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Nature of the Repair of Methyl Methanesulfonate-Induced Damage in *Bacillus subtilis*

H. REITER[1], B. STRAUSS[2], M. ROBBINS, AND R. MARONE
Department of Microbiology, The University of Chicago, Chicago, Illinois

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A nuclease present in extracts of *Bacillus subtilis* inserts breaks in deoxyribonucleic acid (DNA) treated with the monofunctional alkylating agent, methyl methanesulfonate (MMS), but the nature of the sites within the alkylated macromolecule at which these breaks occur is not known. DNA extracted from *B. subtilis* cells that have recovered from MMS damage has lost its susceptibility to enzyme action. The recovery process is accompanied by some DNA breakdown and by the incorporation of thymidine. Some recovery from ultraviolet irradiation (UV) and MMS occurred in organisms starved for thymine or adenine, but UV recovery was stimulated by their addition. It is possible that MMS recovery proceeds by a process of excision and repair similar to, but not identical with, UV repair.

Treatment of *Bacillus subtilis* with the monofunctional alkylating agent methyl methanesulfonate (MMS) results in a decrease in the specific transforming activity of the deoxyribonucleic acid (DNA) extracted from alkylated cells (20). The decrease in transforming activity is particularly striking when compared with the results of alkylation in vitro, a process which reduces the transforming activity only slightly (22). This difference between in vivo and in vitro effects suggested the interposition of cellular enzymes between the initial lesions produced by the alkylating agent and the final state of the DNA, and one such enzyme, a nuclease specific for alkylated DNA, was reported (22).

Brookes and Lawley (4) showed that the primary product of reaction between MMS and DNA is a DNA containing methylated purines with the 7′ position of the guanine residues as the primary site of reaction. The evidence suggests that this methylated DNA remains biologically active (5, 22). However, Brookes and Lawley also showed that methylated DNA is unstable in vitro and undergoes a series of spontaneous reactions in which depurination occurs first, followed by rupture of a phosphodiester bond adjacent to the apurinic site. Since DNA with single strand breaks, however produced, is not efficient for transformation in *B. subtilis* (2, 22), either the spontaneous series of reactions or the alkyl-specific nuclease may destroy the activity of alkylated DNA.

Some of the lesions produced by MMS in the DNA of *B. subtilis* cells are repaired when the cells are incubated in growth medium for a short time after treatment (13), but the way in which the repair is effected has not as yet been determined. It has been shown that the repair mechanism(s) is not completely identical with the mechanisms that repair ultraviolet irradiation (UV) damage (13, 15). This paper reports on some additional characteristics of the repair of MMS-induced damage.

MATERIALS AND METHODS

The *ind*$^+$ *thy*$^-$ strain was a derivative of strain 168 *thy*$^-$ provided by Frank Rothman. The adenine-requiring strain U-16 was provided by N. Sueoka. Transformation was by the protocol of Anagnostopoulos and Spizizen (1), with either *ind*$^-$ *uvr*$^+$ or *ind*$^-$ *uvr*$^-$ strains as recipients. The *Micrococcus lysodeikticus* and *B. subtilis* extracts were prepared as described by Strauss (19), except that DNA was removed from the crude preparations by precipitation with streptomycin sulfate. The streptomycin was then removed during ammonium sulfate precipitation and passage through Sephadex G75. Sucrose gradient centrifugation of native and alkali-denatured preparations was performed in 5 to 20% (w/w) linear gradients as described by Strauss, Searashi, and Robbins (21). The method of Davison, Freifelder, and Holloway (6) was used for the alkali denaturation and centrifugation as follows: one volume of DNA or reaction mixture was mixed with one volume of 0.2 M NaOH. After 30 sec, one volume of neutralized 37%

[1] Trainee on Graduate Training Grant (USPHS 2-TOl GM-603). Present address: Department of Bacteriology, Kansas State University, Manhattan.

