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Alterations in *Bacillus subtilis* Transforming DNA Induced by β-Propiolactone and 1,3-Propane Sultone, Two Mutagenic and Carcinogenic Alkylating Agents

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Transforming DNA was exposed to either β-propiolactone or 1,3-propane sultone and then used for transformation of competent bacteria to nutritional independence from tyrosine and tryptophan (linked markers) and leucine (an unlinked marker). The ability to transform was progressively lost by the DNA during incubation with either of these two chemicals. For all three markers the inactivation curve was biphasic, with a short period of rapid inactivation followed by one characterized by a much slower rate. The overall rate of inactivation was different for all three markers and presumably was related to the size of the marker. The decrease in the transforming activity was in part due to the slower rate of penetration of alkylated DNA through the cellular membrane and its inability to enter the recipient bacteria. This decrease in the rate of cellular uptake, even for DNA eventually destined to enter the cell, began almost immediately after its exposure to the chemical and ended up with an almost complete lack of recognition of the heavily alkylated DNA by the specific surface receptors of competent cells. Such DNA attached to sites on the surface of competent bacteria which were different from receptors specific for the untreated nucleic acid. This attachment was not followed by uptake of the altered DNA. Presence of albumin during the incubation with a carcinogen further increased the degree of inactivation, indicating that the artificial nucleoproteins produced under such conditions were less efficient in the transformation assay than was the naked DNA. Cotransformation of close markers progressively decreased, beginning immediately after the start of incubation of DNA with the chemicals. Extensively alkylated DNA fractionated by sedimentation through sucrose density gradients showed a peculiar distribution of cotransforming activity for such markers; namely, molecules larger than the bulk of DNA ("megamolecules") showed less ability to transform the second marker than did some of the apparently smaller molecules which sedimented more slowly through the gradient. An increase in cotransformation of distant markers was evident in DNA molecules after a short exposure to an alkylating agent, but cotransformation of such markers was absent in DNA treated for longer periods. The observed changes in the transforming and cotransforming activities of the alkylated DNA can be explained by what is known about the physicochemistry of such DNA and in particular about the propensity of the alkylated and broken molecules to form complexes with themselves and with other macromolecules.

Beta-propiolactone (BPL) and 1,3-propane sultone (PS) are two chemically related, small-ring, monoalkylating carcinogens and mutagens. The chemical structures of both compounds are shown in Fig. 1. An excellent review on the chemical and biological properties of BPL has been published recently (3). A list of relevant publications on the mutagenic and carcinogenic activities of PS has been compiled in one of our recent reports (26). Our studies on the effects of BPL on purified DNA, carried out with physical, chemical, and electron microscopic techniques, revealed a well-defined sequence of events (12).

After a brief exposure to BPL, the conformation of DNA detectably changes. When viewed under the electron microscope, the polymer appears smoother and stiffer than the control DNA, and the molecule is coiled into a small number of rigid loops, held together by some intramolecular bridges of unknown chemical nature. Its contour length is increased by as much as 10 to 15%. In parallel experiments the same

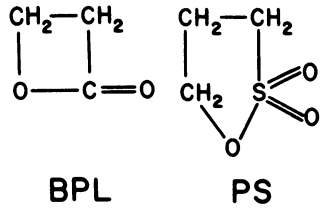


FIG. 1. *Chemical structure of BPL and PS.*

DNA shows rather unusual hydrodynamic properties by sedimenting somewhat faster in sucrose gradients and moving more rapidly through gels during electrophoresis than does the untreated DNA. We call this form of alkylated DNA "early" (E-DNA), to distinguish it from DNA seen during later stages of incubation ("late"; L-DNA). The latter form is the result of a continuously progressing breakdown of the strained E-DNA polymer, with the formation of single-stranded "whiskers" which then react with other macromolecules, including other DNAs, to form sometimes very extensive networks of interconnected DNA chains.

Thus, L-DNA exists in two forms. One fraction is composed of small, broken DNA pieces, sedimenting slowly in sucrose and moving ahead of the rest of DNA during gel electrophoresis. In the study described in the present report we concentrated more on the other form of L-DNA, the large complexes of DNA-DNA adducts ("megamolecules") made of the broken and some unbroken DNA of various sizes, kept together by covalent-like linkages (12). Such DNA moves rapidly through sucrose gradients and slowly through gels. DNA with these physicochemical characteristics appeared at about 10 min after the beginning of the coincubation of DNA with the alkylating agent under the experimental conditions used in the present study. In the presence of proteins such as serum albumin, still larger complexes are formed, composed of both the nucleic acid and the protein (20). Although no electron microscopic studies were carried out on PS-treated DNA, all other analytical techniques indicated that DNA exposed to this chemical reacts in a manner very similar to that observed with BPL (26).

We decided to study the genetic properties of such altered DNA and to attempt a correlation between the observed physicochemical changes in the DNA and its transforming capabilities. For example, we expected that the breakdown of the DNA molecule induced by the alkylating agents should decrease the rate of cotransformation for both the close and the distant markers; and we speculated that the large clusters of DNA (megamolecules) may cotransform relatively more of the distant markers as compared with the close ones, if the breakdown and reassembly of this DNA results in a biologically viable complex molecule.

A part of these observations has been published in a preliminary form (13).

MATERIALS AND METHODS

Bacterial strains. Bacterial strains were obtained from Richard S. Hanson of this university. *Bacillus subtilis* 746 prototroph was used for the isolation of transforming DNA. The recipient strain SB238 carries markers *trpC*$_2$, *tyrA* and *leu* (5) and is dependent for growth on the presence in the medium of tryptophan, tyrosine, and leucine. *Escherichia coli* strain Q13 was used in our earlier studies (19).

Media. The minimal medium of Vogel and Bonner (VB) (25) supplemented with Mg^{2+} and glucose and the minimum medium of Anagnostopoulos and Spizizen (Spizizen medium) supplemented with glucose (1) and agar were used as competence and selective solid media, respectively. The solid medium was, in addition, supplemented with the required L-amino acids at concentrations of 50 μg/ml each.

