by dry distillation of wood) (*6*). Reports of the analysis of the pyrolysates of cigarette paper (C, H, and O only) have given no evidence of the presence of nitrogen heterocycles (*7*).

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Mitomycins and Porfiromycin: Chemical Mechanism of Activation and Cross-linking of DNA

Abstract. *Mitomycins and porfiromycin, generally nonreactive in the natural oxidized state, behave as bifunctional "alkylating" agents upon chemical or enzymatic reduction, followed by spontaneous loss of the tertiary methoxy (hydroxyl) group and formation of an aromatic indole system. Thus activated, mitomycins and porfiromycin react in vitro with purified DNA, linking its complementary strands. A high content of guanine and cytosine favors this cross-linking reaction, which is the basis of the lethal effect in vivo of these antibiotics. The activation and cross-linking reactions are discussed in terms of reactive sites on the mitomycin and DNA molecules.*

In a paper (*1*) on the mechanism of action of the antibiotic mitomycin C (*2*) evidence was presented that mitomycin in lethal concentrations cross-links the complementary strands of intracellular DNA and that it does not react in vitro with purified DNA unless this reaction mixture is supplemented with a homologous or heterologous cell lysate. The results presented here provide information concerning the chemical nature of mitomycin activation, both in vivo and in vitro. The degree of the resultant cross-linking of DNA was determined from the proportion of spontaneously renaturable DNA molecules under conditions of irreversible denaturation, by the method of equilibrium density-gradient centrifugation in CsCl or Cs_2SO_4 or by biological assay of residual transforming activity. The methods have already been described (*1*).

The mitomycins and porfiromycins (all five forms listed in Fig. 1*A*) are metabolically activated, as demonstrated by cross-linking in vitro of purified native DNA, when 1 volume of a freshly prepared cell lysate (10^9 cells of *Sarcina lutea* per milliliter exposed for 10 minutes to 100 μg of lysozyme per milliliter of SSC) is added for each 1 to 4 volumes of DNA (100 to 200 μg of DNA from *Bacillus subtilis* per milliliter) containing 10 to 200 μg of mitomycin per milliliter; the DNA of *S. lutea* is cross-linked at the same time. Heating (10 minutes at 70°C) or dialysis against SSC abolishes the mitomycin-activating capacity of the *S. lutea* lysate. The activity of the dialyzed lysate can be almost completely restored by the addition of TPNH (100 to 200 μg/ml) or of a TPNH-generating system, but not by DPNH, TPN or DPN (*2*); however, TPNH in the absence of lysate is ineffective as a mitomycin-activating agent. Approximately 90 percent of the activity is associated with the cell-free lysate fraction sedimenting at 198,000*g* for 90 minutes. Besides bacterial lysates, a commercial preparation of the *Clostridium kluyveri* diaphorase (*3*) was found to activate mitomycin in the presence of TPNH, whereas several reducing mitochondrial systems or smooth membrane liver fractions (*4*) were ineffective. Dicumarol, a reported inhibitor of some quinone reductases (*5*), did not interfere with the enzymatic activation of mitomycin. Several properties of the mitomycin-activating system of bacterial origin are analogous to the similar mitomycin-reducing system present in liver homogenates (*6*). One could conclude that TPNH-dependent enzymatic reduction of mitomycin appears a necessary prerequisite to DNA cross-linking.

Cross-linking of DNA in vitro can also be accomplished with chemically reduced mitomycins. Native, high molecular weight DNA of viral, bacterial, or mammalian origin (100 to 200 μg/ml of SSC) was mixed with various concentrations of mitomycin (2 to 200 μg/ml), and the reaction was started by

