JOURNAL OF BACTERIOLOGY, June 1970, p. 760-766
Vol. 102, No. 3

Repair of Alkylation Damage: Stability of Methyl Groups in *Bacillus subtilis* Treated with Methyl Methanesulfonate

LOUISE PRAKASH[1] AND BERNARD STRAUSS
Department of Microbiology, The University of Chicago, Chicago, Illinois 60637

Received for publication 6 March 1970

Bacillus subtilis was not inactivated and was able to replicate even though approximately 3×10^4 methyl groups added by methyl methanesulfonate (MMS) were bound to the deoxyribonucleic acid (DNA) of each organism. No significant loss of methyl groups from the DNA occurred for several generations upon incubation of methylated wild-type or MMS-sensitive cells. Single-strand breaks were not observed in the DNA from cells treated at this low MMS dose. Higher doses of MMS resulted in significant killing of both wild-type and MMS-sensitive strains, and the DNA extracted from such treated cells sedimented more slowly than control DNA through alkaline sucrose gradients, indicating the presence of breaks or apurinic sites (or both). These breaks were repaired upon incubation of wild-type but not of MMS-sensitive strains. Repair of damage induced by alkylating agents is probably the repair of breaks which occur as a consequence of high levels of alkylation.

Bacteria and other organisms possess a system which enables them to repair at least a portion of the damage induced by monofunctional alkylating agents (5, 22, 26). The existence of bacterial mutants sensitive to methyl methanesulfonate (MMS), for example, is prima facie evidence for the existence of repair mechanisms in the wild type (25). However, although it is well established that repair of alkylation damage does occur, what is actually repaired is not at all clear. Alkylated deoxyribonucleic acid (DNA) is unstable and is degraded both spontaneously (7, 12) and enzymatically (10, 27). As a result of the stepwise nature of the spontaneous degradation, "alkylated DNA" may be considered to be a mixture of DNA with alkylated sites, apurinic sites, and single-strand breaks. The present study was an attempt to determine exactly what was repaired in repair of alkylation damage. Specifically, we asked the following questions. (i) Are methyl groups added to DNA by alkylation with MMS removed during rapair? (ii) If the repair process does not involve removal of methyl groups from DNA, what damage is repaired? (iii) Does alkylation per se affect the ability of cells to replicate? (iv) How do wild-type and MMS-sensitive strains compare in their ability to repair damage?

[1] Present address: Department of Radiation Biology and Biophysics, The University of Rochester School of Medicine and Dentistry, Rochester, N.Y. 14620.

MATERIALS AND METHODS

MMS was obtained from Eastman Organic Chemicals and was vacuum-redistilled before use. A 0.05 M solution was prepared by dissolving 0.085 ml of MMS in 20 ml of cold 0.05 M phosphate buffer immediately before use. $^{14}CH_3$-MMS (45.2 mc/mM) was obtained from Amersham Searle. Deuterated water, D_2O, and $^{15}NH_4Cl$ were obtained from Bio-Rad Laboratories, Richmond, Cal.; deuterated sugars and a deuterated amino acid mixture were purchased from Merck, Sharpe and Dohme of Canada, Ltd.

Bacillus subtilis strains were obtained as follows: 168M (*ind*$^-$) from J. Spizizen; 168MT$^+$ (*ind*$^+$) by transformation of 168M; 168 *ind*$^-$*thy*$^-$ from Frank Rothman. The MMS-sensitive mutant 168M-5 was isolated by nitrosoguanidine treatment of 168M by the method of Balassa (1) with MMS as a selective agent. Strain 168M-5 is ultraviolet (UV) sensitive, rec$^-$ as determined by its comparative ability to serve as a recipient in transformation and transfection (we thank Kenneth Bott for this determination), and *hcr*$^+$ as determined by its ability to reactivate MMS-treated SP01 phage. Nothing is known about the genetic basis of the MMS sensitivity in this strain.

Cultures were grown in CHT50 medium (4). Lysates were prepared by collecting the cells by centrifugation, washing, and suspending in 10 ml of 0.01 M tris(hydroxymethyl)aminomethane - ethylenediamine - tetra - acetate, *p*H 8.3. They were then incubated with 0.5 ml of lysozyme (20 mg/ml) for 10 to 15 min at

37 C, followed by 0.5 ml of 1% sodium lauryl sulfate at 37 C until clearing occurred. Lysates were sedimented through alkaline sucrose gradients by layering 0.1 ml of lysate (containing 0.1 to 0.7 μg of DNA) onto a 5 to 20% (w/w) linear sucrose gradient in 0.9 M NaCl plus 0.1 M NaOH. Centrifuaation was in the SW50L rotor of a Beckman L-2 ultracentrifuge for 2 hr at 35,000 rev/min at 20 C. Sixteen-drop fractions were collected from the bottom of the tube. Samples were precipitated with cold 5% trichloroacetic acid in the presence of albumin carrier and collected on nitrocellulose filters. The filters were dried and counted in a Packard scintillation counter with a scintillation fluid made up of 19 g of "Premix" (Packard Instrument Co.) in 1 gal (4.5 liters) of toluene. Trichloroacetic acid supernatants were counted in a scintillation cocktail made with Triton X-100 (2).