Chemicals. BPL was purchased from Fellows-Testagar, Oak Park, Mich., and was used without further purification. PS (more than 99% pure) was obtained from Tridom-Fluka Chemical Inc., Hauppauge, N.Y. Our attempts at further purification by vacuum distillation (26) did not appreciably increase the activity of this compound. Bovine serum albumin fraction V was purchased from Armour Pharmaceutical Corp., Kankakee, Ill. All other chemicals used were of analytical grade.

Labeling of *B. subtilis* DNA with radioactive phosphorus. A 10-ml quantity of an overnight culture of bacteria was transferred to 600 ml of Grossman medium (7) supplemented with 1% glucose and 15 mCi of carrier-free $^{32}P_i$. The cells were incubated for at least 8 h in a shaker at 37°C and harvested by centrifugation. The radioactive DNA was subsequently extracted from the lysed cells in a manner identical to that used for non-radioactive DNA. We did not routinely check the purity of the isolated radioactive DNA. However, in the few instances when such DNA was analyzed in CsCl density gradients, more than 98% of the radioactive material was found at the density corresponding to that of *B. subtilis* DNA.

Isolation of transforming DNA. Donor *B. subtilis* 746 bacteria were grown overnight in nutrient broth with vigorous shaking, harvested, and suspended in a small volume of 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate). Lysis was achieved by a short treatment of cells with 20 μg of lysozyme per ml (37°C for 20 to 30 min with constant but gentle stirring) followed by treatment with sodium dodecyl sulfate (final concentration, 1%). DNA was then extracted five to six times with chloroform–butanol as described previously (11). The DNA was precipitated with 95% ice-cold ethyl alcohol, collected on a glass rod, redissolved in 1× SSC, and treated for 30 min at 37°C with a mixture of ribonucleases (20 μg of ribonuclease A per ml and 20 U of ribonuclease T1 per ml; purchased form Worthington Biochemicals Corp. Freehold, N.J., and Sigma Chemical Co., Saint Louis, Mo., respec-

tively). Subsequently, the DNA was extracted once with an equal volume of freshly redistilled, water-saturated phenol in the presence of small amounts of bentonite. After centrifugation, DNA was precipitated with ice-cold 95% ethyl alcohol, redissolved in 1× SSC, and then filtered through a Sephadex G25 column (2.5 by 30 cm) to remove low-molecular-weight contaminants. In some experiments this step was followed by further fractionation of the DNA by preparative centrifugation in sucrose gradients to obtain relatively homogenous DNA fractions (5 to 20% sucrose dissolved in 1× SSC for 8 h at 22,000 rpm in an SW27 Spinco rotor, kept at 4°C during centrifugation). The samples were then dialyzed against 1× SSC, and the purified material was stored frozen at −30°C until use. Occasionally, all these purification steps resulted in a DNA solution that was too dilute for further experimentation. After testing several techniques, we found that the mildest way to concentrate a DNA solution was by gently shaking it with an excess of freshly distilled, water-unsaturated phenol, which efficiently removed water from the solution without any appreciable loss of DNA. All other methods tested significantly decreased the amounts of DNA present due to losses on the dialysis membrane, gel surfaces, etc. The transforming DNA prepared in this manner produced, under the experimental conditions described below, between 1×10^4 and 10×10^4 transformants per μg of DNA; the exact number depended on the size of DNA, the degree of competence of the bacterial culture used on that day, and the marker tested.

Transforming assay. The overnight culture of *B. subtilis* SB238 cells in nutrient broth was transferred into 2 volumes of fresh nutrient broth and incubated with shaking for 2 h at 37°C. One volume of this culture was inoculated into 3.5 volumes of minimal VB or Spizizen medium. The cells were then grown at 37°C for an additional 2.5 h. At this time the culture was distributed into a number of sterile test tubes, the transforming DNA was added, and the samples were incubated with shaking for 1 h at 37°C. At the end of the incubation period, the cells were plated on various solid media as described above. After 48 h of incubation at 37°C, cotransformation of linked and distant markers (5) was scored by replica plating (14) the transformed colonies on appropriate solid media. In the present report we represent this kind of experiment by an arrow showing the order of analysis. For example, *tyr* → *trp* means that the tested culture was first grown on a medium lacking tyrosine, followed by replica plating of the colonies onto a second medium lacking tryptophan.

The noncompetent *B. subtilis* cells were obtained by harvesting by centrifugation an overnight culture grown in nutrient broth and suspending the sediment in an appropriate medium for incubation with DNA.

Inactivation of the transforming DNA by chemical agents and by UV. A quantity of 2 to 20 μg of DNA was mixed with either one of the two carcinogenic chemicals, BPL or PS, and incubated at 37°C with constant shaking. Samples were withdrawn at various times, immediately precipitated with 4 volumes of cold ethanol, and centrifuged for 5 min at 2,600 × *g*, and the sediments were resuspended in 1× SSC.

The dissolved DNA samples were adjusted to a common concentration of DNA by using their optical densities (measured at 260 nm) or their radioactivity as a guide. The slight hyperchromicity of alkylated DNA seen after extensive treatment with the two carcinogens (12; unpublished data) was not taken into consideration when these adjustments were made.

The same procedure was applied in experiments in which the treated DNA was used for subsequent centrifugation in sucrose density gradients. Relatively homogeneous fractions of *B. subtilis* DNA prepurified by preparative sucrose gradient centrifugation as dscribed above, were exposed to the alkylating agent. In some of these tests the alcohol precipitation step was omitted, and the chilled DNA-alkylating agent mixture was placed directly on the top of cold 15 to 30% sucrose gradients (the 1% solution of BPL sinks into a 5% sucrose solution). The tubes were then immediately transferred into cold buckets and centrifuged in a Spinco SW50.1 rotor for 150 min at 42,000 rpm. As in our earlier studies, we found that the losses of alkylated DNA on tube walls were minimized by the use of centrifuge tubes made of polyallomer.

After the collection of fractions, the optical density was read at 260 nm in a spectrophotometer, the radioactivity (if present) was measured, and, finally, 20- and 50-μl samples were withdrawn for transformation assays. Several different volumes of the transformed bacteria were then plated, usually on a variety of selective media.

