ability along with native and

renatured DNA controls, HN_2-D-DNA exhibited a greater drop than its undenatured counterpart (HN_2-N-DNA), indicating that crosslinks caused r to decrease. The decrease in cotransforming ability was not accompanied by an increase in mutagenicity. These results suggested that HN_2-D-DNA cotransformed poorly due to cleavage of crosslinked regions by competent cells. The possible nature of such a cleavage was considered within the framework of events which may normally occur during transformation in B. subtilis. A model was developed which proposes that during the conversion of donor DNA to single-stranded fragments by competent cells, crosslinks are recognized by an endonuclease which operates in the pericellular space and acts to eliminate them. The resultant cleavage of the donor molecule reduces both r and single marker transforming activity. Predictions arising from this model of crosslink-induced scissions were in satisfactory agreement with the observed r and single marker activity of crosslinked DNA.

ACKOWLEDGEMENTS

I wish to acknowledge the guidance and steadfast support of Dr. Rivka Rudner, whose dedicated service to science and education will serve as a model for me throughout my career. I also wish to acknowledge the encouragement, patience and understanding supplied in abundance by my wife Ellen, which made this work possible.

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LIST OF ABBREVIATIONS

HN_2	nitrogen mustard
HN_2-N-DNA	DNA treated with HN_2
HN_2-D-DNA	DNA treated with HN_2 and alkali denatured
r	cotransformation index
SSF	single-stranded DNA fragment
DSF	double-stranded DNA fragment
DRC	donor-recipient DNA complex
HA	hydroxyapatite
KP	potassium phosphate buffer, pH 6.7
MMS	methyl methane sulfonate
MAK	methylated albumin-Kieselguhr
MC	mitomycin C

INTRODUCTION

Interstrand covalent crosslinks have been found in a minor fraction of DNA molecules where they prevent complete strand separation during exposure to denaturing conditions, thereby allowing the strands to snap back upon removal of the denaturant (Alberts, 1968; Alberts and Doty, 1968; Rownd, et al., 1968; Mulder and Doty, 1968). These "natural" crosslinks were shown to occur only at one end of the DNA molecules, while chemically induced crosslinks were positioned randomly along the helix (Alberts, 1968). Following exposure to denaturing conditions, molecules containing either type of crosslink were active in transformation, whereas noncrosslinked molecules remained single stranded and could not transform (Alberts, 1968; Mulder and Doty, 1968; Chevallier and Bernardi, 1968; Kohn and Green, 1966).

During transformation, double-stranded molecules have been shown to be taken up by competent Bacillus subtilis cells and degraded to single-stranded fragments (SSF) and 5' mononucleotides as they pass through the cell membrane (Dubnau and Cirigliano, 1972a; Davidoff-Abelson and Dubnau, 1973a). The SSF can then integrate with the host genome, a process which may occur along single-stranded gaps produced within the recipient chromosome (Harris and Barr, 1971). Transformation with crosslinked molecules could not occur in this manner, since the presence of

covalent crosslinks prevents strand separation of donor DNA and would interfere with normal SSF production. However, formation of the single-stranded intermediate might be achieved if the competent cells first eliminated the crosslinks from the incoming DNA. This could arise by cleavage of the molecule at or near the crosslinked region which would disrupt the continuity of donor molecules. A genetic consequence of this event would be a reduction in genetic linkage between markers. On the other hand, if crosslinks are not removed at an early stage, their presence during integration may produce an aberrant form of donor-recipient recombination which could be potentially mutagenic. This could occur via a repair mechanism similar to postreplication repair of UV damage (Rupp and Howard-Flanders, 1968; Rupp, et al., 1971). This repair process is thought to contain an error-prone component which is responsible for UV mutagenesis (see Section II).

This study deals with the genetic consequences arising from transformation with nitrogen mustard-crosslinked B. subtilis DNA. Two parameters were examined to determine whether competent cells eliminate crosslinks from donor DNA. These were:

1) Measurement of the linkage between two markers
2) Assay of forward mutants induced by crosslinked DNA.

I. PROPERTIES OF CROSSLINKED DNA:

A) Naturally Crosslinked DNA:

Early studies on bacterial transformation showed that denaturation of DNA did not completely inactivate transforming ability (Marmur and Lane, 1960; Doty, et al., 1960; Ginoza and Zimm, 1961; Roger and Hotchkiss, 1961). Since single strands are not taken up by competent Pneumococcus or B. subtilis cells (Lerman and Tolmach, 1959; Rownd, et al., 1968), the residual transforming activity could be due to a DNA fraction which either does not strand separate or can renature when the denaturing condition is removed. Marmur and Lane (1960) and Ginoza and Zimm (1961) concluded that strand separation never occurred, based on the fact that the residual activity of Pneumococcus DNA, heat-denatured and fast-cooled, was independent of DNA concentration. Other studies confirmed this by using density labeled B. subtilis and Hemophilus influenza DNA to demonstrate that residually active DNA molecules of hybrid bouyant density did not separate into heavy and light components following thermal denaturation and quenching (Rownd, et al., 1968; Mulder and Doty, 1968). The amount of residual transforming activity was generally observed to be in the range of 1-8% of native DNA (Ginoza and Zimm, 1961; Alberts and Doty, 1968; Kohn and Green, 1966; Mulder and Doty, 1968; Chevallier and Bernardi, 1968).

Investigations of the physical properties of this denaturation-resistant fraction showed that it behaved

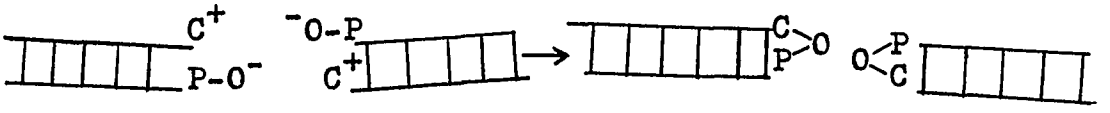
like native DNA with respect to chromatographic behavior, melting profile, partition coefficient, sedimentation velocity and bouyant density (Chevallier and Bernardi, 1968; Alberts and Doty, 1968; Mulder and Doty, 1968; Rownd, et al., 1968). More importantly, the thermal transition of residually active molecules was characteristic of native DNA and the former regained a substantial degree of hypochromicity upon cooling which was not observed for the latter (Alberts and Doty, 1968). This, coupled with the fact that the molecular weight of the residually active molecules was twice that of single strands (Mulder and Doty, 1968; Alberts and Doty, 1968), means that these molecules do unwind but never completely separate and can therefore snap back upon removal of the denaturing conditions. The prevention of strand separation was postulated to result from the production of covalent crosslinks during DNA isolation (Alberts and Doty, 1968).

Of the techniques used to purify naturally crosslinked DNA, hydroxyapatite chromatography has provided one of the better separations of crosslinked molecules from the bulk of denatured DNA. This enabled Chevallier and Bernardi (1968) to determine that the naturally crosslinked fraction comprised 10% of the total DNA and exhibited a specific transforming activity ranging between 30-50%. This range is close to the value of 25% obtained by purification on methylated albumin-Kieselguhr (MAK)

columns, (Rudner and Remeza, 1973). Naturally cross-linked molecules purified either by banding in CsCl gradients or partition separation in dextran-polyethylene glycol were reported to exhibit specific transforming activities of 94% and 81% respectively (Mulder and Doty, 1968; Alberts and Doty, 1968).

The origin of naturally crosslinked B. subtilis DNA molecules was studied by Alberts who found that the amount of crosslinked DNA was invariant to metabolic activity and remained unchanged in newly replicated DNA (Alberts, 1968; Alberts and Doty, 1968). He concluded that natural crosslinks were not produced in vivo, and occurred randomly because markers from different regions of the chromosome exhibited similar levels of residual activity. He also observed that nitrous acid-cross-linked DNA sedimented somewhat faster in alkaline sucrose than naturally crosslinked molecules and he could induce crosslinks by shearing of very high molecular weight DNA (130×10^6 daltons). These findings led him to conclude that the earliest breaks occurring during DNA isolation lead to the formation of a natural crosslink at one end of a molecule (Alberts, 1968). Alberts (1968) proposed that the greater shearing force generated along very large molecules might cause a rupture of a carbon-phosphate bond which would result in $P\text{-}O^-$ and C^+ ion

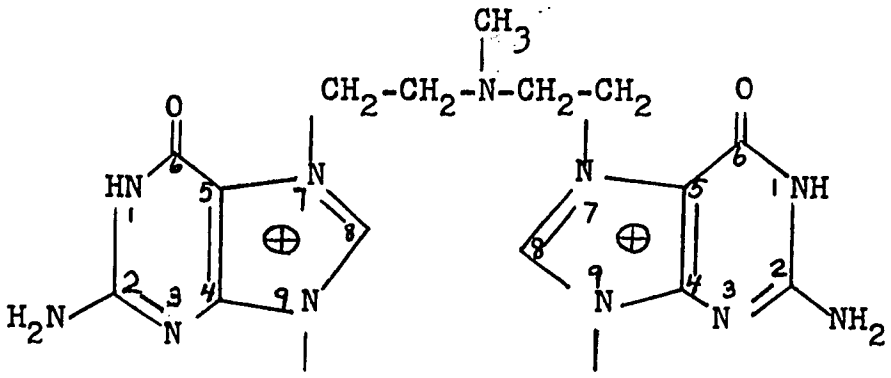
pairs. Such ion pairs could form a new bond connecting complementary strands and would result in a terminal crosslink at one end of a newly formed DNA fragment.



Naturally-occurring terminal crosslinks have been reported elsewhere. For example, an enzyme fraction capable of producing terminal crosslinks has been purified from T4-infected Escherichia coli (Weiss, 1970). The purified enzyme system requires ATP, Mg^{++}, and T4-induced ligase, and can convert terminal 5' monophosphates of T7 DNA into a phosphatase-resistant form, yielding denaturation-resistant molecules. When T4 DNA was used it was a poor substrate for this reaction, so that the biological role of the enzyme is unclear. As another example, vaccinia virus DNA molecules have been found to be terminally crosslinked. Each linear duplex contained a crosslink at each end as shown by the appearance of single-stranded circles in electron micrographs of denatured viral DNA (Geshelin and Berns, 1974). Since the molecular weight of these circles (about 1.2×10^7 daltons) equaled that of the intact double-stranded genome (Geshelin and Berns, 1974), shear could not have produced these crosslinks.

B) Induced Crosslinks:

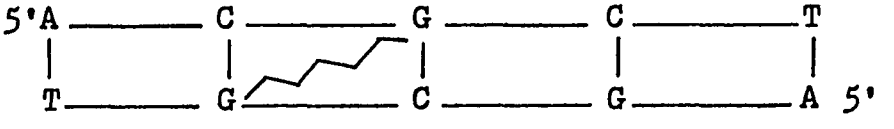
A number of agents have been used to induce interstrand crosslinking either *in vivo* or *in vitro*. These include nitrous acid (Geiduschek, 1961; Becker, *et al.*, 1964; Alberts, 1968), low pH (Freese and Cashel, 1964), UV irradiation (Marmur and Grossman, 1961), mitomycin C (or MC) (Iyer and Szybalski, 1963), the furocoumarin 4,5',8,-trimethylpsoralen (Cole, 1970; 1971) as well as the bifunctional alkylating agents which are best represented by dichloroethyl sulphide (mustard gas) and dichloroethylmethylamine (nitrogen mustard or HN_2). The reaction of the last two compounds with DNA has been extensively studied. The mechanism of crosslinking by these two-armed mustard compounds was postulated by Brookes and Lawley (1961a and b) to involve alkylation at the N_7 position of the guanines of two adjacent G-C pairs so as to create a bridge between the complementary strands. An HN_2 crosslink involving two guanine residues is illustrated below.



This hypothesis was based on the fact that whereas all the alkylating agents tested yielded 7-alkylguanines, the

diguanyl derivative was found only when bifunctional agents were used. This moiety was later shown to be associated with denaturation-resistant DNA and was not recovered after reaction with single-stranded DNA (Lawley and Brookes, 1967). A further indication that bifunctionality is required to form a crosslink was observed when a monofunctional analogue of HN_2, dimethyl chloroethylamine, did not crosslink even though it had reacted with the DNA (Kohn, et al., 1966). In addition, the onset of HN_2-induced denaturation resistance exhibited a slight lag indicating that one arm must first bind, yielding a monoadduct, before the second arm can react to form the crosslink or diadduct (Kohn and Green, 1966). Thus crosslinking results from two sites on the HN_2 molecule which can form a covalent bond with guanine residues on opposite DNA strands.

By using three-dimensional models, Lawley and Brookes (1961b) concluded that in order for the mustard chain to extend across the helix, the base sequence must be 5' cytosine→guanine 3' on both strands as illustrated below.

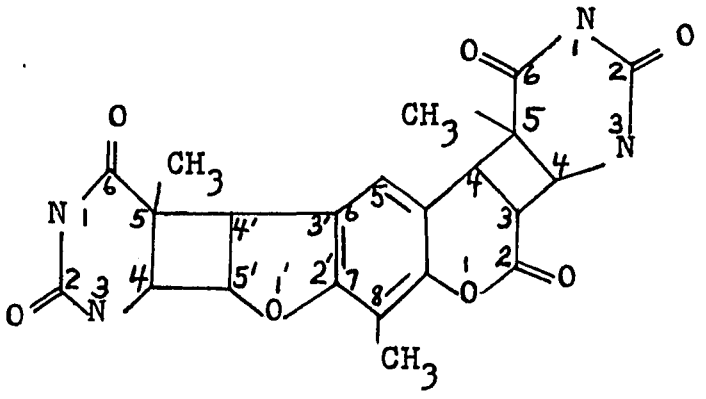


Assuming that the four usual nucleotides are equal in number and distributed randomly, their model predicts that one quarter of the alkylated guanines yields a crosslink. This was observed when calf thymus DNA was

reacted with mustard gas (Brookes and Lawley, 1961b). Using isotopically labeled HN_2, Kohn, et al. (1966) found a correlation between the HN_2 which was bound to B. subtilis DNA and the proportion of denaturation-resistant material. Their results indicated that only about 5% of the alkylated sites yielded a crosslink. DNA reacted in vitro with HN_2 could be exposed to denaturing conditions and still exhibit properties such as bouyant density, viscosity, and hyperchromicity similar to native DNA. The crosslinked fraction persisted even after exposure to high temperatures (up to 99°C), indicating the covalent nature of the crosslinks (Kohn, et al., 1966).

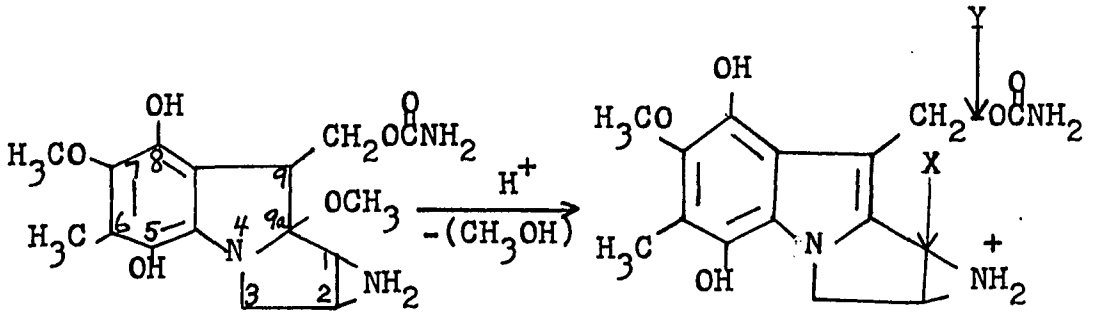
Other than HN_2, only psoralen and MC have been studied in any detail with respect to the crosslinking reaction. Reaction with psoralen was observed following irradiation of a psoralen-DNA mixture with 360nm light (Cole, 1970). The mechanism of crosslinking was revealed by binding studies with isotopically labeled psoralen which showed that the drug was initially associated with denaturable DNA which subsequently became denaturation resistant (Cole, 1971). This indicated that, as with HN_2, crosslinking by psoralen occurs subsequent to monoadduct formation (Cole, 1971). Since psoralen can form two types of monoadducts with pyrimidines (Musajo, et al., 1967), it was suggested that its reaction with pyrimidines on opposite DNA strands would yield a cross-

link (Cole, 1970). The probable structure of a psoralen-thymine crosslink is illustrated below.



