REPAIR OF E. COLI B130 DNA, DAMAGED BY MITOMYCIN-C

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ABSTRACT

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by Irith Ginzburg

E. coli B130 DNA was damaged by treatment with the antibiotic mitomycin-c in concentration of 1 γ/ml which gave 75% survival. Dark repair mechanism was detected by alkaline sucrose gradients which enables detection of single strand breaks. Elevation of endonuclease activity was shown in the treated cells in the time of beginning of the reapir process.

REPAIR OF *E. COLI* B130 DNA, DAMAGED BY MITOMYCIN-C

By

Irith Ginzburg

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INTRODUCTION

The dark repair mechanism was postulated by Setlow (10) and Boyce et al. (7) to consist of a sequence of steps involving excision of damage, DNA degradation, DNA repair replication and joining of the repaired ends. However, evidence establishing the overall sequence is lacking, and in general showing the existence of one of these steps was regarded as evidence for the entire repair process.

Since this postulated mechanism uses enzymes which are available in the cell, nucleases, DNA polymerase and joining enzyme, it is assumed to be a general repair mechanism for radiation and chemical damage.

This work aimes at testing the above hypothesis, and after hypothesis attempts to give a more complete description of the phenomenon of repair.

LITERATURE REVIEW

The concept of "repair of damage to deoxyribonucleic acid - DNA," caused by physical and chemical agents came from the demonstration that such measurable damage which appears as lesions can be reversed or repaired. Thus, in order that the organism will be able to survive the different "lethal effects" to which it is exposed in the environment, the organism must be equipped with a mechanism which enables it to correct different kinds of damage caused to DNA. Two classes of agents which can cause damage to cells are radiation and chemicals.

I will first review the literature concerned with radiation and later the literature dealing with chemical damage with more detailed information on mitomycins, the antibiotic used in this study.

Radiation damage includes damage caused by both ultraviolet radiation (UV), and ionizing radiation. DNA is presumably the primary target for radiation damage of biological consequence (Haynes, 1).

The most studied photoproduct of UV irradiation are pyrimidine dimers; adjacent pyrimidine residues in the same nucleic acid strand that are linked together by cyclobutane ring (Beukers et al., 2). Formation of a dimer results in decreased spacing between the pyrimidine rings and a change

in their orientation as compared to those found in a Watson-Crick structure. Therefore, these pyrimidine dimers would be expected to cause a localized distortion of the helix.

Setlow and Carrier (8) reported that the primary photochemical products in DNA were the $\hat{TT}$, $\hat{CT}$, and $\hat{CC}$ dimers. In acid hydrolysates of irradiated DNA, $\hat{TT}$, $\hat{UT}$ and $\hat{UU}$ were detected. The uracil containing dimers presumably arose from deamination of $\hat{CT}$ and $\hat{CC}$.

All the above dimers have similar photochemical properties in that they are monomerized by short wavelength irradiation (Setlow and Carrier, 5). They are also monomerized by the photoreactivating enzyme in presence of visible light, Rupert (15) and Muhammed (59). All dimers seem to inhibit enzymatic degradation and synthesis of DNA *in vitro*, and presumably in some organisms, they act in a similar manner *in vivo* (Bollum and Setlow, 4). Both $\hat{TT}$ and $\hat{CT}$ dimers are excised from DNA of irradiated resistant bacteria upon further incubation in fresh media (Setlow, 10; Boyce and Howard Flanders, 7).

The repair systems seem to work on double stranded DNA. This was shown by Rörch et al. (9) with ϕx174 virus. The recovery of irradiated bacteriophage depends on the strain of bacterial cells used to titer them. Bacteria which give high phage recovery are said to exhibit host cell reactivation (hcr^+) while those which are incapable of repairing the damage are hcr^- (Metzger, 18).

Only the double stranded, replicative, form of ϕx174-,

and not the single stranded phage, is subject to host cell reactivation by hcr^+ cells.

The UV-induced pyrimidine dimers cause temporary inhibition of DNA synthesis in radiation resistant cells, (Setlow and Swenson, 3). It is assumed that the inhibition period represents the time needed for repair of the lesions.

Molecular Mechanisms for Recovery and Repair

There are several mechanisms which can repair UV damage, that is, pyrimidine dimers in the DNA.

Recovery may be divided into:

(1) Reversal of damage

(a) Photoreversal of pyrimidine dimers

(b) Enzymatic photoreactivation

(c) Photoprotection

(2) Bypass of damage

(3) Dark repair mechanism - Removal of damage

(1) Reversal of damage

Nucleic acids are crucial molecules which absorb UV radiation. Low doses of UV radiation are needed to inactivate DNA. The action spectrum for UV is similar to the absorption spectrum of DNA and RNA but not by other cell components. Photoreactivation is possible for DNA but not for RNA and proteins (Jagger, 12).

(a) Photoreversal

The pyrimidine dimers induced by UV radiation at 2800Å are monomerized by subsequent irradiation at 2390Å

(setlow, 13). It was shown by the Setlows (14) that, if transforming DNA, which was inactivated by 2800Å irradiation is fully photoreactivated with yeast extract, it becomes insusceptible to reactivation at 2390Å and vice versa. This shows that those two mechanisms act independently.

(b) enzymatic photoreactivation

Thymine dimers which are produced at 2800Å cannot be reversed by photons of wavelength longer than 3000Å, because they are not absorbed by the dimer. Photoreactivating enzymes, isolated from yeast and bacteria by Rupert et al. (15), and from yeast by Muhammed (59) monomerize pyrimidine dimers when activated by the absorption of photons in the 3000Å-4500Å region. These photons presumably will not be absorbed by the dimer, as are those at 2390Å, but by some chromophore associated with the enzyme or with the enzyme substrate complex.

Rupert (16) showed that the enzyme exhibited the expected dose rate saturation, and temperature dependence as expected from protein-enzyme in usual Michaelis Menten Kinetics. The existence of an enzyme-substrate complex consisting of the photoreactivating enzyme and UV irradiated DNA was shown. Illumination of the complex broke it, releasing intact enzyme and repaired DNA. The overall process can be summarized as:

$$E + DNApr \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} E - DNApr \xrightarrow[k_2]{h\nu} E + DNAr$$

where DNApr is DNA containing photoreactivable damage, E-DNApr is the enzyme substrate complex produced by reversible reaction, and DNAr is photoreactivated DNA. The light requiring reaction is not reversible since enzymes will not combine with DNA, free of pyrimidine dimers.

(c) photoprotection

Photoprotection was termed by Jagger (6) as a modification effect which involves treatment before the UV irradiation. After irradiating with near UV (3000-3700Å) some cells show decreased sensitivity to UV damage. Because this modifying treatment occurs before the inactivating treatment it is called "protection" in contrast to "photoreactivation" by a posttreatment. The action spectrum for photoprotection is found to induce growth delay.

In liquid recovery (17), E. coli B-cells, which are held in liquid medium for about six hours after irradiation before plating, also demonstrate a growth delay (Jagger, 11). In view of these two phemonena, he postulated that the growth delay permits greater efficiencies of dark repair process.

(2) Bypass of damage

Kinetic studies, done by Bollum and Setlow (4) on an in vitro DNA polymerase system, using as a primer UV irradiated calf thymus DNA, have been interpreted as indicating that slow polymerization around UV-induced lesions is possible. In such a process, "wrong" bases may be incorporated into DNA. The polymerase product was deficient in ApA

sequences as would be expected if there were noncomplementary incorporation opposite to $\overset{\wedge}{TT}$ dimers.

(3) Removal of damage - dark repair

A theory for the mechanism of enzymatic repair of DNA based mainly on demonstration of removal of damaged regions was proposed by Boyce and Howard Flanders (7) and Setlow (10).

This theory was tested experimentally and there is accumulating evidence for the existence of the dark repair process.

Repair by excision and replacement of damaged region involves a number of enzymatic steps using enzymes which are available in the cell. The general scheme for this mechanism is shown in Figure 1.