RESULTS

In vivo alkylation of DNA. An overnight culture of strain 168MT$^+$ was reacted with 0.22 mM ^{14}C-MMS for 60 min at 37 C. The culture was washed, suspended in fresh CHT50 medium, and incubated overnight with KOH (final concentration 0.5 M) at 37 C. This incubation should have resulted in hydrolysis of ribonucleic acid (RNA). The mixture was then precipitated with trichloroacetic acid and centrifuged. The supernatant, which contained hydrolyzed RNA as well as the initial acid-soluble constituents, was collected and counted. The residue was washed with cold trichloroacetic acid, suspended in trichloroacetic acid, and heated in a boiling-water bath for 15 min. After cooling, the sample was filtered through a nitrocellulose filter. The filtrate, which contained the alkali-stable, hot acid-labile constituents, i.e., DNA, was counted. About 10% of the total number of alkyl groups fixed to cells by reaction with MMS in vivo were found in the DNA fraction (Table 1). In a separate experiment, treatment of an alkali-stabile, hot perchloric acid-soluble fraction with charcoal (30) resulted in adsorption of 99% of the radioactivity, indicating that the methyl groups from MMS in this fraction were really associated with the nucleotides.

Alkylated purines have been identified as a major product of the reaction of DNA with alkylating agents both in vitro (6, 7, 13) and in vivo (12, 16, 17). Since depurination occurs under neutral conditions (13), one would expect to see a loss of ^{14}C counts but not of ^{3}H counts when DNA prepared from cells grown with ^{3}H-thymidine and alkylated with ^{14}C-MMS is heated under neutral conditions (Table 2). When the alkali-stabile cell fraction was carefully dissolved in alkali, neutralized, and heated for 15 min at 100 C, there was almost complete loss of ^{14}C in some experiments, whereas less than 10% of the ^{3}H counts became acid soluble. Retention of a portion of the ^{14}C counts (Table 2, 3rd line) can be accounted for as being due to either the formation of phosphotriesters (21) or to the stabilization of the alkyl groups due to an alkali-catalyzed ring cleavage (13). The differential heat lability of the alkali-stable ^{14}C counts is taken as further evidence that the methyl groups from MMS are bound to the DNA.

TABLE 1. *Reaction of ^{14}C-MMS with Bacillus subtilis*

Determination	Total counts fixed	Fraction of total
	counts/min	
Whole cells[a]	3.1×10^6	
DNA (hot acid-soluble fraction)	0.3×10^6	0.096
RNA (cold acid-soluble + alkali-labile fraction)	1.6×10^6	0.516

[a] A 15-ml amount of 1.58×10^9 cells of *B. subtilis* 168 *ind*$^+$ per ml was incubated with 10 μc of ^{14}C-MMS per ml (45.2 mc/mM) for 60 min.

The measurement of the radioactivity fixed to DNA was used to determine the number of methyl groups added. We used the data of Massie and Zimm (14) for the amount of DNA per cell. The calculations indicate that alkylation with 0.22 mM MMS for 60 min results in the fixation of 2.9×10^4 methyl groups per organism or per DNA molecule, since overnight cultures contain uninucleate bacteria. This corresponds to one methyl group per 400 nucleotides.

Since reaction with this level (0.22 mM, 60 min) of MMS caused no decrease in the number of colony-forming units, we wanted to know whether methyl groups added to the DNA by alkylation were removed during incubation of cells. We therefore determined the percentage of counts remaining bound to DNA as a function of the time of incubation in CHT50 medium. Since we were interested in differentiating specific excision of methylated DNA components from random DNA degradation, wild-type and MMS-sensitive cells were prelabeled with ^{3}H-thymidine before alkylation with ^{14}C-MMS. There was very little loss of ^{14}C counts from DNA for up to 170 min of incubation in CHT50 medium (Tables 3–5). During this time, alkylated cells went through about three divisions (Fig. 1). Since the ratios of ^{14}C/^{3}H were constant, there was no specific excision of methylated bases. Similar results were obtained with *Escherichia coli* B/r (Table 5).

