Recovery of Deoxyribonucleic Acid from the Effects of Alkylation

By B. S. STRAUSS

*Department of Microbiology, The University of Chicago, Chicago 37,
Illinois, U.S.A.*

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SUMMARY

The transforming activity for an indole locus of DNA extracted from a methionine-requiring strain of *Bacillus subtilis* was decreased about tenfold per unit of DNA by treatment of organisms with 0.025 M-methylmethanesulphonate for 30 min. at 37°. The treatment decreased the viable count to one tenth of the original and induced a reversion frequency of 10^{-5} . Transforming activity of DNA prepared from alkylated organisms was sensitive, to heating at 50° as compared to DNA extracted from non-alkylated organisms. Methylation and heating of DNA *in vitro* did not inhibit its uptake by competent organisms. Incubation of treated and non-treated organisms in amino acid supplemented medium led to an increase in extractable transforming activity. A period of incubation which led to a 34 % increase in DNA content more than doubled the transforming activity obtainable from methyl methanesulphonate treated organisms. The heat sensitivity of transforming DNA from alkylated organisms decreased after incubation of the organisms. Synthesis of DNA in the absence of a net DNA increase was demonstrated in methyl methanesulphonate treated organisms since thymidine-2-¹⁴C was incorporated without lag from the start of incubation into an alkali-stable hot acid-soluble form. The experiments are interpreted to mean that alkylated DNA can serve as a template for the replication of DNA of normal activity.

INTRODUCTION

One of the questions to be answered about any mutagenic agent is whether its reaction with the genetic material (deoxyribonucleic acid, DNA) of an organism is direct or indirect. If the mutagen methylmethanesulphonate reacted directly with DNA for example, it would be possible to show that immediately after treatment of organisms with alkylating agent, the DNA of the organism became heat sensitive (Strauss, 1962*a*). Since such a determination would require a biological assay for DNA activity, the experiments would have to be done with an organism capable of undergoing transformation. It seemed likely that the system described by Spizizen (1958) and Anagnostopoulos & Spizizen (1961) with *Bacillus subtilis* would be particularly satisfactory because of the possibility of obtaining auxotrophic mutants of this organism similar to those of *Escherichia coli*. The experiments to be described here show that treatment of *B. subtilis* with an alkylating agent resulted in heat sensitization of the DNA extractable from the treated organism. In addition, the DNA of methylated organisms gradually lost its abnormal heat sensitivity as these organisms were incubated in growth medium. Alkylated DNA appears to serve as a template for the production of normal DNA.

METHODS

Several strains of *Bacillus subtilis* were used. The first, a methionine-requiring mutant, was obtained by replica plating from a culture of the prototrophic *B. subtilis* which had been treated with ethylmethanesulphonate. This strain was used as a DNA donor for the transformations described in this paper with the exception of transformation with ^{32}P labelled DNA when a wild type donor was used. The methionine-requiring strain was selected because it could be induced to revert by treatment with methylmethanesulphonate (Fig. 1) as well as with the ethyl derivative, and because it would serve as a recipient strain for transformation as well as being a satisfactory donor. The strain was heat sensitive after methylation

Table 1. *Loss of the differential heat sensitivity due to alkylation upon incubation of a methionine-requiring mutant of Bacillus subtilis*

A washed suspension of cells from an overnight culture was treated for 30 min. at 37° with 0.025 M-methylmethanesulphonate in salts solution (0.048 ml. methylmethanesulphonate/20 ml. of salts solution). After treatment the organisms were washed and suspended in 10 ml. of salts solution. Two ml. were then added to flasks containing 38 ml. of minimal medium (Spizizen, 1958) supplemented with 0.02 % casein hydrolysate, and 50 $\mu\text{g./ml.}$ each of L-methionine and L-tryptophan. The flasks were incubated for the indicated time at 37° with shaking and then the contents were collected by centrifugation and washed with salts solution. The organisms were suspended in 5 ml. of salts solution and plated for viability before and after a heat shock of 50° for 6 min. The organisms were cooled in an ice bath after heating and were kept cold until plated. Viability was determined on minimal medium supplemented with 50 mg. of methionine and 20 mg. of yeast extract/l. and hardened by the addition of 1.5 % agar.

Pre-treatment of organisms	Incubation time in supplemented minimal medium (min.)	Viable organisms (no. $\times 10^{-8}$)		
		After washing in salts solution (a)	After washing in salts solution and heating 6 min. at 50° (b)	Ratio a/b:
None	0	4.8	0.32	15
	10	7.1	0.5	14
	30	3.9	0.44	8.9
Methylmethane-sulphonate (0.025 M)	0	0.81	0.00053	1530
	10	1.1	0.009	122
	30	1.2	0.12	10

as indicated by the inactivating effect of a 6 min. incubation in buffer at 50° and the differential heat sensitivity disappeared after the start of incubation in minimal medium (Spizizen, 1958) supplemented with 0.02 % acid casein hydrolysate and 50 $\mu\text{g./ml.}$ each of L-tryptophan and L-methionine (Table 1). As the recipient strain for transformation we used the histidine + tyrosine + indole requiring triple mutant described by Nester & Lederberg (1961). This strain, supplied by Dr E. Nester, was used to permit the simultaneous determination of the effects of alkylation on linked markers. In one series of experiments utilizing DNA labelled with ^{32}P , wild type *Bacillus subtilis* was used as the DNA donor and the indole requiring strain (168) was used as recipient.