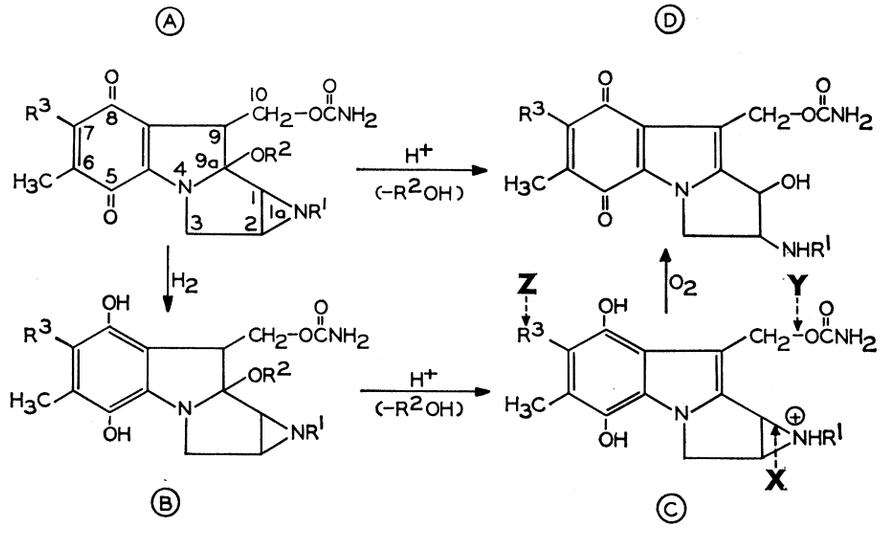

Fig. 1. Structure of mitomycins and porfiromycins, and of their reduction, reoxidation, and protonation products (*7*). *A*, The groups R^1, R^2, and R^3, respectively, are for mitomycin A: H, CH_3, CH_3O; for mitomycin B: CH_3, H, CH_3O; for mitomycin C: H, CH_3, NH_2; for porfiromycin: CH_3, CH_3, NH_2; and for 7-hydroxyporfiromycin: CH_3, CH_3, OH. *B*, Primary reduction product. *C*, Postulated structure after secondary rearrangements (*10*) following the reductive step and protonation of the aziridine nitrogen. Arrows point to the reactive sites and to the points of possible cleavage. *D*, Product of acid degradation (*7*).

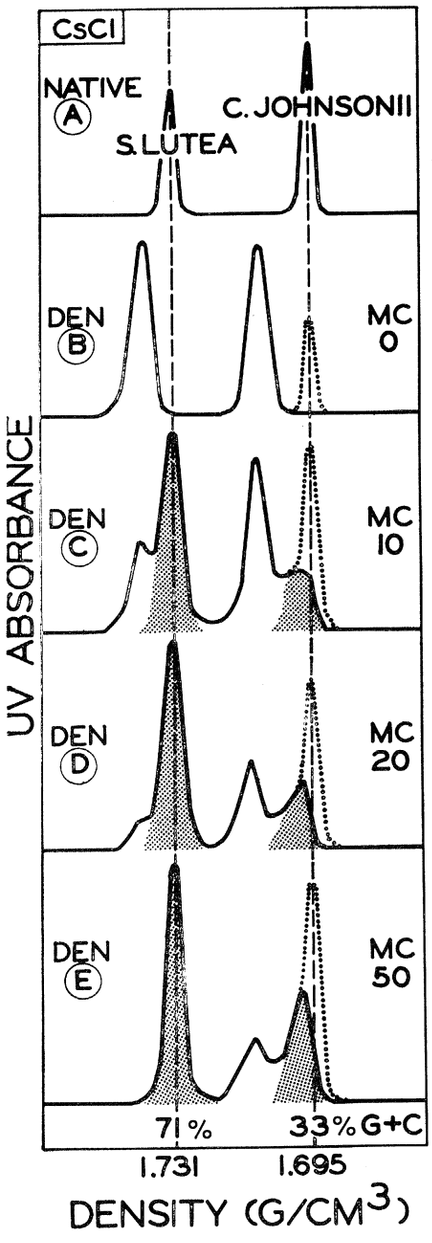

Fig. 2. Microdensitometer tracing after 22 hours of CsCl equilibrium density-gradient centrifugation (44,770 rev/min; 25°C) of a mixture of deproteinized and ribonuclease-treated (*18*) DNA's extracted from *Cytophaga johnsonii* and *Sarcina lutea* cells: *A*, native; *B–E*, denatured DNA (DEN) (heated for 6 minutes at 100°C in DSC and quenched at 0°C) after exposure in vitro to 0, 10, 20, and 50 μg of mitomycin C per milliliter of DSC under reducing conditions (200 μg of DNA + 100 μg of $Na_2S_2O_4$ per milliliter, 1 minute, 20°C), followed by alcohol precipitation of the DNA and dissolving the precipitate in DSC. The shaded areas roughly correspond to the spontaneously renaturable (cross-linked) DNA. The dotted peaks correspond to the native *C. johnsonii* DNA obtained by repeating the centrifugation after addition of 1 μg of this "density-marker" DNA directly to the centrifuge cells.