The failure to excise methyl groups, even though cells containing over 29,000 such groups were able to divide, implied that DNA with methyl groups added by an alkylating agent repli-

TABLE 2. *Heat lability of alkali-stable radioactivity in cells grown with H^3-thymidine and treated with C^{14}-MMS*[a]

Strain[b]	Acid soluble counts/min after hot acid treatment		Acid soluble counts/min after boiling at neutral pH	
	^3H	^{14}C	^3H	^{14}C
168MT+	0.895×10^3	0.634×10^3	0.072×10^3	0.717×10^3
	3.59×10^3	11.4×10^3	0.499×10^3	12.5×10^3
168 *ind⁻thy⁻*	0.524×10^3	0.863×10^3	0.043×10^3	0.530×10^3

[a] Cells prelabeled with H^3-thymidine (overnight growth in 0.1 μc/ml, washed, and concentrated 10-fold the next morning) were incubated with C^{14}-MMS (10 μc/ml, 0.22 mM). Alkali digestion was as described. One pellet was suspended in 0.1 N NaOH and neutralized with 1.1 M HCl-0.2 M tris(hydroxymethyl)aminomethane. The other pellet was suspended in cold 5% trichloroacetic acid. Both samples were placed in a boiling-water bath for 15 min and then cooled. The neutral boiled sample was then precipitated with cold 5% trichloroacetic acid, and filtrates from both the neutral and acid-boiled samples were collected into scintillation vials and counted with Triton X-100 scintillation fluid.

[b] Each line represents an independent determination.

TABLE 3. *Stability of ^{14}C-methyl groups bound to DNA of cells of MMS-resistant strains of Bacillus subtilis*[a]

Strain	Time	^3H	^{14}C	$^{14}C/^3H$
	min	*counts/min*	*counts/min*	
168M[b]	0	8.95×10^2	3.30×10^2	0.365
	80	8.41×10^2	3.15×10^2	0.371
	140	8.78×10^2	3.17×10^2	0.358
	170	8.19×10^2	2.57×10^2	0.309
168M[c]	0	1.48×10^4	1.12×10^4	0.778
	40	1.50×10^4	1.13×10^4	0.776
168 *ind⁻thy⁻*[d]	0	8.78×10^3	1.71×10^3	0.187
	40	7.88×10^3	1.55×10^3	0.188
		8.43×10^3	1.40×10^3	0.157
	80	7.60×10^3	1.28×10^3	0.160
		7.62×10^3	1.20×10^3	0.149
	120	6.73×10^3	1.15×10^3	0.163
		6.21×10^3	1.02×10^3	0.155

[a] MMS treatment was with 0.22 mM ^{14}C throughout. Cells were prelabeled by growth with varying concentrations of ^3H-thymidine. The absolute level of radioactivity represents only the amount of sample taken for analysis. $^{14}C/^3H$ ratios were corrected for carryover of counts.

[b] Incubation was for 90 min; $N_0 = 4.3 \times 10^9$ cells/ml, $N = 3.4 \times 10^9$ cells per ml. $N/N_0 = 0.767$.

[c] Incubation was for 60 min; $N_0 = 3.6 \times 10^9$ cells/ml. $N = 3.6 \times 10^9$ cells/ml, $N/N_0 = 1.0$.

[d] Incubation was for 90 min; $N_0 = 1.88 \times 10^9$ cells/ml, $N = 1.96 \times 10^9$ cells/ml. $N/N = 1.04$.

cated normally. The replication of alkylated DNA was demonstrated by a density transfer experiment. Cells of wild-type strain 168MT+ were adapted to heavy medium containing $^{15}NH_4Cl$, D_2O, deuterated amino acids, and deuterated sugars by the method of Bodmer and Schildkraut

TABLE 4. *Stability of ^{14}C-methyl groups bound to DNA of cells of MMS-sensitive strain 168M-5 of Bacillus subtilis*[a]

Time	^3H	^{14}C	$^{14}C/^3H$
min	*counts/min*	*counts/min*	
0	1.50×10^3	4.34×10^2	0.284
40	1.66×10^3	4.31×10^2	0.254

[a] See footnote *a* of Table 3. Incubation was for 60 min; $N_0 = 9.1 \times 10^7$ cells/ml. $N = 1.25 \times 10^8$ cells/ml. $N/N_0 = 1.37$.

TABLE 5. *Stability of ^{14}C-methyl groups bound to DNA of cells of Escherichia coli B/r*[a]

Time	^3H	^{14}C	$^{14}C/^3H$
min	*counts/min*	*counts/min*	
0	1.80×10^2	6.48×10^2	4.37
45	2.24×10^2	8.64×10^2	4.76
80	2.06×10^2	6.94×10^2	4.04

[a] See footnote *a* of Table 3. Incubation was for 60 min.