In control experiments we established that no toxic or mutagenic effects of traces of the alkylating agents were distorting the experimental results. Such chemicals are relatively unstable in water, especially in the presence of organic materials (2). A 0.15 M solution of BPL was layered on the top of a sucrose gradient and centrifuged, and fractions were collected and stored in a routine manner. The fractions were then used for treatment of *B. subtilis* cells, and the number of colony-forming units was subsequently measured on plates containing either a nutrient broth or a minimum medium. Nowhere in the gradient were we able to detect material which would either decrease the viability of *B. subtilis* cells or increase the mutation rate of the culture.

UV irradiation was carried out basically as recently described (19). A quantity of 8 to 10 μg of DNA was dissolved in 0.14 M NaCl–5 mM phosphate buffer (pH 7.2) and put into a small glass beaker (diameter, 24 mm). The beaker was then placed on ice and under two parallel, 15-W mercury sterilizing lamps, with the peak radiation at 254 nm. The irradiated samples were 3 to 4 mm thick, and the average fluence rate was 0.2 J/m^2 per s, as determined with the aid of a UV meter (Ultraviolet Products, Inc., San Mateo, Calif.). The DNA solution was constantly stirred during the irradiation. Samples were withdrawn at several time points and used for the transformation assay without prior precipitation with alcohol.

DNA binding assay. Radioactively labeled transforming DNAs either treated or nontreated with the alkylating agents were mixed with *B. subtilis* cells and incubated with shaking in a water bath at 37°C. At various times samples were withdrawn, and a part of each sample was used for transformation assays. An-

other portion of the sample was pipetted on a planchet to establish the total amount of radioactive material present in the sample. The remainder was diluted 10 times with TSM buffer (10 mM tris(hydroxymethyl)aminomethane-hydrochloride [pH 7.2], 0.14 M NaCl, 2 mM $MgSO_4$), divided into two equal portions, and centrifuged for 10 min at 4,000 × *g*. The sediments were suspended either in TSM or in TSM containing 10 μg of deoxyribonuclease I per ml (Worthington Biochemicals) and incubated for 10 min at 37°C. The cells were then diluted further with 20 volumes of TSM and centrifuged, and the amounts of radioactivity present in the pellets were measured in a gas flow, low background counter. Except for the time of incubation of cells with the radioactive DNA and with the deoxyribonuclease (or in control samples with TSM alone), all other steps were carried out at 0 to 4°C.

RESULTS

Inactivation kinetics of transforming DNA. Figure 2 shows the results of an experiment in which the transforming DNA was exposed to either BPL or PS. The samples were withdrawn at various times, precipitated with alcohol to remove the alkylating agent, dissolved in 1× SSC to a common concentration, and used for transformation of various markers into the competent cells. The ability to transform all of the markers was rapidly decreased. BPL was consistently and reproducibly more effective as an inactivating agent than PS; a comparable degree of inactivation was achieved with twice the molar amounts (or twice the incubation time) of PS compared with those for BPL. The inactivation curves were multicomponent, with an appreciable decrease in their slopes after the 30 to 50% inactivation point. The reasons for the presence of the slower component are not clear. Such patterns of inactivation had been observed for other mutagenic and carcinogenic chemicals and for UV and ionizing radiation in a variety of systems and for a variety of genetic markers (16–18). In our studies an additional element could play a role, namely, the fact that at this time under our experimental conditions the L-DNA begins to appear (12) and such DNA may perhaps be less sensitive to further chemical insults than are the other DNA forms. Moreover, the L-DNA is highly reactive and tends to attach itself to various surfaces, includng those of glass tubes and vessels (12). Thus, it is conceivable that the extensively alkylated DNA would be selectively lost during precipitation, centrifugation, etc. and that in the transformation assay we would be testing (and scoring) preferentially the relatively undamaged DNA molecules. However, no appreciable change in the inactivation kinetics was observed when siliconized glassware was used for centrifugation and storage of the alkylated DNA.

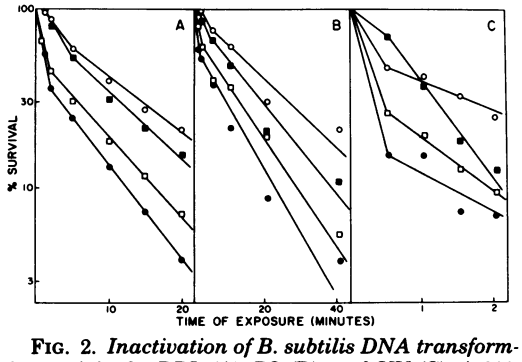


FIG. 2. *Inactivation of B. subtilis DNA transforming activity by BPL (A), PS (B), and UV (C). A 300-μl amount of DNA was exposed to 50 mM BPL or PS or irradiated with UV light. For further details see text. Symbols:* ■, *transformation to tryptophan independence;* ●, *transformation to tyrosine independence;* ○, *cotransformation tyr → trp;* □, *cotransformation trp → tyr.*

Significant differences were observed among the inactivation rates for various markers. The ability of DNA to transform to tyrosine independence was lost most rapidly, whereas the ability to transform to leucine independence (data not shown) was the most resistant, with the tryptophan marker in an intermediate position. An interesting feature of the inactivation curve of the latter is the shoulder visible at the beginning of treatment with BPL or PS.

The inactivation kinetics of linked markers (*tyr* → *trp* and *trp* → *tyr*) showed a similar pattern. A large proportion of colonies already transformed to tyrosine independence did not require tryptophan for growth. A smaller proportion of colonies transformed for the *trp* marker was at the same time cotransformed to tyrosine independence.

We tried to establish whether the difference in sensitivity toward both BPL and PS for various markers is due to differences in their base composition. Both these chemicals react preferentially with guanine (2, 6); thus, segments of DNA rich in this base may be more sensitive than an average DNA is. On the other hand, the differences in sensitivity could be due to differences in the physical size of various genetic loci or to some other characteristics of DNA. To distinguish among these possibilities, we examined the inactivation kinetics for these markers by UV (Fig. 2C), which is believed to preferentially inactivate the thymine-containing DNA (22). The order of inactivation for the two markers in both the transformation and cotransformation assays was identical to that previously observed with BPL and PS. We interpret this observation as an indication that the sensitivity of the marker toward either of the two chemicals

is not related to its base composition.