[2] Research Career Development Awardee (GM-K3-823).

formaldehyde was added and mixed; then 0.125 volume of 1 M KH_2PO_4 was added. A sample of 0.1 ml was layered onto a 5 to 20% (by weight) linear sucrose gradient containing 6% formaldehyde, 0.1 M potassium phosphate (*p*H 7.8), and 1 M NaCl; this preparation was then centrifuged for 3 hr at 35,000 rev/min in the SW 39 rotor of a Spinco L2 ultracentrifuge maintained at 20 C. Sixteen-drop fractions were collected from the bottom of the tube. When both transforming activity and radioactivity of native DNA were to be determined, centrifugation was for 2 hr at 35,000 rev/min and eight-drop fractions were collected. Alternate fractions were assayed for either transforming activity or radioactivity. Radioactivity was determined by precipitating the DNA in the presence of albumin carrier and collecting the precipitate on nitrocellulose filters for counting in a Packard scintillation counter; the scintillation fluid was made up of 4.9 g of 2:5-diphenyloxazole and 0.1 g 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene. Sedimentation constants were calculated by the method of Martin and Ames (11) as previously described (21). Labeled cells were prepared by growing cultures of the thymine-requiring strains in CHT50 medium containing ^{3}H-methyl-labeled thymidine; purified DNA was prepared from such labeled cells as previously described (21). Cells were lysed by treatment at 37 C with muramidase (250 μg/ml, 10 min), pronase (250 μg/ml, 10 min), and sodium lauryl sulfate (0.05%, 5 min) in sequence (13); this procedure completely solubilized the bacteria, yielding a crystal-clear solution.

RESULTS

A nuclease with specificity for alkylated DNA was first demonstrated by showing that the transforming activity of alkylated DNA was reduced by *B. subtilis* extracts (19). In contrast to the extracts from *M. lysodeikticus*, the *B. subtilis* extracts do not cause further inactivation of UV-treated DNA (13).

Alkylated DNA can be a heterogeneous substance containing methyl groups, apurinic sites, and single strand breaks (4); at low doses the number of such pre-existing breaks must be small, since alkylation of DNA to a level equivalent to five inactivating hits per locus has no effect on the sedimentation of native DNA (23). In an attempt to sort out the ability of these various sites (methylated, apurinic, and single strand breaks) to serve as substrates for the degradative enzyme, we carried out a series of experiments in which alkylated or alkylated and heated samples of DNA were alkali-denatured and centrifuged in sucrose gradients before and after enzyme treatment. Alkali denaturation of DNA not only separates the strands but also leads to rapid hydrolysis at apurinic sites (7, 24). We reasoned that, if enzyme-induced breaks were produced at apurinic sites, a further lowering of the sedimentation constant should not be observed after alkali denaturation of enzyme-treated methylated DNA, since all the apurinic sites would be alkali-sensitive to begin with.

MMS-treated DNA denatured with alkali had a lower average sedimentation constant than nontreated, alkali-denatured DNA (Table 1). Heating alkylated DNA at 50 C, a temperature which, although below the thermal denaturation point, is known to accelerate depurination (5), resulted in an even lower $S_{20,w}$ value after denaturation with alkali. It had previously been shown that heating control, nonalkylated DNA for 5 hr at 50 C had no effect on the transforming activity, i.e., did not result in the production of a measurable number of single strand breaks (22). We interpret these data to mean that the pre-existing apurinic sites and single strand breaks present in the MMS-treated *B. subtilis* DNA used in these experiments were exposed by denaturation with alkali but that the remaining initial

TABLE 1. *Sedimentation behavior and transforming activity of alkylated DNA*[a]

Enzyme	$S_{20,w}$ of alkali-denatured DNA			Relative transforming activity		
	Control	Alkylated[b]	Alkylated and heated[c]	Control	Alkylated	Alkylated and heated
None	23.3	17.3	12.3	1.0	0.46	0.041
Micrococcus lysodeikticus extract	21.3	9.2	6.5	1.0	0.022	0
Bacillus subtilis extract	20.4	9.8	7.5	1.0	0.048	0

[a] The details of the enzyme treatment and the method of calculating the weight average $S_{20,w}$ values were as described by Strauss, Searashi, and Robbins (21). Relative transforming activities were calculated from the linear region of the transformation response curves, with the control sample set as 1.0 in each case.