(3). All of the DNA of the cells grown in this medium was heavy, with a density of 1.752 g/ml (Fig. 2a). Heavy cells were treated with ^{14}C-MMS in heavy medium for 60 min. The treated culture was washed, suspended in light medium, and incubated in light medium for 60 min, after which the cells were collected and lysed. A portion of lysate was used for analytical CsCl equilibrium sedimentation (Fig. 2b). A second portion of lysate was assayed for radioactivity bound to DNA; there were 4.07×10^4 methyl groups per cell or one methyl group per 300 nucleotides. The remainder of the lysate was then sedimented to equilibrium in a CsCl gradient in the Beckman L2 preparative ultracentrifuge along with a marker

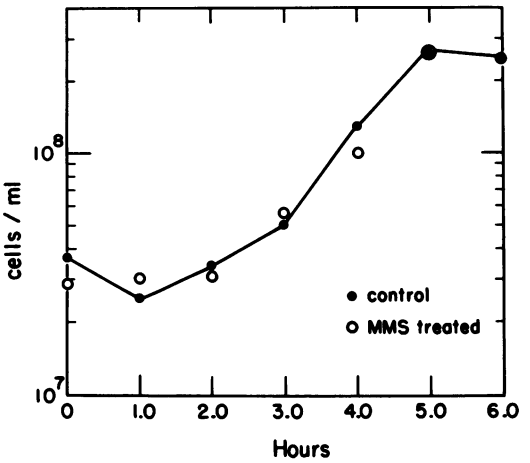

FIG. 1. *Growth of B. subtilis 168 ind⁻ thy⁻ after a 90 min of exposure to 0.22 mM ^{14}C-MMS (10 μc/ml). N/N_0 = 1.1. Symbols: ●, control; ○, MMS-treated. Cells were treated with MMS and kept frozen with 15% glycerol at −60 C for circa 24 hr before use; 2 × 10^8 counts/min were fixed by 2 × 10^9 colony-forming units, which was equivalent to the binding of 2.4 × 10^4 methyl groups per cell.*

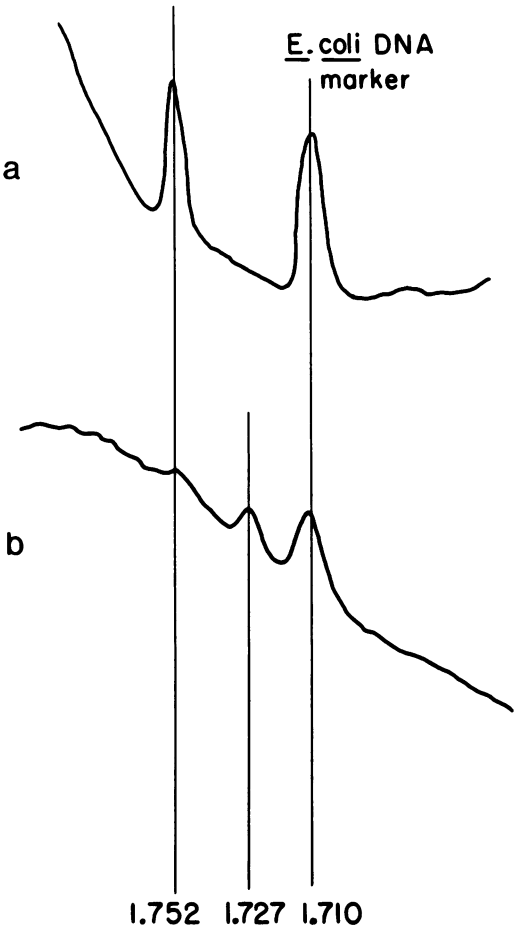

FIG. 2. *Microdensitometer tracings of lysates of B. subtilis 168MT⁺ after equilibrium density gradient centrifugation in CsCl. A 1-ml amount of cells suspended in 0.01 M tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetate, pH 8.3, was lysed by adding 0.1 ml of lysozyme (10 mg/ml) for 20 min at 37 C, succeeded by 0.1 ml of 1% sarkosyl at 37 C until clearing (about 20 min). a, Lysate of cells adapted to heavy medium (HM). b, Lysate of HM cells treated with MMS and then incubated in light medium. A 4-ml amount of cells was treated with 1 ml of ^{14}C-MMS (500 μc in HM) for 60 min at 37 C in heavy medium. Cells were washed, resuspended in 10 ml of light CHT-50 medium, and incubated for 60 min at 37 C. N/N_0 = 1.6. After MMS treatment, cells were harvested and lysed as described in a. A 0.1-ml amount of lysate was subjected to centrifugation in CsCl in a model E analytical ultracentrifuge at 44,000 rev/min, 25 C. Purified E. coli DNA, a gift of Fred Kieras, was used as a marker. A photograph was taken after 20 hr, when equilibrium had been established. Calculations of density were made by the method of Schildkraut et al. (24) with the density of E. coli DNA taken as 1.710 g/ml.*