Adsorption and penetration of ^{32}P-labeled transforming DNA to competent and noncompetent *B. subtilis* cells. Radioactive DNA prepared from *B. subtilis* cells, mixed with competent bacteria, attached to and then penetrated into these cells. (Binding or attachment are operationally defined as an association of DNA with the cells measured by co-sedimentation of the DNA under the influence of moderate centrifugal forces; penetration or uptake are measured by the fraction of the attached DNA which becomes resistant to nucleases). The interaction between normal DNA and competent *B. subtilis* cells is illustrated in Fig. 3. Even under well-controlled experimental conditions there was a certain variability in the amounts of DNA attached to and then taken up by the cells, due mostly to differences among various DNA lots and to the degree of competence of the bacterial culture. After 60 to 90 min of incubation at 37°C in either of the two media used or in TSM, typically 1 to 3% of the added untreated DNA was found associated with the cells. In contrast, the attachment of DNA to noncompetent cells was usually less than 0.1% of the amount added to the incubation mixture, and the amounts of the radioactive DNA which eventually became resistant to deoxyribonuclease were often detected at the level of background radiation. The attachment and penetration ultimately resulted in transformation of a fraction of competent cells. The three markers which we have been studying transformed the recipient cells in the order *leu, trp, tyr.* If the rate of transformation for various markers is related (inversely) to their size, this observation could be interpreted as a further confirmation of the previously postulated correlation between the size of a marker and its sensitivity toward PS or BPL.

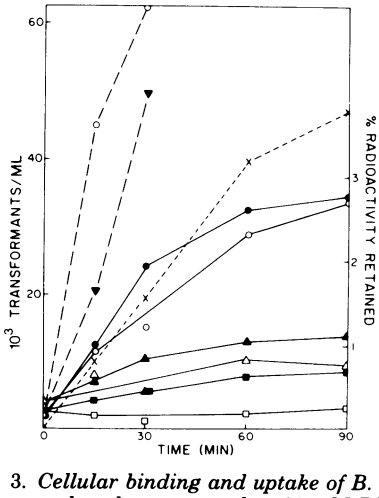


FIG. 3. *Cellular binding and uptake of B. subtilis DNA exposed and not exposed to 20 mM BPL and the kinetics of appearance of various markers after the incubation of cells with untreated DNA. Solid lines show binding and uptake. B. subtilis cells were brought to competence in VB medium and incubated with ^{32}P-labeled transforming DNA. The DNAs used were untreated (circles) or treated at 37°C with BPL for 20 (triangles) or 30 min (squares). The binding and uptake of such DNAs are represented by closed and open symbols, respectively. Dashed lines show the transformation of tyr (×), trp (▼), and leu (○).*

DNA extensively alkylated by BPL attached less readily to the competent bacteria than did the untreated, control DNA (Fig. 3 and Table 1), and its penetration decreased even more dramatically. The decrease in cellular binding of an alkylated DNA plays only a minor role in the decline of the transforming activity of the DNA. From several independent experiments we estimate that a 50% reduction in binding caused by exposure of DNA to BPL or PS corresponds to the decrease in transforming power by a factor of 10 to 20. The state of cellular competence seemed to confer no particular advantage to the alkylated DNA; noncompetent *B. subtilis* cells reacted with such DNA in a way quite similar to that of competent bacteria, and in several cases the relative reactivity (compared with the untreated DNA-noncompetent bacteria system) was significantly increased (Table 1). It is believed that the competence is caused by the presence of specific proteins on the cell surface. These proteins are capable of recognizing and bringing in the DNA (4, 8). Thus, the lack of any enhancement of adsorption and penetration by the state of cellular competence is another example of the alteration of protein-nucleic acid specificity brought about by the alkylation of DNA (10, 15; H. Kubinski and C. B. Kasper, Abstr., 10th International Cancer Congress, Houston, Tex., p. 9–10, 1970).

That the heavily alkylated DNA may indeed bind to a set of receptors different from those preferentially reacting with the normal DNA was further suggested by the experiment shown in Fig. 4. In this experiment, radioactive, untreated transforming DNA and the same DNA treated for 10 min at 26°C with 50 mM BPL were adsorbed to competent cells in the presence of various amounts of unlabeled, untreated *B. subtilis* DNA. We expected to see a progressive inhibition of binding of the radioactive DNA in the presence of increasing amounts of "cold" DNA, if the receptors for the two DNAs are identical (24). Such an expected decrease was, in fact, observed for the untreated, radioactive DNA, and the magnitude of the decrease was related to the amount of the unlabeled, compet-

TABLE 1. *Binding, uptake, and transforming activity of B. subtilis DNA alkylated with either BPL or PS*[a]

Chemical	Time of exposure (min)	Cell type	Medium	Binding (%)	Uptake (%)	Transformation of *tyr* marker (%)
BPL (20 mM)	2	Competent	Spizizen	100	78.1	39.7
	25	Competent	VB	22.7		1.2
	25	Competent	Spizizen	57.3		7.9
	60	Competent	VB	11.5	5.8	0
	60	Competent	TSM	23.9	16.7	0
	60	Competent	TSM + 15 mM $NaNH_4PO_4$	14.2	8.3	
	60	Noncompetent	VB	425		
	60	*E. coli* Q13	VB	108		
PS (50 mM)	2	Competent	Spizizen	86.7		39.8
	25	Competent	VB	37.2	31.4	5.1
	25	Competent	Spizizen	48.7	38.4	
	25	Noncompetent	VB	644	400	
	25	*E. coli* Q13	VB	344		
	40	Competent	VB	13.9	3.5	0
	40	Competent	TSM	24.9	8.2	0.08

[a] Results are expressed as a percentage of the untreated control. Incubation was for 60 min at 37°C.

ing DNA present. No such decrease, however, was seen when the alkylated radioactive DNA was competing against the untreated, unlabeled DNA. At low concentrations, the normal DNA actually enhanced the attachment of BPL-treated DNA, and the degree of that enhancement was related to the time of exposure to BPL.