[b] ^{3}H-labeled DNA was treated for 1 hr at 37 C with 0.025 M MMS in phosphate buffer (0.05 M, *p*H 7.4). The DNA was removed from the reaction mixture by precipitation with ethyl alcohol, and was redissolved in 0.15 M NaCl plus 0.015 M sodium citrate.

[c] Heated for 5 hr at 50 C in potassium phosphate buffer (0.05 M, *p*H 7.4).

products of reaction between MMS and DNA (probably methylated purine nucleotides) were alkali-stable. This hypothesis accounts for the lowered sedimentation constant of heat-treated alkylated DNA, since heating would be expected to increase the sensitivity of methylated DNA to alkali by creating additional apurinic sites. Recently, we have found that phage T7 DNA, cautiously alkylated with MMS, does have the same sedimentation constant in alkali as does a nonalkylated control. The methylated T7 DNA becomes alkali-sensitive on heating, as required by the hypothesis.

Treatment of alkylated DNA with either *M. lysodeikticus* or *B. subtilis* extracts resulted in a large decrease in the average sedimentation constant of the alkali-denatured product (Table 1). Some or all of the apurinic positions therefore survived treatment with extract to give further breakdown on alkali denaturation, since, as discussed above, no decrease in *S* would be expected if the extract produced breaks at all the apurinic sites. If the methylated, but not the apurinic, positions were sites for enzyme action, heated DNA should be a poorer substrate, since it presumably contains fewer methyl groups. However, the number of additional breaks induced by enzyme was practically the same for alkylated and alkylated and heated DNA, as judged by the ratio of the *S* values. Since we do not know the number of methyl groups present or the proportion removed by heating in our experiments, it is not necessary to exclude the methylated nucleotides as possible sites for enzyme action. However, the data are consistent with the idea that breaks can occur in the strand opposite an apurinic or methylated position.

In vivo effects. The action of the nuclease in *B. subtilis* can account for the low transforming activity of DNA extracted from MMS-treated cells. It can also account for our ability to observe an increase in the activity of the DNA extracted from cells incubated for a time after MMS treatment (13). Since DNA is exposed to the action of the degrading nuclease in the course of extraction, we reasoned that those groups which make DNA a substrate for the nuclease would no longer be present in DNA from recovered organisms.

If this analysis is correct, DNA extracted from MMS-treated cells should be of smaller molecular size than DNA from either nontreated cells or from treated cells allowed to recover by incubation prior to lysis. To test this prediction, *ind*$^+$ *thy*$^-$ organisms were labeled by overnight growth in CHT50 medium containing ^{3}H-thymidine. Lysates of nontreated control cells and of MMS-treated cells were prepared immediately after treatment and after 40 min of incubation. The distribution of the radioactivity of native DNA in each of these samples was determined by zone centrifugation in a sucrose gradient (Fig. 1). DNA from nontreated control cells was used as a standard.

DNA from MMS-treated cells was fragmented, compared to the control preparation, and DNA from incubated cells behaved as though it contained larger fragments than those found in lysates of nonincubated cells. Smaller pieces were also found in lysates of incubated cells. When the distribution of transforming activity from incubated cells was examined, more of the activity was found associated with larger pieces than was present in the nonincubated sample, indicating recovery from the MMS-induced damage (Fig. 2).

DNA from organisms which have recovered from MMS treatment is old DNA, that is, DNA a majority of whose atoms are those that were present in the molecule before treatment (13). Since some new molecules (of thymidine) are incorporated into DNA of old density during recovery, it seemed possible that MMS repair might