Fig. 1

Fig. 2

Fig. 1. Effect of methylmethanesulphonate on the viability and reversion frequency of a methionine-requiring mutant of *Bacillus subtilis*. Broken line represents viability; circles indicate organisms treated with 0.025 M-methylmethanesulphonate; points represent non-treated control. Unbroken line, triangles represent reversion frequency of the treated samples; squares represent spontaneous mutation frequencies. Abscissa—time incubated with mutagen at 37°. An overnight culture of organisms was harvested, washed and suspended in 20 ml. salts solution. Ten ml. of suspension were added to 10 ml. of salts solution containing enough methylmethanesulphonate (0.043 ml.) to make the suspension 0.025 M. A second 10 ml. of suspension were added to 10 ml. of salts solution as a control. The resulting suspensions were incubated at 37° with occasional shaking. At the indicated intervals 2.5 ml. of suspension were removed and added to 7.5 ml. of cold salts suspension, centrifuged, washed once and made to 2.5 ml. with salts solution before plating. Revertants were determined on plates of minimal agar supplemented with 100 mg. yeast extract/l. Survivors were determined on plates of minimal agar (Spizizen, 1958) supplemented with yeast extract and 50 mg./l. methionine. Cell dilutions were made in salts solution to which 4 drops of 'Tween 80'/l. were added to prevent aggregation.

Fig. 2. Relation of DNA concentration to the number of transformants. DNA concentration is estimated from the absorption at 260 mμ of the purified preparation assuming an optical density of 200 for a 1% (w/v) DNA solution. An overnight culture of the histidine + indole + tyrosine requiring triple mutant was used to inoculate 10 ml. of CHT 50 medium. After 4 hr. incubation at 37° with aeration the culture was harvested and diluted into 50 ml. of CHT 5 medium. Twenty-five ml. amounts of the suspension in CHT 5 were incubated 90 min. at 37° with shaking in Erlenmeyer flasks. Glycerol (2.5 ml.) was then added to one flask and the culture was quick frozen by immersion in a solid CO₂-acetone bath and stored at -25°. Non-frozen organisms (0.9 ml.) were then added to dilutions of standard DNA (0.1 ml.) and incubated 30 min. Deoxyribonuclease (5 μg./ml.) was added and after 5 min. the suspensions were plated for the determination of transformants at the indole locus. After this plating was concluded the frozen suspension was thawed by suspension with shaking for 15 min. in a water bath at 37° and this 'frozen' suspension was used as a recipient for transformation as described above.

Transformation was by slight modification of the method of Anagnostopoulos & Spizizen (1961). An overnight culture was inoculated into liquid minimal medium (Spizizen, 1958) containing 0.5 % glucose, 0.02 % acid hydrolysed casein and 50 μg . L-tryptophan/ml. (CHT 50 medium). After 4 hr. incubation with aeration at 37° the culture was harvested by centrifugation and diluted ten-fold in fresh minimal medium containing 0.5 % glucose, 0.01 % casein hydrolysate, 5 μg . L-tryptophan and an additional 5 μM /ml. of MgSO_4 (CHT 5 medium). The organisms were incubated for 90 min. at 37° with shaking in 25 ml. of CHT 5 medium in a 250 ml. Erlenmeyer flask. Glycerol (2.5 ml.) was then added and the organisms were frozen by immersion of the flask in a solid CO_2 -acetone mixture. Frozen organisms were stored at -25° for up to 10 days before use. During this storage period the competence of the frozen suspension gradually decreased to about one-tenth the original value for the frozen organisms. Frozen organisms were used for transformation as follows: the suspension was thawed by shaking for 15 min. in a water bath at 37° and then 0.9 ml. of suspension was added to a series of 150 $\times$ 16 mm. test tubes containing 0.1 ml. of the DNA solution to be assayed. The mixture of DNA plus organisms was shaken for 30 min. at 37° before addition of deoxyribonuclease (Worthington Biochemical Co.) to 5 μg . The mixture was incubated with deoxyribonuclease for 5 min. at 37° before being plated for transformants. Transformation was scored on minimal medium (Spizizen, 1958) hardened with 1.5 % agar and containing 20 mg. of Difco yeast extract/l. When only indole transformants were scored this medium was supplemented with 150 mg. casein hydrolysate/l. When histidine and tyrosine transformants were scored the minimal medium plus 20 mg. yeast extract/l. was supplemented with the proper combination of L-tryptophan (20 μg .), L-tyrosine (20 μg .), or DL-histidine (40 μg .). Viable cell count was determined on minimal medium supplemented with 0.02 % casein hydrolysate, 0.02 % yeast extract and 20 μg . L-tryptophan/ml. Freezing decreased the amount of transformation obtainable from a given DNA preparation (Fig. 2) and the total percentage transformation was low (0.01–0.03) in our procedure using the triply auxotrophic mutant. However, the response to DNA was linear and reproducible.