We do not understand the mechanism of this increase of attachment. Possibly, the reaction between normal nucleic acid and specific receptors of a competent cell may uncover other proteins which are capable of reacting with the alkylated DNA. Other explanations are also possible. At very high concentrations of the competing DNA, binding of both kinds of radioactive DNA was inhibited to a comparable degree. This somewhat exotic pattern of enhancement of the binding at low concentrations and an inhibition at higher concentrations of competing DNA was quite reproducible and was found in subsequent experiments not to be influenced by minor variations in the experimental protocol. In all of these experiments, however, the magnitude of the enhancement observed at low concentrations of unlabeled DNA was related to the degree of alkylation of the DNA against which there had been competition.

From a series of other experiments we subsequently learned that the BPL-treated (0.1 M BPL for 60 min at 37°C) unlabeled DNA neither inhibited nor enhanced the binding of untreated radioactive *B. subtilis* DNA. It competed, however, with the radioactive, BPL-treated DNA, further confirming the notion that the normal and the alkylated DNAs react with different receptors on the surface of a competent *B. subtilis* cell. The PS-treated DNA (0.1 M PS for 60 min at 37°C) strongly inhibited the binding of the BPL-treated radioactive DNA and only marginally inhibited that of normal, untreated DNA.

Further evidence indicating different mechanisms of cellular binding for the normal and alkylated DNAs was found when we compared the relative transforming efficiency of the alkylated DNA after its incubation with competent cells in VB and Spizizen media. We observed that consistently higher numbers of transformants were found with the latter medium. Additional experimentation revealed that one of the constituents of the VB medium, sodium ammonium phosphate, specifically inhibited binding of the alkylated DNA (Table 1). No such inhibitory effect was observed with the untreated DNA.

The rates of cellular attachment and penetration for nucleic acids are related to their molecular weights (9). It seemed conceivable, therefore, that some of the observed differences between the normal and the alkylated DNAs merely reflected the differences in their respective molecular weights. To test this possibility, the alkylated and nonalkylated radioactive DNAs were fractionated in sucrose density gradients and the binding kinetics of DNA with similar sedimentation rates were compared. The results (data not shown) confirmed the initial observation of a higher rate of binding to competent cells for normal versus alkylated DNA across the whole spectrum of sizes for *B. subtilis* DNA.

Distribution of single markers and markers closely linked in normal and alkylated DNA after velocity centrifugation.

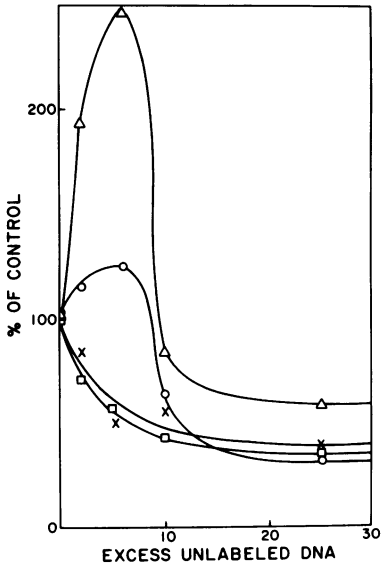


FIG. 4. *Binding of ^{32}P-labeled B. subtilis DNA to competent B. subtilis cells in the presence of various amounts of homologous DNA. The radioactive DNA (4 μg/ml) was divided into two equal portions and incubated for 10 min at 26°C either alone or with 50 mM BPL. Both samples were then precipitated with ice-cold ethyl alcohol, dissolved in equal volumes of 1× SSC, and used for binding to bacterial cells in the presence of various amounts of untreated, unlabeled DNA. Samples were taken at 30 and 60 min and centrifuged, and the sediment was washed once with TSM. The amounts of cell-bound radioactive DNA were then established, and the results were calculated as a percentage of the identical DNA bound under the same conditions in the absence of unlabeled DNA (zero excess). Symbols: △ and ○ binding of the alkylated DNA at the end of 60 and 30 min, respectively; × and □, 30- and 60-min time points for the untreated DNA.*

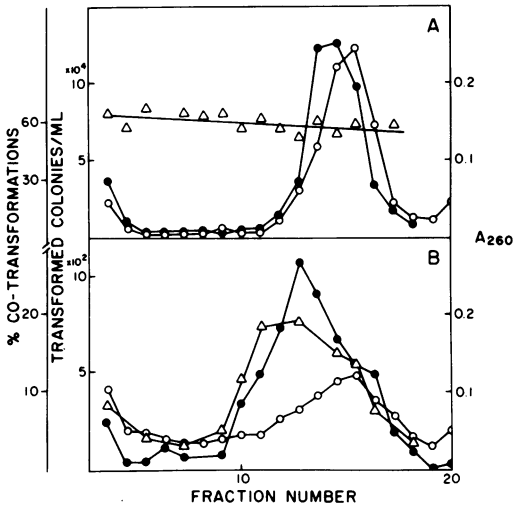


FIG. 5. *Sucrose density gradient centrifugation of 10 μg of B. subtilis transforming DNA, untreated (A) or exposed for 15 min to 0.1 M BPL at 37°C (B). The DNA or the mixture of BPL and DNA were centrifuged for 150 min at 4°C (without a prior alcohol precipitation) in a Spinco SW50.1 rotor at 42,000 rpm. Fractions were collected from the bottom, and the distribution (○) of DNA was measured in a spectrophotometer at 260 nm (A_{260}, absorbance at 260 nm). Subsequently, the ability to transform to tyrosine independence was measured (●) and the cotransformation of the linked marker (trp; △) was established for the transformed colonies. Sedimentation was from right to left.*

DNA samples treated for various lengths of time with BPL were chilled on ice, layered on top of precooled sucrose gradients, and centrifuged. The fractions were collected from the bottom, and the distribution of the UV-absorbing material and the rates of transformation for single and linked markers were established. The results of a typical experiment are shown in Fig. 5. The bulk of untreated *B. subtilis* DNA was recovered from the gradient as a somewhat heterogeneous band with the peak sedimenting at approximately 18 to 20S (as calculated from the rate of sedimentation of the two ribosomal *E. coli* RNAs under identical experimental conditions). The peak of transforming activity for either *trp* or *tyr* markers sedimented slightly ahead of the peak of optical density. Other than being skewed in the direction of heavier DNA, the gradient distribution of tranforming and UV-absorbing activities were quite similar. In contrast, the ability to cotransform linked markers was distributed through the gradient in a linear fashion, its value usually slightly higher close to the bottom and slightly lower in fractions close to the top of the centrifuge tube. The *trp* marker was cotransformed in 50 to 60% of colonies already transformed to tyrosine independence; *tyr* was cotransformed in about 30% of those already transformed for *trp*. As will be shown below, the rate of cotransformation changed with the increased time of incubation of DNA with competent cells. Under a standard set of experimental conditions, however, the rate of cotransformation of these markers was quite reproducible for a given DNA lot. The unlinked (distant) marker (leucine) was cotransformed at a much lower rate than were the two closely linked ones; 0.8% of colonies transformed for the *tyr* marker did not require leucine for growth, compared with a value of 0.5% for colonies already transformed to tryptophan independence.

