THE EFFECTS OF ACTINOMYCIN ON DEOXYRIBONUCLEIC ACID TREATED WITH METHYL METHANESULFONATE

by

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INTRODUCTION

Microorganisms are capable of repairing the damage to DNA induced by the monofunctional alkylating agent, methyl methanesulfonate. Little is known about the nature of this repair, but most of the data are consistent with a model for the repair of UV-induced DNA damage. Since UV repair has been studied extensively, this model has been used as a basis for describing repair of damage to DNA caused by other agents.

The model of excision and repair for UV-induced DNA damage is described as follows:

1. Recognition of the pyrimidine dimers by an endonuclease which breaks DNA near the site of the dimers.
2. Removal of damaged bases along with an unspecified portion of the same strand of DNA.
3. Repair replication by a polymerase enzyme which resynthesizes the portion of the strand which has been removed.
4. A patching operation which covalently links the free end of the newly-synthesized DNA to the "old" DNA strand, completing the sugar-phosphate backbone.

Data suggest that damage to the DNA of microorganisms caused by ionizing radiation, Mitomycin C, and bifunctional alkylating agents can be repaired in a manner which is consistent with this excision-repair mechanism. The repair of DNA damaged by all of these agents can be explained utilizing either part or all of the steps involved in excision-repair. If all of the steps are required

for repair of damage induced by a particular agent (such as Mitomycin C or a bifunctional alkylating agent), a UV-sensitive mutant is sensitive to this agent also. If only the latter steps of excision-repair are required for repair of damage induced by a particular agent (such as ionizing radiation which causes single-strand breaks in DNA, for example) a UV-sensitive mutant may be capable of repairing this particular type of damage.

If Bacillus subtilis 168H is treated with methyl methanesulfonate and subsequently incubated in a growth medium, there is an increase in the transforming activity of DNA from these cells compared to DNA from MMS-treated cells which are lysed immediately Reiter and Strauss, 1965). This indicates there is a repair mechanism in B. subtilis for MMS-induced DNA lesions. There is evidence in support of the hypothesis that MMS repair occurs via the steps of the excision-repair mechanism. Solubilization of DNA occurs if cells are incubated in growth medium after MMS treatment (Searashi and Strauss, 1967). These cells incorporate 3H-thymidine in a nonconservative fashion (i.e., repair replication) with concomitant increase in transforming activity (Reiter and Strauss, 1965).

There are two lines of evidence that this repair is slightly different from the repair of UV-induced lesions in DNA. UV-sensitive cells are capable of repairing the MMS-induced lesions in DNA. MMS causes single-strand breaks in DNA (Strauss and Wahl, 1964). It is possible that repair of MMS-induced damage, similar to the repair of damage due to ionizing radiation, requires only

the latter steps of excision-repair. This hypothesis is consistent with the evidence that MMS-sensitive mutants are also UV-sensitive. To be sensitive to MMS a mutant would have to be defective in a later step of repair. This mutant would still be sensitive to UV, in spite of the fact that it could excise prymidine dimers, since UV repair requires all of the steps in the excision-repair mechanism. Therefore, recovery of MMS-induced lesions may be a result of repair which differs from UV repair by at least one step, but is consistent with the excision-repair mechanism in general.

Reiter and Strauss (1967) reported that MMS repair is different from UV repair in another aspect: repair of UV-induced damage is inhibited by actinomycin, whereas repair of MMS-induced damage apparently occurs in the presence of this antibiotic. The purpose of this research was to attempt to elucidate this anomalous feature of MMS repair.

REVIEW OF LITERATURE

Removal of UV-Induced Lesions in DNA by an Excision-Repair Mechanism

There is an accumulation of evidence that the UV-induced damage to DNA is a result of the formation of pyrimidine dimers. Wacker (1963) was able to demonstrate that lesions were present in DNA after UV-irradiation. At doses that are biologically significant a relationship exists between survival and the amount of thymine dimers present. These dimers are formed from adjacent thymine residues present on the same strand.

UV photoproducts in addition to thymine dimers include cytosine dimers and cytosine-thymine dimers (Setlow, Carrier and Bollum, 1965). Cytosine moieties may also be deaminated, forming uracil dimers or uracil-thymine dimers. The addition of water to 5,6 double bond of these bases frequently occurs (Smith, 1964). The presence of pyrimidine dimers may cause a distortion of the DNA twin helix and local interruption of the hydrogen bonding between base pairs (Setlow, 1966).

It was first recognized that microorganisms are capable of altering the effects of UV light when mutants were isolated with sensitivities toward UV that are different from the wild types (Witkin, 1947; Hill, 1958). A photoreactivation enzyme (Wulff and Rupert, 1962) which splits pyrimidine dimers (Setlow and Setlow, 1963; Setlow, Boling and Bollum, 1965) is capable of

reversing at least 50% of the inactivation of transforming DNA at high doses of UV radiation (Setlow and Setlow, 1962; Setlow, 1964). Immediately after UV-irradiation, if cells are incubated in growth medium, there is a delay in DNA synthesis which is proportional to the dose (Hanawalt and Setlow, 1960). Studies on the rate of synthesis of DNA <u>in vitro</u> indicate that the primer activity of DNA is affected by UV light and part of this damage is due to the formation of thymine dimers (Bollum and Setlow, 1963).

Although both UV-sensitive and UV-resistant cells produce the same number of dimers (Setlow, Swenson and Carrier, 1963), UV-resistant cells are capable of excising thymine dimers during the period when net DNA synthesis does not occur (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). Thymine dimers are not excised <u>per se</u> but are still attached to the phosphodiester backbone (Boyce and Howard-Flanders, 1964). The actual size of the fragments excised cannot be determined since random breakdown by nucleases may occur after excision. DNA synthesis in UV-sensitive cells which are unable to excise thymine dimers is permanently inhibited by low doses of UV light (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). In these mutants one dimer may act as a permanent block to DNA synthesis (Swenson and Setlow, 1966). Pettijohn and Hanawalt (1964) demonstrated that nonconservative replication occurs during the period when there is no net DNA synthesis and DNA degradation is occurring. This nonconservative DNA synthesis may occur at random over the bacterial genome and is not restricted to DNA either in front of or behind the

replication growing point.

UV-sensitive mutants of Escherichia coli K-12 which control excision of thymine dimers have been mapped at three sites on the bacterial chromosome (Howard-Flanders, et al, 1962; Putte, et al, 1965). The gene products from these loci do not act in any sequence (Howard-Flanders, Boyce and Theriot, 1965).

From these results it was concluded that DNA synthesis is prevented by the presence of UV photoproducts, such as thymine dimers. Cells can repair the UV-induced DNA damage by the excision of pyrimidine dimers. This is accompanied by further degradation of DNA and local resynthesis, which occurs by the insertion of new bases pairing with the bases of the opposite strand. If the repair polymerase is identical to the Kornberg enzyme, this synthesis occurs by the addition of a 5' trinucleotide to a 3' OH end of the DNA strand (Chargaff and Davidson, 1960). This last step of repair requires that newly-synthesized DNA be joined to the old part of the same strand to complete the phosphodiester backbone. Recent investigations (Gellert, 1967; Weiss and Richardson, 1967) have demonstrated the existence of an enzyme capable of catalyzing the repair of single-strand breaks with the formation of covalent 3'-5' phosphodiester bonds.

Repair of Other DNA Lesions by an Excision-Repair Mechanism

The model of repair proposed for UV damage (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964) has been expanded to include

repair of damage by other agents (Haynes, 1966). UV light, bifunctional alkylating agents, and Mitomycin C compose a class of agents which damage DNA. This damage probably requires all the steps in the excision-repair mechanism for recovery. These agents affect the DNA molecule by altering its structure in some way. Bifunctional alkylating agents such as nitrogen mustard or mustard gas are capable of forming cross-links in DNA by alkylating the N7 position of guanine in adjacent base pairs, if the guanine moieties occur in opposite strands of the DNA molecule (Brookes and Lawley, 1961; Lawley and Brookes, 1963, 1965).

UV-sensitive cells are sensitive to nitrogen mustard, whereas UV-resistant cells are resistant (Haynes, Patrick and Baptist, 1964). Kohn, Steigbigel and Spears (1965) showed that UV-resistant cells treated with nitrogen mustard are capable of repairing cross-links in their DNA during incubation in growth medium after treatment. DNA synthesis in these cells is only slightly affected after treatment. In contrast, UV-sensitive cells cannot remove cross-links during post-treatment incubation, and DNA synthesis is completely inhibited. In wild type cells nonconservative repair replication occurs during the incubation period after treatment with nitrogen mustard (Hanawalt and Haynes, 1965).

When microorganisms are treated with sulfur mustard, DNA degradation occurs. The rate of loss of sulfur mustard products that are initially bound to DNA is greater than the rate of overall DNA degradation. Several products including mono- and bifunctionally alkylated guanine are eliminated. After a lag, treated cells recover the capacity to synthesize DNA (Papirmeister and Davison, 1964).

In UV-sensitive strains DNA synthesis is inhibited, but the ^{35}S from the sulfur mustard treatment is not removed. In contrast, resistant strains are capable of enzymatically degrading DNA during the inhibition of DNA synthesis with the release of 50% of the ^{35}S. Both strains are alkylated to the same extent. During one hour of post-treatment incubation in growth medium, resistant cells are capable of removing all di- and most mono-functionally alkylated guanine residues (Lawley and Brookes, 1965). Lovelace, Cook and Wheatley (1965) have shown that cells are also capable of recovery after treatment with mustard gas.

Mitomycin C also forms covalent cross-links in the complementary DNA strands. Although the exact site of interaction on the DNA molecule is not known, work with space-filling models is compatible with interstrand linking between the amino group of adenines or cytosines, or between the 06 groups of guanine moieties. Correlation of cell death with GC content favors either G-C, C-C or G-C cross-links (Iyer and Szybalski, 1964; Szybalski and Iyer, 1964). UV-resistant cells treated with Mitomycin C are capable of removing cross-links in DNA, whereas most UV-sensitive cells are not (Terawahi and Greenberg, 1966; Mahler, 1966). The DNA of cells which have been treated with Mitomycin C is degraded after treatment (Reich, Shatkin and Tatum, 1961).

These results certainly suggest that the same mechanism of excision and repair proposed for UV damage may be operating in cells after treatment with bifunctional alkylating agents and Mitomycin C. Cells which are able to excise thymine dimers are

also capable of excising cross-linked, alkylated guanine residues and removing cross-links caused by Mitomycin C. Cells which cannot excise thymine dimers are deficient in these functions also.

Repair of DNA Containing Single-Strand Breaks

A second class of agents which induce DNA damage include ionizing radiation, ^{32}P decay, and monofunctional alkylating agents. These agents all cause single-strand breaks, among other types of damage, in the DNA molecule.

Since UV-sensitive cells are sensitive to bifunctional alkylating agents and Mitomycin C, it is interesting to determine if they are also sensitive to ionizing radiation. One of the major lesions from ionizing radiation at biologically significant doses is single-strand breaks (Shooter, 1957; Freifelder, 1965, 1966). It may be possible that some UV-sensitive mutants can be X-ray resistant even though they lack the excision enzyme, if they are capable of degrading and resynthesizing damaged DNA.

Rörsch, Edleman and Cohen (1963) mapped a gene in _E. coli_ (_hcr_$^-$) that controlled X-ray sensitivity, as well as UV sensitivity. _E. coli_ B_{s-1}, which is UV-sensitive and lacks the ability to excise thymine dimers, is more sensitive to ionizing radiation than _E. coli_ B/r (Hill and Simson, 1961). The initial rate of enzymatic degradation of DNA is the same for B_{s-1} and B/r, but eventually B_{s-1} suffers two to three times as great a loss. Three times as much X-irradiation is required to produce the same reduction of

incorporation of ^{3}H-thymidine in B/r as in B_{s-1} (McGrath, Williams and Swartzendruber, 1966). B/r is capable of repairing single-strand breaks caused by ionizing radiation, but B_{s-1} cannot (McGrath and Williams, 1965). Since E. coli B_{s-1} is a double mutant (Mattern, van Winden and Rörsch, 1965), it is apparently defective in a later step of the excision-repair mechanism, as well as defective in its ability to excise thymine dimers. It is sensitive to both MMS and nitrogen mustard; repair does not occur even though breaks may already be present in the DNA (Bridges and Munson, 1966). There is a synergistic interaction between UV radiation and X-rays in B/r, which also implies that the same repair mechanism is effective in the recovery from both types of damage (Haynes, 1964).

UV-sensitive organisms that are X-ray resistant have also been isolated. Bridges and Munson (1966) isolated a UV-sensitive mutant, E. coli WP2 hcr$^-$, which lacks the ability to excise thymine dimers but is no more sensitive to ionizing radiation than B/r. This mutant is also sensitive to nitrogen mustard but not to methyl methanesulfonate, which causes single-strand breaks in DNA (Strauss and Wahl, 1964; Wahl, 1965). Another UV-sensitive mutant of E. coli K-12 which cannot excise thymine dimers (Boyce and Howard-Flanders, 1964) is capable of degrading DNA that has been X-irradiated to the same extent as the wild type (Emmerson and Howard-Flanders, 1965). These data are consistent with the hypothesis that the degradation step in repair, which requires a break in the phosphodiester backbone of one strand, is separated from the excision step, which causes single-strand breaks in the DNA molecule.

E. coli WP12 hcr$^-$ and E. coli K-12 uvr$^-$ lack the endonuclease capable of causing interruptions in the backbone, but they are still capable of repairing damage from agents which produce single-strand breaks in DNA. Loci which are defective for excision are not necessarily defective for degradation.

^{32}P causes single-strand breaks in DNA by transmutations (Strauss, 1958). Cairns and Davern (1966) have found that DNA synthesis is delayed slightly in normal cells after heavy labeling with ^{32}P but ceases immediately in rec$^-$ (UV-sensitive) strains. Degradation of DNA occurs to the same extent that DNA synthesis has been reduced, but it is not restricted to the part of the chromosome containing the ^{32}P label. In other words, post-treatment behavior of microorganisms after ^{32}P labeling is very similar to that occurring after UV- or X-irradiation. Mutants sensitive to UV light and X-ray are also more sensitive to ^{32}P decay, whereas resistant mutants are less affected by this treatment than wild types (Hill and Simson, 1961).