Purified DNA for transformation experiments was prepared by the method of Marmur (1961) and was treated with alkylating agent *in vitro* by diluting the DNA in 0.1 M phosphate buffer (pH 7.0) containing either methyl- or ethyl-methanesulphonate to give a final concentration of DNA of 60 μg . and allowing the reaction to proceed at 37°. Two volumes of 95 % ethanol in water were then added and the suspension centrifuged after standing in the cold for 15 min. The precipitate was taken up in 0.015 M-sodium chloride + 0.0015 M-trisodium citrate (dilute saline + citrate; Marmur, 1961), the solution made 0.15 M-NaCl + 0.015 M-citrate (saline + citrate) and the DNA reprecipitated with ethanol and taken up in saline + citrate once again. This procedure was considered necessary to remove unreacted alkylating agent. DNA labelled with ^{32}P was prepared by growing wild type *Bacillus subtilis* in low phosphorus medium supplemented with $^{32}\text{PO}_4$ as described by Young & Spizizen (1961). The DNA was extracted from these organisms by the method of Marmur (1961).

In some of these experiments, organisms were treated with the mutagen and the DNA extracted from treated cells by a modification of the method of Stuy (1960)

who extracted DNA from an *Haemophilus* after X-ray irradiation. *Bacillus subtilis* was incubated overnight on slopes of a minimal salts medium (Spizizen, 1958) supplemented with yeast extract (0.15%), acid hydrolysed casein (0.1%), Difco nutrient broth (0.8%) and DL-tryptophan (40 mg./l.). This minimal salts medium had the following composition per litre of medium: $(\text{NH}_4)_2\text{SO}_4$, 2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; K_2HPO_4 , 14 g.; KH_2PO_4 , 6 g.; $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$, 1 g.; glucose (sterilized separately), 5 g. This solution without glucose will be called 'salts solution' throughout this paper. Organisms were harvested by suspension in salts solution which was passed through a gauze filter to remove agar fragments. The organisms were washed and resuspended in salts solution and portions of the suspension were then added to salts solution in which sufficient methylmethanesulphonate was dissolved to make the final solution 0.025 M (0.085 ml./40 ml.). After reaction for 30 min. at 37°, the suspensions were harvested, suspended in 0.15 M-NaCl + 0.1 M-ethylene diaminetetra-acetate (pH 8; saline EDTA), centrifuged and suspended in a small volume of saline EDTA. Crystalline lysozyme was added to 1 mg./ml. and the suspension incubated for 30 min. at 37°. Sodium lauryl sulphate (0.5 ml. of a 25%, w/v, solution) was added, followed in 5 min. by sodium perchlorate to make the solution M. After deproteinization by shaking for 30 min. at room temperature with chloroform and isoamyl alcohol (24+1) the suspension was centrifuged for 10 min. at 5000 g and the upper layer transferred to a centrifuge tube. The tube was cooled and two volumes of cold 95% (v/v) ethanol added; after mixing and standing for 15 min. in the cold the suspension was centrifuged for 10 min. at 25,000 g, the supernatant poured off and the residue taken up in dilute saline citrate. The solution was made 0.15 M-NaCl + 0.015 M-citrate and clarified by centrifugation. The clarified supernatant was used as crude DNA extract.

DNA was determined by the diphenylamine reaction (Burton, 1956) with purified salmon sperm DNA as a standard. When small amounts of DNA were to be determined, 2 mg. bovine serum albumin were added as a carrier for the acid precipitate. Thymidine-2-¹⁴C incorporation into organisms was determined on Millipore membrane filters as described by Kahan (1960). DNA was identified as material which could still be precipitated by cold perchloric acid after incubation for 1.5 hr. at 37° in N-NaOH but which was solubilized by treatment at 70° for 30 min. in 0.5 N-perchloric acid.