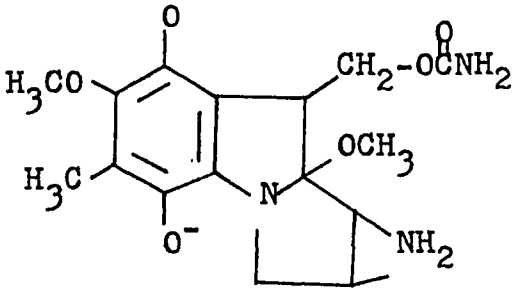
Crosslinking by MC was shown to occur <u>in vivo</u> (Iyer and Szybalski, 1963) while the <u>in vitro</u> reaction required the presence of a reducing agent such as sodium borohydride, (Iyer and Szybalski, 1964a), suggesting that the antibiotic must be activated. Like HN_2, reaction with guanine is involved in crosslink formation (Iyer and Szybalski, 1964b; Lipsett and Weissbach, 1965). Iyer and Szybalski (1964b) originally proposed that the reduced or hydroquinine form of MC could act like a bifunctional alkylating agent reacting with guanine at the C_6 oxygen. They postulated, as shown below, that nucleophilic displacement of the aziridine ring at C_1 (X) and the carbamate group at C_{10} (Y) by guanines on opposite DNA strands would yield a crosslink. The highly unstable nature of the activated form of MC has prevented its isolation; however, more recent evidence suggested that the

partially reduced semiquinone form may combine with DNA in a noncovalent manner with subsequent crosslink formation (Tomasz, et al., 1974).



Reduced MC
(Hydroquinone form)

Activated form



Semiquinone form

In summary, the mode of action of these three cross-linking agents involves reaction with bases on opposite DNA strands to form the interstrand diadduct and in two of these cases it is guanine that is attacked.

II. REPAIR OF CROSSLINKED DNA AND ITS POTENTIAL MUTAGENICITY:

Genetic and biochemical evidence has suggested that chemically induced interstrand crosslinks are repaired via pathways which are similar, if not identical, to

those which act to repair UV damage. The susceptibility of both types of damage, *i.e.* crosslinks and pyrimidine dimers, to the same repair systems, indicates that a structural alteration of the helix is probably recognized. Marmur and Grossman (1961) showed that UV-induced crosslinks in *E. coli* DNA could be repaired by exposure to *Saccharomyces cerevisiae* photoreactivating enzyme. This enzyme repairs UV damage by monomerizing intrastrand thymine dimers in the presence of light (Wulff and Rupert, 1962). These results indicate that interstrand dimers form the crosslinks and are also monomerized. Another UV repair system, namely excision repair, operates in the dark to excise UV-induced dimers (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). In *E. coli* the first step in this process is thought to be mediated by an endonuclease which creates an incision 5' to a dimer and yields a 3'OH terminus (Braun and Grossman, 1974a). The role of this enzyme in excision repair was indicated by its absence in *uvrA* and *uvrB* mutants and by its specific binding to DNA which contained dimers (Braun and Grossman, 1974b). Efficient repair of these incision breaks requires the activity of DNA polymerase I (Paterson, *et al.*, 1971) which may act by nick translation to remove the dimer-containing region and resynthesize the correct sequence using the undamaged strand as a template. However *polA* cells, lacking DNA polymerase I activity, retain some repair

ability which can be eliminated by incubating a polA, temperature sensitive dnaE double mutant at the non-permissive temperature (Youngs and Smith, 1973). Thus at least two enzymes (DNA polymerases I and III) are involved in the resynthesis step. The final step in the process, that of sealing the nick, would be accomplished by polynucleotide ligase.

Excision repair of MC-induced crosslinks was demonstrated when MC-treated E. coli and B. subtilis cells exhibited a loss of denaturation-resistant DNA during post-treatment incubation in the dark (Terawaki and Greenberg, 1966; Mahler, 1966). This loss was not detected when mutants unable to perform excision repair were used. Similar results were obtained for E. coli treated with HN_2 (Kohn, et al., 1965; Hanawalt and Haynes, 1965). In addition, repair of the crosslinks involved the same type of nonconserved replication observed (Pettijohn and Hanawalt, 1964) during excision repair of UV-induced dimers. Thus crosslinks as well as UV-induced dimers can be repaired in vivo by two different processes, photoreactivation and excision repair.

Dimers that escape excision are present during postirradiation replication and cause gaps in the newly synthesized daughter strands. In this situation a third process, known as postreplication repair can restore the strands to their original molecular weight, free of dimers, by recombination between old and new sister strands (Rupp and Howard-Flanders, 1968; Rupp, et al., 1971).

Like excision repair, postreplication repair was shown to be absent in cells lacking both DNA polymerase I and III (Sedgwick and Bridges, 1974). However, additional enzymes must be involved in strand rejoining, since the process was abolished in E. coli by mutation in the recA gene and was reduced in varying degrees by mutations in the recB, recF, uvrD, and exrA genes (Smith and Meun, 1970; Sedgwick, 1975a; Youngs and Smith, 1976). Recently it was proposed that each of the latter four gene products controls an independent pathway which originates from a recA requiring step, and a fifth pathway, under the common control of the recB, uvrD, and exrA genes was also postulated (Youngs and Smith, 1976). At the present time, only the recBC gene product has been identified (Lieberman and Oishi, 1973).

Witkin (1969) proposed that errors in the process of postreplication repair are responsible for UV-induced mutagenesis. This was based on the fact that exrA E. coli cells yielded no mutants after UV irradiation (Witkin, 1967). More recent evidence suggested that mutagenesis may arise from an error-prone step which can be blocked while recombination continues (Eyfjord, et al., 1975). This process, under control of the recA and exr genes, is thought to be induced by UV irradiation and may compete with the recombination step(s) involved in strand rejoining to fill daughter strand gaps opposite dimers in a highly inaccurate manner (Radman, 1974).

Support for an inducible system was obtained by the isolation of a protein, following UV irradiation, which was lacking in the irradiated recA exr mutant (Sedgwick, 1975b). It was further demonstrated that chloramphenicol treatment prior to irradiation blocked UV-induced mutagenesis but not strand rejoining (Sedgwick, 1975c).

Cole (1973) demonstrated that psoralen-induced crosslinks in E. coli could be removed by a form of repair which required excision followed by recombination. He observed that a uvrA or uvrB requiring step produced single-stranded scissions in cells treated with psoralen. This was followed by a strand-rejoining step involving recombination between sister strands of homologous duplexes, which was absent in a recA mutant. To explain these results he proposed that one arm of a cross-link is subjected to an incision on both sides, creating a gap in one strand and a partially excised crosslinking residue. Recombination between the gap-containing strand and its sister from a homologous duplex restores continuity, while the remaining arm of the crosslink, still attached to the complementary strand, can now be removed by the normal excision repair process. This model extends the findings described earlier (p. 13) which showed that excision repair was required for removal of HN_2 and MC-induced crosslinks.

The crosslink repair model presented by Cole (1973) may, like postreplication repair, have a mutagenic component. The partial excision of a crosslink could be followed by a gap-filling step rather than recombination. This resynthesis would be highly error-prone, since the

remaining arm of the crosslink would prevent that region of the complementary strand from serving as a template. In that case, crosslinks, like dimers would be mutagenic as a result of their reparability. Evidence that repair of crosslinks is mutagenic was obtained by Kondo, et al. (1970), who observed that mutant production due to MC exposure of E. coli dropped ten-fold when uvrA or recA mutants were used. They also found that acriflavine, which blocks excision repair, also suppressed MC mutagenesis but only if applied shortly before the first post-MC DNA replication. They concluded that repair of MC-induced crosslinks via excision and recombination was mutagenic.

A crosslink repair process operating on donor DNA might yield mutants during transformation. Mutagenic events during transformation have been reported. For example, transformation of B. subtilis with heteroduplex DNA yielded an increased mutation frequency among the transformants (Rudner and Remeza, unpublished). In addition, reverse (or back) mutations also increased when competent cells were transformed with homologous DNA (Yoshikawa, 1966). One aim of the study presented here is to establish whether error-promoting repair processes operate to remove HN_2-induced crosslinks during transformation and yield an increase in mutation frequency.

Bifunctional alkylating agents produce more monoalkylations than crosslinks (Kohn, et al., 1966; Brookes and Lawley, 1961b), but only the latter were shown to be excised in vivo while the former were not (Lawley

and Brookes, 1965). Therefore monoalkylations are not excised by the same pathway as crosslinks, but are attacked by another enzyme, present in extracts of Micrococcus lysodeikticus and B. subtilis, which causes single-stranded breaks in methyl methane sulfonate (MMS) treated DNA at or near the methylated sites (Strauss and Robbins, 1968; Strauss, et al., 1968). The breaks were observed after incubating methylated DNA with the extracts or following lysis of MMS treated cells which exposes the DNA to the nuclease. Methylation in vitro of B. subtilis DNA had no effect on its transforming activity. This last result indicates that the single-strand breaks do not occur during methylation or transformation. The breaks were postulated to be the first step in the repair of methylated DNA which is similar to excision repair of UV damage in that it involves nonconservative replication (Reiter and Strauss, 1965). In conclusion, DNA subjected to certain kinds of damage can be repaired by a number of pathways, at least one of which is mutagenic.

III. FATE OF TRANSFORMING DNA:

The events which lead to the formation of a stable transformant have been investigated in many laboratories. By following the fate of labeled donor DNA, evidence for a single-stranded intermediate was found in Pneumococcus (Lacks, 1962; Lacks and Greenberg, 1967; Lacks, et al., 1975) and more recently in B. subtilis (Dubnau and Cirigliano, 1972a). No such intermediate has been detected during transformation of Hemophilus (Notani and

Goodgal, 1966), although recipient Hemophilus cells do contain single-stranded DNA regions (LeClerc and Setlow, 1975). Further support for a single-stranded model of integration in the three most frequently used transforming systems (Pneumococcus, Hemophilus, and B. subtilis) comes from the observation that a single-stranded fragment of donor DNA was covalently bound to the recipient genome (Fox and Allen, 1964; Bodmer and Ganesan, 1964; Notani and Goodgal, 1966; Dubnau and Davidoff-Abelson, 1971). In B. subtilis this donor-recipient complex (DRC) was preceded by a noncovalent association of donor and recipient DNA (Dubnau and Cirigliano, 1973).

Kinetic analyses showed that 30 seconds after DNA addition to competent cells, double-stranded fragments (DSF) of an average molecular weight of 9×10^6 daltons appeared which must have resulted from double-stranded cleavage of incoming molecules. The fragments were followed by the concomitant appearance of single-stranded fragments (SSF), of an average molecular weight of 3 to 5×10^6 daltons and 5' mononucleotides (Davidoff-Abelson and Dubnau, 1973a and b; Dubnau and Cirigliano, 1972a). The mononucleotides probably resulted from the degradation of one of the donor strands, while the remaining strand paired with the host genome to form the DRC, the latter containing a single-stranded donor segment of 1 to 5×10^6 daltons with an average molecular weight of about 2.8×10^6 daltons (Dubnau and Cirigliano, 1973 and 1972c). Sensitivity to pancreatic

DNase and shear indicated that the DSF were still outside the cell permeability barrier and were probably formed within the periplasmic space, while the SSF were no longer DNase sensitive, implying that they had passed through the membrane (Dubnau and Cirigliano, 1972a and b). Passage of the SSF from cell membrane to nuclear body may occur within mesosomes. Electron micrographs revealed the presence of mesosomes which extended from the cytoplasmic membrane to the nuclear bodies and were associated with transforming DNA as it accumulated in the nuclear bodies (Vermuelen and Venema, 1974). Thus much of the processing of transforming DNA seems associated with membrane structures.

The role of the recipient cell membrane in transformation has been studied by examining a vesicle fraction prepared after lysis of B. subtilis (Konings, et al., 1973). The vesicles were capable of transport and contained a cosedimenting endonucleolytic enzyme activity (Joenje, et al., 1974). Joenje and Venema (1975) found this activity in vesicle preparations from both competent and noncompetent cell fractions indicating that it was not required for transformation. In contrast, an exonucleolytic activity, loosely bound to the cell membrane, was only found in vesicle preparations from competent cells. Moreover, EDTA treatment blocked this activity and inhibited transformation. Joenje and Venema (1975)

also demonstrated that the exonucleolytic activity appeared 2-3 minutes after the addition of DNA to a competent cell culture, corresponding with the time when SSF first appear in B. subtilis lysates (Dubnau and Cirigliano, 1973). Thus the exonuclease activity of competent B. subtilis, which is loosely membrane bound, most likely produces the SSF. This differs slightly from the situation found in Pneumococcus, where an endonuclease thought to produce SSF was tightly bound to the cell membrane (Lacks, et al., 1975; Lacks and Neuberger, 1975).

The membrane association of these enzymes indicates that the conversion of SSF occurs as donor DNA penetrates the cell membrane. Since much of the normal processing of transforming DNA occurs outside the cell membrane and in close proximity to it, one may assume that the various DNA repair mechanisms would also operate there (see previous section). The present study addresses itself to two possibilities concerning the repair of HN_2-crosslinked DNA by competent cells;

1) During or prior to membrane penetration, crosslinks in transforming DNA may be removed by a process which breaks the molecules and results in a loss of genetic linkage. Support for such a process comes from the observation that in vitro exposure of transforming DNA to various crosslinking agents namely MC, low pH, nitrous acid or UV irradiation reduced linkage (Iyer and

Szybalski, 1963; Strack, et al., 1964; Combes, 1975).

2) If crosslinks are not removed earlier, they would be present during recombination between donor DNA and the recipient genome. An error-prone repair process operating at this stage may result in an increased mutation frequency among the transformants.

This study examines these two possibilities by measuring the effect of HN_2-induced crosslinks on the *trpC2*-*hisB2* linkage in *B. subtilis* and by observing the production of *trp*$^-$ mutants among transformants. To ensure that only crosslinked molecules were examined, purification of crosslinked-denatured DNA was performed by the use of hydroxyapatite columns. The cotransforming ability (r) of this fraction was compared with noncrosslinked molecules purified from DNA which was renatured following alkali denaturation. The effect of crosslinking on mutation frequency was determined by measuring the frequency of *trp*$^-$ mutants among *trp*$^+$, *his*$^-$ recipients that were transformed to *his*$^+$ with purified, crosslinked *trp*$^+$, *his*$^+$ DNA. The evidence to be presented indicates that:

1) HN_2-crosslinked DNA is corrected during transformation as judged by the drop in cotransforming ability.
2) Modification of HN_2-crosslinked DNA during transformation is not mutagenic.

A model involving cleavage of HN_2-crosslinked DNA during transformation is presented. It is concluded that the correction event probably occurs during membrane penetration.

MATERIALS AND METHODS

I. BACTERIAL STRAINS:

SB-25 (BD-55), a derivative of B. subtilis strain 168, was used as the recipient for transformation. This strain carries the closely linked genes hisB2, trpC2 and was kindly provided by D. Dubnau. The prototrophs W23 ($S^rE^rM^r$) and SB-25 (trp^+, his^+) were used as DNA sources, the latter having been made by transformation of SB-25 (trpC2, hisB2) with wild type DNA from strain W23. Strain H3 (hisB2) was used as the recipient for assays of mutagenesis and was prepared by transforming strain SB-25 (trpC2, hisB2) with W23 DNA.

II. DNA EXTRACTION AND PURIFICATION:

DNA isolation was carried out according to a modification (Rudner, et al., 1967) of the Marmur (1961) method. Cells were grown overnight in veal infusion (Difco) plus 0.5% yeast extract (Difco), washed twice in saline - EDTA (0.15M NaCl, 0.1M EDTA, pH 8.0), and resuspended in saline-EDTA (5.0 ml per gm. wet weight of cells). Lysis was carried out by shaking with 2.0 mg/ml lysozyme (Worthington) at 37°C for approximately 90 minutes in 2.0% SLS (Duponol - Sigma). The suspension was then cooled to 25°C and brought to 1.0M $NaClO_4$. Following deproteinization with chloroform-

isoamyl alcohol (24:1), the upper aqueous phase was mixed with 2 volumes ethanol and the resultant precipitate redissolved in 1.0 ml 0.1xSSC per gm. wet weight of the original cell pellet (SSC equals 0.15M NaCl, 0.015 Na citrate, pH 7) overnight at 4°C. Incubation at 37°C for 30 minutes with RNase A (50 µg/ml) and RNase T1 (50 u/ml) was followed by digestion with pronase (50 µg/ml) at 37°C for 90 minutes with shaking. After an extraction with chloroform-isoamyl alcohol, the aqueous phase was further deproteinized with an equal volume of phenol by shaking for 10 minutes. Traces of phenol were removed by shaking for 20 minutes with a half volume of ether. The bottom aqueous layer was then subjected to further deproteinization with chloroform-isoamyl, ethanol precipatation, and dissolved in 0.1xSSC. All steps from the enzyme treatment to solution in 0.1xSSC were repeated once more and the purified DNA (about 1 mg/ml) was dialyzed against 1.0xSSC.