In conclusion, damage induced by agents which cause single-strand breaks in DNA may be repaired by utilizing the latter steps of the excision-repair mechanism. Therefore, UV-sensitive mutants defective in the first steps of this mechanism are still able to repair damage caused by ionizing radiation, MMS, and ^{32}P, but not damage cuased by bifunctional alkylating agents or Mitomycin C. Mutants which are defective in a later step of excision-repair are unable to repair either type of damage.

The Relationship of Recombination and Excision-Repair

One of the current models for recombination envisions a breaking and rejoining of the particular chromosomes involved. This phenomenon may occur by the same mechanism of excision and repair which results in the removal of DNA lesions. Mutants which are unable to form recombinants, although DNA is taken up by the cell, are also UV sensitive (Clarke and Margulies, 1965). In *B. subtilis*, Mitomycin C and UV sensitivity are controlled by a single genetic event and are directly associated with the cell's ability to undergo recombination (Okubo and Romig, 1966).

A recombination-deficient mutant of *E. coli* K-12, which is UV-sensitive in spite of the fact that it is capable of excising thymine dimers, is also sensitive to ionizing radiation. DNA degradation after exposure to UV light occurs at a tremendous rate (Howard-Flanders and Theriot, 1966). Apparently this particular mutant is unable to regulate the breakdown and resynthesis of DNA during excision-repair. However, another UV-sensitive *rec*$^-$ mutant which has an intermediate sensitivity to ionizing radiation undergoes normal DNA degradation after exposure to UV light (Howard-Flanders and Boyce, 1966).

Van de Putte, Zwenk and Rörsch (1966) have mapped four *rec*$^-$ mutants which were UV-sensitive, X-ray sensitive, and *hcr*$^+$ at three different sites on the bacterial chromosome. The nuclease activity in these mutants is no different from wild type. Two *rec*$^-$

mutants are also able to form covalent links at the site of single-strand breaks at the same rate as the normal cells (Gellert, 1967).

Although the phenomenon of recombination is not completely understood, it can be concluded that it is related to excision and repair. Recombination requires the latter steps of this mechanism only. This is similar to the repair of DNA damage induced by agents which cause single-strand breaks in the DNA molecule.

Repair of MMS-Induced DNA Damage

Lawley and Brookes (1963) have shown that methyl methanesulfonate (MMS) causes damage to DNA by alkylating the nucleophilic centers in the DNA molecule at the N7 of guanine and more slowly at the N3 of adenine. Alkylation of the N1 position in adenine is suppressed because of its involvement in hydrogen bonding. Upon alkylation, a quaternary nitrogen is formed, depleting the electrons in the ring structure of the alkylated base. This causes a destabilization of the alkylated moieties. Apurinic sites may then appear if hydrolysis occurs (Lawley and Brookes, 1963). Further hydrolysis at the phosphodiester bonds adjacent to the apurinic sites result in single-strand breaks (Strauss and Wahl, 1964).

A series of mutants of B. subtilis with sensitivities analogous to those in E. coli have been isolated. These mutants behave in a manner which is in agreement with the model of excision-repair. Since B. subtilis can be transformed, it is possible to use this

organism to assay for biological activity DNA treated in various ways.

An enzyme present in Micrococcus lysodeikticus extracts is capable of inactivating and degrading DNA treated with UV light or MMS (Strauss, 1962; Strauss, Searashi and Robbins, 1966). A similar enzyme in B. subtilis is capable of inactivating DNA treated with MMS but not with UV light (Reiter and Strauss, 1965).

UV-sensitive mutants are unable to repair UV-induced lesions as measured by a continued sensitivity of the extracted DNA to the M. lysodeikticus extract when treated cells have been incubated prior to lysis (Strauss, Searashi and Robbins, 1966). Absence of repair can also be measured by a lack of increase in the transforming activity of DNA extracted from UV-treated, incubated cells to be transformed by UV-irradiated transforming DNA (Strauss, Searashi and Robbins, 1966), and by an inability of UV-irradiated phage SP01 to survive on a UV-sensitive host (Reiter and Strauss, 1965). UV-resistant cells solubilize DNA after UV treatment, but UV-sensitive cells do not (Strauss, Reiter and Searashi, 1966).

UV-sensitive mutants are able to recover from MMS-induced damage. This is measured by an increase in extractable transforming activity of MMS-treated cells which have been incubated in medium and by an ability of MMS-treated SP01 phage to survive on these UV-sensitive cells (Reiter and Strauss, 1965).

In contrast, mms^- strain is sensitive to UV light and cannot support growth of either UV- or MMS-treated SP01 (Searashi and Strauss, 1965; Strauss, Reiter and Searashi, 1966). This mutant

degrades its DNA after UV treatment to a greater extent than normal cells do. After MMS treatment even more DNA breakdown occurs (Searashi and Strauss, 1967). The mms$^-$ strain is poorly transformed compared to the UV-resistant or UV-sensitive strains (Strauss, Reiter and Searashi, 1966).

Repair replication similar to that occurring in E. coli treated with UV light (Pettijohn and Hanawalt, 1964) occurs in B. subtilis uvr$^+$ treated with MMS (Reiter and Strauss, 1965). If $^2H^{15}N$ density-labeled cells are treated with MMS and then incubated in $^1H^{14}N$ medium with ^3H-thymidine, incorporation of radioactivity is accompanied by an increase in transforming activity at the position of the "old" (heavy) DNA as determined in CsCl density gradients.

Since there is a nuclease present in B. subtilis which degrades DNA containing MMS induced lesions at the time of lysis, it is possible to measure recovery of MMS-induced damage as an increase in extractable transforming activity from incubated cells compared to MMS-treated cells lysed immediately (Reiter and Strauss, 1965). Recovery may also be determined by measuring an increase in molecular weight of DNA extracted from incubated cells compared to that of MMS-treated cells lysed immediately (Reiter, Strauss, Robbins and Marone, 1967). If cells are prelabeled with radioactive thymidine, DNA from recovered cells appears in an intermediate position in sucrose gradients between DNA from untreated cells and DNA from MMS-treated cells which have been lysed immediately. Transforming activity is found at the position corresponding to the radioactive peak for each DNA sample. If cells are labeled during the recovery period, transforming activity and newly-incorporated label of recovered cells again occurs at a position intermediate

of the transforming activity of control and unrecovered DNA samples.

The above data suggest that MMS recovery is similar to repair except that it may require one less early step in repair, as seen by the resistance of UV-sensitive cells to MMS and the sensitivity of *mms*⁻ mutants to UV light as well as to MMS. It is similar since nonconservative repair replication occurs and some DNA is solubilized in the process. Apparently the excision step is not required for MMS repair since the UV-sensitive strain can repair MMS-induced damage. The *mms*⁻ strain must be deficient in a later step in repair since it cannot repair either UV- or MMS-induced damage.

Reiter and Strauss (1967) have shown that MMS repair seems to differ from UV repair in at least one other characteristic. Actinomycin inhibits repair of UV damage. The DNA from UV-irradiated cells incubated in growth medium plus actinomycin remains susceptible to the *M. lysodeikticus* enzyme. There is a lag in the solubilization of DNA in these cells during the incubation period after treatment which is equivalent to the period required for DNA to become resistant to the *M. lysodeikticus* enzyme in the absence of actinomycin. ^{3}H-thymidine added at the time of incubation after UV treatment is not incorporated in the presence of actinomycin. Actinomycin may be preventing repair replication, or the need of replication if it is preventing excision from occurring. Whatever mechanism of inhibition is being utilized, it seems to be avoided in the repair of MMS-induced damage. If MMS-treated cells are incubated in growth medium plus actinomycin, there is an increase in extractable transforming activity compared to the DNA extracted from MMS-treated cells which have been lysed immediately after treatment. Thus, it appears that cells are able to repair MMS-damaged DNA in the presence of actinomycin.

The Effects of Actinomycin on DNA

Actinomycin binds to the N2 amino group of the guanine moiety in the double-stranded, helical DNA molecule (Cerami, Reich, Ward and Goldberg, 1967). The bound actinomycin molecule lies in the minor groove of the DNA molecule (Reich and Goldberg, 1964). The quinoidal oxygen, the amino group, and the intact peptide lactones of actinomycin are required for this complexing to occur (Cerami, *et al*, 1967).

Actinomycin does not inhibit alkylation of the guanine by mustard gas or other alkylating agents (Cerami, *et al*, 1967). When actinomycin forms complexes with native DNA, the spectrum of the antibiotic changes (Goldberg, Rabinowitz and Reich, 1966), the bouyant density of the DNA is decreased (Kersten, Kersten and Szybalski, 1966), and the thermal stability of the DNA molecule is increased (Haselkorn 1964: Reich, 1964). In microorganisms RNA synthesis is inhibited at much lower concentrations of actinomycin than DNA synthesis (Reich and Goldberg, 1964). Evidence suggests that actinomycin is capable of replacing RNA polymerase in the minor groove and hindering the function of DNA polymerase by changing the physical characteristics of DNA (Reich and Goldberg, 1964). The actinomycin-DNA complex is fairly stable (Reich and Goldberg, 1964). The association may be reversed by 5 M urea, and the actinomycin may be extracted with ether (Hartman, Coy and Kniese, 1963).

MATERIAL AND METHODS

a. Bacterial Strains

Bacillus subtilis 168H *ind*$^+$ thy$^-$, a UV-sensitive mutant of 168M which requires thymidine for growth, was the major organism used in this investigation. *B. subtilis* #40 mms$^-$ind$^+$thy$^-$, an MMS sensitive mutant of 168M also requiring thymidine for growth, was used in some experiments. *B. subtilis* 168H *ind*$^-$, which requires tryptophan for growth, was used as a recipient in transformation experiments. This organism was also used as a source of an enzyme extract.

b. Culture Media

SS(a minimal salts medium composed of $(NH_4)_2SO_4$ (2.0gm), K_2HPO_4 (14.0 gm), KH_2PO_4 (6.0 gm), sodium citrate (1.0 gm), and $MgSO_4 \cdot 7H_2O$ (0.2 gm) in a total volume of one liter) was used for all cell dilutions. CH (minimal salts medium containing a final concentration of 0.5% glucose and 0.04% casein hydrolysate), CHT_{50} (minimal salts medium containing a final concentration of 0.5% glucose, 0.04% casein hydrolysate, and 50μg/ml of tryptophan), or CHT_5 (minimal salts medium containing 0.5% glucose, 0.02% casein hydrolysate, and 5 μg/ml of tryptophan) were used as growth media in all experiments.

Transformants were assayed on CH plates (minimal salts plus 15 gm of Bacto-agar per liter of de-ionized water containing a final concentration of 0.5% glucose and 0.04% casein hydrolysate). Cell counts were made on NBS plates (nutrient agar plates supplemented with 0.5% glucose and minimal salts). BHI (37 gm of brain heart infusion plus 15 gm of Bacto-agar per liter of de-ionized water) or TBAB (33 gm of tryptose blood agar base per liter of de-ionized water) were used to plate cells for overnight growth as a source of inocula.

c. Chemicals

Methyl methanesulfonate (MMS) from Eastman Organic Chemicals was vacuum-redistilled before use. If necessary, the MMS was diluted immediately before use in 0.05 M phosphate buffer (pH 7.4). Actinomycin D, a gift from Merck, Sharpe and Dohme, was first dissolved in absolute alcohol and then diluted to the desired concentration with distilled water.

d. Procedure for Transformation Experiments

B. subtilis 168H _ind_⁻ was grown overnight on either a BHI or a TBAB plate. In the morning a heavy inoculum from the plate was added to 10 ml of CHT_{50} and mixed well. The cells were aerated by bubbling air through the medium for four hours at 37 C. A 10^{-1} dilution was made into CHT_5, and aeration was continued for 90 minutes. At this time 0.9 ml of these competent cells was quickly

added to 0.1 ml of transforming DNA in a test tube 14 mm in diameter. The cells were shaken vigorously for 30 minutes at 37 C, then diluted in SS and plated on CH for an assay of the number of transformants present. Each dilution was plated in duplicate, and an average of the two counts was used.

e. The effects of Actinomycin on Transformation

Competent cells were prepared by adding 5.0 ml of CHT_{50} to an overnight culture of B. subtilis 168H ind⁻ on a BHI plate. Cells were scraped from the agar and suspended in the CHT_{50}. 0.15 ml of this cell suspension was added to 5.0 ml of CHT_{50} and aerated for four hours at 37 C. 1.0 ml of this culture was then added to 9.0 ml of CHT_5 and aerated for 90 minutes. At this time 0.9 ml of these competent cells was added to each of seven tubes containing the following in 0.1 ml volumes:

#1 8.0 μg of DNA from B. subtilis W23 ind^+.

#2 8.0 μg of DNA + 200 μg of DNase added 15 minutes before the competent cells were added.

#3 8.0 μg of DNA + 200 μg of DNase added at the same time as the competent cells.

#4 8.0 μg of DNA + 200 μg of DNase added 3 minutes after the competent cells.

#5 8.0 μg of DNA + 1.6 μg of actinomycin added at the same time as the competent cells.

#6 8.0 μg of DNA + 1.6 μg of actinomycin added 3 minutes after the competent cells.

#7 8.0 μg of DNA + 1.6 μg of actinomycin added 10 minutes after the competent cells.

The tubes were shaken vigorously for 30 minutes at 37 C. 0.1 ml, 10^{-1}, and 10^{-2} dilutions of each were plated on CH to assay for the number of transformants.