RESULTS

Purified DNA preparations were inactivated exponentially by heating at 50° after alkylation *in vitro* with either ethyl- or methyl-methanesulphonate (Fig. 3). Alkylation decreased the initial transforming activity and methylmethanesulphonate caused a greater inactivation on a molar basis than did the ethyl derivative. Inactivation of the linked indole and histidine markers occurred at the same rate on heating alkylated DNA. Labilization of DNA to heat after preliminary alkylation was first demonstrated by Zamenhof, Leidy, Hahn & Alexander (1956) who showed a complete inactivation of alkylated *Haemophilus* DNA after heating for 1 hr. at 76°. The decrease in activity of alkylated DNA and of DNA alkylated and heated for 5 hr. at 50° was not due to the failure of competent cells to take up this treated DNA (Table 2). DNA labelled with ³²P was fixed in a deoxyribonuclease-resistant form almost as efficiently after alkylation, and alkylation and heating as was control

labelled DNA in spite of the great difference in transforming efficiency of the treated and untreated preparations. In this experiment competent organisms of the indole-requiring strain (168) were prepared by the method of Young & Spizizen (1961) and used without preliminary freezing. Similar results showing that *Pneumococcus* transforming DNA inactivated by nitrogen mustard is taken up by competent cells have been obtained by Lerman & Tolmach (1959).

To determine whether alkylation and heating led to drastic degradation, the sedimentation coefficient of *Bacillus subtilis* DNA was determined in the Spinco

Fig. 3. Effect of heating at 50° on transforming activity. *A*, control, non-treated DNA; *B*, DNA which has been treated with 0.025 M-methylmethanesulphonate and freed from excess alkylating agent by precipitation twice with ethanol as described in the text; *C*, DNA treated with 0.1 M-ethylmethanesulphonate and treated as in *B*; *D*, DNA treated with 0.2 M-ethylmethanesulphonate and treated as in *B*. Four ml. of DNA at a concentration of 60 μ g./ml. were reacted with alkylating agents for 1 hr. at 37° in 0.1 M-phosphate buffer (pH 7.0). After precipitation with ethanol and redissolving the precipitate in dilute saline + citrate the samples in 0.15 M-NaCl + 0.015 M-citrate were diluted 1:10 in salts solution before heating. Similar results were obtained when the heating at 50° was carried out in 0.1 M-phosphate buffer (pH 7.0).

Transformation at the indole locus was determined as described in the text using several dilutions of the treated DNA. The values for transformants were determined at DNA dilutions giving about 100 transformants per plate and were then corrected to the same dilution of DNA.

ultracentrifuge at a speed of 42,040 rev./min. with ultraviolet optics. The DNA was centrifuged in a solution made 0.15 M-NaCl + 0.015 M-citrate and the rotor temperature was kept below 8° to retard any further decomposition of alkylated DNA. The S_{20W} value of non-alkylated DNA was 26; DNA treated with 0.025 M-methylmethanesulphonate for 1 hr. at 37° gave an S_{20W} value of 25. After 6 hr. at 48° (a treatment which inactivated the DNA) the methylmethanesulphonate treated material had a S_{20W} value of 28.

When organisms were treated with 0.025 M-methylmethanesulphonate for 30 min.

Table 2. *Effect of alkylation and heating on the uptake of ^{32}P labelled Bacillus subtilis DNA*

Bacillus subtilis labelled with ^{32}P was prepared by growing wild type organisms on the medium described by Young & Spizizen (1961) and containing 5 mc. of $^{32}PO_4$ in 400 ml. of medium. DNA was prepared from these labelled cells by the method of Marmur (1961). The DNA was stored in saline citrate and after standing one day 76 % of the counts were precipitable with cold trichloroacetic acid plus carrier. The labelled DNA was alkylated as follows: 1 ml. of ^{32}P labelled DNA (optical density at 260 $m\mu$ = 2.14) was incubated for 90 min. with 1 ml. of 0.05 M-methylmethanesulphonate in 0.1 M phosphate buffer (pH 7.0). Two volumes of cold 95 % ethanol in water were added, the precipitate was collected by centrifugation and dissolved by adding 0.9 ml. of dilute saline citrate followed by 0.1 ml. of 1.5 M-sodium chloride + 0.15 M-sodium citrate. The resulting solution was diluted 1 to 6.25 and divided into two parts. One portion was heated for 5 hr. at 50° while the second was stored in the cold. Control DNA was taken through the same procedure with only the methylmethanesulphonate eliminated from the initial treatment.

Competent indole *Bacillus subtilis* organisms were prepared following the procedure of Anagnostopoulos & Spizizen (1961). After 90 min. incubation in CHT 5 medium (see text) this suspension was incubated with ^{32}P labelled DNA, 0.9 ml. of suspension was incubated with 0.1 ml. of the DNA solutions described above as well as with 1/10 and 1/100 DNA dilutions. At the conclusion of a 30 min. incubation period, 0.1 ml. of a 500 μ g./ml. solution of crystalline deoxyribonuclease (Worthington Biochemical Co.) was added to the mixture of organisms and DNA. As a control separate portions of the DNA solutions were pretreated for 30 min. at 37° with deoxyribonuclease (plus 0.1 ml. of 0.1 M-MgSO₄) and then 0.9 ml. of organisms in CHT 5 medium (which was supplemented with extra Mg ion as described by Anagnostopoulos & Spizizen (1961)) was added. The difference between the counts fixed when deoxyribonuclease treatment of the DNA followed or preceded incubation of the organisms with DNA was taken as representing the DNA taken up. After incubation the organisms were either filtered through Millipore membrane filters, washed with salts solution and counted or used for the determination of transformants at the indole locus. The concentration of DNA was calculated from the optical density of its solutions at 260 $m\mu$ assuming that a 1 % DNA solution has an optical density of 200. The data are given for incubation of 1.16×10^8 viable organisms with 0.16 μ g. of DNA labelled with ^{32}P (6.06×10^4 c.p.m./ μ g. DNA) in 1.0 ml. of medium.