adding 100 to 200 μg of the reducing agent (sodium hydrosulfite—$Na_2S_2O_4$, or sodium borohydride—$NaBH_4$) per milliliter. Nitrogen was bubbled through all the components and the reaction mixture. Since mitomycin reduction and DNA cross-linking is very rapid (less than 1 minute), the reaction was terminated after 1 to 2 minutes by precipitating the DNA with alcohol and immediately dissolving the precipitate in DSC (*2*). The results of one of many such experiments, demonstrating the relationship of mitomycin concentration to degree of DNA cross-linking, are illustrated in Fig. 2. The proportion of the spontaneously renaturable (cross-linked) DNA (shaded area in Fig. 2), determined by comparing the integrated areas under the tracings (corrected for hypochromicity), is considerably higher in the case of the *S. lutea* DNA (71 percent G + C) than in the case of DNA from *Cytophaga johnsonii* (33 percent G + C), and amounts to 79 as against 36 percent (10 μg of mitomycin per milliliter; Fig. 2*C*), 91 as against 49 percent (20 μg of mitomycin per milliliter; Fig. 2*D*), and 100 as against 62 percent (50 μg of mitomycin per milliliter; Fig. 2*E*), respectively.

Since reduced mitomycin is highly unstable and rapidly loses its biological activity, cross-linking was most effective when DNA and the antibiotic were present in the reaction mixture together with the reducing agent. Mitomycin reduced and reoxidized (5 to 10 minutes later) in aqueous solution had no biological activity, and its ultraviolet spectrum was similar to that of the acid degradation product (Fig. 1*D*). Among mild reducing compounds molecular hydrogen (20°C, atmospheric pressure, in SSC) in the presence of a palladium catalyst (5 percent palladium on charcoal) was also an efficient mitomycin-activating and cross-link–initiating agent, with otherwise little or no effect on the integrity of DNA. Cysteine, glutathione, and ascorbic acid were ineffective as mitomycin-activating agents, ascorbic acid causing degradation of the DNA.

The cross-linking reaction requires at least two reactive sites on the mitomycin molecule, five forms of which, including mitomycins A, B, C, porfiromycin and 7-hydroxyporfiromycin (*7*), were compared in this study (Fig. 1*A*). In their natural oxidized form all five antibiotics exhibit hardly any alkylating function when reacted with thiosulfate

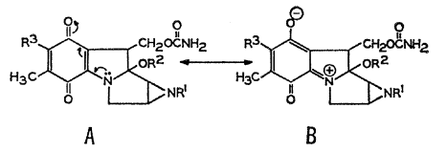

Fig. 3. Resonant forms of mitomycins (compare Fig. 1*A*) (*9*).

at acid or neutral *p*H or with γ-(4-nitrobenzyl)pyridine (*6*, *8*), although they all contain the aziridine ring, unique in a natural metabolite but readily demonstrable by these reagents in a variety of synthetic compounds. This lack of reactivity might be related to the partial withdrawal of electrons from the nitrogen-4 into the quinone ring (Fig. 3, *A* ⟷ *B*) (*9*). The postulated rearrangements upon reduction of the quinone ring (*A* ⟶ *B*) are outlined in Fig. 1. Spontaneous loss of the tertiary 9a-methoxy or hydroxyl group (*10*), probably caused by the regaining of electrons by the nitrogen-4 coupled with the high driving force for the formation of the fully aromatic indole system (*B* ⟶ *C*), would facilitate the fission of the aziridine ring (*C* ⟶ *D*) because of the stabilization of the positive charge by the indole ring in the transition state of the reaction before the ring opening (Fig. 4, *B* ⟷ *C*), thus providing an activated "alkylating" group (carbonium ion, Fig. 4*C*) at position "X" (Fig. 1*C*). Release of methanol during reduction of mitomycin was practically quantitative, as analytically determined after its separation by a microdiffusion technique, with saturated Na_2CO_3 as the liberating agent and 10 percent H_2SO_4 as the absorbing agent. Absorbed methanol was oxidized to formaldehyde by $KMnO_4$, and the formaldehyde was determined by a color reaction with chromotropic acid (*11*).