In another experiment, actinomycin was added to cells before they were lysed. These lysates were then assayed for transforming activity. The protocal was as follows:

B. subtilis 168H $ind^{+}thy^{-}$ was grown overnight in CH medium + 10 μg/ml of thymidine. 0.2 ml of this culture was added to 10 ml of CH + 4 μg/ml of thymidine and aerated for five hours at 37 C. These cells were collected on a membrane filter, resuspended in 24 ml of CH and aerated for 10 minutes at 37 C. Three 2.0 ml samples were removed and added to each of three centrifuge tubes containing 2.0 ml of ice cold SS. The cells were centrifuged in the cold. One sample was resuspended in 1.0 ml of 0.01 M tris + 0.01 M EDTA (pH 8.3). Another sample was resuspended in 1.0 ml of the tris-EDTA buffer containing 10.0 μg of actinomycin, and the last sample was resuspended in the same buffer containing 2.7 μg of actinomycin. The cells were lysed with 50 μg/ml of lysozyme for 15 minutes at 37 C/ 100 μg/ml of pronase was added for 10 minutes at 37 C. Finally, 0.05 ml of 1% Duapnol was added. Without further purification 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions of these lysates were assayed for transforming activity as described in Section d, p 19.

f. Survival Curves

A 10^{-2} dilution of an overnight culture of B. subtilis 168H $ind^{+}thy^{-}$ or B. subtilis #40 $mms^{-}ind^{+}thy^{-}$ was aerated for three hours at 37 C in CHT_{50} + 10 µg/ml of thymidine. Cells were centrifuged, washed, and resuspended in SS + 10 µg/ml of thymidine to a final concentration of 1 x 10^{8} cells /ml. The culture was bubbled for 5 minutes at 37 C. At 0 minutes a sample was removed for an assay of viable cells, and MMS to a final concentration of 0.025 M or 0.05 M was added. At 2, 5, 10, 15 and 20 minute samples were removed, diluted, and assayed for viable cells in duplicate on NBS plates.

g. Extraction Procedure for B. subtilis Enzyme

Three liters of an overnight culture of B. subtilis 168H in nutrient broth + 0.5% glucose was centrifuged, washed with 0.5% NaCl + 0.5% KCl, and resuspended on 80 ml of 0.05 M tris + 10^{-3} M EDTA buffer (pH 7.5). The cell suspension was incubated with 50 mg of crystalline lysozyme in 10 ml of the same solution for 30 minutes at 37 C. The lysate was cooled and sonicated with a Bronson sonifier, Model S-75, in 10 to 15 second pulses with intermitent cooling for a total of 3 to 4 minutes, or until there was a decrease in viscosity. 10 ml of a 10% solution of streptomycin sulfate in 0.05 M phosphate buffer (pH 7.4) was added very

slowly with stirring over a period of one hour. The precipitate was removed by centrifugation in the cold at 16,000 rpm for 30 minutes using a refrigerated Servall certrifuge. Also in the cold, 48 gm of ammonium sulfate was added slowly, with gentle stirring to avoid foaming, to the supernatant over a period of one hour. The precipitate was collected by cold centrifugation at 16,000 rpm for 15 minutes and dissolved in 20 ml of 0.05 M phosphate buffer (pH 7.4). Two millititer quantities were stored at -15 C until used, at which time one of the fractions was thawed at room temperature.

h. DNA Treated With MMS _In Vitro_;
General Procedure

DNA from _B. subtilis_ 168M _tryp_$^+$, purified by the Marmur procedure and redissolved in SS, was diluted in 0.05 M phosphate buffer (pH 7.4) to a final concentration of 400 μg/ml. One half of the DNA was treated with 0.012 M MMS for 30 minutes at 37 C. Untreated and treated DNA samples were each divided into four equal parts. 2.7 μg/ml of actinomycin was added to two fractions of untreated DNA and to two fractions of treated DNA. An equal volume of SS was added to the other two fractions of each. To one tube containing actinomycin and to another tube containing SS, of both treated and untreated DNA, an extract of _B. subtilis_ was added to a final concentration which was a 10^{-1} dilution of the original extract. A volume of SS equal to the volume of extract was added to the two remaining tubes of each set. All tubes were incubated

for 15 minutes at 37 C. 1% Dupanol (sodium lauryl sulfate) was added to a final concentration of 0.01% to stop the enzyme reaction. The reaction mixtures were diluted and added to competent cells. In some experiments, actinomycin was incubated with DNA for 15 minutes at 37 C before treatment with MMS.

When samples were to be centrifuged on sucrose gradients, the same basic protocal was used as described above except the DNA was labeled with ^{3}H-thymidine. Actinomycin was added to one sample 15 minutes before treatment and to one sample immediately after treatment with 0.05 M MMS. Immediately after enzyme treatment, Dupanol was added, and 0.1 ml of each sample was carefully layered on top of a sucrose gradient as described in Section k, p 26.

i. DNA Treated With MMS <u>In Vivo</u>

General Procedure

<u>B</u>. <u>subtilis</u> 168H $\underline{ind}^+\underline{thy}^-$ was grown overnight in CH + 10 μg/ml of thymidine. Cells were diluted 1/50 in fresh Ch + 4 μg/ml of thymidine + 4 μc/ml of ^{3}H-thymidine and bubbled for five hours at 37 C. At this time they were collected on a membrane filter, washed with CH, and resuspended in an equal volume of CH + 4 μg/ml of thymidine. The cells were bubbled for 10 minutes at 37 C and assayed for viable cells. Two samples were removed; one was added to an equal volume of ice-cold SS and another to ice-cold SS + 2.7 μg/ml of actinomycin. 0.025 M MMS was added to the remaining culture. Cells were bubbled for 5 minutes, then chilled immediately and assayed for viable cells. Two samples were quickly diluted into

an equal volume of ice-cold SS, and two were diluted into ice-cold SS + 2.7 μg/ml of actinomycin. All of the samples were then centrifuged in the cold. One MMS-treated sample was resuspended CH + 10 μg/ml of thymidine. Another treated sample which had been diluted in the presence of actinomycin was resuspended in CH + 10 μg/ml of thymidine + 2.7 μg/ml of actinomycin. Both of these samples were bubbled at 37 C for 50 minutes and then centrifuged in the cold. All samples which contained actinomycin were resuspended in a small volume (1.0 to 1.5 ml) of 0.01 M tris buffer containing 0.01 M EDTA (pH 8.3) + 2.7 μg/ml of actinomycin. The other samples were resuspended in the same volume of 0.01 M tris buffer + 0.01 M EDTA (pH 8.3). The cells were lysed with a final concentration of 50 μg/ml of lysozyme at 37 C for 15 minutes. A final concentration of 100 μg/ml of pronase was added for 10 minutes, then 0.05 ml of 1% Dupanol was added per ml of cell suspension. These lysates were diluted 10^{-1} in order to be assayed for transforming activity and centrifuged on sucrose gradients.

The same procedure was used for B. subtilis #40 $mms^- ind^+ thy^-$ except that ^{3}H-thymidine was omitted, and the lysates were analyzed for transforming activity only.

j. Dissociation of Actinomycin-DNA Complex

Lysates from an in vivo experiment were diluted 10^{-1} in an urea solution (48gm dissolved in 100 ml of distilled water and brought to pH 8.3 with NaOH). Samples were mixed very slowly in a shaker waterbath at 37 C for 20 minutes. The actinomycin was

carefully extracted, to avoid shearing the DNA, four times with anhydrous ethyl ether. Samples were dialyzed at room temperature against 0.01 M tris + 0.01 M EDTA (pH 8.3) to remove the urea. The samples were centrifuged on sucrose gradients as described in the next section.

k. Sucrose Gradient Centrifugation

0.1 ml of each reaction mixture from an in vitro experiment was carefully layered on a 5 ml, 5.7% to 17.6% (w/w) sucrose gradient in 0.1 M phosphate buffer (pH 7.4). Samples were centrifuged at 35,000 rpm (100,000 g) for two hours in a SW 50L rotor in a Beckman, Model L-4, ultracentrifuge at 18 C. Seventeen drops per fraction were collected from the bottom of the tube. Each fraction was diluted with 0.5 ml of SS, and DNA was precipitated, in the presence of 50 to 100 μg/ml of bovine albumin carrier, with an equal volume of 1% CETAB (hexadecyltrimethylammonium bromide). Fifteen minutes was allowed for complete precipitation to occur before samples were filtered on 2.4 cm glass fiber filters and washed with distilled water. Samples were dried and 2.0 ml of scintillation fluid (5 gm of Packard Pre-Mix "P" in one liter of toluene) was added for counting in a Series 3000 Packard Tricarb Liquid Scintillation Counter.

Lysates from an in vivo experiment were diluted 10^{-1} in the tris-EDTA buffer. 0.10 ml of each sample was very carefully layered onto a 5% to 17% sucrose gradient in the same buffer. Samples were centrifuged for 90 minutes at 35,000 rpm (100,000 g) at 18 C.

Fractions were collected, precipitated, and filtered as above. In some cases 0.2 ml of SS was added to each fraction, and 0.1 ml of each fraction was put onto a glass fiber filter, dried, and counted.

From an in vivo experiment, a 0.13 ml sample of each lysate which had been treated with urea and ether extracted was carefully layered onto a 8 ml gradient of 5% to 17% sucrose in tris-EDTA buffer formed on a 3.5 ml layer of 30% sucrose in a 9/16 x 3 ½ inch nitrocellulose tube. The gradients were centrifuged for 135 minutes at 35,000 (150,000 g) at 18 C in SW 41 rotor in a Beckman, Model 12-50, ultracentrifuge. A hole was punctured in the bottom of the tube with a # 25 guage needle. Six drops per fraction were collected. The first 48 drops corresponding to the 30% sucrose layer were discarded. 0.2 ml of SS was added to each fraction. 0.1 ml from each fraction was put on a glass fiber filter, dried, and assayed for radioactivity. 0.1 ml of each fraction was added to competent cells to be assayed for transforming activity.

RESULTS

The Effects of Actinomycin on Transformation

In some experiments, the ability of MMS-treated cells to repair alkylated DNA in the presence of actinomycin was assayed as the ability of the DNA from these cells to transform competent cultures of B. subtilis. It was necessary to determine the effect of actinomycin on transformation in order to distinguish a reduction in transforming activity of the DNA due to damage induced by MMS from a reduction caused by the effect of actinomycin on transformation.

1.6 μg/ml of actinomycin reduces the number of transformants obtained from a competent culture of B. subtilis 168H ind^- being transformed by 8.0 μg/ml of DNA from B. subtilis W23 ind^+ (Table 1). If actinomycin is added to DNA at the same time as the competent cells, the number of transformants is reduced to 0.2% of those obtained when competent cells are transformed by 8.0 μg/ml of DNA alone (i.e., control). If actinomycin is added 3 minutes after the competent cells have been added to the DNA, the number of transformants increases from 0.2% to 0.4% of the control. In contrast, if 200 μg/ml of DNase is added to 8.0 μg/ml of transforming DNA at the same time as the competent cells, no transformants occur. If DNase is added 3 minutes after the competent cells have been added to the DNA, the number of transformants increases to 7.0% of the control (see Material and Methods, section e, p 20).

TABLE 1. The Effects on Transformation of Actinomycin Added at Various Times after Transformation Begins

Material Added	Time of Addition	Final Cell Dilution[a]	Number of Transformation/ml	Relative Frequency[b]
None		10^{-2}	848×10^2	
		10^{-3}	81×10^3	
		10^{-4}	7×10^4	
		Average	8.3×10^4	1.0
DNase	15' Before[c]	10^{-1}	0	
DNase	0' Before	10^{-1}	0	
DNase	3' After[d]	10^{-1}	616×10^1	
		10^{-2}	57×10^2	
		Average	5.9×10^3	.07
Actinomycin	o' Before	10^{-1}	9×10^1	
		10^{-2}	3×10^2	
		Average	1.9×10^2	.002
Actinomycin	3'After	10^{-1}	16×10^1	
		10^{-2}	5×10^2	
		Average	3.3×10^2	.004
Actinomycin	10'After	10^{-2}	160×10^2	
		10^{-3}	14×10^3	
		Average	1.5×10^4	.18

[a]Competent cells were diluted and plated on CH after transformation.
[b]Relative to the untreated sample.
[c]Before competent cells are added to transforming DNA.
[d]After competent cells are added to transforming DNA.

In conclusion, if DNase or actinomycin is added to transforming DNA the the same time as the competent cells, DNAse is more effective than actinomycin in reducing transformation. If either of these substances is added 3 minutes after competent cells have been added to the transforming DNA, actinomycin is about 20 times more effective than DNase in inhibiting transformation. This suggests that the effect of actinomycin is more than an immediate halt in the uptake of DNA by competent cells. Otherwise, the number of transformants obtained when actinomycin is added 3 minutes after competent cells have been added to DNA should be equal to or greater than the number obtained when DNase is added 3 minutes after the competent cells have been added to DNA. Since the reverse is true, actinomycin must affect some other aspect of transformation besides the ability of the competent cells to take up DNA.

Since the number of transformants increases as the time of addition of actinomycin to transforming DNA and competent cells is delayed, the effect of actinomycin on transformation must be more than just a general lethal affect on any cell growing in its presence. The frequency of transformation is 18% of control if actinomycin is added 10 minutes after competent cells have been added to the transforming DNA. If actinomycin is added to the control sample after transformation has occurred but before the cells are plated on CH without further dilution, very few cells will grow.

If 0.1 ml of a 10^{-1} dilution is plated, the number of tranformants obtained is equal to that of the control sample plated prior to the addition of actinomycin.

In a similar experiment, 10 μg/ml or 2.7 μg/ml of actinomycin was added to whole cells of B. subtilis 168H $ind^{+}thy^{-}$ prior to lysis (See Material and Methods, Section e, p 20). Without further purification, each of these lysates was diluted, and each dilution was assayed for transforming activity. As a control, lysates of cells which had been lysed without actinomycin were also assayed for transforming activity. The results are shown in Table 2.