DNA sample	Transformed cells/ml.* at a DNA concentration of 0.16 μ g./ml.	Deoxyribonuclease added			μ g. DNA taken up/ transformed cell (no. $\times 10^6$)	μ g. DNA taken up viable cell (no. $\times 10^{10}$)
		After incubation (c.p.m. fixed)	Before incubation (c.p.m. fixed)	Difference as μ g. DNA		
Control	6.97×10^5	1518	382	0.019	0.027	1.6
Control-heated	5.51×10^5	1057	198	0.014	0.026	1.2
Methylated	1.80×10^5	1626	310	0.022	0.121	1.9
Methylated-heated	0.0537×10^5	1056	189	0.014	2.67	1.2

* Calculated from the numbers obtained at a DNA concentration of 0.0016 μ g./ml. assuming linearity of response. The high values for transformants in this experiment are due to the use of fresh (unfrozen) cells of a highly competent indole strain.

at 37° (a treatment which decreased the fraction of viable organisms to 0.1, Fig. 1) the amount of transforming activity extractable was decreased as compared with the amount extracted from untreated organisms. In one experiment (Table 4) the transforming activity obtained from methylated organisms was 530 units (transformed colonies/ μ g. DNA) as compared to 6150 units for material from control organisms. The transforming DNA obtained from methylated organisms was inactivated by heating at 50° (Fig. 4).

Further purification of the extracts from treated and untreated organisms by continued chloroform + isoamyl alcohol deproteinization followed by a second ethanol precipitation yielded preparations with a ratio in specific activity, untreated: treated of 2.5 (data in Table 3). The more purified preparation of DNA from methylmethanesulphonate-treated organisms retained its heat sensitivity as compared to DNA from control organisms. The DNA from the treated organisms used in the experiment shown in Table 3 had a transforming activity 0.22 the original after 3 hr. heating at 50° whereas the DNA from the control culture was completely unaffected by the heating. Admixture of partially purified DNA prepared from methylated organisms did not sensitize untreated DNA to heat. There was a similar lack of sensitization when crude DNA preparations from treated and untreated organisms were mixed. These mixing experiments showed that extracts from alkylated organisms did not contain a substance which could produce a change in DNA resulting in heat sensitization as a secondary reaction. It should be recalled that even the crude preparations have been treated so as to inactivate any protein (enzymes) by denaturation. There was some evidence (Table 3) of competition between treated and untreated DNA at higher DNA concentrations since mixtures of DNA prepared from methylated and control organisms did not give the number of transformants expected on the basis of additivity at the higher DNA concentrations.

Crude preparations of DNA from methylmethanesulphonate-treated organisms did not wind around a glass rod when ethanol was added, although they were precipitated and collected by the centrifugation procedure. There was no significant difference in the amount of acid-precipitable diphenylamine-reacting material present in the crude DNA preparations from control and methylmethanesulphonate treated organisms, indicating that the DNA from alkylated organisms did not undergo differential breakdown to an acid soluble stage during isolation.

Effects of incubation in growth medium

Organisms were treated with methylmethanesulphonate and after washing out the mutagen the organisms were incubated in CHT 50 medium supplemented with L-methionine (50 μ g./ml.). The organisms were incubated with rapid shaking at 37° in 250 ml. Erlenmeyer flasks containing 40 ml. medium. At intervals the organisms were harvested and washed with saline EDTA (pH 8). Crude DNA extracts were prepared and tested for transforming activity. As incubation proceeded the total amount of extractable transforming activity increased and the heat sensitivity of the extracted DNA diminished (Fig. 4).

The DNA content of the extracts and of the organisms from which the extracts were made did not show a comparable increase. For example, the DNA content of the organisms from which the extracts of Fig. 4 were made did not increase during the 150 min. incubation of this rather concentrated suspension. There was

an increase in the transforming units (transformed colonies/ μ g. DNA) in extract from alkylated organisms as compared with the controls while incubation proceeded. In the experiment shown in Table 4 the DNA extracted from the methylmethanesulphonate-treated organisms increased in activity from 53 to 131 units, an increase

Table 3. *Competition between DNA extracted from methylated and control (untreated) organisms*

DNA was extracted from *Bacillus subtilis* treated for 30 min. at 37° with 0.025 M-methylmethanesulphonate in salts solution or incubated in salts solution as a control. The cells were washed, lysed with lysozyme as described in the text and deproteinized by treatment with sodium perchlorate and chloroform+isoamyl alcohol. After the initial precipitation with ethanol as described in the text the DNA preparation in saline-citrate was deproteinized by an additional three treatments with chloroform+isoamyl alcohol, reprecipitated with ethanol and dissolved in dilute saline citrate. The solution was made to 0.15 M-sodium chloride+0.015 M-sodium citrate and stored in the cold. The transforming activity of the DNA from methylated organisms was decreased to 0.22 the original by heating for 3 hr. at 50°. The transforming activity of the DNA from control organisms retained all of its original activity after 3 hr. heating at 50°.