Exposure of transforming DNA to BPL or PS before its centrifugation in sucrose gradients changed its sedimentation pattern, and, predictably, the degree of change was related to the

concentration of the chemical and the length of exposure to it. The transforming activity of DNA and its ability to cotransform both the linked and unlinked markers were rapidly lost (although, as will be shown below, not at the same rate). The peak of transforming activity was recovered at roughly the same position in the gradient irrespective of the degree of alkylation. The distribution of cotransformation of close markers, however, underwent a change. As indicated above, there was a weak but reproducible correlation between the size of the untreated DNA (as defined by its sedimentation rate) and its ability to cotransform, although the differences between the largest and the smallest molecules were not substantial (Fig. 5A) due to a relative homogeneity of fractions chosen from preparative sucrose gradients which were pooled and then used for this second round of sucrose gradient centrifugations (see above). On the other hand, BPL-treated DNA progressively presented a bell-shaped distribution pattern of cotransformation of close markers (Fig. 5B), with the peak of cotransformation roughly at the same position in the gradient as that of the transforming activity. Evidently, in contrast to the normal DNA, some of the alkylated molecules apparently larger than the bulk of DNA were less likely to cotransform close markers. The large complexes of carcinogen-treated DNA (megamolecules; 12) are recovered in this region of the gradient under our experimental conditions.

We examined the colonies transformed with alkylated DNA for the cotransformation of distant markers. None was found in bacteria transformed with DNA exposed for longer than 10 min to 0.1 to 0.15 M BPL or PS at 37°C.

Inactivation of transforming DNA by BPL in the presence of albumin. Addition of a protein to the mixture of nucleic acids and BPL or PS increases the rate of formation of macromolecular complexes, presumably due to the production of nucleic acid-protein and nucleic acid-protein-nucleic acid adducts (20, 26). In a series of tests we established that the presence of a protein during the treatment of transforming DNA with BPL alters the rate of inactivation of such DNA. In one typical experiment, 20 μg of *B. subtilis* DNA was exposed at 37°C to 0.1 M BPL in the presence and in the absence of 800 μg of albumin per ml. At various times (5, 10, and 15 min) samples were withdrawn, chilled, layered on the top of precooled sucrose gradients, and centrifuged. After the collection of fractions and spectrophotometric reading of their density, 20-μl samples were taken from each fraction to transform competent bacteria to tyrosine independence. No major differences in the sedimentation patterns of these two groups of alkylated DNA samples were observed under such experimental conditions. However, significantly fewer colonies were found on plates with cells treated with DNA alkylated in the presence of albumin. The total number of transformants was, on average, only 35.4% of the number of transformants produced under identical conditions by DNA exposed to BPL alone. In control experiments we established that transforming DNA is not inactivated by albumin in the absence of alkylating agents.

Short exposure of transforming DNA to alkylating agents: properties of E-DNA. *B. subtilis* transforming DNA was exposed to either 0.1 M BPL or 0.1 M PS for 2 to 5 min at 26°C; this was followed by the removal of the chemical by alcohol precipitation. As expected, the ability to transform to tyrosine independence was lost more rapidly than was the *trp* marker (Fig. 6). The cotransformation of close markers was similarly affected (data not shown). The appearance of the second marker, however, in the transformed colonies was significantly delayed for DNA even briefly treated with the alkylating agent, as indicated by the slopes of the curves shown in Fig. 7. We believe that this

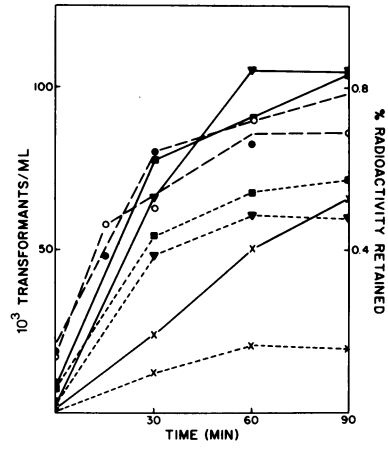


FIG. 6. *Cellular uptake of and transformation by B. subtilis ^{32}P-labeled DNA, untreated or briefly treated with 50 mM BPL (3 min at 26°C). The cells, brought to competence in Spizizen medium, were incubated with the DNA at 37°C with constant stirring. At various times samples were withdrawn and tested for the ability to transform various markers and for the uptake of deoxyribonuclease-resistant, ^{32}P-labeled DNA. Symbols: ○, uptake of ^{32}P-labeled, BPL-treated DNA; ●, uptake of ^{32}P-labeled untreated DNA; ×, transformation of tyr marker; ▼, transformation of trp marker; ■, transformation of leu marker. For ×, ▼, and ■ solid lines indicate untreated DNA and dashed lines indicate BPL-treated DNA.*

slowdown in penetration through the cell membrane was caused either by the presence of "knots" of alkylated bases or by the decrease in flexibility of the E-DNA (12). It is also possible that the increase in length of the E-DNA molecule may physically increase the distance between markers and thus in part be responsible for the delay of the appearance of the second marker in the E-DNA treated culture.