Excision Process

The first step in the repair of affected DNA is the excision of damaged regions. Setlow (7) and Howard Flanders (10) showed that when UV irradiated bacteria are incubated after irradiation, dimers are gradually released and are found in a trichloroacetic (TCA) soluble fraction. The excision of pyrimidine dimers appears to be a part of the repair process required for colony formation, since it occurs in wild type *E. coli* K_{12} but not in mutant strains sensitive to irradiation.

Degradation of DNA

During the period that thymine dimers become acid

Figure 1: Postulated steps in the enzymatic dark repair hypothesis

(1) intact double stranded DNA

(2) modified region in the DNA (•)

(3) opening of phosphate ester bond by enzyme A (endonuclease)

(4) exonuclease action which cut several nucleotides around the lesion

(5) polymerase mediated DNA synthesis starting at the 3'OH end

(6) closing of the final 3'OH - 5'P link by enzyme D (Ligase or polynucleotide joining enzyme)

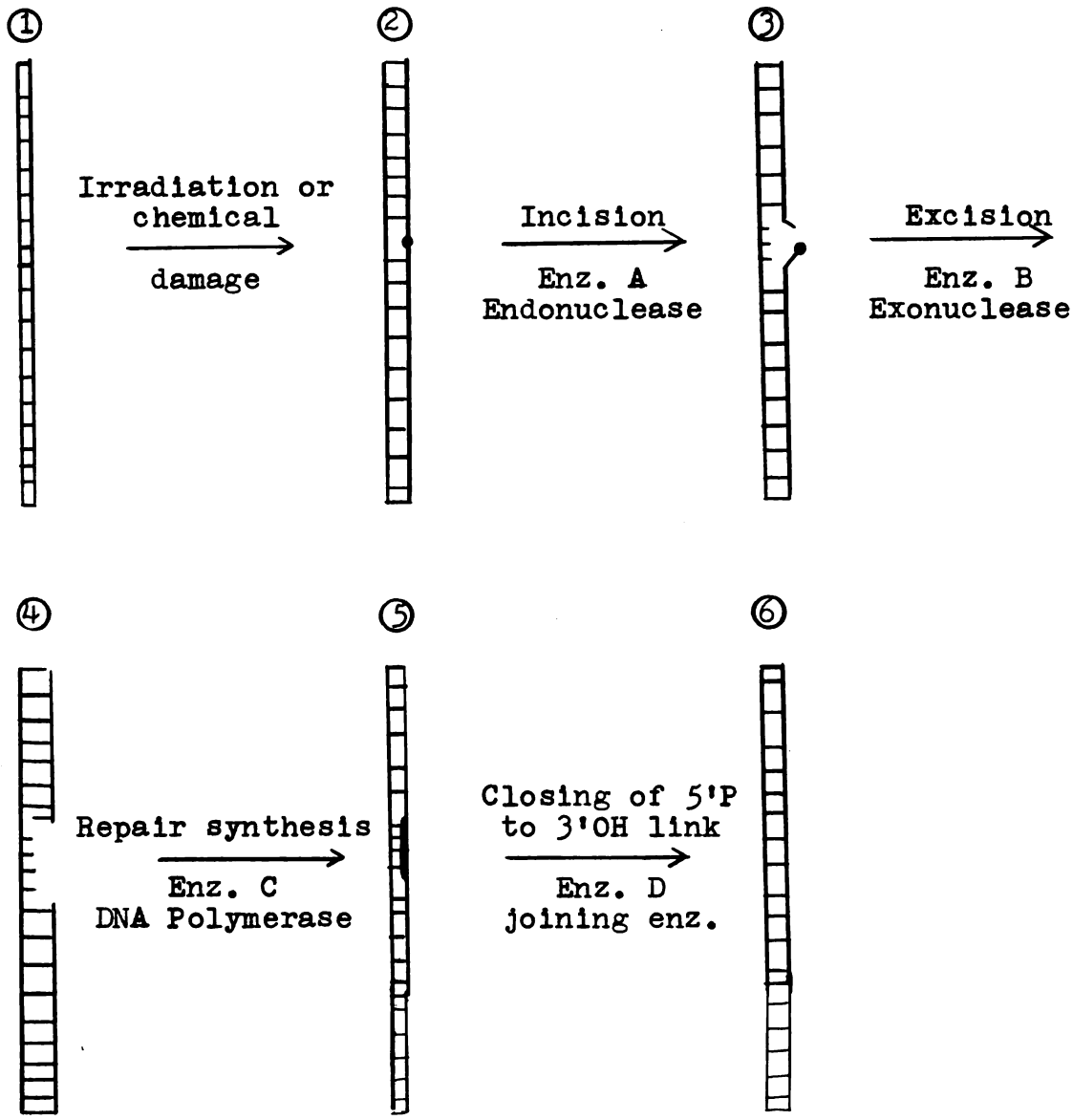
①
②
③
Irradiation or chemical
damage
Incision
Enz. A
Endonuclease
Excision
Enz. B
Exonuclease
④
⑤
⑥
Repair synthesis
Enz. C
DNA Polymerase
Closing of 5'P to 3'OH link
Enz. D
joining enz.

Figure 1

soluble, there is also a substantial release of nucleotides from the DNA to the medium (7, 10). This degradation may reflect the action of exonuclease on one or both free ends of single strands cut during excision.

DNA Repair Synthesis

Any material that is removed in local single-strand breakdown must be replaced by insertion of nucleotides complementary to those of the intact opposite strand by a DNA polymerase. Evidence for repair replication, incorporation of nucleotides into parts of the chromosome other than the normal growing point following UV irradiation was obtained by Pettijohn and Hanawalt (19).

When E. coli is grown with 5-bromo uracil (Bu) after irradiation, the 5-Bu - analog of thymine - is incorporated into the DNA at a number of sites along the molecule, possibly in the repair areas. Heat denaturing following by density gradient centrifugation does not lead to separation of normal and Bu-containing strands, as occurs in the DNA from unirradiated cells. Thus the Bu containing zones appear to be joined into DNA by heat stable bonds.

The same type of replication was shown by Brunk and Hanawalt (20) in DNA from eucaryotic cell damaged by UV or x-rays. This was also confirmed by . Rupp and Howard Flanders (21).

Formation of Phosphodiester Bonds

The final stage in the repair process is the rejoin-

ing of the sugar phosphate backbone. Recently, a few enzymes capable of repairing single strand breaks, which occured in double stranded DNA molecule, were reported by Weiss and Richardson (22), Gefter et al. (23), Olivera and Lehman (24) and Zimmerman et al. (29). The joining enzyme forms a phosphodiester linkage between adjacent 5' phosphoryl and 3' hydroxyl groups.

Bautz (26) reported a biological study that the single strand damages which occured in phage T_4 were repaired by polynucleotide Ligase. J. Tukagi et al. (27) report the repair of single strand breaks in transforming DNA of B. Subtilis by the above enzyme. The breaks were induced by two nucleases.

McGrath and Williams (46) showed repair occuring in E. coli strain B/r (resistant) after x-ray treatment. X-rays induce single strand breaks which were detected upon denaturing DNA in alkaline sucrose gradients. These breaks disappeared upon incubation of the resistant cells after treatment.

Freifelder (28) observed double and single strand breaks produced in bacteriophage T_7 by x-rays. He concluded that the single strand breaks are not lethal to T_7 because, presumably, they are repaired.

Chemical Damage

Chemicals, which are mutagens, act by forming abnormal products in DNA, some of which appear to be subject to repair.

Brooks and Lawley (29) investigated the reaction of mono and difunctional alkylating agents with nucleic acids. Mono functional alkylating agents are chemicals with one reactive center which thus attach to one residue of the DNA. Difunctional alkylating agents possess two reactive centers and thus can react simultaneously with two residues of the DNA. The bifunctional agents generally exert a markedly more cytotoxic action than the corresponding monofunctional agents. The bifunctional chemicals, by binding two residues in the DNA, usually on opposite strands, cross-link the DNA. The cross linkage prevents the two strands of the DNA to separate upon heat denaturing and rapid cooling which is termed "renaturing."