If the lysate is not diluted sufficiently before being assayed for transforming activity, actinomycin inhibits transformation. For example, if the lysate containing 2.7 μg/ml of actinomycin is diluted 1,000-fold, or until the final concentration of actinomycin is 0.0027 μg/ml, there is very little inhibition of transformation compared to that of control lysates lacking actinomycin but diluted to the same extent. If the dilution is only 100-fold, or until the final concentration of actinomycin is 0.027 μg/ml, the transformation frequency is only 14% of a 100-fold dilution of the control lysate. A lysate containing 10 μg/ml must be diluted 10,000-fold, or until the final concentration of actinomycin is 0.001 μg/ml, before there is no inhibition of transformation. A 10^{-2} (0.1 μg/ml of actinomycin) and 10^{-3} (0.01 μg/ml of actinomycin) yield 6% and 47%, respectively. From this experiment it was concluded that at a concentration of 2.7 μg/ml of actinomycin, any reduction of transforming activity in experimental lysates would reflect a result of

TABLE 2. The Effect of Actinomycin on Transformation: Transforming Activity of Cell Lysates Containing This Antibiotic

DNA Dilution	Control		10 μg/ml of Actinomycin		2.7 μg/ml of Actinomycin	
	No. of Transformants/ml	Relative Frequency[a]	No. of Transformants/ml	Relative Frequency	No. of Transformants/ml	Relative Frequency
10^{-2}	4.22×10^4	1.0	2.6×10^3	0.061	6.0×10^3	0.142
10^{-3}	1.20×10^4	1.0	5.7×10^3	0.475	1.04×10^4	0.867
10^{-4}	1.26×10^3	1.0	1.2×10^3	0.960	1.54×10^3	1.22
10^{-5}	1.10×10^2	1.0	1.3×10^2	1.35	1.00×10^2	0.909

[a]Relative to the transforming activity of the control lysate for each dilution.

NNS damage and not an effect of actinomycin, if a 10^{-3} dilution of lysate was assayed. As a precaution in all experiments, various dilutions of lysate from untreated (control) cells lysed in the presence of actinomycin was always assayed for transforming activity. The dilution of this lysate which did not yield reduced transforming activity was considered a valid dilution to use for assaying the experimental lysates which contained actinomycin.

To determine what effect actinomycin might have on growing cells, 2.7 μg/ml of actinomycin was added to a log-phase culture of B. subtilis 168H. The culture was assayed for viable cells at various times thereafter. The results are shown on Table 3. Although actinomycin seems to prevent the cells from dividing when it is present in a culture up to one hour, it is not lethal to these cells. After actinomycin is diluted out of a culture, cell growth resumes (i.e., colonies appear after overnight growth on NBS).

This result is in agreement with the transformation experiment cited above. When actinomycin is added to a control sample containing transforming DNA and competent cells which have been reacted together for 30 minutes (a period of time sufficient for complete transformation), no inhibition of transformation occurs. This is observed only if the transformed cells are diluted before being plated on CH. Note also (Table 1) that in samples containing actinomycin, the number of transformants obtained is consistantly smaller at the 10^{-1} cell dilution (0.1 ml plated directly on CH) than at 10^{-2} or 10^{-3} dilutions.

In conclusion, actinomycin inhibits transformation, but this

TABLE 3. The Effect of Actinomycin on Growing Cells

Time of Assay[a]	Viable Cell Count
0 minutes	2.9×10^8
10 minutes	3.2×10^8
20 minutes	4.2×10^8
30 minutes	3.6×10^8
40 minutes	3.7×10^8
60 minutes	4.1×10^8

[a]The time the assay sample was taken after actinomycin had been added to the culture.

effect may be avoided if the actinomycin is diluted sufficiently before transformation begins.

DNA Treated with MMS *In Vitro*

There is a nuclease active in extracts of *B. subtilis* which specifically inactivates DNA methylated by MMS. When transforming DNA is alkylated with 0.012 M MMS for 30 minutes at 37 C and treated with an extract from *B. subtilis* (see Material and Methods, section h, p 23), there is a hundred-fold drop in transforming activity compared to less than a two-fold drop in that of non-alkylated (control) DNA treated with the same extract (Table 4). The presence of 2.7 μg/ml of actinomycin does not inhibit the inactivation of alkylated DNA by this enzyme. If DNA is incubated with actinomycin for 15 minutes before being treated with MMS and then the enzyme, inactivation still occurs.

Figure 1 shows the degradative effect of enzyme treatment on DNA. Non-alkylated DNA and non-alkylated DNA treated with the extract band in the same position when centrifuged on 5.7% to 17.6% sucrose gradients (see Material and Methods, section k, p 26). Alkylated DNA, alkylated DNA + actinomycin, and DNA incubated with actinomycin and then treated with MMS all band at the same position as control (non-alkylated) DNA. The three alkylated DNA samples all shift to a position of lower molecular weight after treatment with the extract (Fig's. 1 and 2). This shift is the expected consequence of nuclease activity on alkylated DNA. Since this

TABLE 4. Treatment of Alkylated DNA with a B. subtilis Extract, *In Vitro*, in the Presence and Absence of Actinomycin

	Relative Transforming Activity[a]			
Expt. No.	Control[b]	Control + Extract	Control + Actinomycin	Control + Actinomycin + Extract
1	1.0	0.59	1.3	0.56
2	1.0	0.43		
	MMS-DNA[c]	MMS-DNA + Extract	MMS-DNA + Actinomycin	MMS-DNA + Actinomycin + Extract
1	0.19	0.0023	0.27	0.005
2	0.56	0.075	0.28	0.045
			Actinomycin-DNA[d] + MMS	Actinomycin-DNA + MMS + Extract
1				
2			0.23	0.06

[a]Relative to the control DNA in each experiment.

[b]DNA that has not been treated with MMS.

[c]DNA that has been treated with MMS.

[d]DNA that has been incubated with actinomycin prior to treatment with MMS.

PLATE I

The effect of an enzyme extract of B. subtilis 168H on the molecular weight of DNA and of DNA alkylated with MMS prior to treatment with the extract. O—O, DNA; O---O DNA treated with the extract; Δ—Δ, MMS-treated DNA; Δ---Δ, MMS-treated DNA treated with the extract (See Material and Methods, section h, p 23). Samples were centrifuged on 5.7% to 17.6% sucrose gradients at 35,000 rpm for 2 hours at 18 C (see Material and Methods, section k, p 26).

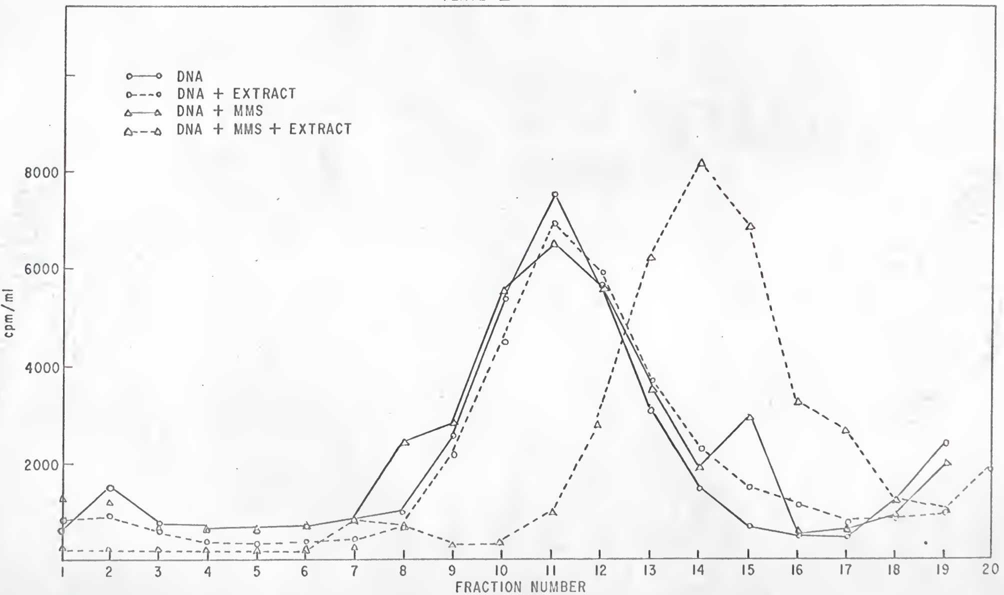


PLATE I
DNA
DNA + EXTRACT
DNA + MMS
DNA + MMS + EXTRACT
cpm/ml
8000
6000
4000
2000
FRACTION NUMBER
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

PLATE II

The effect of an enzyme extract of B. subtilis 168H on the molecular weight of DNA alkylated with MMS and then incubated with actinomycin prior to enzyme treatment and of DNA incubated with actinomycin and alkylated with MMS prior to enzyme treatment. O—O , DNA incubated with actinomycin prior to treatment with MMS; O--O , DNA incubated with actinomycin, then treated with MMS prior to treatment with the enzyme; Δ—Δ , DNA treated with MMS prior to incubation with actinomycin; Δ--Δ, DNA treated with MMS, then incubated with actinomycin prior to enzyme treatment; X—X, DNA treated with MMS (see Material and Methods, section h, p 23). Samples were centrifuged on 5.7% to 17.6% sucrose gradients at 35,000 rpm for 2 hours at 18 C (see Material and Methods, section k, p 26).

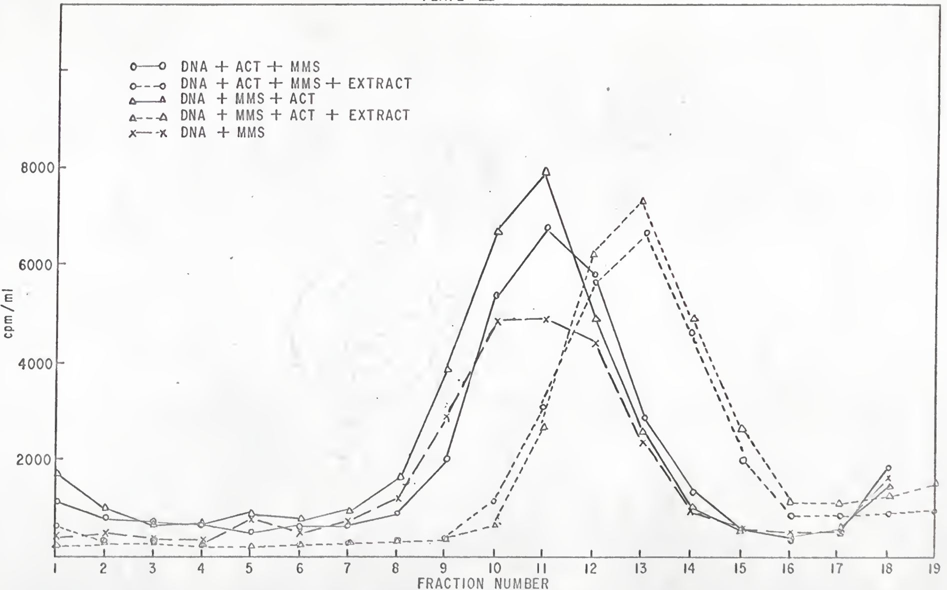


PLATE II
DNA + ACT + MMS
DNA + ACT + MMS + EXTRACT
DNA + MMS + ACT
DNA + MMS + ACT + EXTRACT
DNA + MMS
cpm / ml
8000
6000
4000
2000
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
FRACTION NUMBER

shift occurs, it is clear that actinomycin does not inhibit the alkylation of DNA _in vitro_, nor does it inhibit the nuclease activity of the enzyme on alkylated DNA.

DNA Treated with MMS _In Vivo_

The extent of DNA inactivation produced by treating cells with MMS may be determined by lysing the treated cells and comparing the transforming activity of the DNA from these cells with that of the DNA from cells of the same culture which have been removed prior to treatment and lysed in the same manner.

Alkylated DNA (i.e., methylated DNA) is a substrate for a nuclease present in _B. subtilis_. This nuclease degrades alkylated DNA during the time the cells are being lysed to extract their DNA. If the number of methylated bases (i.e., enzyme substrates) have been reduced prior to lysis by cellular repair processes, the amount of degradation that occurs during lysis is equally reduced (Reiter and Strauss, 1965). The degraded DNA does not act as transforming DNA. Thus, it is possible to determine whether repair has occurred during the incubation after MMS treatment.

As seen in Table 5, the transforming activity of DNA in lysates of cells lysed immediately after treatment with 0.025 M MMS for 5 minutes at 37 C (see Material and Methods, section i, p 24) is 0.6% of the transforming activity in lysates of untreated cells. When the treated cells are incubated for 50 minutes prior to lysis, there is a 15-to 20-fold increase in the transforming activity of the DNA.

When treated cells are chilled so that they cannot repair MMS lesions and 2.7 μg/ml of actinomycin is added to the culture prior to lysis, the transforming activity in this lysate is 3.0% of that in lysates of untreated cells (control). In other words, if treated cells are lysed in the presence of actinomycin, the transforming activity of this lysate (3.0% of control) is 5 times higher than that obtained from a lysate of treated cells lysed in the absence of actinomycin (0.6% of control). Apparently actinomycin alone is able to increase the transforming activity of treated cells under conditions which prevent cellular repair processes from functioning. But this transforming activity is not as high as that occurring in a lysate of treated cells which have been incubated in growth medium + 2.7 μg/ml of actinomycin for 50 minutes prior to lysis. The transforming activity of this lysate is 8.0% of control, or 2 to 3 times higher than the activity in the lysate of treated cells lysed in the presence of actinomycin immediately after treatment.

In summary, repair of MMS-induced damage seems to occur in the presence of actinomycin as measured by an increase in transforming activity in lysates of treated cells which have been incubated in growth medium plus actinomycin prior to lysis. This repair is not so dramatic when transforming activity is compared to that from a lysate of treated cells in which repair was prevented, but actinomycin was also present during lysis. This is true for two reasons: (1) because transforming activity in lysates of treated but non-recovered cells lysed in the presence of actinomycin is 5 times greater than the transforming activity in

TABLE 5. Recovery of *B. subtilis* 168H from MMS Treatment

Treatment	Surviving Fraction[a] N/N_o	Recovery Period	Relative Transforming Activity[b]	
			-Actinomycin	+ Actinomycin
MMS Before Actinomycin Was Added	.003	None	.004	.01
		50 min.	.04	.03
	.06	None	.0004	.015
		50 min.	.06	.075
	.5	None	.041	.06
		50 min.	.22	.14
MMS After Actinomycin Was Added	.14	None	.02	.08
		50 min.	.42	.09

[a]Surviving fraction of MMS-treated cells compared to untreated cells.