Figure 8 summarizes an experiment in which transforming DNA was briefly treated with BPL and then fractionated in a sucrose density gradient. Compared with normal DNA (Fig. 5A), the ability to transform single markers and to cotransform close markers was significantly decreased. Interestingly, the cotransformation of a distant marker (*leu*) actually increased, and after 2 to 3 min of inactivation under these conditions up to three times more cotransformed colonies were found in samples treated with certain fractions taken from sucrose gradients in which such E-DNA was analyzed, compared with corresponding fractions of identical size and optical density but containing an untreated DNA. This increase was highly reproducible. An analysis across the gradient of the inactivation rates for cotransformation of distant markers compared with that of the close ones (which perhaps should be considered a better indicator of inactivation of DNA than is the transformation of a single marker) revealed that the treated DNA showed a very narrow band of increased rate of

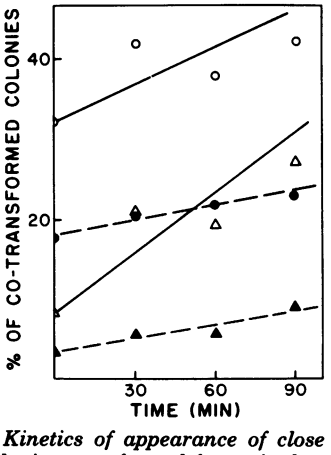


FIG. 7. *Kinetics of appearance of close markers among colonies transformed for a single marker by untreated B. subtilis DNA (solid lines) or the same DNA treated with 50 mM BPL for 3 min at 26°C (dashed lines). The cells were incubated with DNA under standard conditions, and samples were withdrawn at the indicated times and tested for transformations of single markers. After 48 h, cotransformation of the second marker was tested by replica plating. Symbols: △ and ▲, trp → tyr; ○ and ●, tyr → trp.*

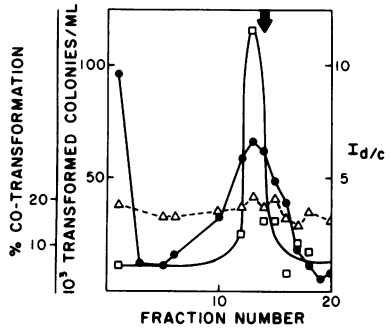


FIG. 8. *Distribution in a sucrose density gradient of B. subtilis DNA (20 μg) transforming and cotransforming activities after its exposure to 50 mM BPL for 2 min at 26°C. The DNA was then cooled on ice, precipitated with cold ethanol, dissolved in 1× SSC, and centrifuged in the sucrose gradient. The distribution of transforming activity (tyr marker; ●) and cotransforming activities for trp (△) and leu (data not shown) were established as described in the text. □, Relative proportion of cotransformations of distant and close markers ($I_{d/c}$ index; see text). The heavy arrow shows the position of the peak of optical density at 260 nm. Sedimentation was from right to left. For further details of centrifugation see the legend to Fig. 5.*

cotransformation of distant markers relative to that of linked markers (Fig. 8). In some experiments and in some fractions the $I_{d/c}$ index was increased by as much at 15 to 20 times over that of the control. (The $I_{d/c}$ index expresses the relative proportion between cotransformation of distant and close markers and is measured as follows: $I_{d/c}$ = [percent cotransformation of distant markers, treated DNA/percent cotransformation of distant markers, untreated DNA]/[percent cotransformation of close markers, treated DNA/percent cotransformation of close markers, untreated DNA].) The close and distant marker cotransformation indexes are measured for an untreated DNA of identical sedimentation rate. Such DNA was centrifuged and analyzed as part of the same experiment. The value of $I_{d/c}$ for 0-min incubation time with BPL is, by definition, 1. Although in all those runs we were unable to distinguish between the gradient positions of normal and E-DNA (because of similarities in their sedimentation rates [13]), the position of the band corresponds to that expected for E-DNA. The sedimentation rate of this high $I_{d/c}$ band did not change appreciably during the course of incubation with BPL. The value of the index, however, changed very rapidly, to reach the peak about 2 min after the beginning of incubation, and then plunged below 1 (control) after some 3 min (Fig. 9). This experiment also explains why the relative increase in

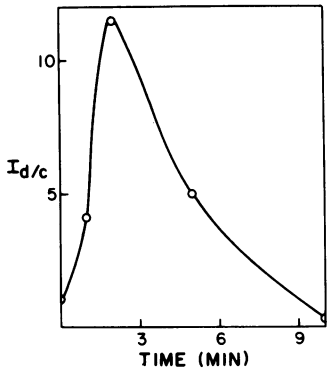


FIG. 9. *Changes in the relative proportions between cotransformation of distant and close markers ($I_{d/c}$ index; see text) during incubation of B. subtilis DNA with 50 mM BPL at 26°C. The samples were withdrawn at various times, precipitated with alcohol, and sedimented in sucrose gradients, and the distribution of distant (tyr → leu) and close (tyr → trp) markers was established. For further details see the legend to Fig. 8. The peak values for $I_{d/c}$ from each gradient were then used to plot distribution versus time of incubation of DNA with the chemical.*

$I_{d/c}$ is barely noticeable in experiments (data not shown) in which unfractionated DNA is examined after its brief exposure to BPL; an $I_{d/c}$ averaged for a heterogeneous sample of DNA is seldom higher than 1.5, even at the 2-min maximum, when it is so apparent during the sucrose gradient analysis.

DISCUSSION

In this study, we attempted to correlate the known physicochemical changes in the alkylated DNA with changes in the biological activity of these molecules. After the exposure of DNA to a simple alkylating agent, such as BPL or PS, several independent changes are observed, beginning with a rapid inactivation of the transforming activity. The rate of inactivation of individual markers does not seem to correlate with differences in base composition, as suggested by essentially similar inactivation rates of these markers by BPL, PS, and UV. Most likely, the differences in their sensitivity are due to the differences in the size of the marker, although some other causes may be responsible. However, the sequence of sizes of these markers has been confirmed by the experiment in which the frequency of their appearance in the population of transformed colonies was tested (Fig. 3). A correlation between the size of a marker and the rate of inactivation of transforming DNA has been also noted in other studies (24).

The shapes of the inactivation curves for the *tyr* and *trp* markers are appreciably and reproducibly different, with the presence of a shoulder in the *trp* inactivation curve. The reasons for this difference are unclear and require further study. A preferential repair or an intrinsic heterogeneity (duplication?) of the *trp* locus may perhaps be responsible for the observed shoulder.

