Alkylation of Nucleic Acids by Mitomycin C and Porfiromycin*

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ABSTRACT: [^{3}H]Mitomycin C and [^{14}C]porfiromycin, after reduction, are shown to attach directly to DNA *in vitro*. Up to one antibiotic molecule per 500 nucleotide residues can be found. RNA and ribosomes are also alkylated by porfiromycin to the same extent as is DNA. Bovine plasma albumin, starch, and glycogen are alkylated to a lesser degree than the nucleic acids under the experimental conditions used.

Mitomycin C is known to cause a rapid cessation of DNA synthesis in bacteria while apparently not affecting directly either RNA or protein synthesis (Shiba *et al.*, 1959). This antibiotic is also an excellent lysogenic inducer of *Escherichia coli* K12λ (Otsuji *et al.*, 1959; Korn and Weissbach, 1962). In addition, the closely related antibiotics, mitomycin A, mitomycin B, and porfiromycin (Webb *et al.*, 1962) are also capable of lysogenically inducing *E. coli* K12λ (A. Lisio and A. Weissbach, unpublished).

Iyer and Szybalski (1963) have previously shown that mitomycin C can cause the formation *in vivo* or *in vitro* of a "cross-linked" DNA which may be the result of alkylation of the DNA by the antibiotic. These workers also showed that the formation of the cross-linked DNA requires the prior reduction of the antibiotic (Iyer and Szybalski, 1964). This reduction can be accomplished enzymatically, *in vivo*, or with chemical reducers such as sodium hydrosulfite *in vitro*.

During investigations on lysogenic induction we prepared [^{3}H]mitomycin C (randomly labeled) and [1a-^{14}C]porfiromycin and studied the direct attachment, *in vitro*, of the antibiotics to nucleic acid. This paper will show that the mitomycin antibiotics do, in fact, alkylate nucleic acids and become fixed to the nucleic acid molecule. *In vitro*, the number of antibiotic residues attached to nucleic acids will exceed 1 per 1000 nucleotide units. Protein and polysaccharides are also alkylated by reduced porfiromycin. The following paper (Pricer and Weissbach, 1965) will discuss the ability of various enzymes to utilize or degrade DNA which contains mitomycin residues.

Materials and Methods

Reagents. Mitomycin C was obtained from Kyowa Hakko Kogyo Co., Tokyo. Porfiromycin was obtained from the Lederle Laboratories Div., American Cyanamid Co. DNA was prepared from cells of *E. coli* K12λ by the method of Marmur (1961). Heat-denatured DNA was prepared by heating K12λ DNA, 0.5 mg/ml, in 0.01 M Tris, *p*H 7.4, 0.02 M NaCl for 10 minutes at 100° followed by rapid cooling in ice. Soluble RNA (*E. coli* B) was a product of General Biochemicals. Ribosomes were prepared from *E. coli* K12λ as described by Tissières *et al.* (1959) and stored in 0.01 M Tris, *p*H 7.4, containing 0.01 M $MgCl_2$ or no Mg^{2+}. Ribosomal RNA was prepared by the method of Nirenberg and Matthei (1961). Bovine plasma albumin, fraction V, was a product of the Armour Laboratories. Soluble starch and glycogen were obtained from the Fisher Scientific Co. Pancreatic deoxyribonuclease and ribonuclease were products of the Worthington Biochemical Corp.; pronase was obtained from Calbiochem. Whatman cellulose powder (standard grade) or No. 1 filter paper was used for chromatography. Hyamine hydroxide, 2,5-diphenyloxazole, and 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene were obtained from the Packard Instrument Corp. [^{14}C]Methyl iodide was purchased from the New England Nuclear Corp.