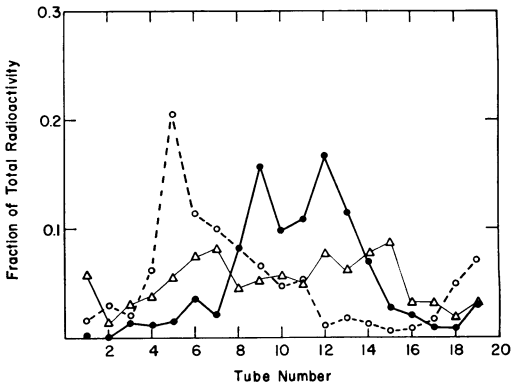

FIG. 1. *Distribution of ^{3}H-labeled DNA from an MMS-treated culture. Cultures of the ind$^+$ thy$^-$ strain were labeled by overnight growth in medium containing 1 μc (per ml) of ^{3}H-thymidine (20 μg/ml). The cells were harvested and washed. A portion was treated with MMS to a survival N/N_0 of 3.6×10^{-3}. One portion of the MMS-treated cells was incubated for 40 min in CHT50 medium before lysis. The cells were lysed by treatment for 10 min with 1 mg (per ml) of lysozyme, followed by the addition of sodium lauryl sulfate to 0.05%. After standing at 37 C until complete clearing occurred, the lysates were diluted and used for zone centrifugation in sucrose gradients. Symbols: ○, control; ●, MMS-treated; △, MMS-treated and recovered. The relative ind$^+$ transforming activity in the linear range: control, 1.0; MMS-treated, 0.097; MMS-treated and recovered, 0.326. The respective ^{3}H activity (counts per min per 0.1 ml): control, 410; MMS-treated, 577; MMS-treated and recovered, 555.*

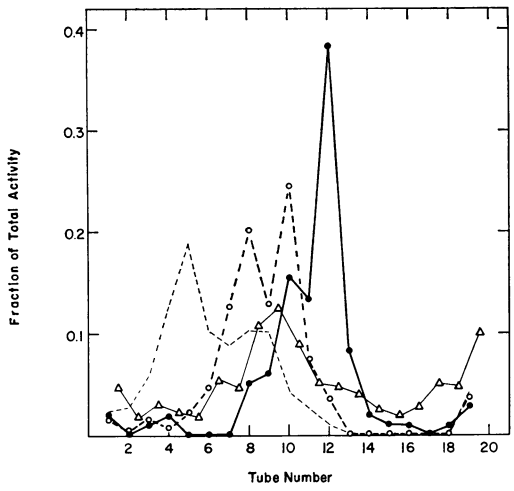

FIG. 2. *Sedimentation of lysates of MMS-treated and MMS-treated and recovered cells: distribution of transforming activity. An overnight culture of ind$^+$ thy$^-$ organisms was harvested, washed, and treated with MMS (0.54 ml per 40 ml of suspension for 10 min at 37 C) to give a surviving fraction N/N_0 of 5.2 $\times$ 10^{-2}. One portion of the mixture was then washed and lysed. This had 0.15 of the transforming activity of the control, nontreated preparation. A second portion was incubated for 40 min in CHT50 medium containing 4 μc (5 μg) of thymidine per ml and then lysed. This lysate had 0.32 of the transforming activity of the control. The lysates were diluted 1:5, and 0.1 ml was centrifuged for 2 hr at 35,000 rev/min in the SW39 rotor of a Spinco L2 centrifuge on a 5 to 20% (w/w) sucrose gradient. Eight-drop fractions were collected. Alternate fractions were used to assay transforming activity and acid-insoluble radioactivity. Symbols: ●, transforming activity of MMS-treated cells; ○, transforming activity of MMS-treated and recovered cells; dotted line, transforming activity of control, nontreated cells (from a separate experiment); △, radioactivity.*

proceed by some minor variation of the steps used to repair UV-treated DNA, that is, by removal of damaged material followed by repair replication (12). The hypothesis that MMS repair proceeds in a manner similar to the repair of UV-induced damage has several attractive features, not the least of which is the prediction of several reactions.

First, a portion of the damaged DNA must be solubilized when the damaged bases are excised. We observed that, in a population treated with MMS to a survival of 10^{-5} and then incubated in CHT50 plus 20 μg (per ml) of thymidine for about 120 min, about 5% of the radioactivity previously incorporated in DNA appeared in an acid-soluble form (Fig. 3). Much greater solubilization was observed during incubation after UV doses producing a lesser degree of inactivation (20*a*).