The predicted values are calculated from the number of transformants given by the extracts when tested separately. Transformation was scored at the indole locus. Each transformation tube had 0.1 ml. of the indicated DNA dilution added per ml.

Mixture	Dilution of mixture added to transformation tubes	Transformants/ml.	
		Found	Predicted on basis of additivity
10.5 μ g. of DNA from treated organisms per ml.	1	3600*	—
	1/10	610	—
	1/100	110	—
9.1 μ g. of DNA from control organisms per ml.	1	7650*	—
	1/10	1850	—
	1/100	240	—
10.5 μ g. DNA from treated organisms + 9.1 μ g. DNA from control organisms per ml.	1	7050*	11,250
	1/10	2280	2460
	1/100	345	350
31.5 μ g. DNA from treated organisms + 9.1 μ g. DNA from control organisms per ml.	1	7300*	18,450
	1/10	2800	3680
	1/100	565	570

* These represent DNA concentrations in the non-linear transformation zone.

of about 150 %, whereas the extracts obtained from control cultures altered by only 35 %. The alkylated DNA lost its heat sensitivity as the incubation proceeded and approached the heat sensitivity of non-alkylated control DNA preparations.

During the period when the transforming units extracted from alkylated organisms increased from 530 to 1310 transformed colonies/ μ g. DNA in extract, the total cellular DNA in the treated culture increased by only 34 % after an initial lag or slight decrease (Fig. 5). More of the total cellular DNA appeared to be extracted from the control non-alkylated organisms by the lysozyme procedure (Table 4). The percentage of DNA extracted was determined by comparison of the DNA values obtained by the analysis of whole organisms with values for DNA in the extracts. Analytical values for DNA in whole organisms are given in Fig. 5 while the concentration of DNA in the extracts is shown in Table 4. Although there was

no measurable increase in the total DNA in the cells during the first 80 min. incubation of methylmethanesulphonate-treated organisms (or even a slight decrease), new DNA was synthesized. Thymidine-2-¹⁴C was linearly incorporated into alkylated organisms incubated in growth medium containing this substance (Fig. 5)

Fig. 4

Fig. 5

Fig. 4. Effect of heat on the transforming activity of crude extracts of DNA prepared from alkylated organisms. The methionine-requiring organisms used were harvested after overnight culture at 37° from 16 slopes of the complete medium described in the text contained in 200 × 25 mm. screw cap tubes. After washing in salts solution the organisms were treated for 30 min. at 37° with 0.025 M-methylmethanesulphonate (0.085 ml./40 ml. of suspension in salts solution) and then washed with salts solution. A suspension of organisms in 12 ml. of salts solution was made and 2 ml. of this suspension were then added to 30 ml. of minimal medium supplemented with 0.02 % casein hydrolysate and 50 µg./ml. of L-methionine. The organisms were incubated with shaking at 37°. The times of incubation in min. are indicated in parenthesis on the figure. After incubation for the indicated time the organisms were harvested by centrifugation and washed with saline EDTA. A crude DNA preparation was then made from these cells as described in the text. The figures represent the number of transformants of the indole locus as determined by the standard assay procedure.

Fig. 5. DNA synthesis and thymidine-2-¹⁴C uptake in alkylated organisms. Circles, thymidine uptake; triangles, DNA by diphenylamine reaction. Solid line, alkylated cells; broken line, controls. The thin line represents the amount of DNA present at the start of incubation. Two µc. thymidine (4×10^6 c.p.m.) of specific activity 10 µc./mg. were added to 40 ml. medium. After washing the organisms were taken up in 8 ml. saline EDTA for analysis. C.p.m. are recorded for material precipitated by perchloric acid after a suspension of organisms was incubated 90 min. at 37° in N-NaOH. The DNA determinations represent diphenylamine-reacting material extracted from whole cells by hot 0.5 M-perchloric acid as described in the text. Additional details of this experiment are given in the legend to Table 4.

and the incorporation began without lag from the start of incubation and proceeded in linear fashion. The incorporation was into the DNA since the radioactivity was solubilized by hot acid and was precipitated by cold acid even after 90 min. at 37° in N-NaOH. The absence of a lag in thymidine incorporation as contrasted to the long lag before the start of net DNA synthesis presents a paradox which will be considered in the discussion.