Preparation of [^{3}H]*Mitomycin C.* Mitomycin C (100 mg) was exposed to 15 curies of $^3H_2$ for 1 week at 0°. Water (10 ml) was then added and removed by lyophilization. Part of the residue (40 mg) was dissolved in methanol and placed on a 100-ml cellulose powder column which had been equilibrated with the benzene-methanol-phosphate solvent of Lefemine *et al.* (1962). This solvent (1200 ml) was passed through the column followed by 300 ml of 10% butanol in benzene. The mitomycin was then eluted with 25% methanol. Aliquots of the methanol eluate (total volume 30 ml) were subjected to paper chromatography with 90% methanol as the solvent. The mitomycin band was eluted from the paper with 50% methanol and successively chromatographed on paper with acetone–benzene–0.01 M potassium phosphate buffer, *p*H 7.0(4.5:2:0.5), and then 90% ethanol. The [^{3}H]mitomycin C obtained from the latter chromatogram showed no change in specific activity (0.5 μc/μmole) after rechromatography in 90% methanol or benzene-methanol-phosphate buffer (Lefemine *et al.*, 1962). It had the same ultraviolet and visible absorption spectrum as authentic mitomycin C and possessed the same biological activity for lysogenically inducing *E. coli* K12λ (Korn and Weissbach, 1962).

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Preparation of [1a-^{14}C]*Porfiromycin (Methyl-labeled).* To 35 mg of mitomycin C in 1.0 ml of dimethylformamide was added 14.2 mg of $^{14}CH_3I$ (20 μc/mole) in 1.0 ml of dimethylformamide. After the further addition of 8.4 mg of $NaHCO_3$ in 0.5 ml of 40% dimethylformamide in water, the incubation tube was tightly stoppered and placed in a 40° water bath for 21 hours with occasional shaking. Approximately 50% of the mitomycin C was methylated under these conditions. Aliquots of the incubation mixture were chromatographed on paper with 90% methanol. This solvent clearly separates mitomycin C (R_F = 0.63) and porfiromycin (R_F = 0.75). The porfiromycin band so obtained was eluted from the paper with 50% methanol and rechromatographed with benzene-methanol-phosphate buffer (Lefemine *et al.*, 1962). The porfiromycin showed less than a 10% change in specific activity after the second chromatogram and contained 20 μc of ^{14}C/μmole of porfiromycin (1.07×10^6 cpm/μg at 85% counting efficiency). Both the [^{3}H]mitomycin C and [^{14}C]porfiromycin were dissolved in water to a concentration of 500 μg/ml and stored at −20°.

Alkylation of Substrates by the Mitomycin Antibiotics.[1] Substrates, in 0.5 ml of 0.01 M Tris, *p*H 7.4–0.02 M NaCl, and containing the antibiotic, were subjected to a rapid stream of helium gas for 5 minutes at 25°. Enough 0.01 M sodium hydrosulfite (prepared immediately before use under anaerobic conditions) was then added to the sample to equal 2 molar equivalents of the antibiotic present. Bubbling with helium was continued for 5 more minutes. In all of the experiments reported in this paper, controls containing the substrate and antibiotic, but no sodium hydrosulfite, were performed simultaneously.

Isolation of Products from Alkylation Reactions. DNA. Two volumes of ethanol were added to the sample and the DNA was collected by winding the threads on a glass stirring rod. The DNA was washed in 70% alcohol several times and then dissolved in 0.50 ml of 0.01 M Tris, *p*H 7.4–0.1 M NaCl (Tris-NaCl). The DNA was reprecipitated with two volumes of alcohol, collected on a stirring rod, and dissolved in 1 ml. of Tris-NaCl containing 5 μmoles of $MgCl_2$ and 20 μg of pancreatic deoxyribonuclease. After incubation for 2 hours at 37°, the solution was added to 10 ml of a dioxane-naphthalene counting fluid (Bray, 1960) and counted. The enzymatic digestion of DNA to smaller fragments was performed to prevent precipitation of the high molecular weight DNA in the dioxane-naphthalene counting fluid.

RNA. The precipitate formed by the addition of two volumes of ethanol was collected by centrifugation and redissolved in 0.50 ml of Tris-NaCl. The precipitation with alcohol was repeated twice more. The final precipitate was dissolved in 1 ml of Tris-NaCl and incubated at 37° for 2 hours with 20 μg of pancreatic ribonuclease. The samples were lyophilized to dryness, dissolved in 1 ml of hyamine, and heated at 57° for 3–5 hours. Toluene-PPO-POPOP (10 ml) (Weissbach and Korn, 1964) scintillation fluid was then added and the radioactivity of the sample was determined.