Second, the recovery should be accompanied by the incorporation of new material into old DNA, and the product of the reaction should have biological activity. It had been shown that thymidine was incorporated into old DNA during incubation after MMS treatment and that this DNA had transforming activity (13). In these experiments, we decided to determine the size of the DNA molecules in which ^{3}H-labeled thymidine was found when MMS-treated organisms were allowed to recover by incubation in medium containing thymidine. MMS-treated cells were allowed to incubate for 40 min in the presence of ^{3}H-labeled thymidine, the cells were lysed, and the lysates were subjected to zone centrifugation in a sucrose gradient (Fig. 2). The transforming activity in this experiment increased from 15% of the untreated control to 32% after recovery. Most of the thymidine incorporated during incubation was found to be associated with fragments of a size characteristic of the recovered transforming DNA. It would appear that thymidine is incorporated preferen-

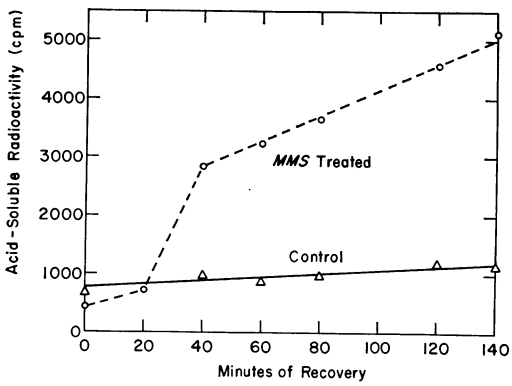

FIG. 3. *Solubilization of DNA in MMS-treated cells. Strain 168 ind$^+$ thy$^-$ cells were labeled by growth in medium containing ^{3}H-thymidine, washed on a membrane filter, and then grown for 90 min in a medium with nonradioactive thymidine. The labeled cells were divided into two portions: one was treated with 0.26 M MMS at 37 C to a survival of 10^{-5}; the other served as a nontreated control. Both were then incubated with aeration at 37 C, and samples were removed at intervals. The samples were chilled, treated with cold 5% trichloroacetic acid, and centrifuged. The acid was removed from the supernatant fluids with ether, and the radioactivity was determined in a scintillation counter. Total radioactivity in the cultures, determined in the same way after heating a sample of the culture in 5% trichloroacetic acid at 90 C for 15 min, equalled 9.2 $\times$ 10^4 counts/min.*

tially into repaired regions or that the process of repair requires such incorporation.

If repair replication of excised regions of damaged DNA were required for recovery from MMS-induced damage, starvation for thymine or other bases would be expected to inhibit the repair process. Absolute starvation of treated cells for either thymine or adenine is difficult to achieve, since, as noted, the breakdown of preexisting DNA to acid-soluble constituents may provide a pool of DNA precursors. We therefore decided to compare the effects of starvation for adenine (Table 2) and for thymine (Table 3) on recovery from both UV and MMS treatment. UV recovery, which presumably requires extensive resynthesis (3, 16), should require both adenine and thymine; these would have to be supplied from either endogenous or exogenous sources. Exponential cultures of *thy*$^-$ *ind*$^+$ and of *ade*$^-$ *ind*$^+$ strains were prepared, starved, treated, and allowed to recover for 40 min in the presence or absence of adenine or thymine. The transforming activity of the resulting extracts was determined by use of a *uvr*$^-$ recipient. Considerable recovery from both UV and MMS treatment was observed, even when the cultures were starved for adenine or thymine. Addition of adenine or thymine increased the recovery from UV damage but had no effect on recovery from MMS damage. The thymine starvation experiment was complicated by an effect of starvation on the control, but the results followed the pattern of adenine starvation: a greater effect of addition of thymine on UV recovery than on MMS recovery. The relative extent of recovery from UV damage in the adenine and thymine starvation experiments was the same whether the *uvr*$^-$ strain or the *M. lysodeikticus* enzyme (21) was used to assay for the presence of UV-induced lesions.