Bifunctional alkylating agents, such as nitrogen mustard (di-2-chloroethyl-methylamine), and sulfur mustard (di-2-chloroethyl-sulfide), act on DNA mainly by alkylating guanine at the N_7 position, thus, a mono and diguanyl product are formed. One such possible linkage is shown diagrammatically in Figure 2.

Two guanine residues, in order to be crossed linked by an alkylating agent of 4 or 5 atoms, must be situated 8Å apart. According to the Watson-Crick model of DNA the N_7 of two guanines will be stereochemically available to cross

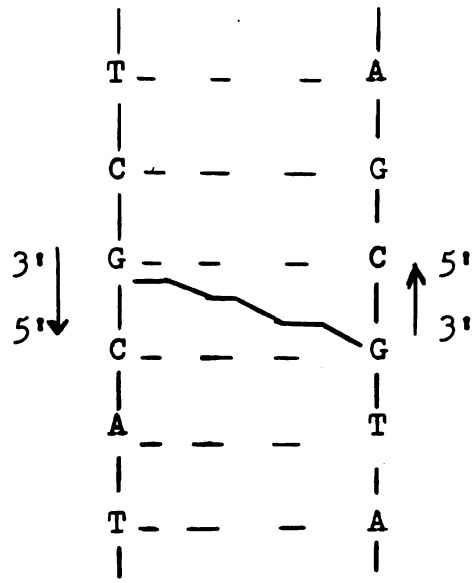

Figure 2

linkage if the sequence of bases in one strand will be guanine cytosine and on the antiparallel strand cytosine guanine.

Linkage of adjacent guanine moieties on the same DNA strand cannot be eliminated on steric grounds but would require the alkyl chain to assume a less probable non-extended configuration.

There is indirect evidence that alkylated bases are released from DNA in bacteria (Kohn et al., 30). They treated bacteria with nitrogen mustard. They found that DNA extracted from treated E. coli B/s_1 strain, maintains its ability to renature after heating during extended incubation. In contrast, DNA from E. coli B cells, upon the same conditions, loses the renaturing ability after 90 minutes. Upon incubation after treatment, the cross linked DNA of E. coli B strain becomes converted to its normal denaturable form. This change in reversibility of denaturation can be explained as an indication of removal or breakage of the cross links from E. coli B.

Lawley and Brooks (31) provided further evidence for excision of bifunctional mustard damage from DNA. Using S^{35}-sulphur mustard they showed that about 50% of the radioactivity was released into the medium from E. coli resistant cells, during incubation after treatment.

E. coli sensitive strains showed release of only about 10% of the radioactivity during incubation. By chromatography it was shown that diguanyl derivatives appear

to be released preferentially, although there is some loss of the monoguanyl products.

Howard Flanders (32) showed that UV sensitive mutants are also more sensitive to killing by nitrous acid than the wild type strain, as judged by colony forming ability.

Nitrous acid is an oxidizing and cross linking agent, (Geiduschek, 33 and Becker et al., 34), but it is not known whether the defects induced by it are excised.

Reiter and Strauss (35) showed repair in cells treated with the monoalkylating agent methyl methane sulfonate (MMS). They found that the activity of transforming DNA, extracted from MMS-treated B. subtilis cells, increases if the cells are incubated after treatment before extraction. This may imply that DNA containing bases alkylated by MMS is repaired during incubation.

Hanawalt and Haynes (36) showed that nitrogen mustard induces the same non-conservative mode of DNA replication, i.e. repair replication, as found in E. coli UV resistant cells (19), after treatment with UV irradiation.

Mitomycin

The mitomycins form a group of closely related bactericidal and cytotoxic antibiotics produced by several Streptomyces species and have the general formula shown in Figure 3, where R_1, R_2, R_3 are different substituents specific for each of the mitomycins, A, B, and C. Mitomycins are generally non-reactive in the natural oxidized state. Upon chemical or enzymatic reduction, followed by spontaneous loss of tertiary methoxy (hydroxyl) group and formation of an aromatic indole system, they become bifunctional alkylating agents, (Iyer and Szybalski, 37) which cross link DNA *in vivo* and *in vitro*. The cross-linking reaction requires at least two reactive sites on the mitomycin molecule. Upon reduction and spontaneous loss of a 9a methoxy or hydroxyl group, position X (Figure 3) becomes an active site of alkylating position. A second alkylating center is at position Y which is highly reactive towards nucleophilic substitution. Although two reactive sites are sufficient to explain the bifunctional cross-linking activity of the mitomycins, it is difficult to exclude the possibility of a third reactive site Z.

Shiba (38) reported the selective action of mitomycin-C (MC) on DNA. The preferential inhibition of bacterial DNA synthesis by MC, accompanied by progressive breakdown of DNA, indicates that DNA is the principal target. Iyer and Szybalski (39) looked for the molecular mechanism of MC action and showed that MC links the complementary DNA strands.

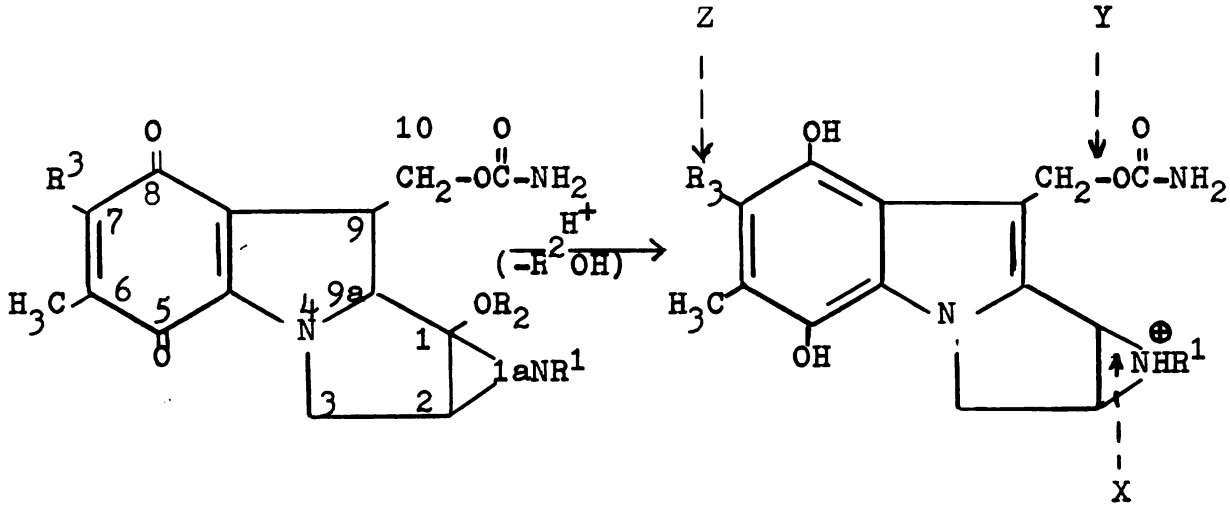

Figure 3

General Formula of the Mitomycins

Native DNA, extracted from MC-exposed cells and examined by equilibrium density-gradient centrifugation, was indistinguishable from DNA extracted from unexposed cells. However, when the DNA's from control and MC-exposed cells were heat-denatured and rapidly cooled, a significant difference in the banding pattern in C_sCl and in Cs_2SO_4 gradients was revealed. Only a small fraction of the denatured DNA from MC-exposed cells banded in position normally occupied by the denatured normal DNA, while the main band formed at the density characteristic for the native or renatured state. Such behavior by analogy to nitrous acid (33) is interpreted as thermostabe linking of complementary strands. By measuring the fraction of DNA rendered non denaturable and assuming a random distribution of cross-links, one finds that the average frequency of MC-induced cross-links corresponds to one per 20,000 nucleotides pair, i.e. one cross-link per molecular weight of 12×10^6 (40).

Weissbach (41), by using (H^3)MC showed that *in vitro* up to one molecule of antibiotic can be attached per 500 nucleotides.