Table 4. *Effect of incubation on extractable transforming activity*

This table represents data from the same experiment shown in Fig. 5. The methionine-requiring organisms used were harvested after overnight culture at 37° from 24 slopes of the complete medium described in the text contained in 200 × 25 mm. screw cap tubes. The organisms were washed in salts solution and suspended in salts solution made 0.025M with respect to methylmethanesulphonate (0.085 ml./40 ml. of salts solution). Control organisms were incubated in salts solution alone. After 30 min. incubation at 37° with gentle shaking the organisms were harvested, washed and resuspended in salts solution. Equal portions of this suspension of organisms were then incubated in 40 ml. minimal medium supplemented with casein hydrolysate (0.02 %), L-methionine (50 µg./ml.) and L-tryptophan (50 µg./ml.) and containing thymidine-2-¹⁴C. The thymidine was added to give a concentration of 5 µg./ml. in a total volume of 40 ml. Each 40 ml. flask contained 2 µc. of thymidine-2-¹⁴C. The organisms were incubated at 37° in a shaking water bath for the indicated time periods, washed with saline EDTA and then made to 8 ml. 4 ml. were used for the extraction of DNA while the remaining portion was used for the analysis of DNA and for the distribution of incorporated radioactivity. The values for transformants are from the linear portion of the response curve since tenfold dilution of the treated DNA gave a tenfold decrease in number of transformants. Transformation was scored at the indole locus.

Treatment	Time incubated in growth medium (min.)	DNA conc. of extract (µg./ml.)	% of all DNA extracted	DNA in assay tubes (µg.)	Transformants/0.1 ml.			Transformed colonies per µg. DNA (before heating)
					Non-heated	After 5 hr. at 50°	Ratio unheated: heated	
Methylmethanesulphonate	0	18.5	42	1.9	98	15	6.5	530
	40	14.5	35	1.5	167	18	9.3	1150
	80	16	36	1.6	188	44	4.3	1180
	120	18.5	31	1.9	241	90	2.7	1810
Control (untreated)	0	20	47	0.2	120	123	0.95	6150
	40	25.5	53	0.26	104	111	0.94	4080
	80	30.5	56	0.30	217	176	1.2	7100
	120	37.5	57	0.38	313	238	1.3	8300

DISCUSSION

The data can be analysed in terms of the following model. DNA is sensitized to heat by concentrations of methylmethanesulphonate which are lethal for the *Bacillus subtilis* used. The reaction of the cellular DNA with methylmethanesulphonate is assumed to occur in at least two main stages (Strauss, 1962a). The initial stage produces an alkylated DNA which participates in transformation and in replication (Green & Krieg, 1961). The second stage occurs after depurination of the alkylated DNA and may be assumed to be the formation of single strand breaks in the DNA. Treatment of the alkylated DNA at 50° accelerates the formation of single strand breaks.

The loss of activity at 50° in our experiments cannot be related to the thermal

denaturation of *Bacillus subtilis* DNA since heating the alkylated DNA for 5 hr. at 50° does not cause an increase in its absorption at 259 m μ (Strauss, 1962*b*) and since the T_m of *Bacillus subtilis* DNA in saline + citrate is 87.4° (Marmur, 1961). Alkylation of DNA does lead to a slight decrease in its thermal denaturation temperature (Strauss, 1962*b*) but the value (67.4° in 0.005*M* buffer compared to 70.5° for control DNA) is still well above 50°. Furthermore, the lack of any major

Fig. 6. Interpretation of the loss of heat sensitivity. The shaded areas represent DNA with single + double strand breaks which is supposed to be inactive for transformation of the indole locus and unable to replicate. Unshaded areas represent both alkylated and non-alkylated DNA able to replicate and of normal transforming activity. MMS = methylmethanesulphonate.

change in the sedimentation constant of alkylated or alkylated and heated *B. subtilis* DNA compared to non-treated material shows that the heating reaction does not cause any major degradation of the treated DNA into small molecules. Lerman & Tolmach (1957, 1959) showed that DNA with single strand breaks produced by deoxyribonuclease was inactive for transformation but was taken up by competent cells. Methylated, and methylated and heated *Bacillus subtilis* DNA is also taken up by competent cells almost as efficiently as untreated DNA (Table 2). The DNA extracted from alkylated organisms must consist of a mixture of alkylated DNA and of DNA with single-strand breaks, but there can be very little normal DNA in the extract. This follows from the heat sensitivity of extracts from alkylated organisms (Fig. 4); less than 3% of the original transforming activity remained after 5 hr. at 50°.