III. DENATURATION AND RENATURATION:

DNA was diluted to 25 µg per ml in triethanolamine-HCl buffer (0.025M), pH 7.3. Samples were denatured by gentle addition of 1/4 volume NaOH (0.16N), EDTA (0.002M) to yield pH 12.3. After 3 minutes at room temperature without stirring, an equal volume of citric acid (0.2N), Tris (0.06M) was added to neutralize the solution (pH 7.0-7.3). When dilution of the DNA was a critical

factor, 1/8 volume NaOH (0.32),EDTA (0.004M) was neutralized by an equal volume of citric acid (0.4N), Tris (0.12M).

Thermal renaturation of DNA was performed in 2xSSC at 68°C for 4-5 hours at a concentration of about 18 µg DNA/ml (Rudner, et al., 1967). Samples were cooled by plunging in ice.

IV. NITROGEN MUSTARD TREATMENT:

N-methyl-bis (2 chlorethyl) amine HCl (HN_2), a bifunctional nitrogen mustard, donated by Merck, Sharp and Dohme Research Laboratories, was hydrated to 400 µM and used fresh except for one instance where it had been stored for three weeks (a decline in activity was observed after several months storage at -20°C).

Crosslinking was performed according to Kohn and Green (1966). HN_2 was diluted in 0.025M triethanolamine-HCl, pH 7.3 and allowed to stand 30 minutes at 25°C to yield the ethyleneimmonium ion. DNA was then added to yield 25 µg DNA per ml and 10 µM HN_2. The solution was denatured as described above and the reaction terminated by addition of 1/25 volume $Na_2S_2O_3$ (1.0M) which inactivates unreacted alkyl groups. Undenatured samples were treated in an identical manner except that alkali and acid were mixed before being added. Samples to be used for transformation were added directly to recipient cells after being sterilized

with a drop of chloroform.

V. HYDROXYAPATITE PREPARATION:

Hydroxyapatite (HA) was prepared according to the method of Tisilius, et al. (1956) as modified by Miyazawa and Thomas (1965). Four hundred ml H_2O was added to a four liter beaker and two liters each of 0.5M Na_2HPO_4 and 0.5M $CaCl_2$ were simultaneously dropped in by means of a peristaltic pump (Buchler) at a flow rate of 260 ml per hour (the mixture being stirred overnight). The precipitated brushite ($CaHPO_4.2\ H_2O$) was collected by allowing it to sediment and then decanting the supernatant. It was then washed 4 times in 4 liters H_2O and the mixture was boiled for 1 hour with stirring to convert brushite to hydroxyapatite. The crystals were washed 4 times with 4 liters H_2O to remove fines and resuspended in 4 liters 0.2M sodium phosphate buffer, (NaP) pH 6.8. This mixture was brought to a boil with stirring and the fines were decanted. The process was repeated twice more with the length of boiling increased to 5 and then 15 minutes. The HA obtained was stored in an equal volume of 0.001M NaP at 4^oC. Such preparations had earlier been found to be more reliable than commercial preparations (Rudner and Remeza, 1973).

VI. HYDROXYAPATITE CHROMATOGRAPHY:

Columns of 1.6 cm diameter were packed with HA at room temperature to a height in cm which was generally equal to the total A_{260} units of DNA to be applied. If less HA was used, clogging sometimes ensued. This especially happened when renatured samples were applied. After equlibrating the column with the starting buffer, about 10 ml of a DNA sample was applied which had been dialyzed 3x against 2 liters 0.05M potassium phosphate buffer (KP) pH 6.7 for 4, 18 and 3 hours.

Elution was performed by using a linear gradient (0.05-1.0M KP, pH 6.7). The total eluent was 300 ml. A flow rate of 32-35 ml per hour was maintained by a peristaltic pump which was also used to pack the column and apply the sample. At this fairly slow flow rate it was observed that the gradient was often interrupted by a loss of pressure difference between the two buffer reservoirs. This was overcome by using connecting tubing of 1.0mm in diameter so as to keep the flow rate slow enought to maintain the pressure difference.

Generally 4 ml fractions were collected. Absorbance of the fraction was read at 260 nm in the automatic recording spectrophotometer (Gilford) using an automatic transferator (Gilson). Peak fractions to be used in transformations where DNA concentrations was critical were also read individually in 3 ml cuvettes. The point

at which half the moving front of a peak had been eluted was determined as the elution molarity. This was done by calculating the midpoint between the base-line and highest peak fraction.

Fractions used for transformation were sterilized over chloroform and used immediately or stored at 4°C. Under these conditions HA-purified, renatured DNA showed a loss in transforming activity of about 30% after three weeks.

VII. PREPARATION OF FROZEN COMPETENT CELLS:

Competent cells were prepared by a modification (Rudner, et al., 1967) of the two step growth cycle (Anagnostopoulos and Spizizen, 1961). A colony from a Tryptose Blood Agar Base (Difco) plate was grown at 37°C in the roller shaker during the day in 5 ml of antibiotic medium #3 (Difco) supplemented with 50 µg per ml of the required amino acids. One ml of a 1:100 dilution of this day culture was added to 10 ml of supplemented antibiotic medium #3 and grown overnight. The cells were then collected. One hundred ml of medium I was inoculated to a Klett reading of 20-25 units (Klett-Summerson colorimeter, red filter #66). Medium I consists of minimal medium (Anagnostopoulos and Spizizen, 1961) containing 0.5% glucose, 0.02% vitamin free casamino acids (Difco), 0.10% yeast extract (Difco), 0.8% L-arginine, and 100 µg per ml of each amino acid required by the auxotroph.

After incubation with aeration at 37°C for 4-4.5 hours in the metabolyte shaker (New Brunswick), the culture had grown to about 200 Klett units. It was then diluted 1:10 into a liter of medium II consisting of minimal medium plus 0.5% glucose, 0.01% vitamin free casamino acids, 0.05% yeast extract, 2.5×10^{-3} M $MgCl_2$, 1×10^{-3}M $CaCl_2$, 5.0×10^{-4}M spermine tetrahydrochloride and 50 µg per ml of each of the required amino acids. This was divided into four equal portions and incubated at 37°C for 90 minutes with aeration in the NBS Gyrorotary shaker (New Brunswick). At this time the culture had reached maximum competency and generally doubled in Klett units. The culture was prepared for freezing while a small portion was used to assay the transformability of fresh cells. The cells were collected by centrifugation in a Sorvall RC2-B at 7,000 RPM for 5 minutes. They were resuspended to a 20x concentration in the original supernatant containing 10% glycerol, quickly frozen over dry ice-acetone, and stored at -70°F in a Revco deep freeze. The use of a common batch of frozen cells aided in eliminating the variability in transformability often encountered among fresh preparations and allowed for comparisons between experiments. Five batches of cells were prepared in this manner and Table I presents transformation assays obtained during the use of one batch. As shown in Table I, freezing caused a drop in viability by about 50% and competency dropped an additional 50% as compared with fresh cells. No subsequent losses

were observed during the five week period that this batch was used.

VIII. TRANSFORMATION ASSAYS:

Frozen cells were thawed, diluted ten-fold in medium II and 0.9 ml was incubated with 0.1 ml transforming DNA for 30 minutes at 37°C. The process was terminated by addition of 10 µg per ml pancreatic DNase (Worthington) in 0.2M $MgSO_4$ with 10 minutes further incubation. Samples were stored on ice until plated. Plates contained minimal medium (Anagnostopoulos and Spizizen, 1961) 1.5% Bacto Agar (Difco), and 50 µg/ml L-tryptophan and/or 50 µg/ml L-histidine.

IX. DETERMINATION OF COTRANSFORMATION INDEX:

The cotransformation index (r), is a measure of the frequency of the simultaneous transfer of two genetic markers during transformation as compared to the total number of transformants of any type. If donor markers are designated "1" and recipient "0", then r = 11/(10 + 01 + 11) (Nester and Lederberg, 1961; Nester, et al., 1963). In these experiments r was obtained in one of two ways:

a) Direct plating-

Transformants were plated and incubated at 37°C for 48 hours so as to select for the double transformant

class and either single transformant class. Since in this strain the number of *his*$^+$ always equaled the number of *trp*$^+$ transformants, (*i.e.* 01 = 10) it was unnecessary to select for both single classes. The selection method did not distinguish double transformants from among the singles; therefore, a sum of the two single classes would also include twice the number of double transformants. For this reason the doubles had to be subtracted and

$$r = trp^+his^+/[2(trp^+ \text{ or } his^+) - trp^+his^+].$$

In practice, if unsupplemented plates yielded half the colonies of the singly supplemented plates, then r = 0.33.

b) Replica plating-

Transformants were selected for either *trp*$^+$ or *his*$^+$ on plates. After 48 hours at 37°C plates containing 50-150 well spread colonies were replica plated to a selective medium so as to directly determine the fraction of double transformants among the singles. In some instances replica plating was performed by transferring at least 100 colonies to nutrient agar (Difco) with sterile toothpicks. Since this method distinguishes between double and single transformants

$$r = trp^+his^+/[2(trp^+ \text{ or } his^+) + trp^+his^+].$$

X. MUTAGENESIS ASSAY:

Frozen competent H3 cells were transformed with

crosslinked or renatured *trp*$^+$, *his*$^+$ DNA and plated on minimal medium containing 2.5 µg per ml L-tryptophan and 250 µg per ml histidine assay (Difco). On this medium, mutations in the *trpD*, *F* or *C* regions of the *trp* operon which respectively block conversion of anthranilic acid, phosphoribosylanthranlic acid and Amadory compound. (1 (O-carboxyphenylamine) 1-deoxyribulose 5-PO_4) to indoleglycerol 3-PO_4, will yield cells which accumulate these fluorescent intermediates and can thus be detected (Anagnostopoulos and Crawford, 1961; Herriott, 1971; Freese and Strack, 1962). Since fluorescent colonies were rare, samples were generally plated so as to obtain 200-400 colonies/plate. Fluorescent colonies among the transformants were scored as mutants after 40 hours incubation at 37°C by viewing the plates under a UV lamp at 254 nm.

RESULTS AND DISCUSSION

I. CROSSLINKING TREATMENT:

When DNA is crosslinked by HN_2 and then denatured, the molecules will snap back to the double-stranded state upon removal of the denaturing condition. Since single strands do not normally transform (Lerman and Tolmach, 1959), the extent of crosslinking as a function of HN_2 treatment can be followed by measuring the increase in single marker transforming activity after alkali denaturation.

In the present study experiments were first performed to determine the HN_2 dose which would yield the highest transforming activity following transient exposure to alkali. DNA samples were exposed to increasing concentrations of HN_2 for two hours. Following treatment, half of each sample was denatured for three minutes with alkali and neutralized (HN_2-D-DNA), while the other half remained undenatured (HN_2-N-DNA). Transforming activities of the samples were compared to the native sample at zero time. Figure 1 shows that HN_2-N-DNA exhibited a continuous drop in transforming activity as a function of HN_2 concentration and declined to 6.2% after exposure to 50 μM HN_2. The pattern exhibited by HN_2-D-DNA in Figure 1 was quite different. The DNA treated only with alkali retained 1.5% of the original transforming activity which represents the naturally crosslinked fraction. Exposure

to low HN_2 concentrations, followed by alkali treatment, caused the transforming activity to rise to 22% at 10 μM HN_2, representing a fifteen-fold increase. Higher HN_2 concentrations caused a drop in activity and, as Figure 1 shows, this drop paralleled the fall in HN_2-N-DNA activity. If crosslinking of DNA by HN_2 resulted only in protection against denaturation then the transforming activity of HN_2-D-DNA should continue to increase to 100% of native DNA as more molecules are crosslinked. However, evidence to be presented in this study indicates that this is not the case. The presence of a crosslink has a two-fold effect; it causes the transforming ability of a molecule to drop by 50% and it produces denaturation resistance. This loss of activity was demonstrated by HN_2-N-DNA as shown in Figure 1 (also see Fig. 2) and as a result HN_2-D-DNA activity would not reach 100%.

Another kinetic experiment using the optimal concentration of 10 μM HN_2 was then done to find the maximum transforming activity produced as a function of HN_2 exposure time. Figure 2 shows that with increasing time, HN_2-D-DNA and HN_2-N-DNA samples again exhibited opposite responses. HN_2-D-DNA samples showed an initial value of 1.6% which reached 33% by 100 minutes where the rise in transforming activity began leveling off. The activity of the HN_2-N-DNA samples showed a continual drop to 64% of the original acitivity at the end of treatment. In another experiment, HN_2 exposure was continued

for three hours. This is presented in Figure 3 where the activities of HN_2-D-DNA and HN_2-N-DNA showed little change after 100 minutes. Treatment with 10 μM HN_2 for 100 minutes was therefore established as the lowest dose which yields the highest HN_2-D-DNA transforming activities. Unless otherwise noted, all HN_2-DNA samples were obtained by this treatment which generally inactivated HN_2-N-DNA samples by about 50% and yielded HN_2-D-DNA samples with approximately 25% of the activity of native DNA.

II. HYDROXYAPATITE CHROMATOGRAPHY OF CROSSLINKED AND NON-CROSSLINKED DNA:

To show that the increased transforming activity of HN_2-D-DNA was due to a denaturation-resistant fraction resulting from HN_2 treatment, a physical parameter was required to identify this double-stranded fraction. In addition, a purification process was needed which would yield homogenous populations of bihelical molecules from samples containing single and double-stranded DNA. These purified molecules were subsequently used to study the effects of crosslinking on transforming DNA. Since all HN_2-D-DNA samples were denatured and allowed to spontaneously renature, thermally renatured DNA, as well as native samples were used as controls. Use of such renatured DNA controls could allow for a distinction between effects due to the presence of crosslinks and those due to strand unwinding and rewinding.

Hydroxyapatite (HA) chromatography can resolve DNA molecules according to their secondary structure and was chosen as the method of purification (Bernardi, 1969a and b; Bernardi and Chevallier, 1968). In the present study native, denatured, HN_2-D-DNA and renatured DNA samples were fractionated on HA columns and assayed for transforming activity. The results illustrated in Figures 4-7 showed that these samples yielded a fraction (peak 1) which generally eluted at about 0.17M potassium phosphate buffer pH 6.7 (KP) and was lacking in transforming activity. The elution profiles revealed a second fraction (peak 2) eluting between 0.23-0.26M KP, which exhibited transforming activity. Under similar conditions, Bernardi (1969b) observed that peak 1 of denatured bacterial DNA eluted at 0.15M KP, while peak 2 eluted at 0.20-0.22M KP. These slight differences arise from the variation among batches of HA (Rudner and Remeza, 1973). Figure 4 shows that when native DNA was chromatographed, practically all the DNA (92%) was recovered in peak 2 (see also Table 2).

Figure 5 represents an HA chromatograph of denatured DNA which showed that 10% of the recovered material was found in peak 2 (see also Table 2) and that it contained the residual transforming activity normally exhibited by denatured DNA (see Introduction). Peak 1 of this HA-eluted DNA exhibited a low level of activity - about 15 times background levels - which may be due to saturation of

an extracellular exonuclease at this high DNA concentration (3.5 μg/ml) thereby allowing some single strands to transform. This exonuclease degrades single-stranded DNA and was observed to be saturated by DNA concentrations above 1.0 μg/ml (Tevethia and Mandel, 1970). Treatment of DNA with HN_2 prior to denaturation resulted in an approximate four-fold increase in the amount recovered in peak 2 as shown in Figure 6 (compare with Figure 5; also see Table 2). Since a corresponding increase in transforming activity was observed, it is clear that the HN_2-cross-linked molecules do transform. These results show that the HN_2 treatment converted a portion of the DNA to a denaturation-resistant form. When denatured DNA was thermally renatured and chromatographed, a large fraction of the recovered material eluted in peak 2. This is seen in Figure 7 which shows an approximate seven-fold increase in the amount recovered in peak 2 compared to that of denatured DNA. A comparison with Figure 6 shows that thermal renaturation produced more double-stranded molecules than did the HN_2 treatment used in these studies. Renatured DNA was the most difficult to chromatograph and yielded the poorest recoveries (45-83%). After applying the DNA to the column, an apparent packing down of the HA crystals resulted in a decreased column height and a concomitant block in eluent flow. This problem was overcome by using about 25% more HA when chromatographing

renatured DNA. Some packing was also observed for native samples but it was not great enough to block the flow of the eluent. Difficulties in chromatographing renatured DNA may have been due to the formation of high molecular weight aggregates during renaturation by base pairing of single strand "tails". High molecular weight DNA (2.5×10^7 daltons) is known to cause clogging of HA columns (Pakroppa and Muller, 1974). Presumably the large molecules bind to many HA crystals and pack them down as they move through the column.