The cellular attachment and penetration of the alkylated DNA is severly impaired. Shortly after the beginning of incubation of DNA with BPL, a marked decrease is observed in the rate of uptake of even this DNA, which is destined to eventually enter the cell, as judged by the rate of appearance of close markers among the transformed colonies (Fig. 7). Further exposure to alkylating agents alters even more drastically the binding specificity of the alkylated DNA, which then no longer is being recognized by the specific surface receptors of a competent cell. Instead, such DNA appears to bind to some other nonspecific site(s) on the surface of either the competent or noncompetent cells. Understandably, this binding is not followed by penetration into and the transformation of the bacterial cell. A moderate excess of normal DNA no longer interferes with this interaction. Such binding, however, is inhibited by the presence of sodium ammonium phosphate, which shows no effect on binding of normal, untreated DNA. The mechanisms of this inhibition are obscure. Possibly, this salt specifically alters the molecular conformation of the receptor protein(s) for the alkylated DNA. If this interpretation is indeed correct, this observation may prove useful in the isolation of this as yet hypothetical protein molecule(s).

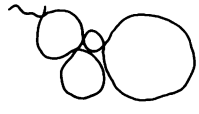
DNA exposed to BPL or PS in the presence of a model protein (albumin) is inactivated more rapidly than is DNA which has been exposed to the carcinogen alone. Such an increase in the degree of inactivation due to the presence of a protein which by itself has no effect on transformation of an untreated DNA appears somewhat paradoxical at first; the alkylating agents, including BPL and PS, react readily with nucleophilic centers in both the proteins and the nucleic acids (2, 3). Thus, a degree of protection rather than an inactivation may be expected. Under the conditions of the experiment, however, a substantial number of artificial nucleoproteins are formed by the action of the chemical on the mixture of albumin and DNA (20, 26). Evidently, the presence of an albumin molecule tightly bound to DNA inhibits transformation, possibly by interfering with the penetration of DNA into the host cell or with its further intracellular processing and chromosomal incorporation (8).

The kinetics of inactivation of linked markers was parallel to that of the single ones. Again,

there was a shoulder in the inactivation curve for the *trp* marker among the colonies already transformed to tyrosine independence. In all experiments based on the experimental protocol outlined in the legend to Fig. 2, carried out with or without minor changes of such protocol, we observed that the second marker was always inhibited to a lesser degree than what could have been anticipated on the basis of the rate of inhibition of single markers and assuming that the second marker had a random and equal chance of being "hit". This is indicated by the slightly but reproducibly lower slope of inactivation for the second marker, compared with that of the single one, as if a marker located on a piece of DNA which as yet suffered no damage elsewhere, had a better chance to survive than an average marker of the same kind in the tested population.

This is not entirely surprising. The DNA molecules in solution are obviously heterogeneous as to their size, base composition, etc., and thus a process of natural selection is likely to remove those molecules which are least likely to survive the chemical or physical insult. These experiments also suggest that any damage to DNA between (or outside) the two studied linked markers must be either quite rare or, alternatively, must affect only marginally the cotransformation of the second marker. This is a rather unexpected observation, because shear and ionizing radiation inactivation experiments indicate

TABLE 2. *Synopsis of physical and biological characteristics of alkylated (E- and L-DNA) versus normal DNA*

Characteristic	Normal (no treatment)	E-DNA (treated for 1 to 5 min at 37°C with 50 mM BPL or PS)	L-DNA (treated for >10 min at 37°C with 50 mM BPL or PS)
Physical properties (see reference 12)			
Sedimentation	18–24S	Slightly increased	4–8S 30S
Electrophoretic mobility	18–24S	Slightly increased	4–8S Not admitted to the gel
Electron microscopy			
Cellular binding and penetration			
Binding to competent cells	Good	Good	Decreased
Uptake by competent cells	Good	Slower	Strongly decreased
Inhibition by $NaNH_4HPO_4$	No		Yes
Competition by normal DNA	Inhibits binding		Increases binding (up to 5× excess)
Competition by alkylated DNA	No inhibition of binding		Inhibits binding
Binding to noncompetent cells	Poor		Increased
Uptake by noncompetent cells	Very little or none		Increased(?)
Biological properties			
Transformation	Good	Slightly decreased for some markers	Strongly decreased
Cotransformation of close markers	Good (30–60%)	Slightly decreased	Very strongly decreased
Cotransformation of distant markers	Good (0.5–0.8%)	Increased	None

that the linkage between linked markers is inactivated more rapidly than are the two single markers themselves (21).

Cotransformation of the leucine, i.e. distant, marker is altered in a different manner during the early stages of DNA inactivation. At the time when cotransformation of the linked markers is already appreciably decreased, this distant marker is cotransformed at a rate higher than that observed for normal DNA. There could be several reasons for this relatively high cotransformation of distant markers by the E-DNA. Most likely, the presence of a limited number of intramolecular bonds (cross-links?) in such DNA (12) may physically bring together the normally unlinked markers and, thus, increase their chances of being cotransformed.

After a longer incubation with the alkylating agent, the DNA progressively breaks down, and some of these broken molecules combine to form large complexes (megamolecules). This L-DNA has a highly decreased ability to transform single markers, and no cotransformation of distant markers is detectable, perhaps in part due to the difficulty of obtaining for further study a sufficient number of colonies transformed for a single marker. The close markers are cotransformed at a much lower rate than they are in the control, untreated DNA. The distribution of the cotransforming activity for close markers in DNA exposed to BPL or PS for long periods and fractionated in sucrose density gradients is different from that of untreated DNA and indicates that some of the large (fast-sedimenting) molecules of alkylated DNA are incapable of cotransforming linked markers. By repeating this type of experiment several times we have excluded the possibility that this apparent decrease of cotransformation with an increasing molecular weight is an artifact of the low numbers of colonies transformed for a single marker and available for further analysis. We interpret the paucity of cotransforming DNA in the areas of the gradient where we would expect to find the megamolecules as an indication that these complexes are composed of short pieces of DNA. Their poor penetration into the bacterial cells may indicate that only the relatively unaltered DNA chains on the periphery of the complex are taken into the cell and eventually incorporated into its genome.

Table 2 summarizes the relationships between the physicochemical changes in DNA induced by the two monoalkylating agents tested and the alterations in the biological activity of such DNA. We believe that the observed correlations not only are useful for a better understanding for the transformation process, but also may help to elucidate the mechanisms of mutagenesis and tumor induction by chemicals.

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