[b]Relative to the extractable transforming activity from control cultures not treated with MMS. Control cultures that were lysed in the presence of actinomycin had essentially the same transforming activity as control cultures lysed alone.

PLATE III

Molecular weight studies on DNA from cells treated with MMS prior to lysis. O—O , MMS-treated cells lysed immediately (M_o); O---O, MMS-treated cells lysed immediately in the presence of actinomycin (M_o + Act. D); Δ—Δ , MMS-treated cells which were incubated in growth medium prior to lysis (MR); Δ---Δ, MMS-treated cells which were incubated in growth medium containing actinomycin prior to lysis (MR + Act. D); X—X, untreated cells lysed in the absence of actinomycin (Control); X--X, untreated cells lysed in the presence of actinomycin (Control + Act. D). Samples were centrifuged on 5% to 17% sucrose gradients at 35,000 rpm for 90 minutes at 18 C (see Material and Methods, section k, p 26).

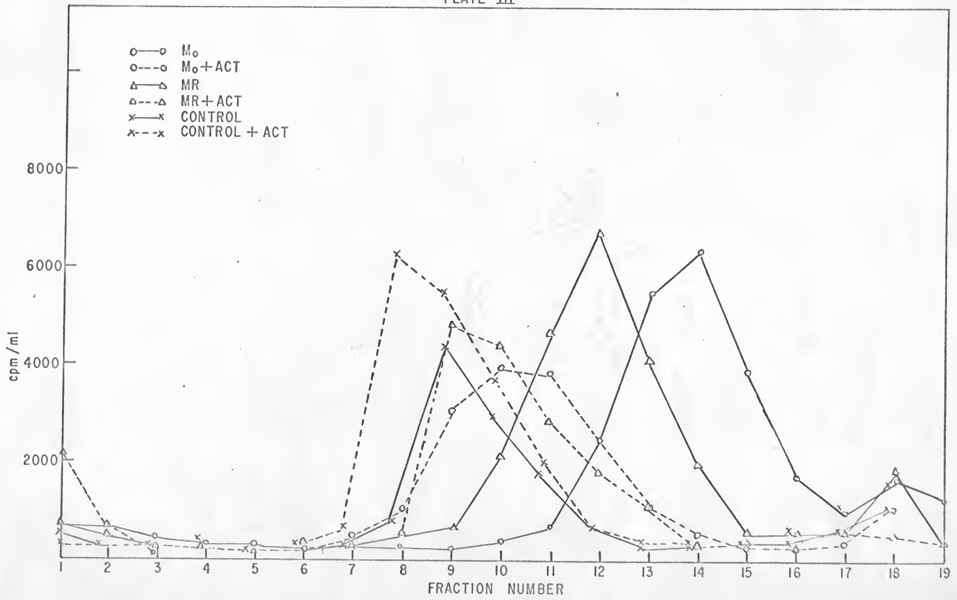


PLATE III
Mo
Mo + ACT
MR
MR + ACT
CONTROL
CONTROL + ACT
cpm / ml
2000
4000
6000
8000
FRACTION NUMBER
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

lysates of treated but non-recovered cells lysed in the absence of actinomycin; and (2) because the transforming activity of lysates from treated cells recovered in the presence of actinomycin is 1.3 times less than the transforming activity of lysates of treated cells recovered in the absence of actinomycin. The presence of actinomycin seems to cause an increase in the transforming activity in lysates of treated, non-recovered cells and a decrease in transforming activity in lysates of treated, recovered cells. In these experiments, transformation activity was assayed at a dilution of the lysates in which there was no inhibition of transformation as determined from lysates of untreated cells lysed in the presence of actinomycin. Therefore, any effect of actinomycin was not on transformation _per se_.

If 2.7 $\mu g/ml$ of actinomycin is added to cells 10 minutes before treatment with 0.01 M MMS, there is no increase in transforming activity in a lysate of these treated cells which have been incubated for 50 minutes at 37 C in growth medium plus actinomycin prior to lysis compared to that in lysates of MMS-treated cells pre-incubated with actinomycin but lysed immediately after treatment in the presence of this antibiotic (Table 5). The transforming activity from both of these lysates is 4 times higher than that from a lysate of treated, non-recovered cells lysed in the absence of actinomycin and 5 times less than that from a lysate of treated cells recovered and then lysed in the absence of actinomycin. This suggests that repair does not occur if actinomycin is added to cells prior to treatment with MMS, although actinomycin

does cause an increase in the transforming activity of both of these lysates.

Molecular Weight Studies on DNA Treated with MMS *In Vivo*

The molecular weights of DNA from treated cells lysed both in the presence and absence of actinomycin have been compared to those of DNA from treated cells which were incubated in growth medium both in the presence and absence of actinomycin prior to lysis. When samples of the lysates are centrifuged on 5% to 17% sucrose gradients (Material and Methods, section k, p 26) differences in molecular weights of the DNA can be seen (Fig. 3). Treated but unrepaired DNA bands at a position indicative of a smaller molecular weight in comparison to untreated (control) DNA. As expected, repaired DNA from incubated cells bands at a position intermediate of control and unrepaired DNA. Surprisingly enough, the DNA in lysates of cells recovered and lysed in the presence of actinomycin and the DNA in lysates of non-recovered cells lysed in the presence of actinomycin both band at a position nearly the same as the control DNA. Actinomycin itself increases the effective molecular weight of untreated DNA slightly. If actinomycin is added to control cells, the DNA from this lysate will band consistently at a slightly lower position, one fraction, than control DNA alone. This slight difference is within the range of error for this particular technique.

In an unpublished experiment Reiter (personal communication) has demonstrated the same result. If actinomycin is added to extracted DNA and centrifuged in either a sucrose gradient containing the same concentration of actinomycin or in a sucrose gradient without actinomycin, the actinomycin-DNA complex will band at a position which is one fraction lower than control DNA without actinomycin.

These data are not able to account for the greater difference, 3 to 4 fractions, obtained when DNA from MMS-treated cells recovered in the presence of actinomycin is compared to the DNA from treated cells recovered alone, or when DNA from non-recovered cells lysed in the presence of actinomycin is compared to DNA from non-recovered cells lysed alone (Fig. 3).

DNA from both the lysate of the recovered cells in actinomycin (i.e., treated cells which have been both incubated and lysed in the presence of actinomycin) and the lysate of the non-recovered cells in actinomycin (i.e., treated cells which have been lysed in the presence of actinomycin immediately after treatment) have increased molecular weights which are nearly equal to the DNA from a lysate of untreated cells. Also, there is a smaller difference in transforming activity between these two lysates than in that between lysates of recovered and non-recovered cells lacking actinomycin. Therefore, it seemed possible that the DNA from the cells recovered in actinomycin was not really being repaired. The two to three-fold difference in transforming activity of this lysate and the lysate from non-recovered cells lysed in actinomycin could be due to the increased concentration of actinomycin inside the recovered cells as a result of a 50 minute incubation period at

37 C before lysis.

The effect of actinomycin on molecular weight and transforming activity of DNA could be due to an inhibition of the alkylated-DNA-specific nuclease, or possibly due to the preservation of the physical integrity of the DNA, in spite of nuclease activity, as a result of actinomycin interacting with DNA and, perhaps, protein. From the in vitro studies it is clear that actinomycin does not inhibit the activity of the nuclease in extract form. But it is possible that the DNA-actinomycin interaction is more complex in vivo and can inhibit enzyme activity, or that the activity of the enzyme is altered as a result of extraction. The best available method for answering both of these questions seemed to be by dissociating the actinomycin-DNA complex, removing the actinomycin, and determining the molecular weight of the DNA. If the nuclease had been effective at the time of lysis, the molecular weight of the DNA should decrease, now banding at higher positions in a sucrose gradient. If this were true, it would now be possible to determine whether repair had occurred during incubation by comparing the positions of recovered and non-recovered samples which had been dissociated from actinomycin.

Urea is capable of removing actinomycin from DNA at concentrations (approximately 5 M) which are not disruptive to the DNA molecule itself (Hartman, Coy, and Kniese, 1963). The actinomycin may then be removed by extraction with ether. Figure 4 shows the effect of just ether extraction and the effect of urea treatment and ether extraction on control DNA from untreated cells lysed

PLATE IV

Molecular weight studies on DNA from untreated cells lysed in the presence or absence of actinomycin. The lysates were treated with ether alone, or with urea first and then ether (see Material and Methods, section j, p 25). Samples were dialysed in tris-EDTA buffer and then centrifuged on 5% to 17% sucrose gradients at 35,000 rpm for 135 minutes at 18 C (see Material and Methods, section k, p 27). O—O , DNA from untreated cells (Control); O—O , DNA from untreated cells that has been treated with ether (Control + Ether); O---O, DNA from untreated cells that has been treated with urea and then ether (Control + Urea + Ether); Δ—Δ, DNA from untreated cells lysed in the presence of actinomycin (Control + Act. D); Δ—Δ, DNA from untreated cells lysed in the presence of actinomycin that has been treated with ether (Control + Act. D + Ether); Δ---Δ, DNA from untreated cells lysed in the presence of actinomycin that has been treated with urea and then ether (Control + Act. D + Urea + Ether).

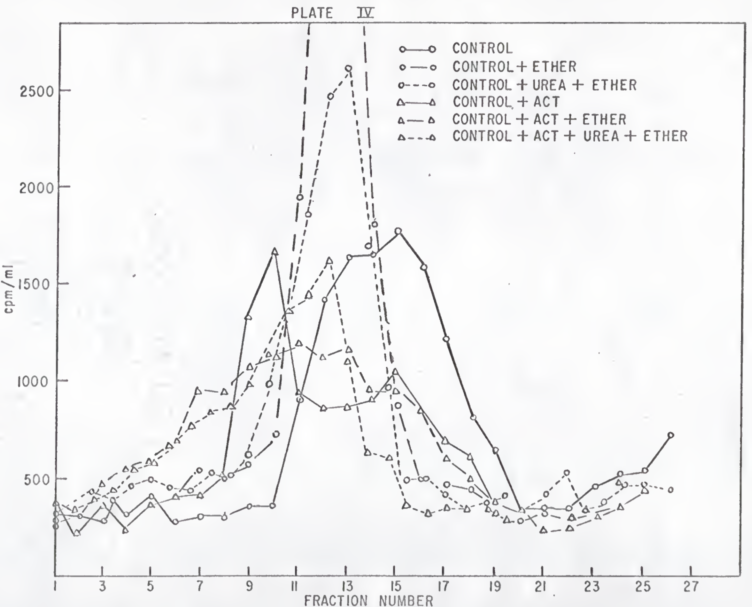


PLATE IV
CONTROL
CONTROL + ETHER
CONTROL + UREA + ETHER
CONTROL + ACT
CONTROL + ACT + ETHER
CONTROL + ACT + UREA + ETHER
cpm/ml
2500
2000
1500
1000
500
1
3
5
7
9
11
13
15
17
19
21
23
25
27
FRACTION NUMBER

PLATE V

Molecular weight studies of DNA from MMS-treated cells that has been treated with urea and extracted with ether (see Material and Methods, section i, p 24; section j, p 25). O——O , DNA from MMS-treated cells lysed immediately (M_o); O — O , DNA from MMS-treated cells lysed immediately in the presence of actinomycin (Mo + Act. D); O---O , DNA from MMS-treated cells lysed immediately in the presence of actinomycin that has been treated with urea and extracted with ether (M_o + Act. D + Urea + Ether); Δ——Δ, DNA from MMS-treated cells incubated in growth medium prior to lysis (MR); Δ — Δ, DNA from MMS-treated cells incubated in growth medium containing actinomycin prior to lysis (MR + Act. D); Δ---Δ, DNA from MMS-treated cells incubated in growth medium containing actinomycin prior to lysis that has been treated with urea and extracted with ether (MR + Act. D + Urea + Ether). Samples were centrifuged in 5% to 17% sucrose gradients at 35,000 rpm for 135 minutes at 18 C (see Material and Methods, section k, p 27).

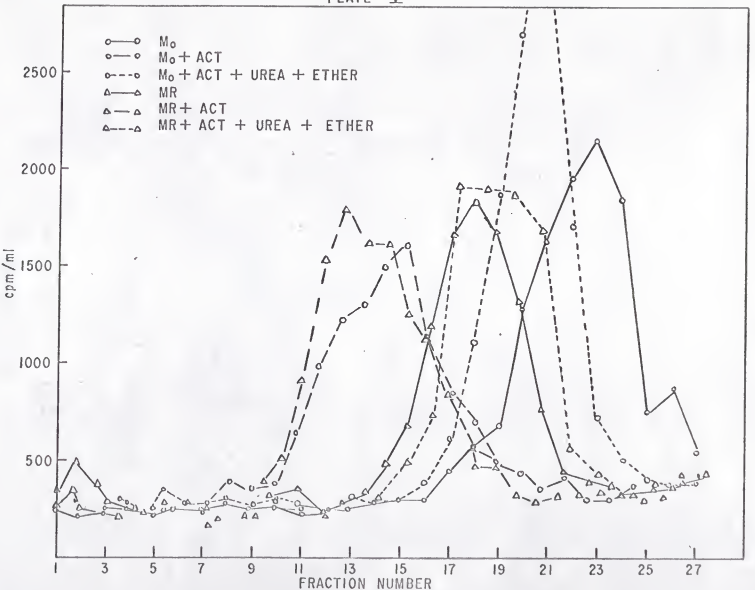


PLATE V
Mo
Mo + ACT
Mo + ACT + UREA + ETHER
MR
MR + ACT
MR + ACT + UREA + ETHER
cpm/ml
2500
2000
1500
1000
500
1
3
5
7
9
11
13
15
17
19
21
23
25
27
FRACTION NUMBER

alone and on control DNA from untreated cells lysed in the presence of actinomycin (see Material and Methods, section j, p 25). It is apparent that this treatment has essentially no effect on the molecular weight of DNA per se.