RIBOSOMES. Samples were isolated as described for RNA except that the enzymatic digestions contained 40 μg/ml of pronase in addition to ribonuclease.

BOVINE PLASMA ALBUMIN. Protein was precipitated as described for the RNA isolation. The enzymatic digestions contained pronase (40 μg/ml) instead of ribonuclease.

GLYCOGEN AND STARCH. The polysaccharide was isolated by alcohol precipitation as described for RNA (performed three times) and then dissolved in 0.5 ml of 0.02 M KCl. The samples were put on copper planchets and dried, and radioactivity was determined on an end-window Geiger counter.

RADIOACTIVE DETERMINATIONS. ^{3}H and ^{14}C determinations were carried out in a liquid scintillation spectrometer with counting efficiencies of 20–30% for ^{3}H and 75–85% for ^{14}C. Some ^{14}C determinations were performed with an end-window Geiger counter at an efficiency of 15–20%.

Phosphate was determined by the method of Ames and Dubin (1960).

TABLE I: Alkylation of DNA by Mitomycin C and Porfiromycin.[a]

mμMoles in Incubation		mμMoles of Antibiotic in Isolated DNA	Nucleotides/ Antibiotic
[^{14}C]-Porfiromycin	K12λ-DNA		
14.2	2000	0.217	9200
28.6	2000	0.379	5280
57.2	2000	1.17	1710
85.8	2000	1.42	1410
57.2[b]	2000 (heat-denatured)	0.259	7700
[^{3}H]-Mitomycin C			
57.2	2000	1.12	1785
85.8	2000	1.14	1750
380[c]	1000	2.21	452
721[c]	4000	4.69	854

[a] Incubations were carried out as described under Materials and Methods. DNA was isolated by winding on a stirring rod. Concentration of DNA is expressed in mμmoles of nucleotide phosphorous. [b] Heat-denatured DNA was isolated by centrifugation, after the addition of ethanol, as described for the isolation of RNA in the methods section. [c] These experiments were carried out in 0.15 M NaCl–0.015 M Na citrate.

[1] We are indebted to Drs. Iyer and Szybalski for a copy of their manuscript before publication (Iyer and Szybalski, 1964).

TABLE II: Alkylation of Bell Components by Porfiromycin.[a]

Sample	Amount	mμMoles of Porfiromycin Fixed	Porfiromycin/ Nucleotide
Soluble RNA	3.85 μmoles (33.9 absorbancy units)	3.0	1:1285
Ribosomal RNA	2.76 μmoles (27.6 absorbancy units)	0.493	1:5600
Ribosomes (10^{-2} M Mg^{2+})	990 μg (15 absorbancy units)	0.188	1:7970
Ribosomes (no Mg^{2+})	1650 μg (27 absorbancy units)	0.510	1:5400
Bovine plasma albumin	1000 μg	0.062	
Glycogen	5000 μg	0.076	
Starch	5000 μg	0.160	

[a]The incubation conditions are described under Materials and Methods. In the calculation of the porfiromycin/nucleotide ratios, full recovery of the nucleic acid was assumed.

Results

Alkylation of DNA. Table I shows the *in vitro* alkylation of DNA by reduced porfiromycin or mitomycin C. The number of antibiotic molecules attached to the DNA range from 1 per 9200 nucleotide residues to 1 per 452 nucleotides depending on the concentration of the antibiotic relative to DNA in the alkylation reaction. Full recovery of the DNA was assumed in these calculations. The results with [^{3}H]mitomycin C have been corrected to include a 13% loss of charcoal-absorbable ^{3}H which occurs when the randomly labeled [^{3}H]mitomycin C by itself is reduced.[2] This loss of tritium is presumably caused by elimination of the methoxy group at the 9a position of the mitosane nucleus (Iyer and Szybalski, 1964). In all of the experiments shown in Table I, both the alkylation reaction and DNA isolations were carried out at ambient temperature. The values obtained in Table I (and subsequent experiments reported in this paper) have been corrected for control experiments containing the DNA substrate and the antibiotic but with no reducing agent. Under these latter conditions the amount of antibiotic associated with the isolated DNA was about 1% of the values shown in Table I and probably reflects the inadequacy of washing during the DNA isolation. It is to be noticed that both the [^{14}C]porfiromycin and [^{3}H]mitomycin C-alkylate DNA to the same extent under comparable conditions.