The results in this section showed that HN_2 treatment prior to alkali exposure increased the denaturation-resistant fraction to a level (40%) which was substantially less than 100%. Since transforming activities were seen to level off in Figures 2 and 3, crosslinking should have been maximal - i.e. the crosslinked fraction should have reached 100%. It is possible that the HN_2 preparations used were unable to produce 100% denaturation resistance because they had lost crosslinking activity during further incubation. It should be noted that exposure to 10 μM HN_2 for 2 hours was shown by others to result in almost 90% denaturation resistance (Kohn, et al., 1966). Given different DNA preparations containing an equivalent number of crosslinks per molecule, the fraction of denaturation-resistant material produced will be a function of the number of nicks per molecule. DNA isolated in this laboratory was shown to contain an average of one

nick per strand (Galloway, 1976) and would require at least twice as many crosslinking hits per molecule to produce the same denaturation resistance as a preparation of molecules with no nicks. Since an almost identical HN_2 treatment provided Kohn, et al., (1966) with approximately twice the denaturation resistance obtained in the present study, it is possible that the DNA preparation used by them contained less than one nick per strand.

III. SPECIFIC TRANSFORMING ACTIVITY OF CROSSLINKED DNA:

Once HA profiles had been assayed for transforming activity, fractions yielding the highest specific activity were saved for further studies. Transforming activities of these fractions are presented in Table 2. The results in Table 2 show that purified HN_2-D-DNA transformed 43% as well as native, while naturally crosslinked and purified renatured molecules exhibited activities of 40% and 29% respectively. Thus, although thermal renaturation restored more molecules to a double-stranded form than did HN_2 exposure, they transformed poorly. It was already suggested (p. 38) that renatured molecules were partially single-stranded. Such molecules would be expected to produce low activities.

A crosslink can provide an effective nucleus for zippering up unwound strands, therefore crosslinked molecules should be nearly perfect in their secondary structure and yield high activities. Since this was not

observed, it is possible that transforming activity was affected by the presence of crosslinks. A model describing how recipient cells may utilize crosslinked DNA during transformation is presented in Section VII. The model predicts that each crosslinking hit (natural or chemical) reduces activity by 50%. As will be seen, the value derived from the model for HN_2-D-DNA is in good agreement with the activity presented in Table 2.

The finding that the specific transforming activity of HN_2-D-DNA was less than that of native is not in agreement with the report of Kohn and Green (1966). Rather than directly measure the activity of purified molecules, they compared the specific transforming activity of unpurified HN_2-D-DNA with the yield of double-stranded DNA obtained by density gradient centrifugation. They observed that the specific activity closely matched the percentage of double-stranded molecules which survived denaturation as a result of HN_2 treatment. This correlation led them to conclude that HN_2-crosslinked molecules were as active as native DNA. However in other experiments specific activity was only high following correction, by as much as 50%, for the HN_2 inactivation of HN_2-N-DNA. An explanation for the inactivation was never provided, but without correction, values dropped to 50% or close to that obtained in the present study.

The specific transforming activity of 40% obtained for naturally crosslinked DNA is in agreement with that

observed after purification by MAK and HA chromatography of 25 and 43% respectively (Rudner and Remeza 1973; Chevallier and Bernardi 1968). It has also been claimed that naturally crosslinked DNA purified by either density gradient centrifugation or dextran-glycol phase partition could transform like native DNA (Mulder and Doty, 1968; Alberts and Doty, 1968).

IV. COTRANSFORMATION INDEX OF HN_2 CROSSLINKED DNA:

To determine if the presence of crosslinks resulted in a disruption of the normal continuity of donor DNA, crosslinked and noncrosslinked DNA fractions, purified by HA chromatography, were compared as to their ability to cotransform linked markers at various DNA concentrations. Recipients bearing the trpC2 and hisB2 markers were used because these two genes are cotransformed with a high frequency of about 50% (Nester and Lederberg, 1961; Nester, et al., 1963; Anagnostopoulos and Crawford, 1961; Carlton, 1967). Assays of the cotransformation index (r) were performed either by replica plating or plating directly on selective media (see Materials and Methods for computation of r). To test the effect of alkali exposure, the r of DNA which was alkali denatured and thermally renatured was also assayed. Table 3 shows the results of three different experiments where native, renatured and HN_2-D-DNA were compared with respect

to r. In all instances the data showed that r values of HN_2-D-DNA samples were substantially lower than those of native or renatured DNA. This was true whether r was measured by direct plating or replica plating. When all r values were averaged, they yielded the following: native = 0.36, renatured = 0.31, and crosslinked = 0.21. In the case of renatured DNA this represents a decrease to 86% of native, while HN_2-D-DNA exhibited a greater decline to 58% of native DNA. The slight drop in renatured DNA r values as compared to those of HN_2-D-DNA rules out the possibility that the latter resulted from strand unwinding and rewinding. In all three experiments HN_2-D-DNA showed a higher transforming activity for individual markers which was greater than the activity of renatured samples. Therefore, although crosslinked molecules can transform single markers better than renatured molecules, they cannot cotransform as well.

At DNA concentrations approaching saturation, it is possible for double transformants to arise from the random coincidence of two separate transforming molecules. This phenomenon, known as congression could produce spurious linkage values (Anagnostopoulos and Crawford, 1961; Nester, et al., 1963). However, Nester, et al., (1963) demonstrated that within the linear response to donor DNA concentration, congression occurred at levels which were too low to influence linkage estimation. In addition, using a mixture of DNA from two strains, (trp^+ his^-,

trp$^-$ his$^+$) Rudner (unpublished) observed that at donor concentrations as high as 13.0 µg DNA/ ml, congression accounted for no more than 0.5% of the total yield of double transformants. Since the present study measured linkage at donor concentrations never exceeding 1.5 µg DNA/ml, the effect of congression was ruled out.

It was of interest to determine whether differences in r values occur along the HA profile. Chevallier and Bernardi (1968) observed that transforming activity and the absorbance (A_{260}) profile did not perfectly coincide. Instead the transforming activity peak often followed the optical density peak by one or two fractions. They interpreted this to mean that later-eluting molecules have a greater bihelical configuration thereby yielding a higher transforming activity. Displacement of activity with respect to A_{260} profile was occasionally observed in the present study (Figure 6) and could cause r to vary among the fractions comprising peak 2. An additional experiment was therefore performed in which individual fractions from peak 2 of renatured and HN_2-D-DNA chromatographs were assayed at two concentrations. The results, presented in Table 4, illustrate that late-eluting fractions did exhibit somewhat higher specific activities but r did not change. It appears that the slight differences in secondary structure which resulted from HA fractionation did not affect r.

The reliability of the two methods used to measure r

was tested by parallel assays (6 times) from the same fraction. These data are presented in Table 5 where the means and standard deviations of the two methods show that they are equivalent. Comparison with the data in Table 3 shows that the variation among the assays performed on fraction 19 was less than the difference between r of renatured (0.31) and HN_2-D-DNA (0.21). It is concluded that the observed differences between renatured and HN_2-D-DNA samples were not due to sampling error.

The data presented in Tables 3 and 4 were obtained from assays carried out at 0.1 µg DNA/ml or greater. It has been demonstrated in both Pneumococcus and B. subtilis that for concentrations up to 0.1-0.2 µg DNA/ml the number of transformants increased proportionately, while at higher concentrations the response curve leveled, indicating that recipients were becoming saturated (Hotchkiss, 1956; Anagnostopoulos and Spizizen, 1961). However in the present study the linear dose response continued into higher DNA concentrations (0.1-0.9 µg DNA/ml, see Table 3). The earlier studies used freshly prepared competent cells, whereas frozen cells were used here. The dose response of both types of cell preparations was therefore investigated. The result is summarized in Table 6 and illustrated in Figure 8. As expected, a break in the response curve of fresh cells occurred at 0.1-0.2 µg DNA/ml. When frozen cells were used however, linearity was obtained up to 1.0-2.5 µg DNA/ml. In

addition, at concentrations where both cell preparations exhibited a linear response, frozen cells transformed one-tenth as well as fresh cells and required ten times more DNA to approach saturation. Linkage values were assessed with frozen cells using more dilute DNA solutions, so that saturating conditions were never used. It was also observed that competency was more sensitive to freezing than viability (see Table 1). This may be due to a combination of effects such as the formation of abortive receptor sites and/or inhibition of cell division by competent cells as a result of surface damage due to freezing and thawing. Tomasz (1969) suggested that inhibition of cell division could explain the loss of transformability in cells extensively treated with lysozyme. Mesosomes communicating with the nuclear body serve for the uptake and transport of transforming DNA in B. subtilis (Vermuelen and Venema, 1974) and disruption of mesosome integrity could yield binding sites incapable of transporting donor DNA to the cell interior.

In summary, crosslinking of DNA results in molecules which exhibit a marked decrease in the ability to cotransform linked markers even though single transformants occur at a higher frequency than in renatured controls. This observation is :

1. Independent of the transforming DNA concentrations used in this study.

2. Independent of the methods used to measure r.
3. Independent of elution position within peak 2 of HA chromatographs.

V. DECREASE IN COTRANSFORMATION INDEX:

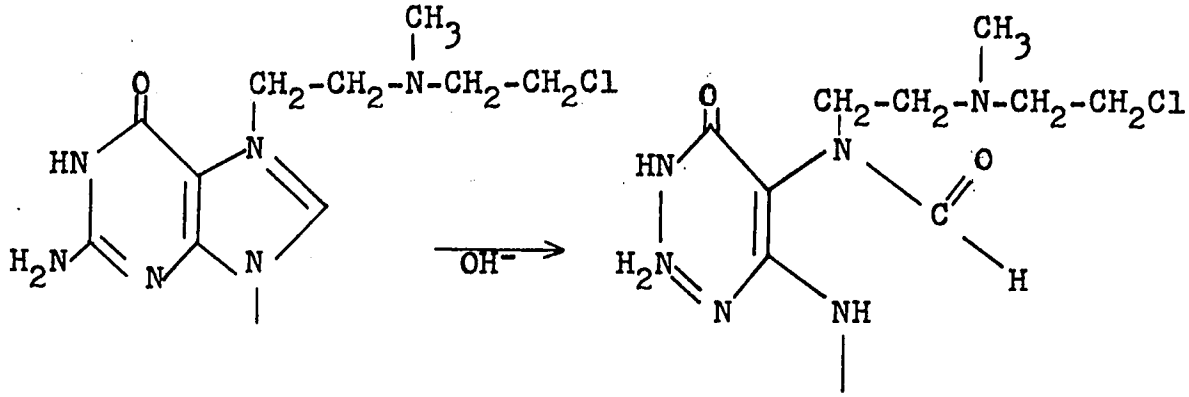
Treatment with HN_2 yields monoalkylated residues as well as crosslinks (Brookes and Lawley, 1961a and b; Kohn, et al., 1966). Alkylated residues can undergo depurination followed by strand scission (Brookes and Lawley, 1961b; Laurence, 1963). However crosslinks may also interfere with the normal events of transformation. Either product could cause r to drop, but a distinction between the two can be made by comparing the response of native DNA to HN_2 exposure (HN_2-N-DNA) with that exhibited by DNA which is denatured following HN_2 treatment (HN_2-D-DNA). Both preparations should be monoalkylated to the same extent, but unequal as to the percent of transforming molecules containing crosslinks. HN_2-N-DNA is a mixture of crosslinked and noncrosslinked transforming molecules, while HN_2-D-DNA is comprised of only crosslinked transforming molecules which survive denaturation and single strands which are unable to transform. Denaturation therefore serves to purify crosslinked molecules for transformation studies. This being the case, native and denatured samples should exhibit equivalent losses in r as a function of HN_2 exposure if r is decreased as a result of monoalkylation. Alternatively, a more pro-

nounced response by HN_2-D-DNA would indicate that crosslinks decrease r and that this effect results from something other than depurination. Time course experiments were performed to distinguish between the effects of monoalkylation and crosslinking. Samples were removed at various times and r was measured in addition to single marker transforming activity. Figure 9 presents the latter results as the means of three independent experiments plus the standard deviations for each point and is similar to the results reported earlier in Figure 2. Additional information is presented in Table 7 and Figure 10. As shown, after a 100 minute treatment, the r of HN_2-D-DNA dropped to an average value of 0.28, while that of HN_2-D-DNA was 0.19. These represent a decrease to 82% and 56% for the r of HN_2-N-DNA and HN_2-D-DNA respectively. The latter agrees well with the value of 58% observed for HA-purified HN_2-D-DNA (Table 3) and is expected if renaturation acts to selectively enrich for crosslinked molecules capable of transformation. Another manifestation of the purification effect can be seen at short reaction times where the r value of HN_2-D-DNA appeared to drop more rapidly than that of HN_2-N-DNA. This is also expected, since at the onset of the reaction few HN_2-N-DNA molecules will be crosslinked, but only crosslinked molecules will comprise the HN_2-D-DNA sample. Figure 10 illustrates that increased exposure to HN_2 caused the r of both HN_2-N-DNA and HN_2-D-DNA to drop, but the decline exhibited by HN_2-D-DNA was greater than that of HN_2-N-DNA. These

results support the notion that the presence of crosslinks, not scissions resulting from depurination, is responsible for the decrease in r; however, they do not rule out the possibility that the latter may contribute to this decrease.

Although the foregoing indicates that crosslinks produce a linkage loss, it is possible that the greater loss in r exhibited by HN_2-D-DNA in Figure 10 was due to denaturation per se. If this were the case, the r of HN_2-D-DNA would have been less than HN_2-N-DNA by a constant amount throughout the period of treatment. This is contrary to the observation that the r of HN_2-D-DNA exhibited a decrease which did not parallel the loss shown by HN_2-N-DNA in Figure 10. Moreover, noncrosslinked samples which were denatured (D-O) would have exhibited lower r values than undenatured samples (N-O). This was not the case as shown by the data in Table 7 where r of the D-O samples was essentially equal to r of the N-O samples. Alkali treatment following depurination can create scissions (Kohn and Spears, 1967a); however, the rate of depurination at 37°C is exceedingly small with a half-life of about 52 hours (Brookes and Lawley, 1961b). In the experiments described in Table 7, crosslinking was performed at 25°C and the time between the first alkylation and alkali exposure was never more than 100 minutes. It is therefore unlikely that r dropped due to the appearance of alkali-labile sites. Rather than decrease r, alkali denaturation probably stabilized

monoalkylations and crosslinks against possible strand scission. As illustrated below, alkali exposure breaks the imidazole ring of 7-alkylguanine moieties, preventing possible depurination (Brookes and Lawley, 1961c; Lawley and Brookes, 1967; Kohn and Spears, 1967b). Stabilization of alkylated residues by rupture of the purine ring at high pH makes them resistant to depurination-induced scissions and further strengthens the argument that crosslinks alone caused r to drop.



VI. MUTAGENICITY OF CROSSLINKED TRANSFORMING DNA:

It is known that crosslinks can be repaired by processes similar to those which repair UV damage and that at least one repair process is thought to be mutagenic (see Introduction). Crosslinks could therefore be mutagenic by virtue of their reparability during transformation. Using a technique developed by Anagnostopoulos and Crawford (1961), experiments were performed to determine whether crosslinks are mutagenic. The technique makes use of two factors:

1. The hisB2 locus is closely linked to the six genes shown below which comprise the tryptophan operon in B. subtilis (Anagnostopoulos and Crawford, 1961; Nester, et al., 1963; Carlton, 1967; Carlton and Whitt,1969).

   trpE trpD trpC trpF trpB trpA hisB

2. The accumulation of fluorescent intermediates by certain trp mutants allows the mutants to be readily detected among his^+ transformants.

Prototrophic (trp^+his^+) DNA was used to transform hisB2 recipients and the his^+ transformants were analyzed for the appearance of forward trpC, trpD or trpF mutants. These auxotrophs accumulate 1-carboxyphenyl-amino-deoxyribulose-PO_4, anthranilic acid and phosphoribosyl anthranilic acid respectively and fluoresce under UV light. By initially selecting for his^+ transformants it was possible to eliminate all those cells which did not incorporate donor DNA at a site linked to the trp region. Adding a suboptimal amount of tryptophan to the selective plates allowed the desired trp mutants to grow slowly and accumulate fluorescent intermediates. Since the hisB2 gene is about 50% linked to the trp region ($r = 0.33$) when using native DNA, about half of the newly originated trp mutants can be recovered among the transformants (Anagnostopolous and Crawford 1961; also see Table 3 and 7). In order to focus on the effect of crosslinks and eliminate any effects of strand unwinding and rewinding, HN_2-D-DNA was tested with renatured DNA as

a control. HN_2-D-DNA cotransformed trpC2 and hisB2 only 68% as often as renatured DNA did (see r values in Table 3), therefore any comparison of the mutation frequency of these two DNA samples must take this difference into account by multiplying the mutation frequency of HN_2-D-DNA by 1.00/0.68 or 1.47.