Information about the sites of mitomycin attachment on the DNA is still far from complete. Earlier evidence of indirect nature suggested that the guanine, cytosine or both moieties of DNA might preferentially react with MC, since the degree of cross-linking increases with increasing guanine and cytosine (G + C) content. Thus a higher degree of cross-linking was observed with *S. lutea* DNA (71% [G + C])

than with C. johnsonii (33% [G + C]) when both were treated simultaneously with reduced MC. Similar observations have been made with other DNA's of mammalian and viral origin (40) having various [G + C] contents.

The experiment of Iyer and Szybalski, (37) demonstrating in vitro reaction between nucleic acid and activated (reduced) MC, have prompted several attempts to measure directly the binding of radioactive mitomycins to DNA. The reaction between 20 μg labeled MC and 200 μg of purified B. subtilis DNA showed one mitomycin molecule bound per 2500 nucleotide pairs, i.e. per 1.5×10^6 molecular weight units. These results show directly that MC can be covalently bound to DNA with only 5-10 antibiotic molecules participating in the cross-link and the other molecules reacting with one strand only.

Lipset and Weissbach (45) have further shown preferential reaction with guanine residues, although the reaction with other bases is not negligible.

By experiments with space filling models of DNA and mitomycin (40), the cross linking involving site Y and X of the antibiotic limits the choice of the hypothetical linking sites on the DNA molecule compatible with preservation of the largely undistorted double-helical configuration of the native DNA. Cross-links between N_7 position of the guanines on the opposite DNA strands similar to those postulated for nitrogen or sulfur mustards (Lawley and Brookes, 31) are probable, though with gross distortion.

Boyce and Howard Flanders (42) found that UVR genes in E. coli K-12 control the level of survival after MC treatment and also MC-induced breakdown of DNA. Upon treatment of bacteria labeled with C^{14} thymine with MC they found release of about 30% of total radioactivity into TCA acid soluble fraction in the UVR^{+} strain, whereas 6% release occured in the mutant strains. These observations, they conclude, can be explained by stating that MC-induced lesions are exised from the DNA and the breakdown of DNA is only secondary to the excision.

MATERIAL AND METHODS

Bacteria and Phage Strains

E. coli B130, a thymidineless mutant, was used throughout these experiments. The bacteria were grown at 37° in medium containing per one liter: 1 g NH_4Cl; 0.49 g $MgSO_4 \cdot 7H_2O$; $FeSO_4 \cdot 7H_2O$ 0.5 mg; 3 g KH_2PO_4, 6 g Na_2HPO_4, 0.5% glucose (58). This minimal medium was supplemented with thymidine at concentration of 1 γ/ml.

T_4 phage was used as a DNA marker for alkaline sucrose gradients. *E. coli* B130 strain and the T_4 phage were kindly given by Dr. J. Boezi.

Labeled Thymidine

Methyl H^3 thymidine 5 mC in 5 ml spec. act. 3 C/mmole and C^{14} thymidine 0.25 μc/ml spec. activity 12 mC/ml were purchased from Schwarz Co. Mitomycin C- isolated from Streptomyces Caespitosus in crystalline form was purchased from Sigma Co. In all experiments where MC- was used, it was in concentration of 1 γ/ml which gives 70-75% survival as found by colony counts.

Alkaline Sucrose Gradient

The method used was that described by McGrath and Williams (46).

E. coli B130 cells, growing exponentially, (generation

time 45 minutes) were fully labeled by H^3 thymidine 1 γ/ml. When the cells reached an optical density of 0.5, which is approximately 5 x 10^8 cells/ml. They were divided into three samples. One sample was kept as the control; another sample was treated with MC for 15 minutes and collected immediately; and the third sample was treated with MC for 15 minutes, then collected and resuspended in fresh medium supplemented with unlabeled TdR 1 γ/ml for the various periods as specified in each experiment. The collected cells were washed twice in 0.1M Tris buffer pH 7.5.

Cells were transformed into protoplasts by a modification of the lysozyme-versene method (47). After washing, the cells were suspended in protoplasting medium, containing per liter: 171 g sucrose; 30 ml of 1M Tris pH 8. Lysozyme at conc of 0.1 mg/ml was added and the cells were swirled gently for two minutes. Then 0.2 ml 1% EDTA per ml of bacteria was added. In this way protoplasting is completed in 10 minutes.

The cells are lysed by slowly pipetting them into 0.1 ml of 0.5M NaOH which had been layered on top of a 4.8 ml 5%-20% alkaline sucrose gradient - in 0.1M NaOH 0.9M NaCl adjusted to pH 12.3 by NaOH. Approximately 5 x 10^6 protoplasts were placed on the gradient. Gradients were centrifuged at 30,000 rpm for 90 minutes at 20°C in a SW-39 swinging-bucket rotor on a Spinco model L2-50 centrifuge. The nitocellulose tubes were pierced with a hypodermic needle, No. 26, which is supported by a teflon tubing. The teflon

tubing was mounted on the hypodermic needle in order to ensure a reproducible pore size and to prevent gradient disturbance which might be caused by inserting the needle too high. One drop fractions were collected on Whatman 3MM filter paper discs 2.3 cm diameter, which were then washed three times in 5% trichloacetic acid in order to remove acid soluble material, washing once with 95% ethanol in order to remove TCA and with ether to facilitate drying (48). The dried discs were counted in toluene - BBOT - 4 g per liter (2,5 bis [2(5-tert-Butylbenzo x azolyl)] thiophene scintillation fluid in Packard Tricarb Scintillation counter.

Cosedimentation of Alkaline Sucrose Gradients of E. coli Cells and T_4

The same procedures as above were followed but 0.1 ml of T_4 bacteriophage (approximately 5000 cpm) were layered together with the E. coli protoplasts on top of the 0.1 ml of 0.5M NaOH on the sucrose gradient. Discrimination between the two isotopes was done at the setting:

for C^{14} windows from 200-1000, gain 16%

for H^3 windows from 30-200, gain 60%

Less than 10% of C^{14} counts were found in the H^3 channel.

Preparation of ^{14}C-TdR Labeled T_4 Marker

Labeled T_4 phage, used as a marker for alkaline sucrose gradients, were prepared by modification of the procedure of T. Kano-Sueoka (49).

Four hundred ml of E. coli B130 were grown in medium

supplemented with 1 γ/ml TdR, to O.D. 0.4. Ten minutes before the infection 100 μg/ml of D-L tryptophan was added. Tryptophan is needed for better adsorption of the phage to the bacteria. The culture was infected by T_4 at multiplicity of 0.1. At the time of infection, C^{14} TdR 0.25 μc/ml was added and the culture shaken until lysis.

When the lysis was completed, the cell debris was spun down at 6000 rpm for 15 minutes. The supernatnat fraction was passed through kieselguhr (40 g/l of supernatant) and then through a millipore filter (pore size 0.45 μ). Phage was collected by centrifugation at 22000 g for 1 hour and then suspended in buffered salt solution: 2 g NH_4Cl; 5 g NaCl; 0.37 g KCl; 0.01 g $MgCl_2 \cdot 6H_2O$; 0.026 g Na_2SO_4; 0.09 g Na_2HPO_4; and 0.046 g KH_2PO_4 in 1 liter of 0.1M Tris-HCl buffer pH 7.3. The phage suspension was then treated with DNase and RNase (5 μg/ml each), and the phage was spun down again at 22000 g for 1 hour. The buffered salt solution was poured onto the phage pellet and kept in cold for at least 24 hours before resuspending the phage.

In order to prevent aggregation of phage due to divalent cations, 10 mM of EDTA was added to buffered salt solution.

The specific activity of T_4 bacteriophage was 1×10^5 cpm per unit of optical density at 260 mμ.

Preparations of ^{3}H-TdR-Labeled DNA

One liter of E. coli B130 was grown in 37°C in medium supplemented with TdR: 0.919 mg of unlabeled TdR, and 1 ml

of H^3 TdR (equal to 81 μg). Cells were grown to O.D. 1.9 then were collected by centrifugation and suspended at 15 ml/g cells in .01M Tris-HCl pH 8.0 buffer containing 0.2M sucrose. This suspension was brought to 2% of sodium dodecyl sulfate (SDS) and the suspension shaken until lysis occured. An equal volume of buffered phenol is added and the suspension shaken gently for 15 minutes. The emulsion is centrifuged at 10000 rpm for 10 minutes to separate the aqueous phase from the phenol phase. The aqueous solution is collected and the phenol extraction was repeated again.