of light, ^{3}H-thymidine-labeled *B. subtilis* DNA. Most of the ^{14}C counts were found in a band with a peak density of 1.727 g/ml corresponding to hybrid DNA (Fig. 3). A fraction, representing about 20% of the total counts, was found at the heavy density, 1.752 g/ml. Since there had been a 60% increase in the number of cells during the period of incubation in light medium, this 20% most likely represents DNA from unreplicated cells. [Actually 25% of the DNA would be unreplicated if there had been a 60% increase in the number of cells. If R represents the fraction of cells which replicate and U the fraction which do not replicate, then: $R + U = 1$, $N/N_0 = 1.6 = (2R + U)/(R + U)$ and $R = 0.6$. The DNA extracted from the mixture of replicated and unreplicated cells will therefore be a mixture of 75% (1.2/1.6) replicated and 25% (0.4/1.6) unreplicated DNA.] We assume that ^{14}C counts in the region of light DNA are most likely due to protein or other material present at the top of the gradient. Clearly alkylated DNA is able to replicate.

Repair after high doses of MMS. Although alkyl groups are not removed from DNA and alkylated DNA does replicate, we know that alkylation "damage" is repaired since MMS-sensitive mutants exist. What is repaired? DNA treated with higher concentrations of MMS in vitro contains apurinic sites and single-strand breaks (7, 28). Apurinic sites and single-strand breaks can be demonstrated by alkaline sucrose

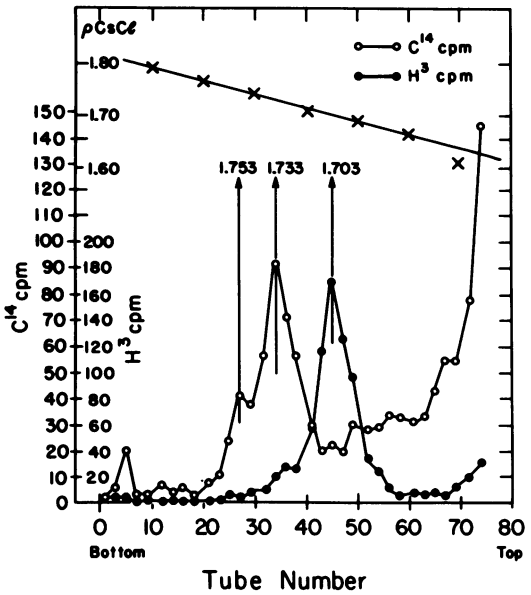

FIG. 3. *Preparative equilibrium density gradient centrifugation in CsCl of DNA from lysates of HM cells treated with ^{14}C-MMS for 60 min and then incubated in light medium for 60 min (same sample as that used for tracing in Fig. 4b). ^{3}H-thymidine-labeled B. subtilis DNA, a gift of K. Bott, was used as a marker. Symbols: ○, ^{14}C counts per minute from HM cells; ●, ^{3}H counts per minute from marker DNA; ×, density of CsCl calculated from refractive index. Centrifugation was carried out in the SW50L rotor of a model L-2 ultracentrifuge at 30,000 rev/min, 20 C, for 48 hr. Five-drop fractions were collected from the bottom of the tube; fractions were incubated overnight in an equal volume of 1 N KOH at 37 C. The next day, fractions were cooled, precipitated with cold trichloroacetic acid with albumin carrier, filtered through Millipore filters (Millipore Corp., Bedford, Mass.), dried, and counted with premix scintillation fluid by using double label settings. The numbers above the arrows represent the density of each peak. Densities were determined from measurements of refractive index at room temperature corrected to the known density (1.703) of the light B. subtilis DNA marker; 2.9 × 10^{2} counts/min were fixed by 10^{8} colony-forming units, which is equivalent to the binding of 4.07 × 10^{4} methyl groups per DNA molecule.*

sedimentation since alkali produces breaks at apurinic sites (9, 29) and exposes single-strand breaks by denaturing the DNA. Apurinic sites or single-strand breaks (or both) were not detected in either wild-type or MMS-sensitive strains after a low dose of MMS (Fig. 4a). However, at a dose of MMS (25 mM, 20 min) high enough to inactivate, apurinic sites or single-strand breaks (or both) were detected by a decrease in the sedimentation rate through alkaline sucrose (Fig. 4b,c). Incubation of highly alkylated wild-type cells for 40 min, after alkylation but prior to lysis, resulted in an increase in the sedimentation rate of the DNA to a position intermediate bebetween control and alkylated DNA (Fig. 4b). Such a shift to a faster-sedimenting component was not seen when alkylated cells of the MMS-sensitive strain 168M-5 were incubated (Fig. 4c).