Table 8 summarizes the results of four such separate experiments. Taken all together, the results yielded a mutation frequency of 4.17×10^{-5} and 2.86×10^{-5} for cross-linked and renatured DNA respectively, A spontaneous frequency as high as $1.0\text{-}2.0 \times 10^{-4}$ was reported for native DNA when the more closely linked trpA locus was used instead of hisB (Freese and Strack, 1962; Bresler, et al., 1968). This higher value would also result from the higher r exhibited by native DNA (Table 3). After correcting for differences in the r values of HN_2-D-DNA and renatured DNA, the mutation frequency of HN_2-D-DNA was higher than that of renatured DNA by a factor of 2.2 (see Table 8). This is smaller than the variation observed for the same DNA sample assayed at different times as in the case of sample D in Table 8. Therefore, the 2.2-fold increase is not considered significant. Moreover, other instances of mutant production during transformation have yielded much greater increases of from ten to thirty-fold (Yoshikawa, 1966; Rudner and Remeza, unpublished). In addition, other authors who studied the action of various mutagenic agents on trans-

forming DNA by measuring the induction of fluorescent mutants have observed increases of ten-fold or more (Freese and Strack, 1962; Strack, et al., 1964; Bresler, et al., 1968). These results indicate that the modification of HN_2-crosslinked DNA which caused r to drop was not mutagenic. The fact that HN_2-crosslinked DNA did not induce mutants during transformation indicates that the process which reduced r does not involve an error-prone type of repair.

VII. POSSIBLE OCCURRENCE OF CROSSLINK-INDUCED SCISSIONS DURING TRANSFORMATION:

A reduction in r following HN_2-crosslinking could result from a scission at or near the crosslinked site during transformation. There are two ways this could occur.

1. Mechanical breaks during transformation caused by the presence of crosslinks.
2. Scissions resulting from the activity of an endonuclease which creates breaks at crosslinked regions during transformation.

The first possibility assumes that a crosslink can act as a barrier to uptake of a linear molecule beginning at one end. This could cause tension-induced breaks as the proximal region of the molecule is drawn into a competent cell. Tension exerted on a molecule during

simultaneous uptake by a recipient at each end was postulated to reduce the linkage of distant markers in a very high molecular weight DNA preparation (Kelly, 1967). It is possible that similar shearing forces could also be generated along short DNA stretches so as to cause breaks during uptake of a crosslinked DNA molecule. The second possibility, requiring enzymatic activity to yield breaks, is presented as a model along with the normal events of transformation. Figure 11-I depicts the processing of native B. subtilis DNA during transformation and illustrates how an endonuclease may alter the final product when crosslinked DNA is involved. As shown, the sequence begins with the first intermediate in the process, the DSF, formed by endonucleolytic cleavage of both strands of a native molecule as it enters the periplasmic space (Dubnau and Cirigliano, 1972a). Figure 11-I-A illustrates the onset of the exonucleolytic degradation of the DSF with the release of 5' monophosphates as it penetrates the cell membrane (Dubnau and Cirigliano, 1972a and b). Digestion is assumed to begin only at the 3' OH end of a strand. Degradation of this strand continues while the other strand - now an SSF - enters the cytoplasm as shown in Figure 11-I-B and I-C. If the DSF is crosslinked, as illustrated in Figure 11-II, progressive single-stranded degradation would be blocked unless the crosslink was

removed. Removal could arise from the activity of an endonuclease able to produce either single (II-B) or double-stranded (II-B') breaks at or near the crosslinked sites by recognizing helix distortions due to crosslinks. Either type of cleavage would reduce r when a crosslink is located between the trpC2 and hisB2 markers as illustrated in II-C and II-C'. The extent of linkage loss would be a function of the fraction of molecules containing one or more crosslinks between the two markers. Crosslinks outside the trpC2-hisB2 region would not affect r but would reduce the specific activity of individual markers. Since uptake from either end of donor molecules is equally likely, single marker activity should decrease by 50% per crosslink if each crosslinked region is cleaved and distal fragments can no longer transform.

The crosslink-induced scissions could occur at one or another stage of the transformation process depending upon the location of the postulated endonuclease. If the enzyme were membrane bound, scissions could occur as a DSF is converted to an SSF. The sequence in Figure 11-II illustrates this possibility. An extra-membranal site of action could yield scissions at an earlier stage, perhaps during DSF production, which is known to involve double-stranded cleavages (Dubnau and Cirigliano, 1972a). A B. subtilis endonuclease located outside the membrane has been isolated and may be responsible for DSF pro-

duction (Dubnau and Scher, 1976). It would be of interest to determine whether this newly characterized enzyme can use crosslinked DNA as a substrate *in vitro*. Another endonuclease which incises one DNA strand on the 5' side of thymine dimers has been purified from *E. coli* (Braun and Grossman, 1974a and b). A similar endonucleolytic activity in *B. subtilis* could attack crosslinks and represent the first step in repair via recombination between homologous duplexes as postulated by Cole (1973). This being the case, a decrease in r may simply arise from failure to complete the repair process before SSF formation occurs.

Since natural crosslinks occur only at one end of a molecule (Alberts, 1968), cleavage of these regions should not disrupt the *trp-his* linkage. This was illustrated by the high r values obtained for the zero time HN_2-D-DNA samples (Table 7) where the residual activity was derived from the naturally crosslinked fraction. On the other hand, single marker activity of naturally crosslinked DNA should drop to 50% of that exhibited by native DNA because a polar cleavage should prevent integration of the entire molecule 50% of the time, depending upon which end presents itself to the recipient cell. The data in Table 2 showed that the activity of naturally crosslinked molecules did not exceed 50% and is consistent with the model. Thus, a natural crosslink should affect transforming activity to the same extent

as an internal HN_2-induced crosslink, while only the latter is expected to reduce r. Assuming that HN_2-induced crosslinks occur randomly along the helix, the reduction in r per crosslinking hit will depend upon the size of the trpC2-hisB2 segment with respect to the entire molecule. This segment encompasses five cistrons (see p.50) Taking the average cistron as 1000 nucleotides or 3.3×10^5 daltons, we have a minimal single-stranded molecular weight of about 1.7×10^6 daltons for the entire segment. This is about half the single-stranded molecular weight (3.3×10^6 daltons) of DNA isolated in this laboratory (Galloway, 1976) and means that each HN_2 crosslink, on the average, is expected to reduce r by 50%.

If DNA is crosslinked randomly by HN_2, the number of crosslinks per molecule should follow a Poisson distribution. The distribution, based on an exposure to 10 μM HN_2 for 100 minutes, was determined as shown in the Appendix. The predicted r and transforming activity of crosslinked DNA were calculated keeping in mind the following two conclusions:

1. Each HN_2 crosslink decreases r and single marker activity by 50%.
2. Each natural crosslink reduces single marker activity by 50%.

The predictions were compared with the experimental values and, except for HN_2-N-DNA activity, they were

in good agreement (see Table 9). The unexpected low level of HN_2-N-DNA activity may reflect the fact that this DNA was not exposed to alkali treatment which, as stated earlier (p. 49), probably stabilized alkylated residues in HN_2-D-DNA against possible strand scission. On the whole, the comparison in Table 9 is consistent with the notion that competent cells cleave crosslinks during transformation and accounts for the transforming behavior of HN_2-crosslinked DNA.

CONCLUDING REMARKS

The major findings of this study were:

1. HN_2-crosslinking of transforming DNA caused a reduction in r.
2. The specific transforming activity of crosslinked molecules was substantially less than that of native DNA.
3. Introducing crosslinks into transforming DNA did not increase its mutagenicity.

Treatment of transforming DNA with other crosslinking agents such as MC, low pH and nitrous acid also caused a drop in linkage (Iyer and Szybalski, 1963; Strack, et al., 1964); however, no correlation between linkage loss and degree of crosslinking was attempted in these studies. More recently it was reported that UV irradiation lowered r without a corresponding reduction in molecular weight of the irradiated DNA (Combes, 1975). These results support the conclusion of the present study which is that a reduction in cotransformation of linked markers results from the presence of crosslinks. Enzymatic cleavage of crosslinked regions provides a means of effectively removing crosslinks. Whether such a process could occur as shown in Figure 11 has yet to be determined. One way to investigate the possibility that recipients can enzymatically cleave crosslinked DNA is to use B. subtilis mutants defective in repair

functions as recipients and test their ability to lower r. Of particular interest are UV sensitive strains unable to perform excision repair, such as the host cell reactivation mutants (hcr) isolated by Okubo and Romig (1965). If a reduction in r is a manifestation of an aborted excision repair process then these mutants should be unable to decrease the r of donor HN_2-D-DNA. Similar tests could be attempted with another class of UV sensitive mutants (rec) defective in recombination, (Okubo and Romig, 1966; Hoch, et al., 1967; Dubnau, et al., 1973; Sadaie and Kada, 1976) however, the deficiency in transformation usually exhibited by these mutants could make this impractical. Nevertheless, the finding that a rec recipient exhibits no decrease in r would provide indirect evidence that enzymatic cleavage does occur. Direct physical evidence for the presence of a soluble endonuclease located in the pericellular space could come from in vitro examination of the supernatant fraction collected from competent cells following protoplast formation. In addition, membrane vesicles prepared from competent cells according to the method of Konigs, et al. (1973) could be tested for the presence of a membrane bound activity. The supernatant fraction and the vesicle fraction could be assayed for nuclease activity using HN_2-D-DNA and MMS-treated DNA as substrates. Any resultant decrease in

molecular weight could be measured in alkaline sucrose. The isolation of a fraction which reduces the molecular weight of HN_2-D-DNA without similarly affecting MMS-treated DNA would provide direct evidence that crosslinks are enzymatically cleaved. Additional physical evidence to support the crosslink cleavage model might be obtained by molecular weight determinations of SSF produced during transformation with isotopically labeled donor DNA which has been crosslinked in vitro. These SSF are expected to be of a lower molecular weight than those produced when noncrosslinked (native) donor DNA is used. Finally, a search for the presence of the putative crosslink-specific endonuclease in noncompetent cells could reveal whether such an enzyme is always present to repair endogenous DNA or is only found in competent cells where it acts specifically on exogenous (donor) DNA.

APPENDIX

Determination of Cotransformation Index (r) and Specific Transforming Activity of Crosslinked DNA

The fraction of DNA that is crosslinked by HN_2 alone can be determined by subtracting the naturally crosslinked fraction from the total amount of denaturation-resistant DNA found after HN_2 treatment. The latter was observed to be 41.5% in Table 2 (average of two experiments), while the naturally crosslinked fraction was found to be 8.5% (average of three experiments):

$$41.5\% - 8.5\% = 33\%$$

$$33\%/(100 - 8.5\%) = 36\%$$

or, 36% of the DNA which was not previously crosslinked became denaturation-resistant following HN_2 treatment. In addition, 36% of the naturally crosslinked fraction was also HN_2-crosslinked:

$$8.5\% \times 36\% = 3.1$$

$$36\% + 3.1\% = 39.1\% \text{ crosslinked by } HN_2.$$

If HN_2 crosslinks are randomly distributed, then from the Poisson distribution:

$$P(k) = \frac{e^{-m} m^k}{k!}$$

where k = number of crosslinking "hits",

m = multiplicity.

$$P(0) = 0.609 = e^{-m}$$

so: $$m = -\ln 0.609 = 0.496$$

and $P(1) = 0.302$, $P(2) = 0.075$ $P(3) = 0.012$.

Cotransformation Index of HN_2-N-DNA

The trp-his region is about 50% of the average total fragment size (p. 56) so each HN_2 crosslink reduces r by 50% on the average. Therefore a second crosslink would inactivate half of the remaining r yielding a total drop of 0.75, while a third crosslink would result in a 0.88 total decrease. Terminally positioned natural crosslinks are not expected to lower r. The predicted drop in r of HN_2-N-DNA may be calculated:

HN_2 crosslink distribution among fraction not naturally crosslinked	% decrease in r
$P(0) = 0.608 \times 91.5\% = 55.6\%$	$55.6\% \times 0.00 = 0$
$P(1) = 0.302 \times 91.5\% = 27.6\%$	$27.6\% \times 0.50 = 13.8\%$
$P(2) = 0.075 \times 91.5\% = 6.9\%$	$6.9\% \times 0.75 = 5.2\%$
$P(3) = 0.012 \times 91.5\% = 1.1\%$	$1.1\% \times 0.88 = 1.0\%$

HN_2 crosslink distribution among naturally crosslinked fraction	
$P(0) = 0.608 \times 8.5\% = 5.17\%$	$5.17\% \times 0.00 = 0$
$P(1) = 0.302 \times 8.5\% = 2.57\%$	$2.57\% \times 0.50 = 1.29\%$
$P(2) = 0.075 \times 8.5\% = 0.64\%$	$0.64\% \times 0.75 = 0.48\%$
$P(3) = 0.012 \times 8.5\% = 0.10\%$	$0.10\% \times 0.88 = 0.09\%$
	21.9% drop

or r of HN_2-N-DNA = 78.1% of native DNA.

Cotransformation index of HN_2-D-DNA

The noncrosslinked fraction does not survive denaturation and cannot transform. Therefore the predicted loss in r of HN_2-D-DNA equals the drop calculated for HN_2-N-DNA divided by the fraction surviving denaturation:

21.9/41.5 = 52.8% drop

or r of HN_2-D-DNA = 47.2% of native DNA.

Specific Transforming Activity

If the uptake of donor molecules is not polar (i.e. either end can penetrate) then each crosslink (natural or HN_2), on the average, yields a 50% loss in specific transforming activity.

HN_2-N-DNA

Crosslink distribution among fraction not naturally crosslinked	% decrease in activity
P(0) = 55.6%	55.6% x 0.00 = 0
P(1) = 27.6%	27.6% x 0.50 = 13.8%
P(2) = 6.9%	6.9% x 0.75 = 5.1%
P(3) = 1.0%	1.0% x 0.88 = 0.9%
Crosslink distribution among naturally crosslinked fraction	
P(1) = 5.17%	5.17% x 0.50 = 2.58%
P(2) = 2.57%	2.57% x 0.75 = 1.92%
P(3) = 0.64%	0.64% x 0.88 = 0.55%
P(4) = 0.10%	0.10% x 0.94 = 0.09%
	24.94% drop

or specific activity of HN_2-N-DNA = 75.1% of native DNA.

HN_2-D-DNA

The predicted specific activity of HN_2-D-DNA equals the activity calculated for HN_2-N-DNA less the noncrosslinked fraction which is denatured and can no longer transform. Since 41.5% of the HN_2 treated DNA was denaturation-resistant, the noncrosslinked fraction equals 58.5%

75.1% - 58.5% = 16.6%.

HN_2-D-DNA (purified)

The predicted drop in specific activity of HN_2-D-DNA purified by HA chromatography can be determined as follows:

(predicted drop for HN_2-N-DNA)/(denaturation-resistant fraction)

24.9%/41.5% = 60.0%

or specific activity of HN_2-D-DNA (purified) = 40.0%.

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Table 1.

The loss of viability and competency upon freezing of *B. subtilis* cells

Time (Weeks)	No. of *trp*$^+$ transformants/ml[a]	Viable cells/ml	Transformants/viable cells ($\times 10^{-3}$)
Before freezing	1.2×10^6	2.0×10^8	6.0
24 h. post freezing[b]	4.0×10^5	1.4×10^8	2.9
1	3.0×10^5	8.5×10^7	3.5
2	3.0×10^5	9.2×10^7	3.3
3	3.1×10^5	7.2×10^7	4.3
4	3.1×10^5	1.1×10^8	2.8
5	3.5×10^5	1.0×10^8	3.5

[a] Obtained with native DNA at 1.0 μg/ml.

[b] Frozen cells were transformed at 2x concentration. For the sake of comparison all values have been normalized to that obtained for fresh cells by halving them.

Table 2.