The supernatant was collected and 5M NaCl was added to a final concentration of 1M NaCl. An equal volume of 95% ethanol was layered over the solution and the DNA was collected by winding on a glass stirring rod. The collected DNA was dissolved in 1 ml of 0.01M NaCl and treated with RNase 0.02 mg/ml and then a phenol extraction as described above. The collected DNA was then dissolved in 1 ml of 0.01M NaCl and dialysed against .01M NaCl. The DNA thus isolated contained 30000 cpm/γ DNA.

Endonuclease Studies

E. coli cells were grown as before to O.D. 0.5 at 37°C and then were divided into 3 samples of 100 ml. One sample served as control, the other two samples were treated with MC 1 γ/ml for 15 minutes. The second sample was collected immediately after treatment and the third sample was resuspended in fresh medium for 10 minutes.

Preparation of cell extracts for source of enzyme was a modification of the method of I.R. Lehman (50).

The collected cells from 100 ml were suspended in 2 ml of potassium phosphate buffer 0.15N pH 7. The cells, kept in ice, were sonicated 4 times for 15 seconds 2 ampers. The sonicate was then centrifuged at 15000 g for 10 minutes and the supernatant was collected. Before assaying for endonuclease activity, 50 µg of pancreatic RNase was added for about an hour to destroy the inhibition due to RNA present in the crude extract (50).

Assay of Endonuclease

Endonuclease activity was assayed according to E.P. Geiduschek and A. Daniels (51). . This assay, using nitrocellulose filter, is very sensitive to the endonuclease assay since the filter retains only relatively large polynucleotide chains which appear upon denaturing DNA.

^{3}H DNA was incubated in a reaction mixture of total volume of 0.3 ml, containing Tris 1M pH 7.5, 0.1M $MgCl_2$, bovine serum albumin (important for retention on the filter) and various amounts of cell extracts. The mixture was incubated in a 37°C water bath. Aliquots were removed at 0', 5', 10', 20', 25' and 30 minute intervals and were diluted to 1 ml in solvent of low ionic strength containing sufficient EDTA to bind the Mg^{++} in the reaction mixture. This solvent contains NaH_2PO_4 0.004M; Na_2HPO_4 0.007M and Na_2H_2 EDTA 0.001M. The diluted aliquot was heated 5 minutes at 100°C and quickly cooled in an ice bath. It was then diluted

to 15 ml in solvent containing 0.5M KCl and 0.01M Tris buffer pH 7.0. The 15 ml were applied to a nitorcellulose filter previously soaked in the same solvent and washed with 75 ml of the same solvent. The discs were then rinsed with 5 ml of 80% ethanol, dried and counted in BBOT-toluene scintillation liquid. Protein was measured by a method of Lowry et al. (56).

RESULTS

Alkaline Sucrose Gradients

Typical data for *E. coli* B130 strain plotted as cpm against fraction number or distance travelled from the meniscus, are shown in Figures 4-8.

It is clear by comparison with the control experiment (Figure 4) that MC treatment caused a decrease in sedimentation rate. The decrease in sedimentation is not significantly observed at 0' (Figure 5) but is shown very clearly after 10' (Figure 6) of growth in fresh medium. Upon further incubation in fresh medium for 30 minutes (Figure 7) and 45 minutes (Figure 8), the sedimentation rates increase and are again comparable to that of the control - untreated sample.

The decrease in the S value after about 10 minutes and the subsequent increase of S value up to 45 minutes can be attributed to the repair process.

Estimations of molecular weights and sedimentation coefficients of the various DNA samples were done by cosedimentation of cell lysates with the DNA of T_4 phage as a marker.

Quantitative analyses are based on the distance travelled by each DNA species from the meniscus.

Figures 4-8: Alkaline sucrose gradient profile of H^3-DNA of *E. coli* B130 cosedimented with T_4 marker.

Fig. 4. control - untreated

Fig. 5. MC 1 γ/ml, no incubation

Fig. 6. MC 1 γ/ml, 10 minutes incubation

Fig. 7. MC 1 γ/ml, 30 minutes incubation

Fig. 8. MC 1 γ/ml, 45 minutes incubation

d - distance travelled from the meniscus

f - fraction no.