DNA from treated cells recovered and then lysed in actinomycin and DNA from treated, non-recovered cells lysed in actinomycin were treated with urea and extracted with ether. The DNA from each of these lysates now bands at a new position (Fig. 5). It is clear that the DNA from the treated cells recovered and lysed in actinomycin now bands at the position of repaired DNA, whereas the DNA from treated, non-recovered cells lysed in actinomycin is approaching the position occupied by non-repaired DNA. The slight difference in molecular weight between this sample and that of the non-repaired DNA may be due to residual actinomycin on the DNA from these treated, non-recovered cells lysed in the presence of actinomycin, or possibly due to a partial interference with nuclease activity at the time of lysis.

In conclusion, actinomycin does not seem to inhibit the repair of MMS-induced DNA damage occurring during the incubation period in growth medium after treatment, or the activity of the nuclease specific for alkylated DNA which acts at the time of lysis in MMS-treated cells. Actinomycin does cause an increase in the molecular weight of DNA from both treated cells lysed immediately and treated cells incubated prior to lysis. This must be due to some interaction of actinomycin with DNA and, perhaps, protein.

Survival Curves for MMS-Treated *B. subtilis* 168H and *B. subtilis* #40 mms^-

The differences in survival of *B. subtilis* 168H and *B. subtilis* #40 mms^- after treatment with MMS can be seen in Figure 6. It should be noted that the survival curve for #40 mms^- is determined at a dose which is one half of that given for inactivation of 168H (0.025 M instead of 0.05 M MMS). Under these conditions (see Material and Methods, section f, p 22), the survival curve for *B. subtilis* #40 mms^- has a slight shoulder, extrapolating to 4. The survival curve for *B. subtilis* 168H extrapolates to 1.0, suggesting one-hit kinetics. The slope for *B. subtilis* 168H changes at higher doses, whereas the slope for the survival curve for *B. subtilis* #40 mms^- remains constant throughout the highest doses tested. From these results it is clear that this strain is more sensitive to MMS than 168H is.

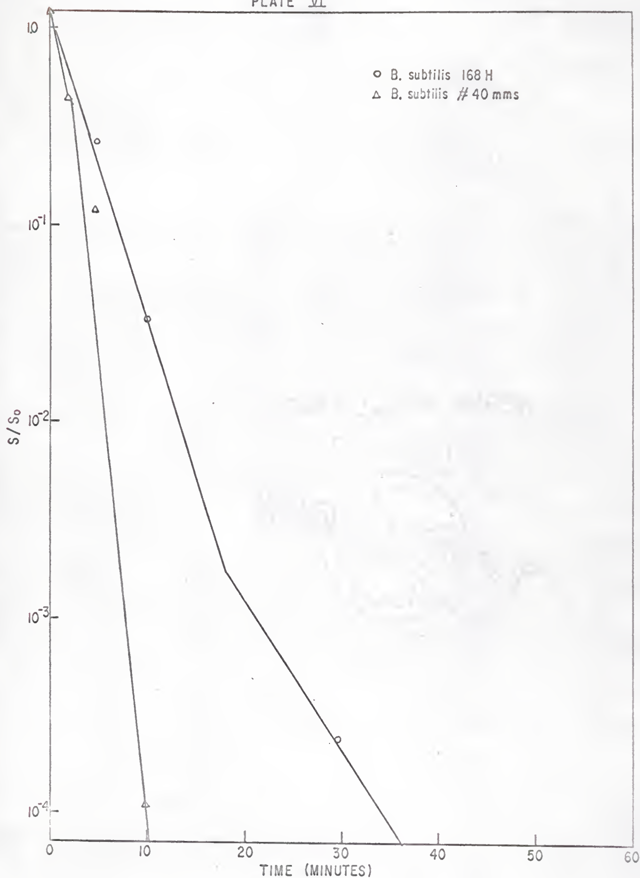
DNA from *B. subtilis* #40 mms^- Treated with MMS *In Vivo*

A culture of *B. subtilis* #40 mms^- was treated with 0.0056 M MMS for 4 minutes at 37 C. A sample of the treated culture was incubated in growth medium for 50 minutes at 37 C prior to lysis. Another sample of the treated culture was lysed immediately after treatment. These lysates were assayed for transforming activity (see Material and Methods, section i, p 24).

PLATE VI

Survival curves for MMS-treated cultures of B. subtilis 168H and B. subtilis #40 mms$^-$ (see Material and Methods, section f, p 22). B. subtilis 168H was treated with 0.05 M MMS and B. subtilis #40 mms$^-$ was treated with 0.025 M MMS. Assay samples were diluted and plated on NBS.

PLATE VI



The transforming activity of lysates of recovered cells is about 4 times greater than that of lysates of non-recovered cells (Table 6). In other words, some repair is occurring after MMS-treatment in this sensitive strain, but it is not as great as that occurring in a MMS-treated culture of B. subtilis 168E.

A lysate of treated cells of B. subtilis #40 mms^- which have been incubated and lysed in the presence of actinomycin has only 2 times more transforming activity than a lysate of treated cells lysed immediately in the presence of actinomycin (Table 6). The transforming activity of this lysate (of treated, non-recovered cells lysed in actinomycin) is the same as that of a lysate of treated, non-recovered cells lysed alone. On the other hand, DNA in a lysate of treated cells recovered and lysed in the presence of actinomycin has 2 times less transforming activity than the DNA in a lysate of treated cells recovered and lysed alone. Again, actinomycin seems to decrease the transforming activity of lysates of recovered cells incubated and lysed in its presence. In this case, it did not affect the transforming activity of lysates of non-recovered cells lysed in its presence compared to that of lysates of non-recovered cells lysed in its absence.

By observing the intensity of color in pellets of centrifuged cells, it was apparent that more actinomycin was present in the MMS-treated cells that were incubated in growth medium containing actinomycin for 50 minutes after treatment than in the cells that were diluted into ice-cold SS containing actinomycin immediately

TABLE 6. Recovery of B. subtilis #40 mms⁻ after MMS Treatment

Treatment	Surviving Fraction[a] N/N_o	Recovery Period	Relative Transforming Activity[b] - Actinomycin	Relative Transforming Activity[b] + Actinomycin
MMS Before Actinomycin Was Added	.027	None	.10	.06
		50 min.	.44	.11
	.039	None	.06	.08
		50 min.	.28	.18
	.04	None	.16	.19
		50 min.	.72	.41
MMS After Actinomycin Was Added	.04	None	.38	.5
		50 min.	.6	.5
	.037	None	.14	.2
		50 min.	.4	.27

[a]Surviving fraction of MMS-treated cells compared to untreated cells.

[b]Relative to the extractable transforming activity from control cultures not treated with MMS. Control cultures that were lysed in the presence of actinomycin had essentially the same transforming activity as control cultures lysed alone.

after MMS treatment. It is possible that the transforming activities of these lysates might be equivalent if the final concentration of actinomycin was equivalent in both samples. To avoid this difference in concentration, 2.7 $\mu g/ml$ of actinomycin was added to the cells 10 minutes prior to treatment with MMS. After treatment one sample was lysed immediately in the same concentration of actinomycin. Another sample was incubated for 50 minutes at 37 C in the same concentration of actinomycin and then lysed as usual. The results of two experiments are shown in Table 6. There is no difference in the transforming activity of DNA in lysates of recovered and non-recovered cells when both are incubated with actinomycin prior to MMS treatment. These results can be compared to the transforming activity from corresponding lysates of cells which have been treated with the same dose of MMS but lysed in the absence of actinomycin. On the average, the transforming activity of the DNA from recovered cells which have been incubated in actinomycin prior to MMS treatment 1.3 times less than the transforming activity of the DNA from recovered cells incubated without actinomycin. The transforming activity of DNA from non-recovered cells incubated in actinomycin prior to MMS treatment is 1.3 times more than the transforming activity of DNA from non-recovered cells treated and lysed in the absence of actinomycin. These results are similar to those obtained for B. subtilis 168H under the same conditions (Table 5). In both instances, the transforming activities of the two lysates of MMS-treated cells (recovered and non-recovered) pre-incubated in

actinomycin are equal and intermediate of that of treated cells lysed immediately in the absence of actinomycin (non-recovered) and of treated cells incubated prior to lysis but in the absence of actinomycin (recovered).

Especially for B. subtilis 168H, the transforming activity is lower than that normally obtained when treated cells are recovered, but not pre-incubated, in the presence of actinomycin. Normally, the transforming activity of lysates of recovered cells containing actinomycin is 1.3 times less than that of lysates of recovered cells alone. When recovered cells are incubated with actinomycin prior to MMS-treatment, and then lysed, the transforming activity of this lysate is 5 times less than that of lysates of recovered cells alone (Table 5).

This suggests that repair is not occuring when actinomycin is added to cells prior to MMS treatment. Also, the transforming activity does not increase when cells incubated in actinomycin prior to MMS treatment have been recovered in growth medium after treatment. It would be interesting to disocciate the actinomycin from the DNA and then compare the molecular weights of these samples.

DISCUSSION

The repair of DNA lesions induced by the monofunctional alkylating agent, methyl methanesulfonate, is similar to the repair of DNA lesions induced by UV light. After MMS treatment, DNA synthesis stops for a period of time. During this period when there is not net increase in DNA, breakdown of DNA into acid-soluble fragments occurs along with the incorportaion of radioactive thymidine as repair replication.

MMS repair differs from UV repair in that UV-sensitive mutants are able to repair MMS damage. It is possible to visulize that the same mechanism of repair may be invoked, but that the repair of MMS damage may require one less step of this mechanism. Therefore, mutants unable to carry out the first step of UV repair (excise thymine dimers) are still capable of repairing MMS-induced lesions, since excision is not required for the repair of this type of damage. In agreement, MMS-sensitive mutants are unable to repair UV-induced lesions. Although these mutants, presumably defective in a later step of repair, may be able to excise thymine dimers, they are still unable to complete the repair process.

MMS repair and UV repair differ in one other aspect. UV repair is inhibited if actinomycin is present during the recovery period after UV treatment. In contrast, repair of MMS damage seems to occur under the same conditions.

In B. subtilis there is a nuclease activated at the time of lysis which specifically degrades alkylated DNA. Therefore, it is possible to detect repair of DNA in MMS-treated cells as an increase in extractable transforming activity after incubation in growth medium. If MMS-treated cells are incubated in growth medium containing actinomycin, the transforming activity of DNA from these cells is now greater than that of DNA from MMS-treated cells which are lysed immediately after treatment. This suggests that the DNA has been repaired (i.e., the methyl groups have been removed) and the DNA is no longer susceptible to the nuclease specific for alkylated DNA. This research was an effort to study this particular aspect of MMS repair in greater detail.

The transformation activity of lysates of MMS-treated cells lysed immediately, both in the presence and absence of actinomycin, and of MMS-treated cells incubated prior to lysis, both in the presence and absence of actinomycin, was determined. These data, at first glance, suggest that repair has occurred both in the presence and absence of actinomycin. The transforming activity of lysates of recovered cells is about 20 times greater than that of lysates of non-recovered cells. When actinomycin is present during the recovery and lysis of MMS-treated cells, the transforming activity of lysates of recovered cells is only 2-3 times greater than that of non-recovered cells lysed in actinomycin. DNA from MMS-treated cells recovered in growth medium containing actinomycin does not have as much transforming activity as DNA from MMS-treated cells recovered in growth medium without actinomycin

(8% of control versus 11% of control). This may be due to an effect of actinomycin on transformation that is not apparent in lysates for control (untreated) cells lysed in the presence of actinomycin. Since control cells have not been incubated for 50 minutes at 37 C, the concentration of actinomycin within these cells may be lower than that of recovered cells. This difference may also be an indication that repair is not as efficient in the presence of actinomycin.

Transforming activity of DNA is higher in lysates of non-recovered cells lysed in the presence of actinomycin than in lysates of non-recovered cells lysed alone (3% of control versus 0.6% of control). Since actinomycin may be holding the DNA molecule intact (see below), it is possible that the recipient organism is capable of taking up this DNA, repairing it, and then being transformed. In the UV repair system, repair of UV-damaged DNA does occur in the recipient cell (Reiter and Strauss, 1965). Reiter also has evidence that competent cells are able to take up DNA in the presence of actinomycin at concentrations which are inhibitory to transformation (personal communication). Another possible explanation may be that degradation (thus inactivation of transforming activity) by the nuclease specific for alkylated DNA may not be as efficient in cells containing actinomycin.

Results from sucrose gradient centrifugation suggest that the molecular weight is the same for DNA present in a lysate of non-recovered cells lysed in the presence of actinomycin and for DNA present in a lysate of cells recovered and lysed in the presence of actinomycin. The DNA in both lysates bands at a position

in sucrose gradients that is very close to the position of DNA from untreated cells. In contrast, DNA from non-recovered cells lysed in the absence of actinomycin bands at a position near the top of a sucrose gradient, indicating it has a smaller molecular weight. DNA from cells recovered in the absence of actinomycin bands at a position intermediate of control and unrepaired DNA.

From the above results, it is impossible to distinguish between the possibility that actinomycin may be inhibiting the nuclease specific for alkylated DNA or merely preserving the physical integrity of the DNA molecule as a result of its attachment to DNA. Since the molecular weight of the DNA in the samples containing actinomycin is the same, it is also impossible to determine whether repair, measured as an increase in molecular weight of repaired DNA compared to unrepaired DNA, has occurred in the presence of actinomycin.

Native DNA in the B conformation is more efficient in binding actinomycin (Reich, 1964), and actinomycin complexed to DNA causes an increase in the irreversible denaturation temperature (Reich, 1964; Haselkorn, 1964). This may be indirect evidence that actinomycin is attached in some way to the strand of DNA opposite to the one containing guanine, which is bound to the quinoidal oxygen of actinomycin by its N2 amino group (Reich and Goldberg, 1964). Perhaps the peptide lactones, which are required to be intact for complexing to occur (Reich and Goldberg, 1964), may be hydrogen bonded to the opposite strand. There are four sites in the peptide

lactones capable of forming hydrogen bonds (Reich and Goldberg, 1964). If the nuclease causes single-strand breaks in the DNA molecule, the attachment of actinomycin to both strands at the site of a single-strand break may prevent the opposite strand from being broken at this fragile point. Alternatively, actinomycin could be responsible for holding together the two free ends resulting from a single-strand break. Peptide lactones could again be involved in this bonding. Since it is probable that all the alkylated guanine moieties have actinomycin bound to them (see below), either of the above possibilities could account for the DNA molecule remaining intact, in spite of single-strand breaks caused by the alkylated-DNA-sepcific nuclease.