The transforming activity of the double-stranded fraction resulting from hydroxyapatite (HA) chromatography of various *B. subtilis* DNA preparations

Exp. no.	DNA	Amount recovered in peak 2 (%)	Concentration of HA-DNA during transformation (μg/ml)	No. of trp^+ transformants/ml		Activity relative to native reference DNA (%)[b]	Average activity relative to chromatographed native DNA (%)
				HA-DNA	Native reference DNA[a]		
1	Native	92	0.9	5.0×10^5	6.0×10^5	93	100
2		92	1.0	7.5×10^5	7.8×10^5	96	
1	HN_2-D-DNA	40	0.8	1.7×10^5	6.0×10^5	35	43
2		43	0.9	3.3×10^5	7.8×10^5	47	
1	Renatured	59	0.8	1.1×10^5	4.5×10^5	31	29
2		75	1.1	1.4×10^5	5.5×10^5	23	
1	Denatured	6.8	0.1	3.3×10^5	6.6×10^{5c}	50	40
2		10.5	0.1	1.6×10^5	4.5×10^{5c}	35	
3		8.2	0.1	1.2×10^5	4.0×10^{5c}	30	

[a] Transformations were carried out with frozen cells using the same native reference DNA at 1.0 μg/ml

[b] Normalized to 1.0 μg DNA/ml

[c] Transformations were carried out with freshly prepared cells using a native reference DNA at 0.1 μg/ml

Table 3.

A comparison of cotransformation index (r) and specific transforming activity from various hydroxyapatite purified DNA samples as a function of DNA concentration

		Native DNA		Renatured DNA		HN_2-D-DNA	
Exp. no.	DNA (µg/ml)	Transformants/ml	r	Specific activity relative to native DNA (%)	r	Specific activity relative to native DNA (%)	r
1[a]	0.8	5.0×10^5	0.37	22	0.31	41	0.19
	0.4	2.0×10^5	0.34	33	0.37	44	0.23
	0.2	8.5×10^4	0.36	34	0.32	48	0.22
	0.1	6.0×10^4	0.39	22	0.33	35	0.17
2[b]	0.8	4.9×10^5	0.33	22	0.28	38	0.20
	0.1	3.4×10^4	0.38	28	0.25	47	0.20
3[b]	0.9	5.4×10^5	0.33	17	0.28	40	0.20
	0.4	2.2×10^5	0.38	23	0.30	33	0.22
	0.1	5.0×10^4	0.32	26	0.35	32	0.27
Mean			$\pm$0.026		$\pm$0.038		$\pm$0.027
			0.36(100)[c]		0.31(86)		0.21(58)

[a] r obtained by replica plating to second selective medium

[b] r obtained by direct plating for *his*$^+$ and double transformants

[c] Numbers in parenthesis are percents

Table 4.

The cotransformation index (r) of various fractions of renatured and HN_2-crosslinked DNA obtained by hydroxyapatite (HA) chromatography

DNA (μg/ml)	Renatured DNA fraction no.[a]			HN_2-D-DNA fraction no.[a]		
	18	19	20	19	20	21
0.5	0.28(23)[b]	0.31(28)	0.27(27)	0.19(35)	0.20(44)	0.19(46)
0.1	0.26(22)	0.28(24)	0.28(30)	0.20(32)	0.17(38)	0.21(38)
Mean		0.28			0.19	

[a] Obtained by direct plating for *his*$^+$ and double transformants.

[b] Numbers in parenthesis refer to specific activity relative to chromatographed native DNA.

Table 5.

A comparison of the two methods used to assay the cotransformation index (r)

Exp. no.	No. of *his*$^+$ transformants/ml ($\times 10^{-4}$)	Direct plating[a]	Replica plating[b]
1	1.8	0.19	0.20
2	2.0	0.17	0.16
3	1.9	0.20	0.17
4	2.1	0.16	0.19
5	1.9	0.19	0.19
6	1.8	0.17	0.16
Mean		0.18	0.18
		$\pm$ 0.017	$\pm$ 0.017

[a] Six separate transformation tubes were prepared from fraction no. 20 shown in Table 4 and the DNA was used at 0.1 µg/ml. Following incubation with competent cells, each sample was plated on *his*$^+$ and *trp*$^+$*his*$^+$ selective plates.

$$r = \textit{trp}^+\textit{his}^+/(\textit{his}^+ \times 2 - \textit{trp}^+\textit{his}^+)$$

[b] Obtained by replica plating 100-200 colonies from *his*$^+$ plates to *trp*$^+$ selective plates to determine double transformants.

$$r = \textit{trp}^+\textit{his}^+/(\textit{his}^+ \times 2 + \textit{trp}^+\textit{his}^+)$$

Table 6.

A comparison of the DNA dose response of fresh and frozen *B. subtilis* recipients

DNA concentration (μg/ml)	Fresh cells (no. of *trp*$^+$ transformants/ml)[a]	Frozen cells (no. of *trp*$^+$ transformants/ml)[b]	Yield with frozen cells relative to fresh cells (%)
5.0	2.8×10^6	1.4×10^6	50.0
2.5	2.2×10^6	8.4×10^5	38.2
1.0	1.6×10^6	3.9×10^5	24.3
0.5	1.7×10^6	2.0×10^5	11.8
0.2	9.3×10^5	9.8×10^4	10.5
0.1	5.2×10^5	5.0×10^4	9.6
0.05	2.5×10^5	2.7×10^4	10.8
0.01	5.0×10^4		
0.005	2.0×10^4		

[a] No. of viable cells was 2.1×10^8/ml

[b] No. of viable cells was 1.9×10^8/ml after being frozen and double concentrated

Table 7.

Changes in cotransformation index (r) as a function of HN_2 exposure time

Time (min.)	HN_2-N-DNA exp. no.[a] 1	2	3	Mean	HN_2-D-DNA exp. no.[a] 1	2	3	Mean
0	0.36	0.29	0.33	0.33 ± 0.035	0.38	0.30	0.35	0.34 ± 0.040
20	0.33	0.27	0.31	0.30 ± 0.030	0.28	0.30	0.27	0.28 ± 0.016
40	0.31	0.24	0.24	0.26 ± 0.041	0.22	0.26	0.25	0.24 ± 0.021
60	0.28	0.30	0.26	0.28 ± 0.032	0.16	0.24	0.19	0.20 ± 0.040
80	0.25	0.25	0.26	0.25 ± 0.007	0.21	0.23	0.21	0.21 ± 0.014
100	0.28	0.29	0.26	0.28 ± 0.016	0.20	0.19	0.17	0.19 ± 0.016

[a] Once all the samples were collected they were immediately transformed at 1.5 μg DNA/ml and r was obtained by direct plating for *trp*$^+$ and double transformants.

Table 8.

Assays of forward mutations accompanying transformation with HN_2-crosslinked and renatured *B. subtilis* DNA

		Renatured DNA		HN_2-D-DNA	
Sample	Assay	No. of mutants	No. of colonies examined	No. of mutants	No. of colonies examined
A	1	0	2.0×10^3	0	7.4×10^3
B	1	2	1.6×10^4	1	2.4×10^4
C	1	0	1.3×10^3	0	3.8×10^3
	2	1	5.6×10^4	1	5.5×10^4
D	1	1	1.6×10^4	2	7.1×10^3
	2	0	3.2×10^4	0	1.9×10^4
	3	0	1.4×10^4	1	1.5×10^3
Total		4	1.4×10^5	5	1.2×10^3
Mutation frequency		2.8×10^{-5}		4.17×10^{-5}	

HN_2-D-DNA/Renatured DNA = 1.5 $\quad 1.5 \times 1.47 = 2.2$[a]

[a] Corrected for the ability of renatured DNA to cotransform better than HN_2-D-DNA by multiplying observed value of 1.5 by 0.31/0.21 (see average r values in Table 3)

Table 9.

A comparison of observed and predicted cotransformation indices (r) and transforming activities of HN_2-crosslinked DNA

	Yield relative to native DNA (%)		
	Predicted (see Appendix)	Observed[a]	Remarks
r			
HN_2-D-DNA	47	56 ± 5	Table 7 (3 samples)
		58 ± 8	Table 3 (9 assays)
HN_2-N-DNA	78	82 ± 5	Table 7 (3 samples)
Activity			
HN_2-D-DNA (purified)	40	43	Table 2
		40 ± 7	Table 3 (9 assays)
HN_2-D-DNA	17	24 ± 6	Figures 2 and 9 (4 samples)
HN_2-N-DNA	75	54 ± 9	Figures 2 and 9 (4 samples)

[a] Mean values

Figure 1. The effect of increasing HN_2 concentration on the transforming activity of *B. subtilis* DNA. Various amounts of HN_2 in 0.025M triethanolamine-HCl buffer, pH 7.3 were mixed with DNA at a final concentration of 25 µg DNA/ml. The reaction was carried out for two hours at 25°C. The transforming activity of a sample incubated in buffer alone and exposed to NaOH previously neutralized by HCl represents 100% relative transforming activity. Samples were transformed at 1.5 µg DNA/ml and assayed for *trp*$^+$ transformants per ml of transformation mix with 100% equal to 7.2×10^5 transformants/ml. X, samples denatured for 3 minutes following HN_2 treatment; • , undenatured samples.

FIG 1

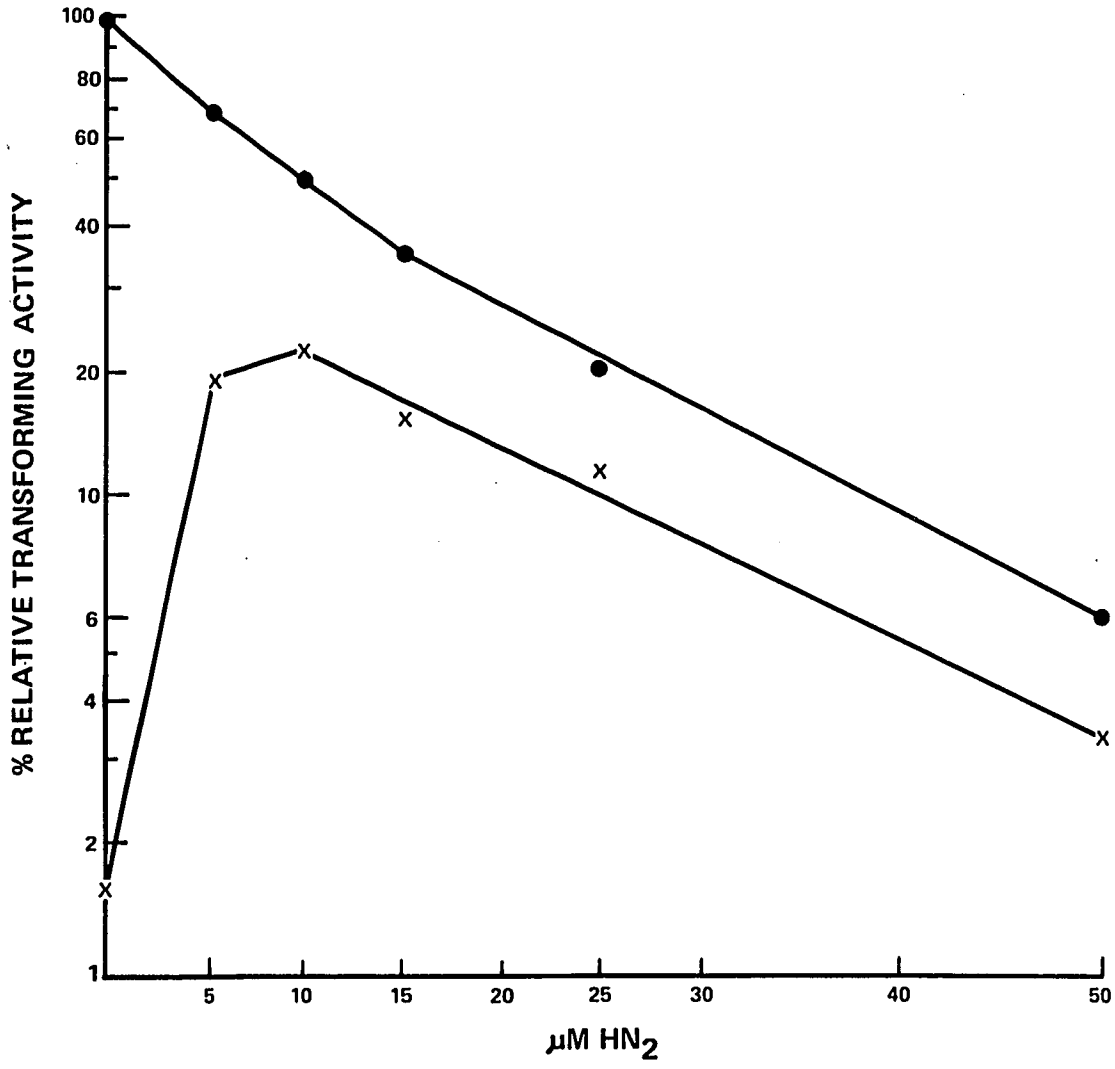
% RELATIVE TRANSFORMING ACTIVITY
100
80
60
40
20
10
6
4
2
1
5
10
15
20
25
30
40
50
μM HN2

Figure 2. The effect of HN_2 on the transforming activity of B. subtilis DNA as a function of the reaction time. The HN_2 concentration in the reaction mixture was 10 μM and the reaction was carried out for 100 minutes. The transforming activity of a sample removed immediately and exposed to NaOH previously neutralized with HCl represents 100% relative activity. Transformations were carried out at 1.5 μg DNA/ml with 100% equal to 9.5×10^5 transformants per ml. X, samples denatured for 3 minutes following HN_2 treatment; . , undenatured samples.

FIG 2

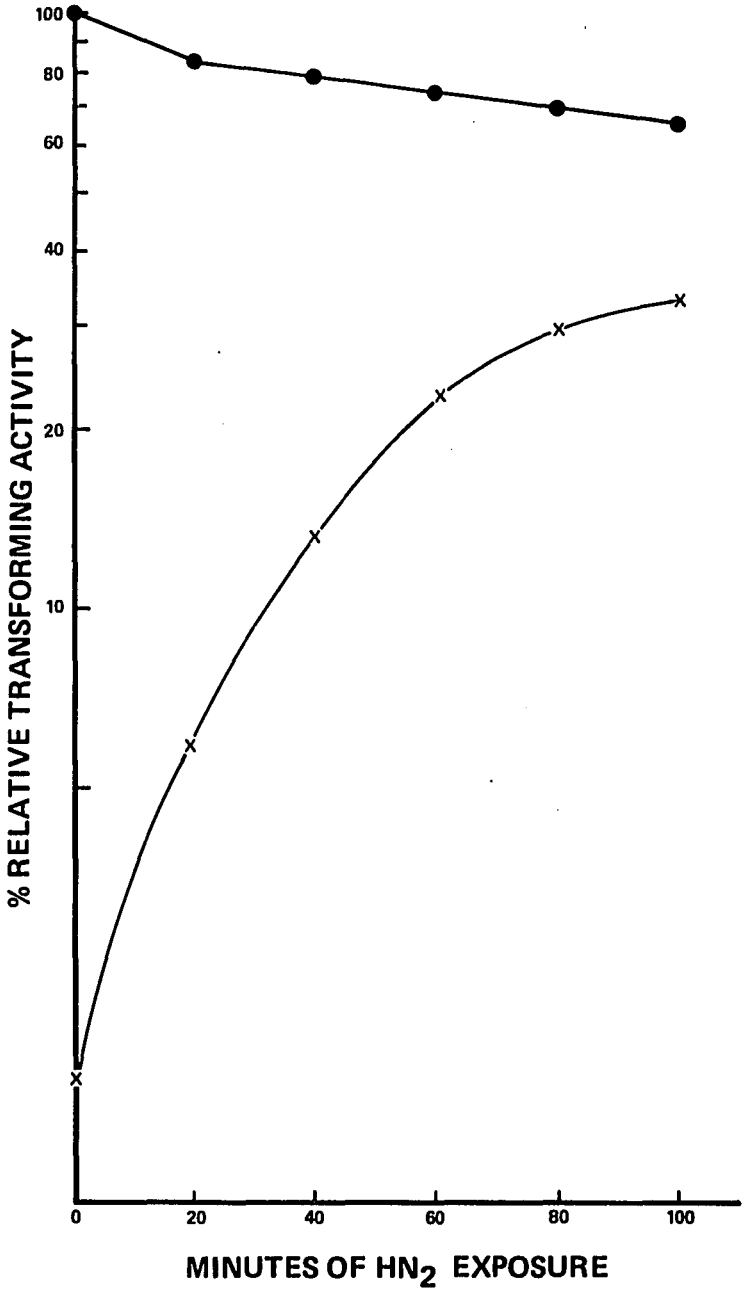
100
80
60
40
20
10
% RELATIVE TRANSFORMING ACTIVITY
0
20
40
60
80
100
MINUTES OF HN_2 EXPOSURE

Figure 3. The effect of HN_2 on the transforming activity of B. subtilis DNA as a function of the reaction time. The HN_2 concentration in the reaction mixture was 10 μM and the reaction was carried out for 180 minutes. Transformations were carried out at 1.5 μg DNA/ml with 100% activity equal to 8.3×10^5 trp^+ transformants per ml. X, samples denatured for 3 minutes following HN_2 treatment; ., undenatured samples.