The DNA synthesized in the first 10 min after treatment with high doses of MMS sedimented more slowly than the DNA synthesized by control cells. At low doses of MMS (0.22 mM, 60 min), newly synthesized DNA sedimented in an equivalent fashion in both control and MMS-treated cultures. At higher doses (25 mM, 20 min), the newly synthesized DNA from both resistant and sensitive strains sedimented more slowly (Fig. 5a,b). Further incubation in the absence of radioactive thymidine, led to a shift of the slowly sedimenting DNA made after MMS treatment to a more rapidly sedimenting component. Some increase in size of newly synthesized DNA did occur on further incubation of the MMS-sensitive strain (Fig. 5b), but there was less transfer into the very rapidly sedimenting component found in wild-type lysates. These "small" pieces sediment much more rapidly than the fragments reported by Okazaki et al. (15), but are about the size of the newly synthesized DNA observed in UV-treated bacteria by Rupp and Howard-Flanders (23).

DISCUSSION

Methyl groups added to DNA by alkylation with MMS are stable for several generations (Tables 3–5), and cells containing methylated DNA are capable of replication (Fig. 1, 3). The low dose of MMS used in these experiments resulted in neither inactivation nor detectable single-strand breaks (Fig. 4a) in either wild-type or MMS-sensitive strains, even though approximately 3 × 10^{4} methyl groups were fixed to the DNA of each organism. Repair of alkylation damage cannot be demonstrated at low dose since there is no recognizable damage to be repaired.

At a high dose of MMS, apurinic sites or single-strand breaks (or both) were detected after treatment of both wild-type and MMS-sensitive strains. Although these two types of damage (apurinic sites and single-strand breaks) cannot be distinguished in alkaline sucrose, it seems likely that many of the lesions are actually single-strand breaks since such breaks have been previously demonstrated at comparable doses (20, 28). Our data suggest that wild-type *B. subtilis* can repair single-strand breaks which arise after alkylation of cells with higher doses of MMS. An MMS-sensitive mutant, on the other hand, was deficient or lacking in this capacity; the MMS-sensitivity

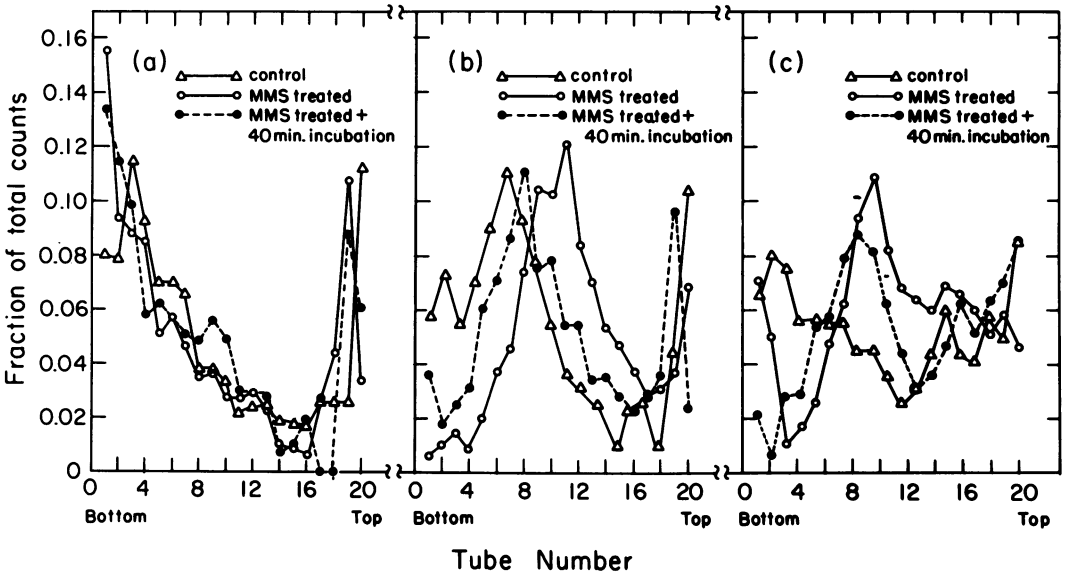

FIG. 4. *Sedimentation in alkaline sucrose of DNA from B. subtilis 168M (MMS-resistant) and 168M-5 (MMS-sensitive) prelabeled with ^{3}H-thymidine after treatment with MMS. a, Strain 168M after 60 min of exposure to 0.22 mM C^{14}-MMS (low dose). N/N_0 = 1.03. b, Strain 168M after 20 min of exposure to 25 mM MMS (high dose). N/N_0 = 0.69. c, Strain 168M-5 after 20 min of exposure to 25 mM MMS (high dose). Symbols: △, control; ○, MMS-treated; ●, MMS-treated + 40 min of incubation in complete medium before lysis.*

of this strain may be attributable to its inability to repair breaks.