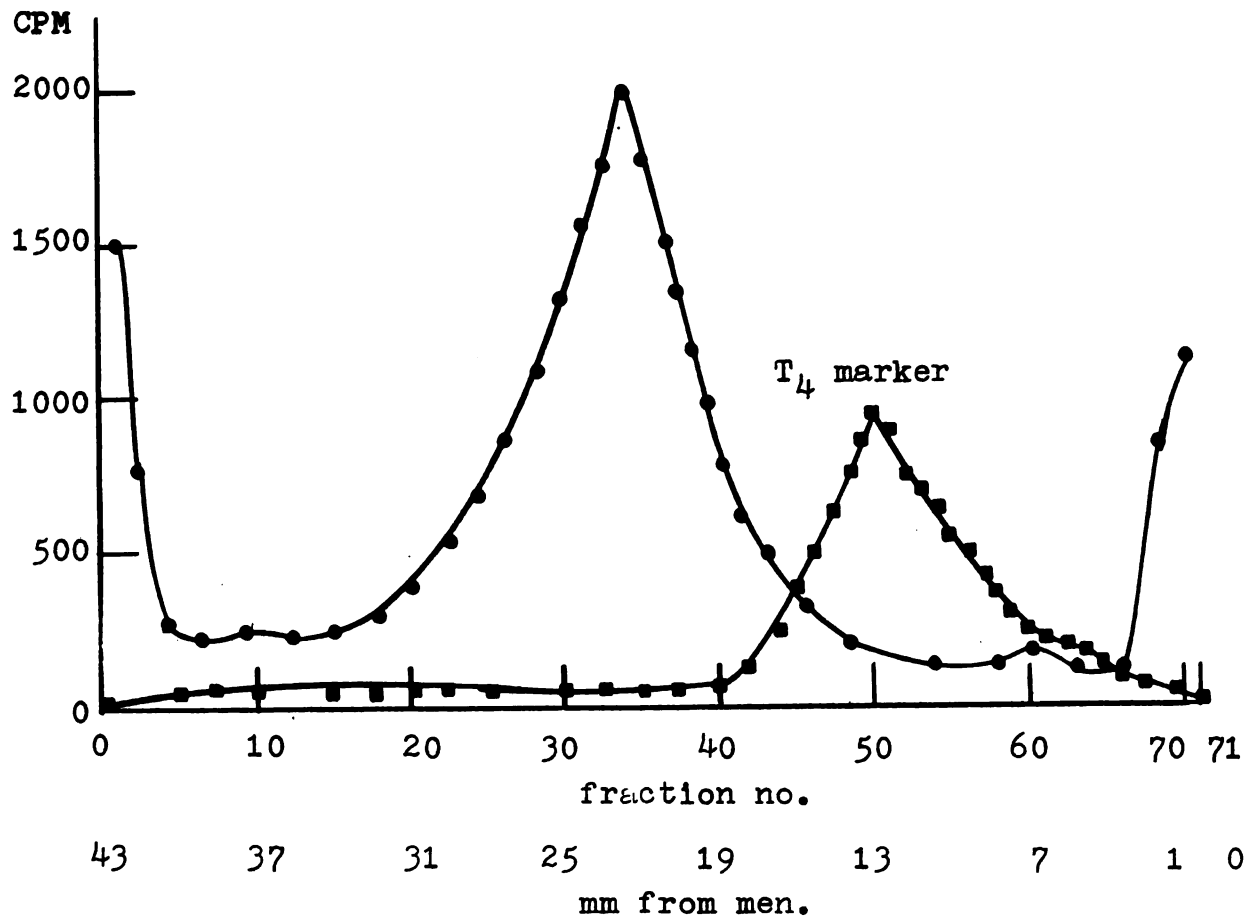

Figure 4: Control

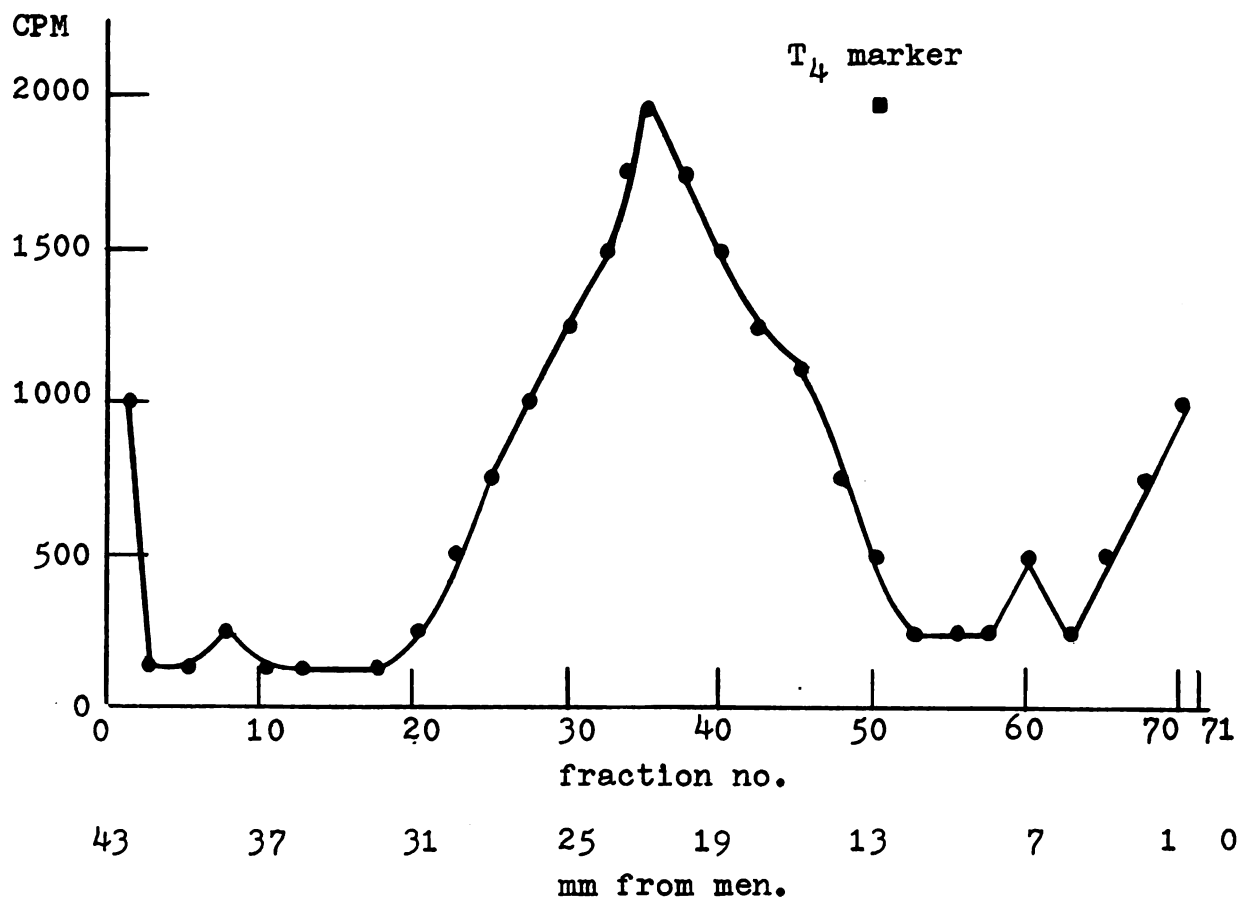

Figure 5: Zero time

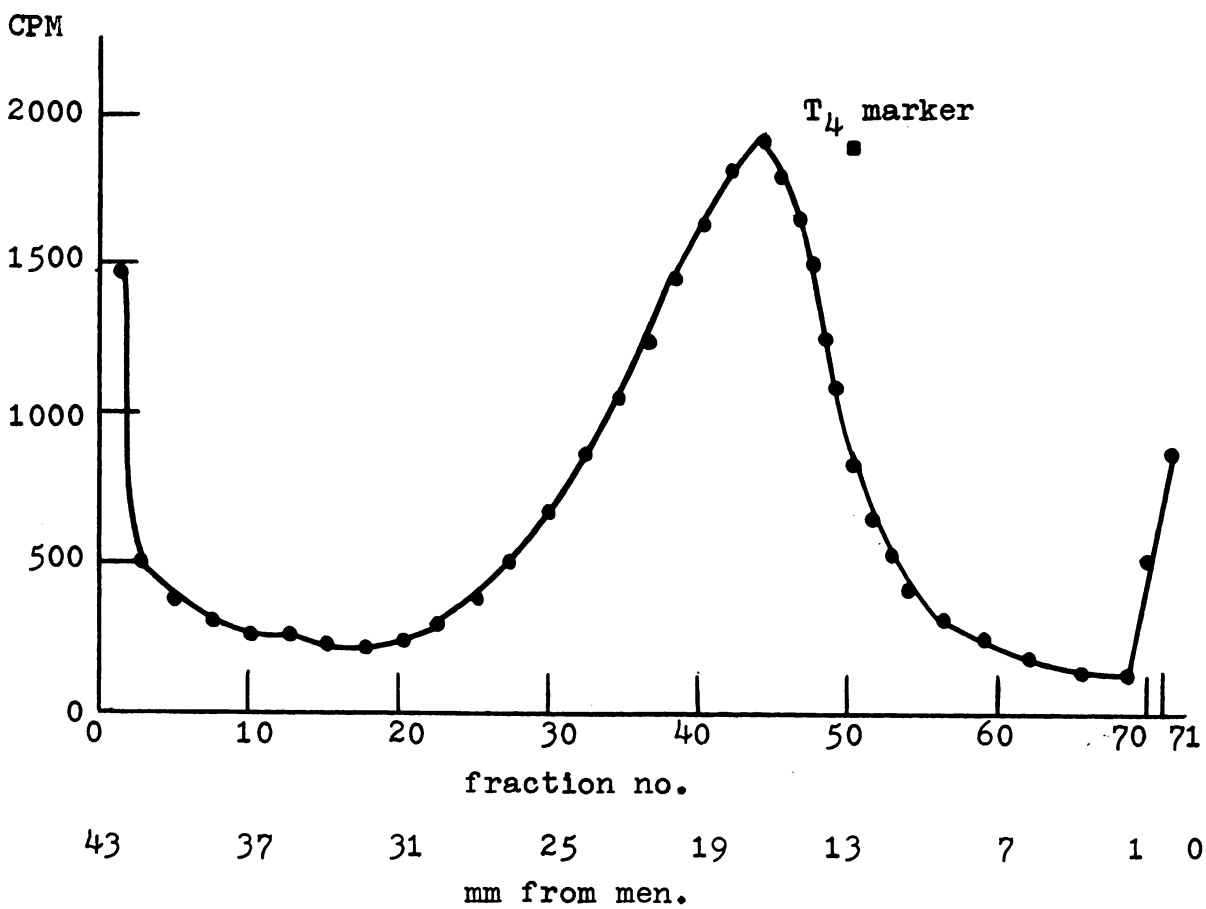

Figure 6: 10 Minutes

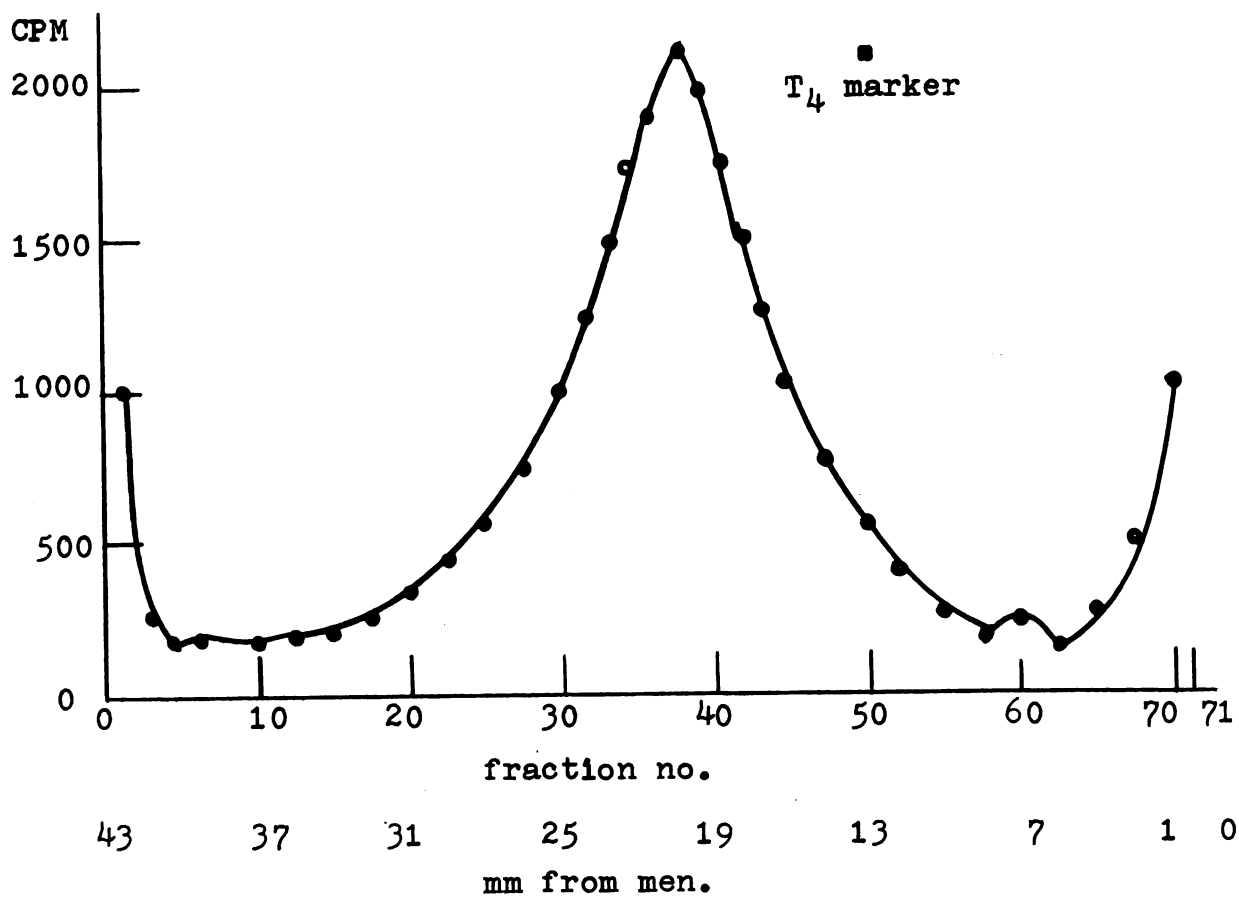

Figure 7: 30 Minutes

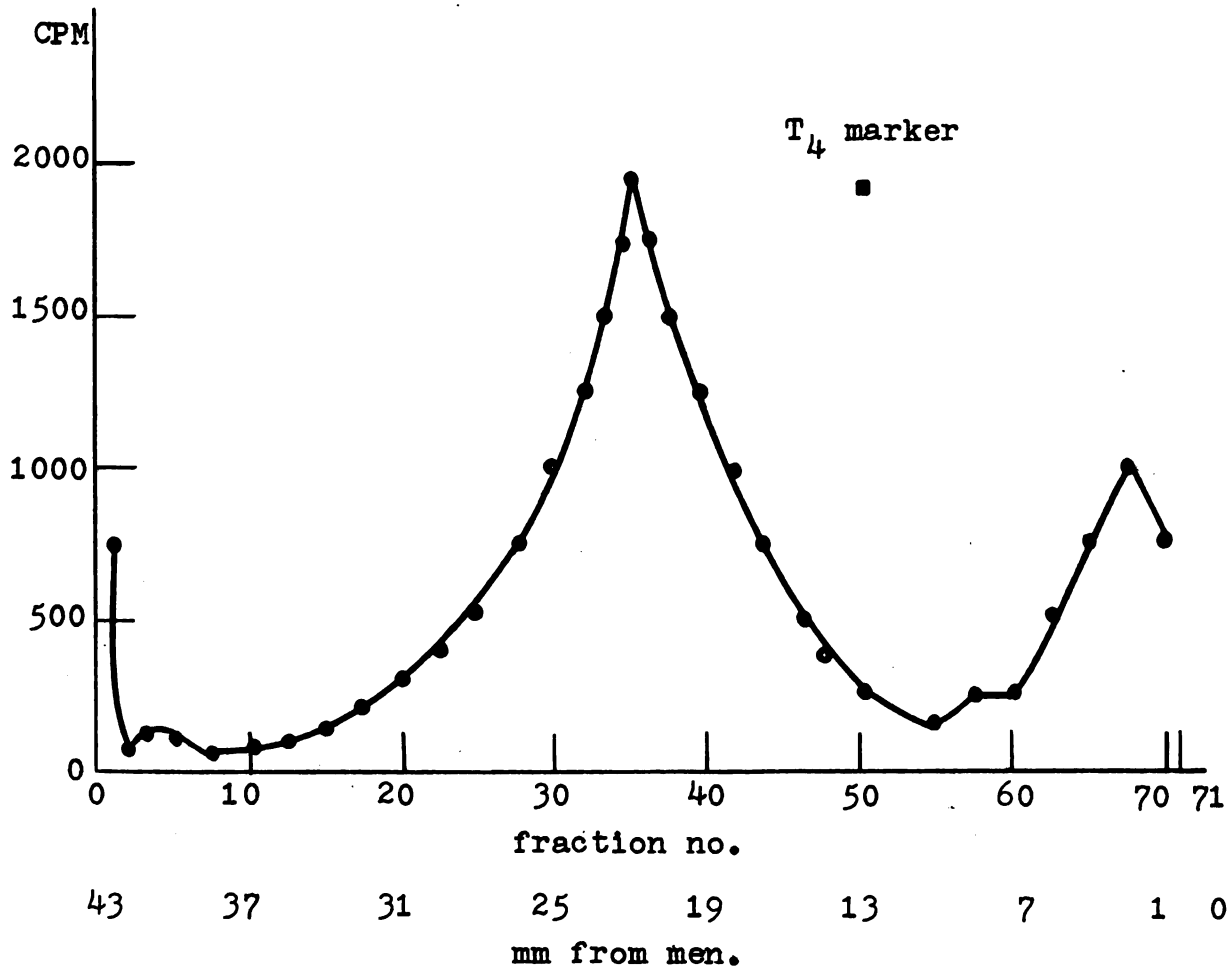

Figure 8: 45 Minutes

Abelson and Thomas (52) derived the following relation:

$$\frac{D}{D_{T_4}} = \left(\frac{M}{M_{T_4}}\right)^{0.38}$$

where D_{T_4} is the distance travelled by T_4 DNA and D the DNA in question, and M and M_{T_4} the corresponding molecular weights when 0.38 is an empirical constant.

This relation is equivalent to:

$$\frac{S}{S_{T_4}} = \frac{D}{D_{T_4}}$$

derived by Burgi and Hershey (53), where S and S_{T_4} are the corresponding sedimentation coefficient values. This is based on the empirical relation proposed by Dotty, McGill and Rice (54):

$$S = aM^k$$

where a and k are empirically derived constants, when the experiments were done on λ phage and half λ phage.