Furthermore, one might predict activation of a second "alkylating" center at carbon-10 (carbonium ion, Fig. 4*E*; "Y," Fig. 1*C*), highly reactive toward nucleophilic substitution, since here again a positive charge in the transition state would be stabilized by the indole nitrogen (Fig. 4, *D* ⟷ *E*), and since the carbamate anion is considered to be a good leaving group. This "alkyl" (carbon-10)–oxygen fission would be similar to that observed in pyrrolizidine alkaloids containing an analogous acyloxymethyldehydropyrrolizidine configuration (*12*).

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") at the carbon-7 position. The presence of the *o*-aminoquinone moiety both in mitomycin C and in the antibiotic streptonigrin (Fig. 5) might indicate only a superficial similarity (*13*) in their mode of action, especially since chemical reduction of streptonigrin does not influence its reactivity in vitro with DNA (*14*).

Irreversible loss of activity upon opening of the aziridine ring (Fig. 1*D*) attests to the primary participation of this moiety in the cross-linking reaction. However, methylation at nitrogen-1a (mitomycin B, porfiromycins) affects neither the stability of the aziridine ring nor the cross-linking activity. Substitution of a methoxy group for the amino group at the carbon-7 position of mitomycin C (mitomycins A and B) somewhat enhances the cross-linking of DNA, whereas hydroxyl substitution (7-hydroxyporfiromycin) results in a slightly less active compound. Similarly, the introduction of a tertiary hydroxyl group in place of the methoxy group at carbon-9a (mitomycin B) has little effect on cross-linking (Fig. 1*A*).

The techniques used in the previous study (*1*) did not permit the exact localization of the reactive sites on the DNA molecule participating in the cross-linking reaction. Also direct proof for the reaction between DNA and mitomycin was missing. The observation that mitomycin-induced cross-links are relatively rare, not exceeding one per 10^3 nucleotide pairs (*1*), renders direct chemical detection of the DNA sites taking part in such links difficult. However, the higher degree of cross-linking observed with *S. lutea* DNA (71 percent G + C) when compared with *C. johnsonii* DNA (34 percent G + C), both treated simultaneously with reduced mitomycin (Fig. 2, *C–E*), indicates that mitomycin cross-links probably involve guanine or cytosine, or both. Similar observations have been made with other DNA's of various contents of G + C, of mammalian, bacterial, and viral origin. If sites "X" and "Y" are indeed the "alkylating" sites, the short span between them (Fig. 1*C*) does not leave much choice in postulating the possible cross-linking sites on native DNA. Experimenting with space-filling models indicated that interstrand linking between the amino groups of the adenines or cytosines and the oxygen-6 groups of guanine (*15*) seems to be the only feasible scheme. Since cross-linking dependence on G+ C content favors rather G-G (or G-C, or C-C) links, the possible competitive

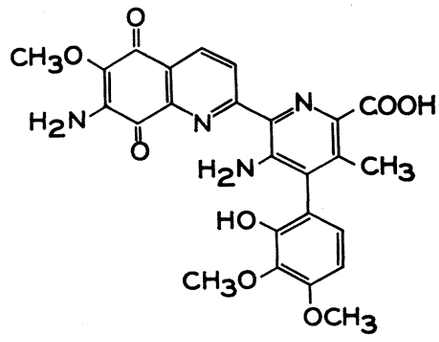

Fig. 5. Structure of the antibiotic streptonigrin (*13*).

effect of high concentrations of deoxycytidylic, deoxyguanylic, deoxyadenylic, and thymidylic acids (10 molar equivalents) on DNA cross-linking in the presence of limiting concentrations of mitomycin was studied. Surprisingly it was found that deoxycytidylic acid actually promoted the cross-linking reaction; the other three deoxynucleotides had no effect under similar conditions. The interpretation of these experiments and direct proof for the covalent bonding between reduced mitomycin and DNA must await the availability of radioactive mitomycin.