The inability of strain 168M-5, and presumably of strains sensitive to monofunctional alkylating agents in general, to repair single-strand breaks probably accounts for their cross-sensitivity to other agents which indirectly (UV-irradiation) or directly (X-ray irradiation) cause single-strand breaks in DNA. The particular enzyme(s) deficient in the MMS-sensitive strain is not known. A mutant of *E. coli* lacking DNA polymerase has been found to be both UV- and MMS-sensitive (8), and mutants with deficient ligase activity are UV sensitive (18, 19). However, strain 168M-5 does have DNA polymerase activity (M. Goulian, *personal communication*). Some excision process may also be required for MMS repair, since the broken DNA chain will lack a purine if the break has occurred as a result of spontaneous depurination (12).

The breaks resulting from alkylation could occur as a result of spontaneous depurination or of enzyme action. It is unlikely that the endonuclease specific for UV damage (11) is involved, since UV-sensitive mutants are invariably cross-sensitive to bifunctional alkylating agents but not necessarily to monofunctional alkylating agents (25). An endonuclease specific for alkylation damage and different from the UV-endonuclease has been described (10, 27), but it is not clear that this alkylation-specific nuclease has anything to do with the in vivo production of alkylation-induced breaks in *B. subtilis*. In our experiments, methylation at low doses of MMS was to the extent of one methyl group per 400 nucleotides. If the enzyme described by Strauss and Robbins

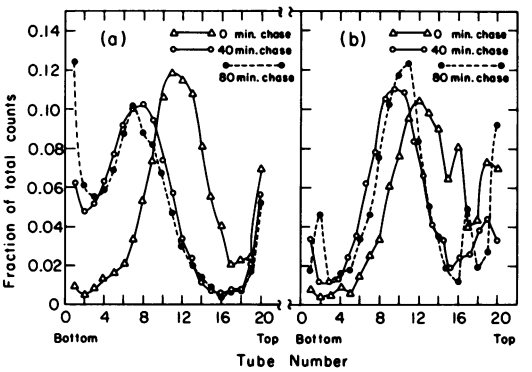

FIG. 5. *Sedimentation in alkaline sucrose of DNA from B. subtilis 168M (MMS-resistant) and 168M-5 (MMS-sensitive) labeled for 10 min with ^{3}H-thymidine after MMS treatment. The cultures were washed and resuspended in nonlabeled medium and incubated for the times indicated before lysis. a, Strain 168M after a 20 min of exposure to 25 mM MMS (high dose). N/N_0 = 0.298. b, Strain 168M-5 after a 20 min of exposure to 25 mM MMS (high dose). N/N_0 = 0.011. Symbols: △, 0-min chase; ○, 40-min chase; ●, 80-min chase.*

(27) were involved, the alkylated DNA contained sufficient methyl groups to serve as a substrate; however, no degradation was observed at the low dose. It may be that this alkylation-specific nuclease, as it occurs in vivo, may be spatially removed from the (alkylated) DNA and may not act until the cells are lysed. Boyce and Farley (5) have shown that high doses of MMS cause single-strand breaks in covalently bonded circular lambda DNA and have also shown that these breaks are repaired in superinfected *E. coli*. These authors conclude that the breaks occur as a result of enzyme action on alkylated sites, but their data indicate that some breaks do occur even in the absence of extract.