Alkylation of RNA and Ribosomes. Table II shows that RNA and ribosomes are also alkylated by reduced porfiromycin using the same conditions as those employed with DNA in Table I. The extent of alkylation of RNA is about the same as that observed with DNA. Soluble and ribosomal RNA are both alkylated as are ribosomes prepared in the presence or absence of Mg^{2+}. Protein (bovine plasma albumin) and the polysaccharides starch and glycogen are alkylated to a lesser extent on a weight basis than are ribosomes.

Effect of Temperature upon Isolation of Porfiromycin–Nucleic Acid Complexes. While these experiments were in progress, other studies by Dr. Marie Lipsett indicated that s-RNA alkylated with reduced mitomycin C showed spontaneous decomposition (Lipsett and Weissbach, 1965). Evidence that spontaneous decomposition of DNA may occur after alkylation with porfiromycin is shown in Table III. In these experiments DNA was isolated by winding on a stirring rod at room temperature as described in the experimental section and as shown in Table I. In addition, the supernatant fluids from the alcohol precipitations of the experiments shown in Table I were stored at −20° for 3 days. A further precipitate was then noticed and this material was isolated, washed three times with cold ethanol, and examined for radioactivity. The results are shown in Table III, and it is apparent that the precipitate isolated at −20° contains an appreciable amount of porfiromycin. This precipitate is either fragmented DNA or intact DNA missed during the winding procedure since it is insoluble in alcohol, soluble in water, and shows an absorption maxima of 260 mμ in water. The total number of porfiromycin molecules now exceeds 1 per 1000 nucleotide residues of the DNA if the material isolated at −20° is included in the calculations, and if full recovery of the DNA is assumed.

To minimize possible breakdown of nucleic acids after porfiromycin attachment, isolation of the alkylated nucleic acid was carried out at low temperatures. Table IV shows experiments in which alkylation of nucleic acids was performed at 25° (for 5 minutes), and subsequent isolations of the nucleic acids were rapidly carried out within 30 minutes at 0° instead of at room temperature. The recovery of both DNA and porfiromycin was measured. The porfiromycin-DNA complex isolated at 0° now contains 1 porfiromycin residue per 550–860 nucleotides, which is considerably higher than the values shown in Table I where the DNA from similar incubation mixtures was isolated over a period of hours at room temperature. It is worth noting that

[2] Lipsett and Weissbach (1965).

TABLE III: Alkylation of DNA by Porfiromycin.[a]

mμMoles in Incubation		mμMoles of Porfiromycin in DNA			
[^{14}C]-Porfiromycin	DNA	Precipitate at 25°	Precipitate at −20°	Total	Nucleotides/ Porfiromycin
57.2	2000	1.17	0.87	2.04	980:1
85.8	2000	1.42	1.46	2.88	694:1
57.2	2000 (heat-denatured)	0.26[b]	2.87	3.13	640:1

[a] These experiments are also shown, in part, in Table I. The precipitate at 25° refers to DNA isolated by winding on a stirring rod; that at −20° refers to DNA isolated from the supernatant fluids of the 25° precipitate. [b] Heat-denatured DNA was isolated by centrifugation, after the addition of ethanol, as described for the isolation of RNA in the methods section.

TABLE IV: Alkylation of Nucleic Acids by Porfiromycin.[a]

mμMoles in Incubation [^{14}C]-Porfiromycin	Nucleotide	mμMoles in Isolated Product Porfiromycin	Nucleotide	Nucleotide/ Porfiromycin
DNA				
57.2	2000	2.1	1860	860:1
85.8	2000	2.46	1700	690:1
Heated DNA				
57.2	2000	3.54	1950	550:1
Soluble RNA				
57.2	4050	6	3950	660:1

[a] The incubations are described under Materials and Methods. Isolation of the nucleic acids was performed at 0°.

native DNA, heated DNA, and soluble RNA are alkylated to the same extent under these conditions.