Exact measurement of the thermal and chemical stability of the cross-links is rather important for determining their chemical nature, since it was reported by Lawley and Brookes (*16*) that cross-links involving the alkylation of the nitrogen-7 position of guanine are thermally quite unstable because of the simultaneous labilization of the purine-deoxyribose bond. On the other hand, the mitomycin-induced cross-links must be thermally stable, having been initially discovered (*1*) because of the resistance they confer on the DNA double helix to thermal denaturation. Comparative studies on denaturation of identical samples of normal and of mitomycin–cross-linked DNA's by formamide (*17*), by alkali (1 minute at 25°C, 0.7 g of Na_2HPO_4, 5 ml of 1*M* NaOH, water up to 100 ml), and by heat under various conditions indicated that mitomycin cross-links exhibit the same degree of stability to all three agents. Subjecting cross-linked DNA to prolonged heating (up to 20 minutes at 100°C) results in some loss of spontaneously renaturable (cross-linked) material. All such losses, however, are accountable for by secondary thermal degradation of the DNA, which affects to a similar degree both the con-

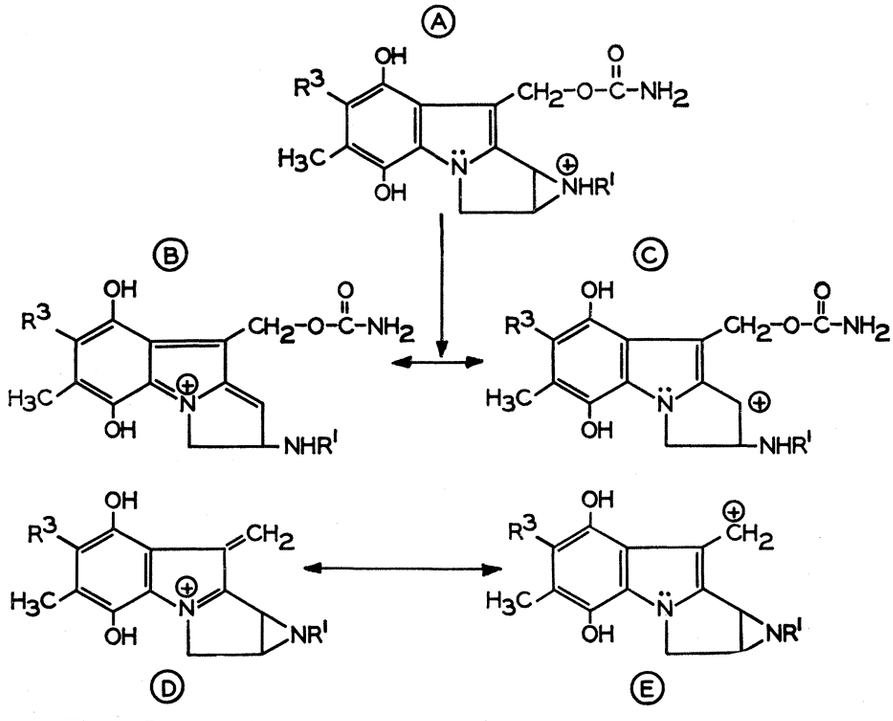

Fig. 4. Resonant forms of reduced mitomycins (compare Fig. 1*C*) (*19*).

trol DNA and that exposed to mitomycin. As discussed earlier (*1*), such scissions would free DNA fragments from their association with covalent cross-links.

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19. Contribution No. 569 from the Microbiology Research Institute, Canada Department of Agriculture, Ottawa. Supported in part by USPHS grant CA-07175 and by NSF grant G-18165. We thank H. H. Muxfeldt, J. A. Miller, C. Heidelberger, and J. D. Scribner for helpful discussion; and W. Schroeder (Upjohn Co.), who supplied us with porfiromycin and the triethylamine salt of 7-hydroxyporfiromycin (U-12, 270E, lot No. 4702-WS-136.1); A. Weissbach (NIH) who provided a sample of mitomycin B; S. Wakaki (Kyowa Hakko Kogyo Co., Tokyo) who supplied us with mitomycins A, B, and C and others; and Mrs. V. G. Arneson, L. Fenton, and Miss S. Becker for technical assistance.