Our data are best accounted for as follows. Single-strand breaks occur spontaneously after in vivo alkylation but can be detected only after treatment with high doses of alkylating agent. Repair of MMS-induced damage involves the repair of single-strand breaks which are produced spontaneously as a result of alkylation. Repair does not involve the removal of a substantial number of methylated bases. Methylated bases do not interfere with DNA replication.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant GM-07816 from the National Institute of General Medical Sciences, and by a grant from the National Science Foundation (GB 8514). L. P. was the recipient of a traineeship from Microbiology Training Grant GM-00603 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Balassa, G. 1969. Biochemical genetics of sporulation. 1. Unidirectional pleiotropic interactions among genes controlling sporulation in *Bacillus subtilis*. Mol. Gen. Genet. **104**:73–103.
2. Benson, R. H. 1966. Limitations of tritium measurements by liquid scintillation counting of emulsions. Anal. Chem. **38**:1353–1356.
3. Bodmer, W., and C. Schildkraut. 1964. Preparation and characterization of $^{15}N^{2}H^{3}$-labeled DNA from *Bacillus subtilis* and *Escherichia coli* phage T2. Anal. Biochem **8**:229–243.
4. Bott, K., and B. Strauss. 1965. The carrier state of *Bacillus subtilis* infected with the transducing bacteriophage SP10. Virology **25**:212–225.
5. Boyce, R., and J. W. Farley. 1968. Production of single-strand breaks in covalent circular lambda phage DNA in superinfected lysogens by monoalkylating agents and the joining of broken DNA strands. Virology **35**:601–609.
6. Brookes, P., and P. D. Lawley. 1961. The reaction of mono- and difunctional alkylating agents with nucleic acids. Biochem. J. **80**:496–503.
7. Brookes, P., and P. D. Lawley. 1963. Effects of alkylating agents on T2 and T4 bacteriophages. Biochem. J. **89**:138–144.
8. DeLucia, P., and J. Cairns. 1969. Isolation of an *E. coli* strain with a mutation affecting DNA polymerase. Nature (London) **224**:1164–1166.
9. Freifelder, D., and R. B. Uretz. 1966. Mechanism of photoinactivation of coliphage T7 sensitized by acridine orange. Virology **30**:97–103.
10. Friedberg, E., and D. Goldthwait. 1969. Endonuclease II of *E. coli*. I. Isolation and purification. Proc. Nat. Acad. Sci. U.S.A. **62**:934–940.
11. Kaplan, J. C., S. R. Kushner, and L. Grossman. 1969. Enzymatic repair of DNA. I. Purification of two enzymes involved in the excision of thymine dimers from ultraviolet-irradiated DNA. Proc. Nat. Acad. Sci. U.S.A. **63**: 144–151.
12. Lawley, P. D. 1966. Effects of some chemical mutagens and carcinogens on nucleic acids. Progr. Nucl. Acid Res. Mol. Biol. **5**:89–131.
13. Lawley, P. D., and P. Brookes. 1963. Further studies on the alkylation of nucleic acids and their constituent nucleotides. Biochem. J. **89**:127–138.
14. Massie, H. R., and B. Zimm. 1965. Molecular weight of the DNA in the chromosomes of *E. coli* and *B. subtilis*. Proc. Nat. Acad. Sci. U.S.A. **54**:1636–1641.
15. Okazaki, R., T. Okazaki, K. Sakabe, K. Sugimoto, and A. Sugino. 1968. Mechanism of DNA chain growth. I. Possible discontinuity and unusual secondary structure of newly synthesized chains. Proc. Nat. Acad. Sci. U.S.A. **59**:598–605.
16. Olson, A. O. 1968. Estimation of the products of DNA alkylation. J. Chromatog. **35**:292–294.
17. Olson, A., and D. R. McCalla. 1969. Excision of 7-methyl guanine from the DNA of *Euglena gracilis*. Biochim. Biophys. Acta **186**:229–231.
18. Pauling, C., and L. Hamm. 1968. Properties of a temperature-sensitive radiation sensitive mutant of *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. **60**:1495–1502.
19. Pauling, G., and L. Hamm. 1969. Properties of a temperature-sensitive, radiation-sensitive mutant of *Escherichia coli*. II. DNA replication. Proc. Nat. Acad. Sci. U.S.A. **64**:1195–1202.
20. Reiter, H., B. Strauss, M. Robbins, and R. Marone. 1967. The nature of the repair of methyl methanesulfonate-induced damage in *Bacillus subtilis*. J. Bacteriol. **93**:1056–1062.
21. Rhaese, H., and E. Freese. 1969. Chemical analysis of DNA alterations. IV. Reactions of oligodeoxynucleotides with monofunctional alkylating agents leading to backbone breakage. Biochim. Biophys. Acta **190**:418–433.
22. Roberts, J. J., A. R. Crathorn, and T. P. Brent. 1968. Repair of alkylated DNA in mammalian cells. Nature (London) **218**:970–972.
23. Rupp, W., and P. Howard-Flanders. 1968. Discontinuities in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet irradiation. J. Mol. Biol. **31**:291–304.
24. Schildkraut, C., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. **4**:430–443.
25. Searashi, T., and B. Strauss. 1965. Relation of the repair of damage induced by a monofunctional alkylating agent to the repair of damage induced by ultraviolet light in *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **20**:680–687.
26. Strauss, B. 1968. DNA repair mechanisms and their relation to mutation and recombination. Curr. Top. Microbiol. Immunol. **44**:1–85.
27. Strauss, B., and M. Robbins. 1968. DNA methylated *in vitro* by a monofunctional alkylating agent as a substrate for a specific nuclease from *Micrococcus lysodeikticus*. Biochim. Biophys. Acta **161**:68–75.
28. Strauss, B., and R. Wahl. 1964. The presence of breaks in the deoxyribonucleic acid of *Bacillus subtilis* treated *in vivo* with the alkylating agent, methyl methanesulfonate. Biochim. Biophys. Acta **80**:116–126.
29. Tamm, C., H. Shapiro, R. Lipshitz, and E. Chargaff. 1953. Distribution density of nucleotides within a deoxyribonucleic acid chain. J. Biol. Chem. **203**:673–688.
30. Tsuboi, K. K., and T. D. Price. 1959. Isolation, detection and measure of microgram quantities of labeled tissue nucleotides. Arch. Biochem. Biophys. **81**:223–237.