If this hypothesis be correct, then the heat sensitivity of DNA formed on an alkylated template will be determined by the average number of alkylated strands in the parental DNA (Fig. 6). If on the average only half of the strands of the marked (indole) region are alkylated, then replication will give a population in

which half the markers are heat sensitive and half are resistant. If both strands are alkylated, then replication will lead to no decrease in heat sensitivity, since it is supposed that a single-strand break can inactivate a marker and each marker will still contain one alkylated heat-labile strand. Further replication will of course produce heat-resistant markers (Fig. 6). This hypothesis may be used for a rough calculation of the degree of resistance to heat and the increase in transforming activity obtained on incubation of alkylated organisms in growth medium. The calculations are presented more to show that the hypothesis does not lead to absurd conclusions than for their quantitative validity. In the experiment shown in Table 4 and Fig. 5, the number of transformed colonies/ μ g. DNA obtained from the treated organisms increased from 530 to 1310 after incubation for 120 min. with a net increase of only 34% in the DNA. Heating for 5 hr. at 50° decreased the activity of the DNA from non-incubated organisms to 8.2 transformed colonies/ μ g. DNA. Consider an extent of methylmethanesulphonate reaction which leaves half of the progeny markers (marked regions in molecules) alkylated and heat sensitive (Fig. 6). The hypothesis requires that only the molecule active for transformation can participate in replication, and since the activity more than doubles I assume that all the active molecules participate in replication. For every 131 DNA molecules after replication there will then be $131/2 = 66$ heat sensitive and 66 non-sensitive molecules. If the ratio of activity before and after heating is the same as before incubation (i.e. 6.5) there will be $66/6.5 + 66 = 76$ active molecules after heating, giving an unheated:heated ratio of $131:76 = 1.7$. If some of the progeny DNA molecules were produced on a template of doubly alkylated DNA (Fig. 6) more of the progeny molecules would be heat sensitive. If only 1/4 of 131 molecules were resistant to heat after replication whereas the others still contained an alkyl group, as would occur if the templates for replication had been an equal mixture of doubly and singly alkylated molecules, there would be $33 + 98/6.5 = 48$ biologically active molecules present after heating for 5 hr. at 50°, which would give a ratio of unheated:heated of $131:48 = 2.7$, which was the ratio obtained (Table 4, line 4). (The situation is complicated by the fact that the 2.5-fold increase in transforming activity requires more than one round of replication for certain of the DNA molecules.) It is also clear that there can be an initial increase in transforming activity obtained from alkylated organisms upon incubation in growth medium without decreased heat sensitivity, if doubly alkylated molecules are used as templates.

The ratio of the transforming activity from treated and non-treated organisms was 530/6150 transformed colonies/ μ g. DNA = 0.086 (Table 4). It may be assumed that this is the fraction of the total DNA in treated organisms which is capable of serving as a template for replication, that is, that the DNA from treated organisms consists of 8.6% of alkylated but biologically active DNA and of 91.4% biologically inactive DNA with single+double strand breaks (Fig. 6). The active alkylated DNA would have the normal transforming activity which in this experiment was 615 transformed colonies/ μ g. DNA. Any new DNA synthesized would also have normal transforming activity. After incubation for 120 min. there was a 34% increase in the DNA of the treated material (Fig. 5). In every 1.34 μ g. DNA extracted after incubation there would then be $0.34 + 0.086 = 0.42$ μ g. active DNA and 0.82 μ g. inactive DNA which had not participated in replication. The active DNA would produce 6150 transformed colonies/ μ g. DNA so that after incubation

one would expect $0.42 \times 615 = 258$ transformed colonies/ $1.84 \mu\text{g.}$ or 193 transformed colonies/ $\mu\text{g.}$ DNA as compared with an actual figure of 181 transformed colonies/ $\mu\text{g.}$ after incubation and 58 transformed colonies/ $\mu\text{g.}$ DNA before incubation. If the newly synthesized DNA still containing an alkyl group had a lower transforming efficiency than normal DNA the calculated value would approach that found more closely.

The physical identification of the state produced by heating alkylated DNA at 50° with single strand breaks cannot be made with assurance in the absence of more physical data. It is still possible that this inactivation might represent the formation of apurinic sites, as suggested by the experiments of Brookes & Lawley (1961) and of Bautz & Freese (1960). It is also likely that the state of organisms after alkylation is more complex than we have indicated. Although the incorporation of thymidine- ^{14}C by treated organisms was linear from the start of incubation, there was no net increase in the DNA of treated organisms until 120 min. of incubation. Actually a slight decrease in DNA content after the start of incubation was a regular feature of our experiments. It seems reasonable to suppose that some process occurs soon after the start of incubation which serves to degrade DNA that has been alkylated. A degradative process affecting alkylated DNA has been described (Strauss, 1962*b*). Breakdown of *Haemophilus* DNA was reported by Stuy (1960) after X-ray treatment. Degradation of DNA would of course introduce another element of uncertainty into the calculations above. Finally, there is no evidence that the 'recovery' from heat sensitivity of the extracted DNA (which actually represents synthesis of new DNA) is related to the rapid and dramatic recovery of viability of organisms from the effects of alkylation after very short periods of incubation in growth medium.

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