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Pyruvate Metabolism and Control: Factors Affecting Pyruvic Carboxylase Activity

Abstract. *Pyruvic carboxylase activated by acetyl coenzyme A is highly active in the mitochondria of rodent liver, and its activity is increased in fasting and alloxan diabetes. In conjunction with acyl carboxylase activated by di- and tricarboxylic acid, it forms a reciprocating control network. Analog models of similar networks tend to correct for perturbations, stabilizing the overall system.*

Utter and Keech have demonstrated pyruvic carboxylase (*1*) in avian and beef liver. This enzyme, activated by acetyl coenzyme A, carboxylates pyruvate to oxalacetate without utilization of the cofactor. Pyruvic carboxylase, therefore, provides a link between fat and carbohydrate metabolism in a manner similar to the activation of acetyl coenzyme A carboxylase by members of the tricarboxylic acid cycle (*2*).

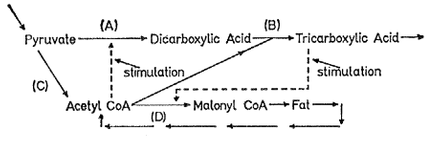

Fig. 1. Proposed control network having crossed over feedback loops.

Since this enzyme is a significant means of formation of dicarboxylic acid in the rat liver, we are reporting the results of food withdrawal and diabetes on its activity.

Several preparations were utilized in the data presented here. Powdered whole liver was prepared by homogenization of mouse or rat liver in 10 times its volume of cold acetone. Each homogenate was dried and, prior to use, was suspended in 50 times its weight of tris buffer. Fresh mitochondria were prepared by homogenization in 0.25*M* sucrose with a loose-fitting Teflon pestle. The debris was removed by centrifugation at 850*g* for 10 minutes; the mitochondria were obtained by centrifugation at 7000*g* for 10 minutes. Just before use they were suspended in an amount of tris buffer (*p*H 7.4) equivalent to the volume of liver from which the mitochondria were obtained. Powdered mitochondria were prepared by suspending the isolated mitochondria in 20 volumes of acetone, drying the precipitate, and grinding the dry powder in a volume of tris buffer (*p*H 7.4) equivalent to the volume of liver from which it was obtained. Reaction mixtures, made as indicated in Table 1, were incubated for 5 minutes at 30°C. The reactions were stopped by freezing the mixture in a bath cooled with solid carbon dioxide and alcohol. The protein was denatured by acid, the precipitate was discarded, and the supernatant was gassed vigorously with carbon dioxide gas. Radioactivity measurements were made on a Packard Tri-Carb scintillation counter.

Table 1. Comparison of enzyme preparations. One milliliter of incubation mixture contained protein obtained from 125 mg of fresh liver; 200 μmole of tris (*p*H 7.4); 2.5 μmole of adenosine triphosphate; 1.4 μmole of acetyl coenzyme A; 10 μmole of pyruvate; 9 μmole of Mg; 10 μmole of $NaHC^{14}O_3$. The mixture was incubated for 5 minutes at 30°C.

Preparation	$C^{14}O_2$ fixed * (mμmole)		
	Per g of liver	Per mg of liver protein	Per mg of mitochondrial protein
Powdered mitochondria	1013	6.0	69
850g supernatant	963	5.6	96
9000g supernatant	913	5.5	119
Fresh mitochondria	162	0.9	11.6

* In nonvolatile compounds.

In agreement with results (*1*) on avian and beef liver, and within the error of the method, all the pyruvic carboxylase activity is located in the mitochondrial fraction of rat and mouse liver cells, and the enzyme is biotin-dependent and avidin-sensitive.

Table 1 indicates the results from various types of enzyme preparations. A much higher degree of activity is present in the powders of mitochondria than in the equivalent amount of fresh mitochondria, thus a control mechanism exists whether it be selective membrane permeability, competing enzymatic reactions, or the interaction of two or more enzymatic sequences.

In preparations of whole liver powders, gradient elution (*3*) indicated that 80 percent of the radioactivity was in citrate, 10 percent in aspartate, 3 percent present in a form easily decarboxylated by $Al_2(SO_4)_3$ (presumably oxalacetate), and about 1 percent each in malate, glutamate, and α-ketoglutarate.