The T_4 marker used in this experiment has MW of 130 x 10^6 daltons according to Rubinstein, Thomas Jr. and Hershey (55). Thus a single strand has a molecular weight of 65 x 10^6 daltons. According to the above authors, the S value for T_4 should be calculated with the constants:

$$S = 0.0244\ M^{0.543}$$

which gives a value of 62.12 for the whole molecule and a value of 42.7S for the half molecule - one polynucleotide chain.

According to the above relations, the MW and S values for the DNA of E. coli cells at different times were

calculated and given in Table 1. This data were reproducible for all the experiments carried out and present an average picture.

TABLE 1

		D/D_{T_4}	MW	S'
Control	Fig. 1	1.73	2.7×10^8	72.8
0'	Fig. 2	1.69	2.58×10^8	72.16
10'	Fig. 3	1.50	1.88×10^8	64.05
30'	Fig. 4	1.55	1.96×10^8	66.18
45'	Fig. 5	1.69	2.58×10^8	72.16

Endonuclease Studies

The results, plotted as cpm/mg protein against time of reaction, are given in Figure 9 and Table 2. These are the average results of the experiments carried out.

The assay is not necessarily linear with time. The time required to reach a certain reduction in counts retained on the filter could be considered as an approximate inverse measure of activity. By this criterion the treated cells which were suspended for 10 minutes in fresh medium show an increase in endonuclease activity of about 50% over the control and the zero time samples. After 5 minutes of incubation, the samples, incubated with lysates of MC treated and suspended cells, show about 50% of the initial counts while the two other samples reached this point only after 10 minutes.

Figure 9: Endonuclease activity in three samples: Control, treated sample - time zero - and treated sample incubated 10 minutes in fresh medium.

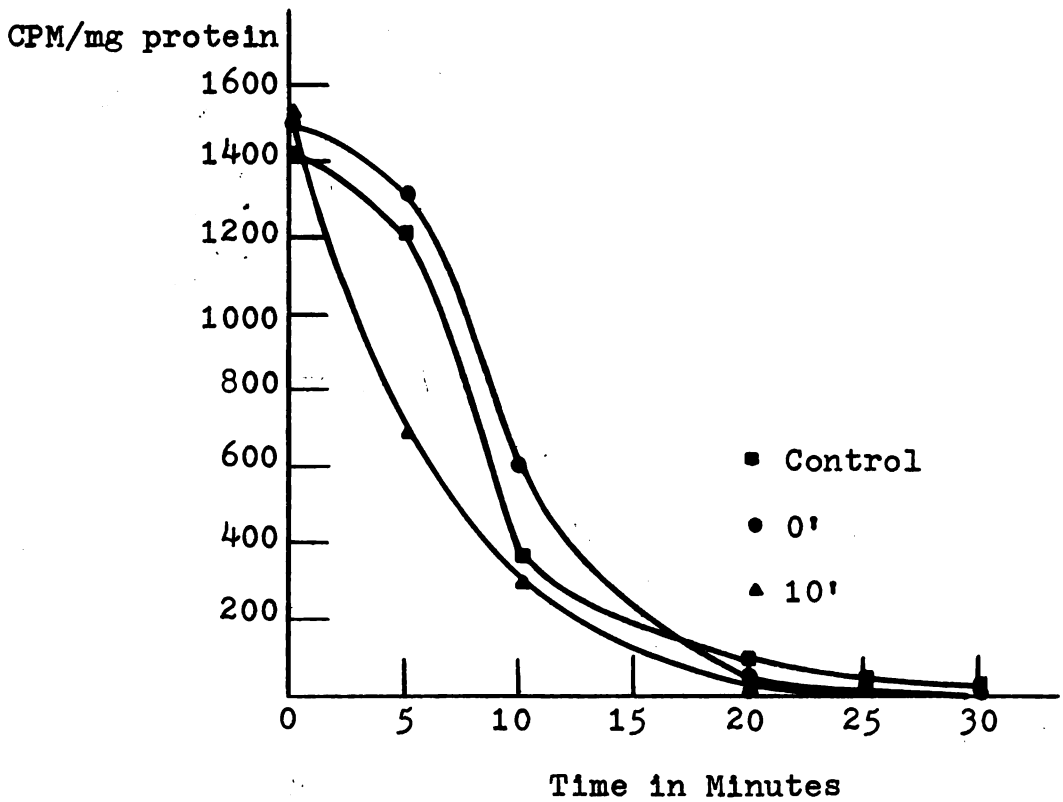
CPM/mg protein
1600
1400
1200
1000
800
600
400
200
0
5
10
15
20
25
30
Time in Minutes
Control
0'
10'

Figure 9

TABLE 2

Endonuclease Activity

Time	Control	MC 1 γ/ml 0'	MC 1 γ/ml 10' in fresh med
0'	1632	1500	1502
5'	1229	1335	683
10'	367	600	332
20'	98	48	83
25'	55	40	63
30'	16	30	30

DISCUSSION

As pointed out by Setlow (43) "The experimental observation of any one of these steps (dark repair mechanism) is often taken as an indication of the existence of a repair mechanism. This may not be justified, as is seen from a brief consideration of what is known about these steps."

This was the prevailing idea during this study. The work reported here tried to demonstrate more comprehensive evidence for the repair process. The dark repair mechanism, involving excision, degradation, repair replication and joining steps, appears to be common for damage caused by UV radiation, x-rays and chemical agents.

MC which can either cross-link DNA (39) or react with a single base and thus inhibit DNA synthesis (38) must be removed before the cells are able to resume normal growth and division.

As was shown in my studies with cells lysed on top of an alkaline sucrose gradient, a decrease in sedimentation value was observed after 10 minutes of incubation in fresh medium, and later this S value increases and reaches the control level. Both these changes are attributed to the overall repair process. Presumably, the damage is removed by the excision and degradation steps - thus a decrease in S value is observed and subsequently it is postulated that as the

repair continues through repair replication and rejoining of the phosphate diester bond, the S value reaches the normal value.

This is also in accordance with the studies on endonuclease activity. The elevated activity was shown after about 10 minutes which is the time period when a decreased S value appeared in the alkaline sucrose gradient.

In contrast to the work with x-ray damaged cells (46) where the decrease in sedimentation is demonstrated at time - zero, since the single strand breaks are a direct effect of the damage, MC-induced single strand breaks appear only after about 10 minutes of incubation in fresh medium. This observation can be explained by the first step in the postulated repair process according to the scheme in Figure 1.

Howard Flanders et al. (42) showed release of radioactivity in UV resistant E. coli K_{12} cells treated with MC. He showed a release of 30% of the DNA's radioactivity into the medium which is three times more than the amount shown by Setlow (10) and Boyce et al. (7) after cells were treated with UV. This release was explained by him as part of their repair process - the degradation following excision. There are, however, some points in his work which are not in agreement with current knowledge. First, this 30% release is much too high to be expected from DNA degradation due to repair. Secondly, this work was done with high concentration of MC (5 μg/ml) which gave only 30% survival. Because of the low survival at this high concentration and long treatment, it

can be inferred that there was either an inhibition of the repair process or secondary effects of the MC treatment. The high release of label from the DNA might be explained as a secondary effect due to degradation in the 70% of dead cells, similar to the effect which was observed first by Reich et al. (44). The inhibitory concentration of MC for variety of strains, as determined by gradient plate technique by Szybalski (45), was found as 0.5 μg/ml for E. coli K_{12} which is far from the 5 μg/ml used in the above studies.

The cross-links are presumably formed between two guanine residues in the opposite strand in adjacent base pairs, similar to those formed by nitrogen mustard (29) and by MC (40, 41). The problem of repair replication thus arises.

In UV damaged cells, the thymine dimers are released leaving intact complementary strand which may serve as template for repair replication. The same mechanism can be explained for cross-links if one strand segment containing one end of the cross-link is excised, allowing it to swing out and permit replacement of that segment by new DNA. Only then, can the other attachment point on the other side be excised (30).

Thus, with the observations of increased nuclease activity and decreased sedimentation rates from DNA in MC-treated cells, it is concluded that a repair of interstrand cross-links occurs, without the loss of the base pair sequence of the genome, by a multistage process, probably involving excision and DNA repair replication.

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