TABLE 2. *Adenine requirement for UV and MMS recovery*[a]

Treatment	Transformants/ml at 10^{-3} dilution			Relative recovery[b]
	Non-incubated	Incubated for 40 min		
		Without adenine	With adenine	
None	109	153	153	1.0
UV	15	28	43	1.5
UV plus extract[c]	0.15	0.45	0.78	1.7
MMS	13	44	41	0.94

[a] Adenine-requiring cells in the exponential phase of growth were starved for adenine for 10 min before the start of the experiment. Transformation for the *ind*$^+$ marker was by use of a *uvr*$^-$ recipient.

[b] Ratio of recovery with adenine to recovery without adenine.

[c] Lysis in presence of *Micrococcus lysodeikticus* extract.

TABLE 3. *Effect of thymine starvation on the recovery of transforming activity in an ind*$^+$ *uvr*$^+$ *thy*$^-$ *strain after treatment with UV or MMS*[a]

Treatment	Transformants/ml at 10^{-2} extract dilution			Relative recovery[b]
	Non-incubated	Incubated for 40 min		
		Without thymine	With thymine	
None	990	475	664	1.4
UV	38.4	42	73	1.7
UV plus extract[c]	1.2	13	26	2.0
MMS	13.2	28	37	1.3

[a] Exponential cells were starved 60 min before the start of the experiment. Transformation for the *ind*$^+$ marker was by use of a *uvr*$^-$ recipient.

[b] Ratio of recovery with thymine to recovery without thymine.

[c] Lysis in presence of *Micrococcus lysodeikticus* extract.

DISCUSSION

DNA extracted from organisms allowed to incubate after treatment with MMS was not as sensitive to the action of a nuclease specific for alkylated DNA as was DNA from nonincubated cells. Recovery occurs on incubation, and includes the removal of the sites of nuclease action from the DNA. Since the possible sites for nuclease action result from an initial methylation, the simplest hypothesis for recovery is that there is a direct demethylation of the DNA. It is possible that there are enzymes which remove *N*-methyl groups from the DNA in a manner similar to the dealkylating enzymes demonstrated in microsomes (10). However, there is no evidence for this idea, and a more orthodox hypothesis is that MMS repair in *B. subtilis* requires excision of the lesion and resynthesis of the excised region in a manner similar to that proposed for the repair of UV damage (7*a*). There have been reports that UV-sensitive mutants are sensitive to bifunctional alkylating agents. These mutants, in contrast to normal cells, are unable to remove the products of reaction of bifunctional agents with DNA (5, 8).

However, it has been shown that damage produced by monofunctional alkylating agents is repaired in a strain unable to repair UV-induced damage (13). Recently, we have shown that, although actinomycin D inhibits the repair of UV-induced damage, it has little or no effect on the repair of MMS-induced damage in *B. subtilis* (14).

Nonetheless, our data are compatible with the idea that excision and repair replication (12) are required for the repair of MMS damage, although the initial stages of the repair differ from those involved in the repair of UV-induced damage. DNA breakdown is observed after MMS treatment, and thymidine incorporation seems to accompany repair. At first sight, the nutritional data appear to contradict the hypothesis, since recovery from MMS treatment is not affected by starvation for either adenine or thymine (which must necessarily be involved in any DNA synthesis). However, the most striking feature of our experiments was the extensive UV repair observed in the absence of added adenine or thymine. Stimlation of UV repair did occur on the addition of adenine or thymine, but the effect was small. There must be a considerable pool of adenine or thymine in the starved cells, resulting perhaps from breakdown of the MMS (or UV-)-treated DNA. This breakdown may account for the observation that dimer excision and other phases of UV repair occur during thymine starvation in *E. coli* (17, 18). No particular significance can be attributed to the negative results of the nutritional experiments as far as MMS recovery is concerned.

Repair from at least some damage induced by MMS is probably similar to repair of UV damage, except for the different mechanisms involved in the production of the original break at the site of damage. Presumably, those experiments which distinguish between UV and MMS repair did so because they involved the earliest stages of UV repair. We suppose that the *uvr*$^-$ strain is unable to excise, and that actinomycin D inhibits one of the early stages in the excision process. As has been pointed out before (15), the *uvr*$^-$ strain is not sensitive to treatment with MMS; the *mms*$^-$ strain, sensitive to MMS treatment, is sensitive to UV, a finding which supports the idea that the two types of repair are related.

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