There are an average of 4.26 lethal hits per locus if the survival of transforming activity for the indole locus of non-recovered cells is 0.014 compared to the transforming activity of DNA from control cells (Table 5). Assuming that each lethal hit is equivalent to a methylated guanine, there would be 1.3×10^3 methylated guanine residues per DNA molecule which is 3×10^6 base pairs long (McCarthy, 1967). This is assuming that a single-strand locus has a molecular weight of 3.5×10^6 daltons (Strauss, Searashi and Robbins, 1966). Assuming one fourth of the bases to be guanine, 0.087% of the guanines would be methylated. At the concentrations of actinomycin used, there are 4×10^6 actinomycin molecules available per DNA molecule. This would be 2.7 actinomycin molecules per each guanine residue. Obviously, there would be a 100% probability of an actinomycin molecule being attached to a methylated guanine.

The number of alkylated guanines would be higher if the nuclease only caused single-strand breaks (Strauss, personal communication to Reiter). In this case, a lethal hit would be the result of a scission in the DNA molecule which occurred when two single-strand breaks were closely located on opposite strands. Assuming the probability of an alkylation occurring is the same on either strand, the probability of two alkylations being nearly opposite would be 0.00087. From this, the probability of a guanine on either strand being alkylated would be 0.03. Although the assumption that the latter calculation is based on (that the nuclease causes single-strand breaks) is probably the correct one, there is still a very high probability that the alkylated moiety will be attached to an actinomycin molecule (since there are 2.7 actinomycin molecules present per each guanine residue).

In conclusion, there was enough actinomycin present in these experiments that each methylated guanine could be attached to a molecule of actinomycin. This supports the hypothesis that actinomycin is capable of preserving the physical integrity of methylated DNA in spite of single-strand breaks caused by the nuclease specific for DNA methylated at the site of guanine and, perhaps, adenine residues. This would explain why the molecular weight of DNA from both recovered and non-recovered cells lysed in actinomycin is nearly the same as that of control DNA. It also suggests an explanation for the fact that transforming activity is higher in lysates of non-recovered cells lysed in actinomycin compared to that of lysates of non-recovered cells lysed

alone (i.e., competent cells are able to take up this "intact" DNA, repair it, and then be transformed). The second possible explanation, that actinomycin prevents the nuclease from acting on methylated DNA, could also account for the fact that the molecular weight of DNA from both recovered and non-recovered cells in actinomycin is nearly the same as control. It would be difficult to explain why the transforming activity of DNA from non-recovered cells lysed in actinomycin is greater than that of DNA from non-recovered cells lysed alone, but not as great as that of DNA from cells recovered and lysed in actinomycin --unless concentration of actinomycin within the cell is a critical factor.

Studies on the effect of actinomycin on the nuclease _in vitro_ suggest that actinomycin does not inhibit the activity of this enzyme. There is the same amount of reduction in transforming activity if the samples of methylated-DNA, methylated-DNA incubated with actinomycin, and DNA incubated with actinomycin prior to methylation are treated with an extract of _B. subtilis_. Although unlikely, it is possible that the activity of the enzyme is altered as a result of extraction, and, _in vivo_, inhibition of the enzyme is occurring.

If these samples are centrifuged on sucrose gradients, it is apparent that actinomycin does not prevent the degradation of methylated-DNA by the nuclease. This is in contrast to the results of _in vivo_ studies. Perhaps the interaction of actinomycin and DNA is more complex _in vivo_. The DNA used in _in vitro_ studies has been purified. Therefore, if another component (such as protein)

is involved in the complex formed *in vivo*, this would not be present in the purified DNA.

To determine: 1) whether DNA from both recovered and non-recovered cells lysed in actinomycin was degraded by the nuclease at the time of lysis; and 2) whether DNA from cells recovered in actinomycin was actually repaired; it is necessary to dissociate the actinomycin from the DNA with 5 M urea, and remove it by repeated extractions with anhydrous ether. This treatment does not alter the molecular weight of control DNA or control DNA complexed with actinomycin. As seen from sucrose gradient centrifugation, there is a large decrease in the molecular weight of DNA from both cells recovered and lysed in actinomycin and non-recovered cells lysed in actinomycin. Originally these DNA samples banded at a position similar to that of control DNA. DNA from these recovered cells now bands at the same position as repaired DNA from treated cells which were recovered in the absence of actinomycin, and the DNA from these non-recovered cells is approaching the position of non-repaired DNA from treated cells lysed immediately but in the absence of actinomycin. The fact that this DNA has not reached the position of non-repaired DNA may be explained in two ways; enough actinomycin may still be remaining on the DNA molecule to partially hold it together; or the presence of actinomycin at the time of lysis has prevented as complete a degradation by the nuclease from occurring. It may be possible to test the first possibility by determining a spectrograph of the sample. If actinomycin is present there will be a change in absorption at

440 mμ (Cerami, et al, 1967).

In conclusion, repair of MMS-induced damage to DNA does occur in the presence of actinomycin. The transforming activity of lysates of MMS-treated cells incubated in growth medium containing actinomycin increases 2-3 fold over that of lysates of MMS-treated cells lysed immdeiately in actinomycin. When actinomycin is dissociated from DNA, the molecular weight of DNA from cells recovered in actinomycin decreases to that of repaired DNA. The molecular weight of DNA from non-recovered cells lysed in actinomycin decreases to nearly that of unrepaired DNA. Repair, measured as an increase in transforming activity after incubation in growth medium, is not as efficient in the presence of actinomycin. The transforming activity of DNA from these cells is never as great as that from MMS-treated cells incubated in growth medium without actinomycin. It is not known for certain if this is an effect of actinomycin on repair.

Earlier data have supported the hypothesis that the mechanism for MMS repair is the same as that of UV repair, but with fewer steps being utilized for MMS repair. Now it appears that at least some of the repair of MMS damage must be different than that of UV repair. UV repair, in contrast of MMS repair, is inhibited by the presence of actinomycin. Also, UV repair requires repair replication. When MMS-treated cells are being incubated in growth medium containing actinomycin, repair occurs without the incorporation of ^{3}H-thymidine. (Reiter, personal communication).

Another type of repair for MMS-induced damage can now be

hypothesized. An enzyme may be present which is capable of removing the methyl group on the N7 of guanine without causing a single-strand break in the sugar-phosphate backbone. It is possible that repair of apurinic sites caused by hydrolysis of the methylated base from the ribose-phosphate backbone occurs via the excision-repair mechanism. These lesions would not be repaired in the presence of actinomycin. Both mechanisms may be functioning normally when actinomycin is not present, since the transforming activity of DNA from MMS-treated cells recovered in its absence is greater than the activity of that from MMS-treated cells recovered in its presence.

Another explanation involving methylated adenine moieties may be considered. Adenine is methylated at the N3 position at one-fourth the frequency of the methylation of guanine at N7, but it is released five times faster than 7-methyl guanine to yield apurinic sites (Lawley, 1966). Since these methyl groups would not be in the same position with respect to the DNA double helix, an enzyme capable of recognizing the 7-methyl guanine and removing the methyl group may not detect the 3-methyl adenine. These lesions may require at least the latter steps of excision and repair mechanism, if the apurinic sites were further hydrolyzed to yield single-strand breaks. This repair would be inhibited by actinomycin.

Results of Treatment of B. subtilis #40 mms⁻ with MMS

From the results of the survival curves, it is apparent that

B. subtilis #40 mms$^-$ is more sensitive to MMS than B. subtilis 168H. Originally this strain was unable to repair DNA damage induced by MMS, but during this investigation it was found that this strain obtained a four-fold increase in transforming activity after post-treatment incubation in growth medium. This is less than half of the repair obtained by B. subtilis 168H under the same conditions. In agreement with the results for B. subtilis 168H, there was a decrease in transforming activity of DNA from MMS-treated cells recovered in actinomycin compared to that for DNA from MMS-treated cells recovered in its absence. In this case, the transforming activity of DNA from non-recovered cells lysed in actinomycin was the same as that obtained for DNA from non-recovered cells lysed in its absence. In this case, transforming activity of DNA from recovered cells in actinomycin was only two-fold higher than that of DNA from non-recovered cells lysed in actinomycin.

It was originally planned to determine the molecular weight distribution of DNA from treated cells unable to repair MMS damage, both in the presence and absence of actinomycin. Since this strain underwent some recovery, this problem was not continued.

It is interesting to note that when actinomycin was added to these cells prior to treatment with MMS, the transforming activity of DNA from recovered and non-recovered cells, both in the presence of actinomycin, was equivalent (Table 6). Although the transforming activity of the lysates of non-recovered cells lysed in actinomycin was increased to even a greater extent compared to the transforming

activity of lysates of non-recovered cells alone, there was a more noteable decrease in the transforming activity of lysates of treated cells recovered in actinomycin compared to that in lysates of treated cells recovered alone.

The same type of results were obtained in an identical experiment with B. subtilis 168H (Table 5). In this case, the transforming activities of the lysates of both recovered and non-recovered cells incubated with actinomycin prior to treatment with MMS were equal and intermediate of the transforming activities obtained for lysates of non-recovered cells and recovered cells, both in the absence of actinomycin.

It appears that the repair of damage induced by MMS that occurs in the presence of actinomycin may be inhibited if the actinomycin is added early enough. Perhaps enzyme induction is required for this type of repair. In other experiments, actinomycin was added after MMS treatment, which requires 4-5 minutes. This is adequate time for enzyme induction to take place. This "inducible" enzyme may be the one suggested earlier which is capable of removing the methyl group from guanine without causing a single-strand break in the sugar-phosphate backbone.

SUMMARY

1. Actinomycin inhibits transformation at concentrations greater than 1 x 10^{-3} μg/ml. Apparently the effect is more complex than inhibition of DNA uptake by competent cells.

2. Growing cells incubated in growth medium containing 2.7 μg/ml of actinomycin for at least one hour at 37 C are not killed, although the number of cells present does not increase during this time.

3. Actinomycin does not inhibit methylation of DNA with methyl methanesulfonate.

4. Actinomycin does not prevent DNA methylated <u>in vitro</u> with MMS from being inactivated by a nuclease present in <u>B. subtilis</u> extracts specific for methylated DNA. If methylated DNA is incubated with actinomycin prior to treatment with the enzyme extract, the actinomycin does not preserve the physical integrity of this DNA.

5. Actinomycin does not prevent repair of MMS-induced damage to DNA from occurring during post-treatment incubation of MMS-treated cells in growth medium containing this antibiotic. This repair is less than that normally occurring when MMS-treated cells are incubated in growth medium without actinomycin.

6. The presence of actinomycin at the time of lysis of MMS-treated cells lysed immediately after treatment enhanced the transforming activity of this lysate compared to that

of MMS-treated cells lysed immediately, but in the absence of actinomycin.

7. Transforming activity of lysates of MMS-treated cells recovered and lysed in the presence of actinomycin is less than that of MMS-treated cells recovered and lysed in the absence of actinomycin.
8. If actinomycin is present in both recovered and non-recovered samples of MMS-treated cells, the original size (molecular weight) of the DNA is preserved, in spite of the presence of single-strand breaks caused by the nuclease specific for alkylated DNA that is activated at the time of lysis.
9. Dissociation of actinomycin from DNA by 5 M urea results in a decrease in molecular weight of DNA from MMS-treated cells, both non-recovered and recovered samples, lysed in the presence of actinomycin. The molecular weight of DNA from the treated cells recovered and lysed in actinomycin is now equivalent to that of repaired DNA. The molecular weight of DNA from treated cells lysed immediately in actinomycin is now nearly equivalent to that of unrepaired DNA.
10. If actinomycin is added to cells prior to treatment with MMS, repair does not occur. Transforming activities of lysates of both recovered and non-recovered samples of this culture are equal and intermediate of the transforming activities of lysates of non-recovered cells and recovered cells alone.
11. B. subtilis #40 mms$^-$ is more sensitive to MMS than B. subtilis 168H. Although repair occurs in this strain, it is less than half of that occurring in B. subtilis 168H.

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LITERATURE CITED

Bollum, F. J., and R. B. Setlow. 1963. Ultraviolet inactivation of DNA primer activity: 1. Effects of different wavelengths and doses. Biochim. Biphys. Acta 68:599-607.

Boyce, R. P., and P. Howard-Flanders. 1964. Release of UV-induced thymine dimers from DNA in E. coli K-12. Proc. Natl. Acad. Sci. 51:293-300.

Bridges, B. A., and R. J. Munson. 1966. Excision-repair of DNA damage in auxotrophic strains of Escherichia coli. Biochem. Biophys. Res. Comm. 22:268-273.

Brookes, P., and P. D. Lawley. 1961. The reaction of mono- and di-functional alkylating agents with nucleic acids. Biochem, J. 80:496-503.

Cairns, J., and C. Davern. 1966. Effect of ^{32}P decay upon DNA synthesis by a rediation-sensitive strain of Escherichia coli. J. Mol. Biol. 17:418-427.

Cerami, A., E. Reich, D. C. Ward, and I. H. Goldberg. 1967. The interaction of actinomycin with DNA: Requirement for the 2-amino group of purines. Proc. Natl. Acad. Sci. 57: 1036-1042.

Chargoff, E., and J. N. Davidson. 1960. The Nucleic Acids: Chemistry and Biology. Vol. III. Academic Press, New York.

Clark, A. J., and A. D. Margulies. 1965. Isolation and characterization of recombination deficient mutants of E. coli K-12. Proc. Natl. Acad. Sci. 53:451-459.