Discussion

The ability of reduced porfiromycin or mitomycin C to alkylate DNA, RNA, or ribosomes is clearly shown in these experiments. *In vitro*, at least, both RNA (soluble or ribosomal) and DNA (either heat-denatured or native) are alkylated to about the same extent, and it is easily possible to obtain about one antibiotic per 500 nucleotide residues in the nucleic acid. This is a higher degree of alkylation than would be expected if all the antibiotic residues attached to DNA were involved in "cross-links" in the DNA. Iyer and Szybalski (1963) have estimated less than one "cross-link" per 2000 nucleotide residues. Hence it is probable that the attachment of the antibiotic molecules to DNA for the most part is not across the double helix to form "cross-links." This view is supported by the fact that heat-denatured DNA is alkylated as well as native DNA. It is to be expected, therefore, that the majority of the mitomycin residues attach to nucleic acids by a monofunctional alkylation in which one antibiotic molecule would be attached to a single base. Whether, in fact, the "cross-linking" observed in DNA alkylated by mitomycin C is caused by bridging of the double helix by the mitomycin molecule itself is still unproved.

Alkylation of DNA by mitomycin might be expected to have an influence on the biological or enzymatic activity of the nucleic acid. The following paper examines the behavior of the DNA-mitomycin complex toward several enzymes which utilize DNA as a substrate. Similarly, it might be expected from the data in this paper that in a cell exposed to mitomycin C the RNA or ribosomes would also be alkylated as much as the DNA. This must be considered in evaluating the effects of the mitomycin antibiotics on the intact cell. Though proteins and polysaccharides are apparently alkylated to lesser extent than the nucleic acids, it is nevertheless not to be overlooked. The effect of mitomycin alkylation on the enzymatic activity of proteins remains to be determined.

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Enzymatic Utilization and Degradation of DNA Treated with Mitomycin C or Ultraviolet Light*

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ABSTRACT: *Escherichia coli* K12λ DNA, alkylated with mitomycin C *in vitro*, is utilized one-third to one-fourth as well as native DNA when tested as primer for *E. coli* DNA or RNA polymerase. The alkylated DNA is also degraded at a slower rate than native DNA by three distinct exonucleases obtained from *E. coli:* the λ-exonuclease associated with λ-phage formation, the phosphodiesterase (Exo I) which is specific for single-stranded DNA, and the exonuclease associated with DNA polymerase (Exo II).

However, the RNA-inhibitable endonuclease, obtained from *E. coli*, and pancreatic endonuclease attack alkylated DNA at almost the same rate as native DNA. DNA, irradiated with ultraviolet light, acts the same as alkylated DNA with the aforementioned enzymes.

The ability of reduced mitomycin C to alkylate and attach to nucleic acids (Weissbach and Lisio, 1965) raises the question of how a DNA molecule containing such attached antibiotic residues will react with the various enzymes concerned with nucleic acid metabolism. This question is of particular relevance in view of the findings of Shiba *et al.* (1959) who reported that bacterial cells exposed to mitomycin C cease to make DNA but continue to synthesize RNA and protein.

In this paper DNA alkylated with mitomycin C has been compared to native DNA with the following enzymes: DNA polymerase, RNA polymerase, endodeoxyribonucleases, and exodeoxyribonucleases. We have also compared in these enzyme systems a DNA which had previously been irradiated with ultraviolet light and found it to resemble the mitomycin C-alkylated DNA in its behavior. In general, the alkylated or irradiated DNA is utilized or degraded at a slower rate than normal DNA by the above-mentioned enzymes. However, endonucleases cannot significantly distinguish normal DNA from alkylated or irradiated DNA.

Materials and Methods

DNA was prepared from *Escherichia coli* K12λ cells by the method of Marmur (1961). The preparation of K12λ [^{3}H]DNA has been previously described (Weissbach and Korn, 1963). Mitomycin C was purchased from the Kyowa Hakko Kogyo Co. (Tokyo). Salmon sperm DNA and nucleoside triphosphates were purchased from Calbiochem. [8-^{14}C]ATP was a product of Schwarz Biochemical Corp. and was adjusted, with nonradioactive ATP, to a specific activity of 0.69 μc/μmole. Crystalline pancreatic DNAase was a product of the Worthington Biochemical Corp. DNA polymerase

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