FIG 3

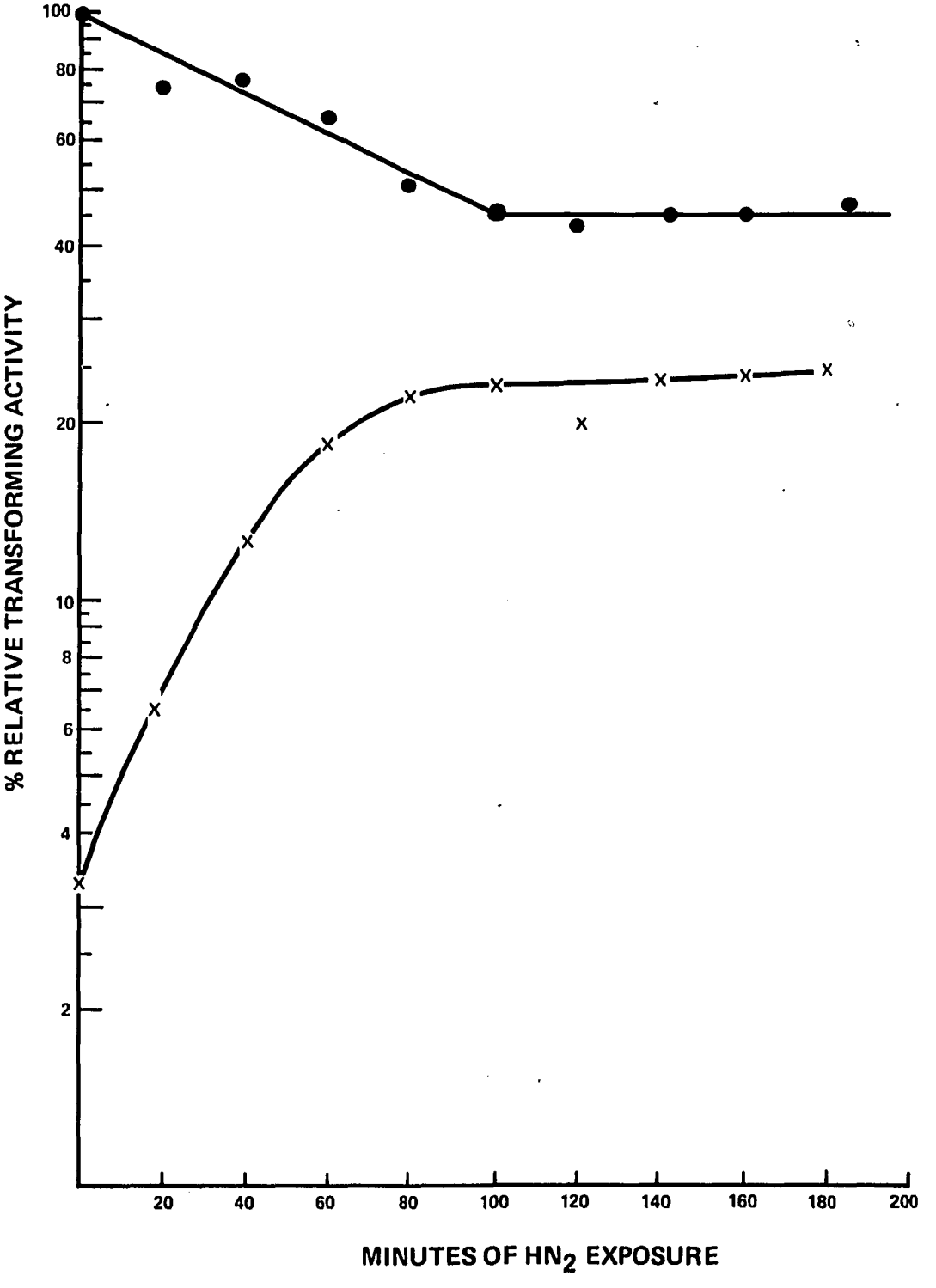
% RELATIVE TRANSFORMING ACTIVITY
100
80
60
40
20
10
8
6
4
2
20
40
60
80
100
120
140
160
180
200
MINUTES OF HN_2 EXPOSURE

Figure 4. Hydroxyapatite chromatography of native B. subtilis DNA. Native DNA (395 µg), dialyzed against the starting buffer, was applied, as indicated by the arrow, to a column 1.6 cm in diameter, packed to a height of 8 cm and eluted by means of a linear gradient of 0.05-1.0M KP (total volume, 300 ml). The values above the peaks indicate elution molarity. The recovery as a % of output DNA was: peak 1, 5; peak 2, 92. Total recovery was 92% of input DNA. ι_____., A_{260}; x_____x, trp^+ transformants/ml.

FIG 4

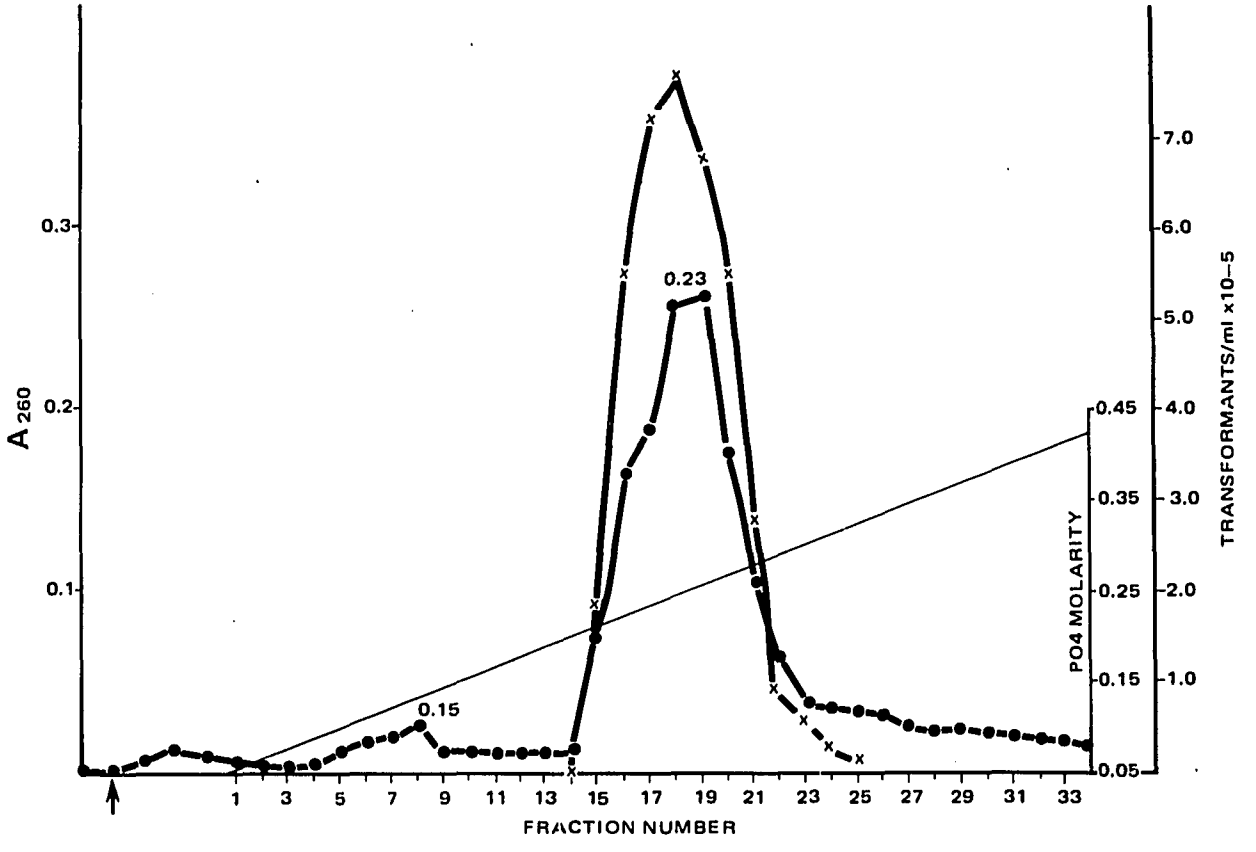
A260
0.3
0.2
0.1
0.23
0.15
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
FRACTION NUMBER
PO4 MOLARITY
0.45
0.35
0.25
0.15
0.05
TRANSFORMANTS/ml x10-5
7.0
6.0
5.0
4.0
3.0
2.0
1.0

Figure 5. Hydroxyapatite chromatography of alkali-denatured B. subtilis DNA. A solution of DNA (25 µg/ml) was alkali denatured. Following neutralization and dialysis, this DNA (490 µg) was applied to the column. The recovery as a % of output DNA was: peak 1, 77; peak 2, 11. Total recovery was 90% of input DNA. For additional information see Figure 4.

FIG 5

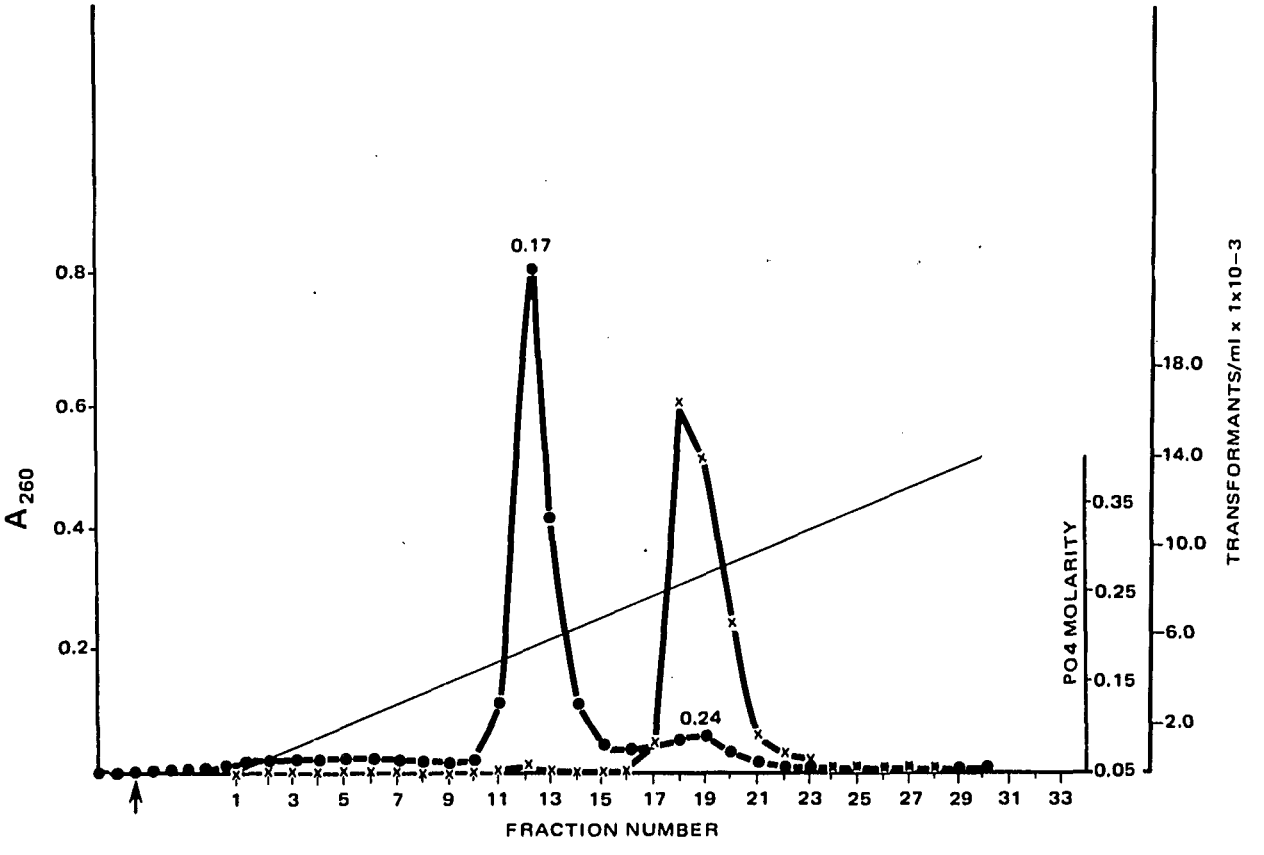
0.17
0.24
0.8
0.6
0.4
0.2
A_{260}
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
FRACTION NUMBER
PO4 MOLARITY
0.35
0.25
0.15
0.05
18.0
14.0
10.0
6.0
2.0
TRANSFORMANTS/ml x 1x10−3

Figure 6. Hydroxyapatite chromatography of B. subtilis DNA treated with HN_2 and alkali denatured. A solution of DNA (25 µg/ml) was exposed to 10 µM HN_2 for 100 minutes and alkali denatured. Following neutralization and dialysis, this DNA (405 µg) was applied to the column. The recovery as a % of output DNA was: peak 1, 45; peak 2, 43. Total recovery was 92% of input DNA. For additional information see Figure 4.

FIG 6

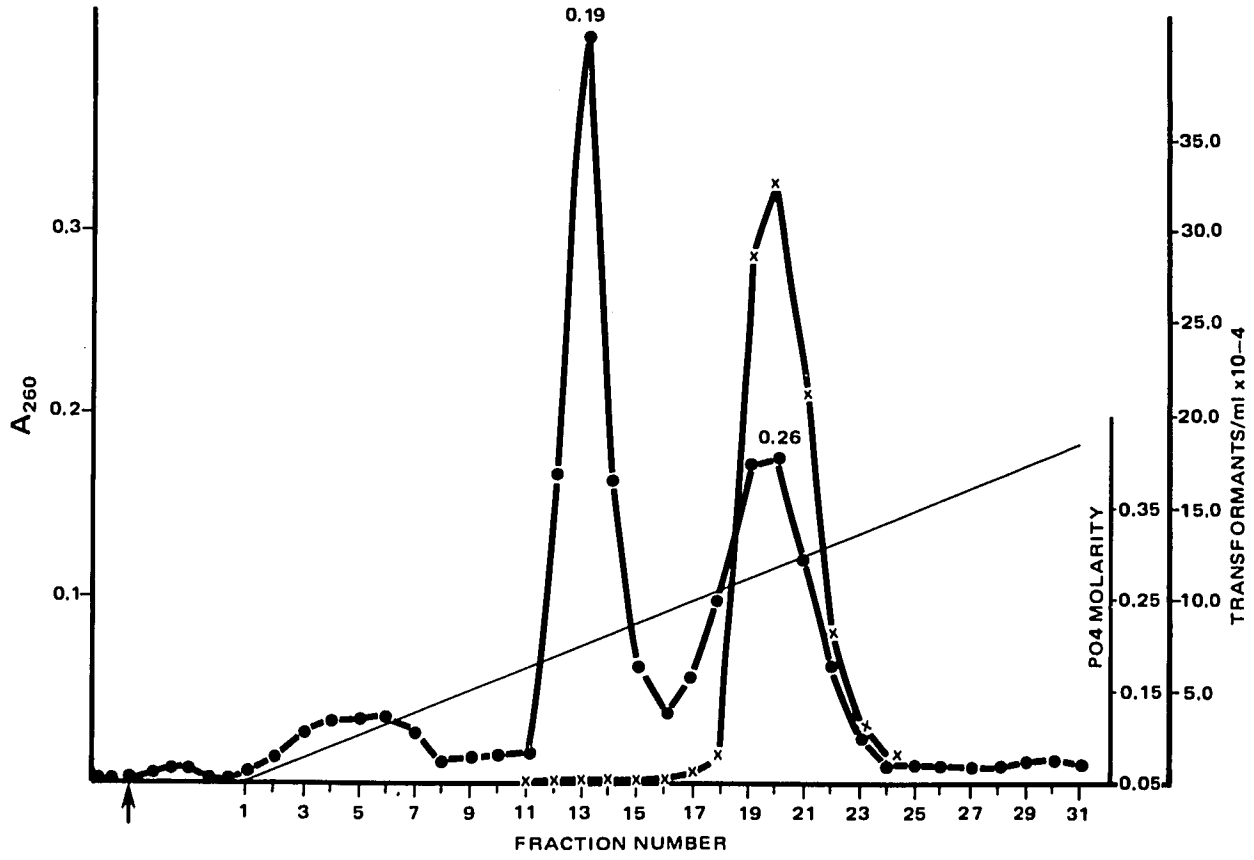
0.19
0.26
A_{260}
0.3
0.2
0.1
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
FRACTION NUMBER
PO4 MOLARITY
0.35
0.25
0.15
0.05
TRANSFORMANTS/ml x10-4
35.0
30.0
25.0
20.0
15.0
10.0
5.0

Figure 7. Hydroxyapatite chromatography of renatured B. subtilis DNA. Thermal renaturation of alkali denatured DNA (18.8 µg/ml) was carried out in 2xSSC at 68°C for 4 hours. Following dialysis, this DNA (375 µg) was applied to a 10 cm column. The recovery as a % of output was: peak 1, 20; peak 2, 75. Total recovery was 67% of input DNA. For additional information see Figure 4.