Emmerson, P. T., and P. Howard-Flanders. 1965. Post-irradiation degradation of DNA following exposure of UV-sensitive and resistant bacteria to X-rays. Biochem. Biophys. Res. Comm. 18:24-29.

Freidfelder, D. 1965. Mechanism of inactivation of coliphage T7 by X-rays. Proc. Natl. Acad. Sci. 54:128-134.

Freidfelder, D. 1966. DNA strand breakage by X-irradiation. Radiation Res. 29:329-338.

Gellert, M. 1967. Formation of covalent circles of lambda DNA by E. coli extracts. Proc. Natl. Acad. Sci. 57:148-155.

Hanawalt, P., and R. B. Haynes. 1965. Repair replication of DNA in bacteria: Irrelevance of chemical nature of base defect. Biochem. Biophys. Res. Comm. 19:462-267.

Hanawalt, P., and R. B. Setlow. 1960. Effect of monochromatic ultraviolet light on macromolecular synthesis in Escherichia coli. Biochim. Biophys. Acta 41:238-294.

Hartman, F., U. Coy, and G. kniese. 1963. Zum biolocischen wirkungs-mechanismus der actinomycin. (Hoppe-Seylor's) Zeitschrift fur Physiolocische Chemie. Bd. 330:227-233.

Haselkorn, R. 1964. Actinomycin D as a probe for nucleic acid secondary structure. Science 143:682-684.

Haynes, R. H. 1964. Role of DNA repair mechanisms in microbial inactivation and recovery phenomena. Photochem. Photobiol. 3:429-450.

Haynes, R. H. 1966. The interpretation of microbial inactivation and recovery phenomena. Radiation Res. Suppl. 6:1-24.

Haynes, R. H., M. H. Patrick, and J. E. Baptist. 1964. Comparative response of radioresistant and radiosensitive strains of Escherichia coli to various inactivation agents. Radiation Res. 22:194.

Hill, R. F. 1958. A radiation-sensitive mutant of Escherichia coli. Biochim. Biophys. Acta 30:636-637.

Hill, R. F., and E. Simson. 1961. A study of radiosensitive and radioresistant mutants of Escherichia coli strain B. J. Gen. Microbiol. 24:1-14.

Howard-Flanders, P., and R. P. Boyce. 1966. DNA repair and genetic recombination: studies on mutants of Escherichia coli defective in these processes. Radiation Res. Suppl. 6:156-184.

Howard-Flanders, P., R. P. Boyce, and L. Theriot. 1966. Three loci in Escherichia coli K-12 that control the excision of thymine dimers and certain other mutagen products from host or phage DNA. Genetics 53:1119-1137.

Howard-Flanders, P., and L. Theriot. 1966. Mutants of E. coli K-12 defective in DNA repair and in genetic recombination. Genetics 53:1137-1150.

Iyer, V. N., and W. Szybalski. 1964. Mitomycins and porfiromycin: chemical mechanism of activation and cross-linking of DNA. Science 145:55-58.

Kersten, W., H. Kersetn, and W. Szybalski. 1966. Physicochemical properties of complexes between deoxyribonucleic acid and antibiotics which affect ribonucleic acid synthesis (Actinomycin, daunomycin, cinerubin, nogalamycin, chromomycin, and olivomycin). Biochemistry 5:236-244.

Kohn, K. W., N. H. Steigbigel, and C. L. Spears. 1965. Cross-linking and repair of DNA in sensitive and resistant strains of E. coli. treated with nitrogen mustard. Proc. Natl. Acad. Sci. 53:1154-1161.

Lawley. P. D. 1966. Effects of some chemical mutagens and carcinogens on nucleaic acids. Eds. J. N. Davidson and W. E. Cohn. Prog. Nucleic Acid Res. and Mol. Biol. 5:89-131.

Lawley. P. D., and P. Brookes. 1965. Molecular mechanism of the cytotoxic action of difunctional alkylating agents and resistance to this action. Nature 206:480-483.

Lawley, P. D., and P. Brookes. 1963. Further studies on the alkylation of nucleic acids and their constituent nucleotides. Biochem. J. 89:127-139.

Lovelace, A., J. Cook, and P. Wheatley. 1965. Recovery from the "lethal" effects of cross-linking alkylation. Nature 205: 980-983.

Mahler, I. 1966. Effect of Mitomycin C on five excision-repair mutants of Bacillus subtilis. Biochem. Biophys. Res. Comm. 25:73-79.

McGrath, R. A., and R. W. Williams. 1965. Degradation of X-ray damaged DNA- A model for repair. Biophysics J. and Abstracts 5:42.

McGrath, R. A., R. W. Williams, and P. C. Swartzendruber. 1966. Breakdown of DNA in X-irridiated Escherichia coli. Biophysics J. 6:113-121.

Matters, I. E., M. P. can Winden, and A. Rörsch. 1965. The range of action of genes controlling radiation sensitivity in E. coli. Mutation Res. 2:111-131.

McCarthy, B. J. 1967. Arrangement of base sequences in deoxyribonucleic acid. Bact. Rev. 31:215-229.

Okubo, S., and W. R. Romig. 1966. Impaired transformability of Bacillus subtilis mutant sensitive to Mitomycin C and ultraviolet radiation. J. Mol. Biol. 15:440-450.

Papirmeister, B., and C. L. Davison. 1964. Elimination of sulfur mustard-induced products from DNA of Escherichia coli Biochem. Biophys. Res. Comm. 17:608-610.

Pettijohn, D., and P. Hanawalt. 1964. Evidence for repair-replication of ultraviolet damaged DNA in bacteria. J. Mol. Biol. 9:395-410.

Van de Putte, P., C. A. van Sluis, J. van Dillewijn, and A. Rörsch. 1965. The location of genes controlling radiation sensitivity in E. coli. Mutation Res. 2:97-110.

Van de Putte, P., H. Zwenki, and A. Rörsch. 1966. Properties of four mutants of Escherichia coli defective in genetic recombination. Mutation Res. 3:382-392.

Reich, E. 1964. Actinomycin: correlation of structure and function of its complexes with purines and DNA. Science 143:684-689.

Reich, E., and I. H. Goldberg. 1964. Actinomycin and nucleic acid function. Eds. J. N. Davidson and W. E. Cohn. Prog. Nucleic Acid Res. and Mol. Biol. 3:183-233.

Reich. E., I. H. Goldberg, and M. Rabinowitz. 1962. Structure-activity correlations of actinomycins and their derivatives. Nature 196:743-748.

Reich, E., A. J. Shatkin, and E. L. Tatum. 1961. Bacteriological action of Mitomycin C. Biochim. Biophys. Acta 53:132-149.

Reiter, H., and B. Strauss. 1965. Repair of damage induced by a mono-functional alkylating agent in a transformable ultraviolet-sensitive strain of Bacillus subtilis. J. Mol. Biol. 14:179-194.

Reiter, H., B. Strauss, R. Marone, and M. Robbins. 1966. Actinomycin D inhibition of UV-dark repair in Bacillus subtilis. Biochem. Biophys. Res. Comm. 24:892-895.

Reiter, H., B. Strauss, M. Robbins, and R. Marone. 1967. Nature of the repair of methyl methanesulfonate-induced damage in Bacillus subtilis. J. Bacteriol. 93:1056-1062.

Rörsch, A., A. Edelman, and J. A. Cohen. 1963. The gene-controlled radiation sensitivity in Escherichia coli. Biochim. Biophys. Acta 68:263-270.

Searashi, T., and B. Strauss. 1965. Relation of the repair of damage induced by a monofunctional agent to the repair of damage induced ultraviolet light in Bacillus subtilis. Biochem. Biophys. Res. Comm. 20:680-687.

Searashi. T., and B. Strauss. 1967. Glucose requirement for DNA breakdown resulting from treatment of *Bacillus subtilis* with ultraviolet radiation or methyl methanesulfonate. Mutation Res. 4:372-375.

Setlow, J. K. 1964. Effects of ultroaiolet on DNA: correlation among biologiacl changes, physical changes, and repair mechanisms. Photochem. Photobiol. 3:405-413.

Setlow, J. K. 1966. Photoreactivation. Radiation Res. Suppl. 6:141-155.

Setlow, R. B. and W. L. Carrier. 1964. The disapperance of thymine dimers from DNA: and error-correcting mechanism. Proc. Natl. Acad. Sci. 51:226-231.

Setlow, J. J., M. Boling, and F. Bollum. 1965. The chemical nature of photoreactivable lesions in DNA. Proc. Natl, Acad. Sci. 53:1430-1436.

Setlow, R. B., W. L. Carrier, and F. J. Bollum. 1965. Pyrinidine dimers in UV-irradiated poly dI:dC. Proc. Natl. Acad. Sci. 53:1111-1118.

Setlow, R. B., and J. K. Setlow. 1962. Evidence that ultraviolet-induced thymine dimers in DNA cause biological damage. Proc. Natl. Acad. Sci. 48:1250-1257.

Setlow, R. B., and J. K. Setlow. 1963. Nature of the photo-reactivable ultraviolet lesion in deoxyribonucleic acid. Nature 197:560-662.

Setlow, R. B., P. A. Swenson, and W. L. Carrier. 1963. Thymine dimers and inhibition of DNA synthesis by radiation of cells. Science 142:1464-1466.

Shooter, K. V. 1957. The physical chemistry of deoxyribosenucleic acid. Prog. Biophys. 8:309-346.

Smith, K. C. 1964. Photochemistry of the nucleic acids. Ed. Giese. Photophysiology. Vol. III:329-388.

Strauss, B. 1958. The genetic effect of incorporated radio-isotopes: the transmutation problem. Radiation Res. 8:235-247.

Strauss, B. 1962. Differential destruction of the transforming activity of damaged DNA by a bacterial enzyme. Proc. Natl. Acad. Sci. 48:1670-1675.

Strauss, B. DNA repair mechanisms and their relation to mutation and recombination. Current Topics in Microbiology and Immunology, (to be published).

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Strauss, B., T. Searashi, and M. Robbins. 1966. Repair of DNA studied with a nuclease specific for UV-induced lesions. Proc. Natl. Acad. Sci. 56:932-939.

Strauss, B., H. Reiter, and T. Searashi. 1966. Recovery from ultraviolet- and alkylating agent-induced damage in *Bacillus subtilis*. Radiation Res. Suppl. 6:201-211.

Strauss, B., and R. Wahl. 1964. The presence of breaks in deoxyribonucleic acid of *Bacillus subtilis* treated *in vivo* with the alkylating agent, methanesulfonate. Biochim. Biophys. Acta 80:116-126.

Swenson. P. S., and R. B. Setlow. 1966. Effects of ultraviolet radiation on macromolecular synthesis in *Escherichia coli*. J. Mol. Biol. 15:201-219.

Szybalski, W., and V. N. Iyer. 1964. Cross-linking of DNA by enzymatically or chemically activated mitomycins and porfiromycins, bifunctional "alkylating" antibiotics. Fed. Proc. 23:946-957.

Terawashi, A., and J. Greenberg. 1966. Post-treatment breakage of Mitomycin C induced cross-links in deoxyribonucleic acid of *Escherichia coli*. Biochim. Biophys. Acta 115:540-545.

Wacker, A. 1963. Molecular mechanisms of radiation effects. Prog. Nucleic Acid Res. 1:369-399.

Wahl. R. 1965. Detection of single-strand breaks in DNA alkylated *in vitro*. Fed. Proc. 24:226.

Weiss, B., and C. C. Richardson. 1967. Enzymatic breakage and joining of deoxyribonucleic acid. I. Repair of single-strand breaks in DNA by an enzyme system from *Escherichia coli* infected with T4 bacteriophage. Proc. Natl. Acad. Sci. 57:1021-1028.

Witkin, E. 1947. Genetics of resistance to radiation in *Escherichia coli*. Genetics 32:221-248.

Wulff, D. L., and C. S. Rupert. 1962. Disappearance of thymine photodimers in ultraviolet irradiated DNA upon treatment with a photoreactivating enzyme from baker's yeast. Biochem. Biophys. Res. Comm. 7:237-240.

THE EFFECTS OF ACTINOMYCIN ON DEOXYRIBONUCLEIC ACID TREATED WITH METHYL METHANESULFONATE

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ABSTRACT

A nuclease present in *Bacillus subtilis* degrades methylated DNA at the time of cell lysis. Since the degraded DNA does not act as transforming DNA, repair may be measured as an increase in transforming activity of DNA from MMS-treated cells incubated in growth medium prior to lysis compared to that of MMS-treated cells lysed immediately.

A culture of *B. subtilis* 168H was treated with a monofunctional alkylating agent, methyl methanesulfonate. Two samples of this culture were lysed immediately after treatment; one in tris-EDTA buffer containing actinomycin and the other in tris-EDTA buffer only. A third sample of the treated culture was recovered in growth medium plus actinomycin, and a fourth sample was recovered in growth medium only.

Transforming activities of these lysates showed that although repair occurs in the presence of actinomycin, it is less efficient than normal repair. Molecular weight studies indicated that actinomycin inhibits degradation of methylated DNA in both recovered and non-recovered cells, although the transforming activity of the DNA has been inactivated by the nuclease. Dissociation of actinomycin from DNA with 5 M urea showed that the DNA had actually been degraded by the nuclease but was being held intact in the presence of actinomycin. DNA from non-recovered cells now bands at a position in sucrose gradients indicative of a small molecular weight. It is nearly equivalent to the molecular weight of unrepaired DNA from MMS-treated cells lysed immediately after

treatment, but in the absence of actinomycin. DNA from recovered cells now bands at an intermediate position in sucrose gradients identical to that of repaired DNA from MMS-treated cells recovered in growth medium in the absence of actinomycin prior to lysis. From these data it was concluded that repair of MMS damage does occur in the presence of actinomycin.

If actinomycin is added to cells prior to treatment with MMS, repair apparently does not occur. The transforming activity of lysates of both recovered and non-recovered cells from this culture are identical and intermediate to the transforming activities of lysates of recovered and non-recovered cells to which actinomycin was not added. This suggests that the enzyme responsible for repair of MMS-induced damage (at least that occurring in the presence of actinomycin) is inducible rather than constitutive.