FIG 7

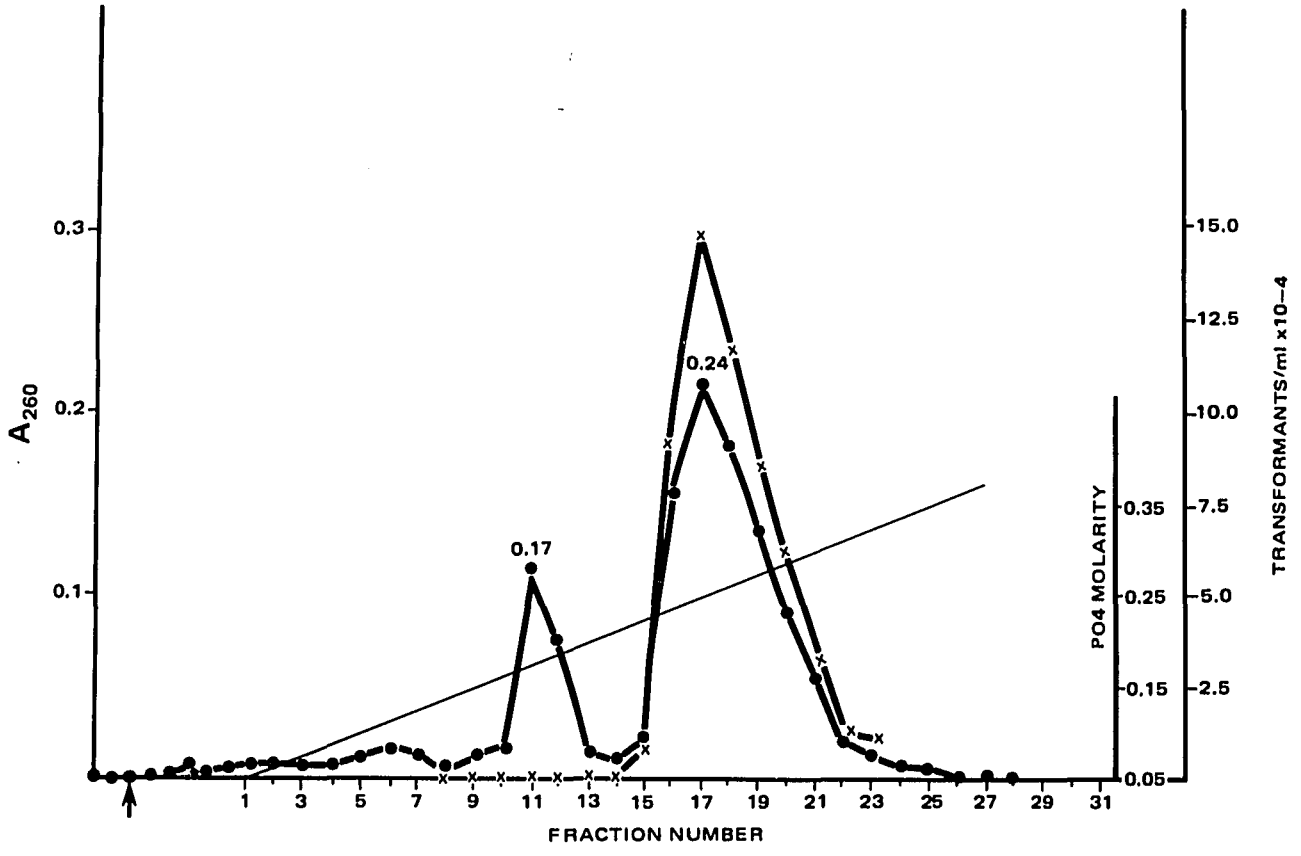
A_{260}
0.3
0.2
0.1
0.17
0.24
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
FRACTION NUMBER
PO4 MOLARITY
0.35
0.25
0.15
0.05
TRANSFORMANTS/ml x10−4
15.0
12.5
10.0
7.5
5.0
2.5

Figure 8. A DNA dose response curve of fresh and frozen B. subtilis recipients. The viable count was as follows: fresh cells, 2.1×10^8/ml; frozen cells, 9.5×10^7/ml. The frozen cells were transformed at a 2-fold concentration and trp^+ transformants were counted. The numerical values are presented in Table 6. X, fresh recipients; ., frozen recipients.

FIG 8

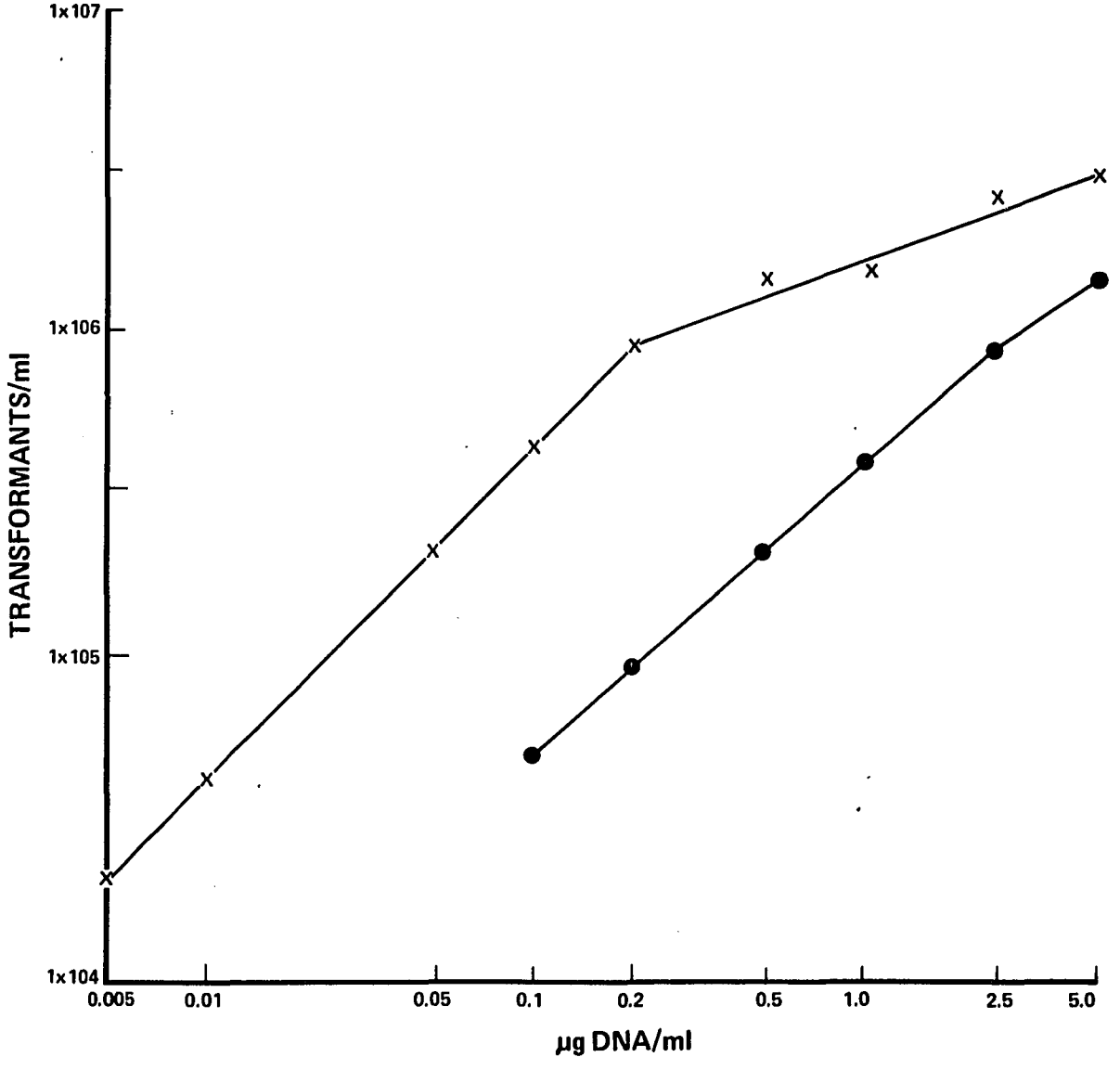
1x107
1x106
1x105
1x104
TRANSFORMANTS/ml
0.005
0.01
0.05
0.1
0.2
0.5
1.0
2.5
5.0
µg DNA/ml

Figure 9. The effect of HN_2 on the transforming activity of B. subtilis DNA as a function of the reaction time. HN_2 (10 µM) was reacted with DNA (25 µg/ml) and transformations were carried out at 1.5 µg DNA/ml. The transforming activity at 100% was 1.0×10^6 trp^+ transformants/ml. The values are the averages of three experiments. X, samples denatured for 3 minutes following HN_2 treatment; ., undenatured samples. For additional information see Figure 2.

FIG 9

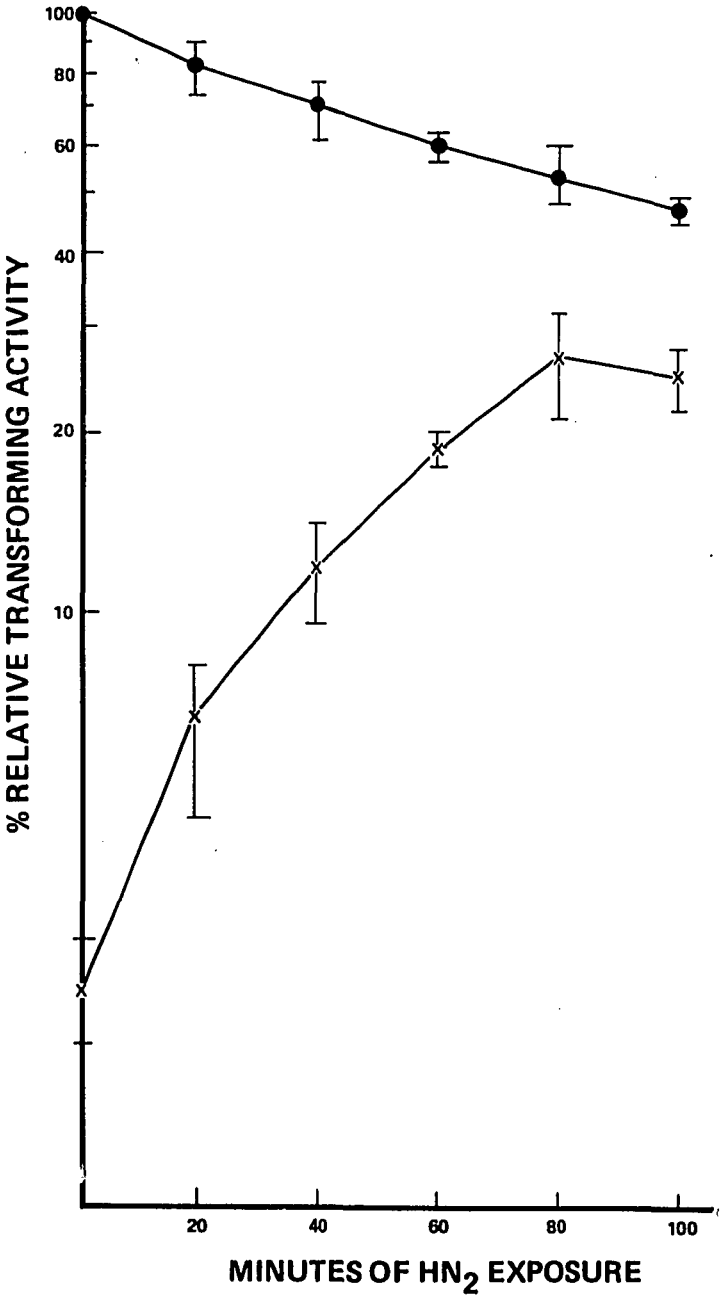
100
80
60
40
20
10
% RELATIVE TRANSFORMING ACTIVITY
20
40
60
80
100
MINUTES OF HN_2 EXPOSURE

Figure 10. The effect of HN_2 on the cotransformation index (r) of B. subtilis DNA as a function of the reaction time. DNA samples obtained from the experiment described in Figure 9 were assayed for their ability to cotransform trpC2 and hisB2. The values are the averages of three experiments. X, samples denatured for 3 minutes following HN_2 treatment; ., undenatured samples. For additional information see Figure 9 and Table 7.

FIG 10

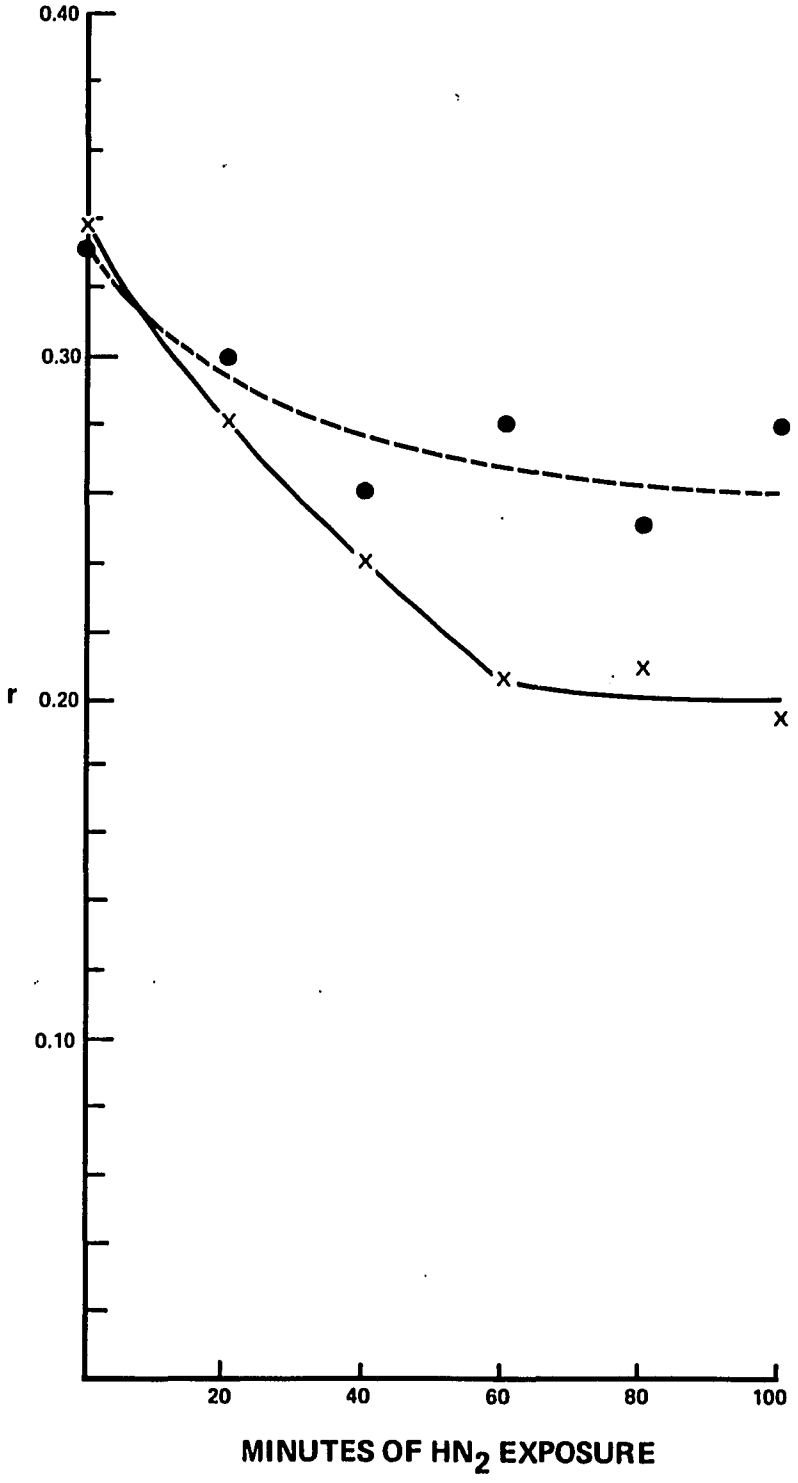

Figure 11. Possible enzymatic events leading to a decrease in the cotransformation index (r) of crosslinked DNA; trp, his, transforming strand for marked loci; pN, 5' nucleoside monophosphate; ---- ◄—,3'——►5' exonucleolytic degradation; ↑, site of cleavage by postulated endonuclease.

FIG 11