A MOLECULAR MECHANISM OF MITOMYCIN ACTION: LINKING OF COMPLEMENTARY DNA STRANDS*†

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The selective action of the antibiotic mitomycin C (MC)1 on deoxyribonucleic acid (DNA),2-4 together with its reported antineoplastic,1, 7 mutagenic,8 and phage-inducingg activities, has stimulated several investigations on the mechanism of its action. The preferential inhibition of bacterial DNA synthesis by MC, accompanied by progressive and extensive breakdown of the DNA, indicates that DNA is the principal target. However, the rapidity of MC-induced “death” seemed to be out of step with the relatively much slower process of DNA breakdown. This suggested that the effects hitherto observed might be secondary to an earlier action of the antibiotic on DNA. Such a primary lesion is described here and interpreted as in vivo MC-induced linking (“cross-linking”) of the complementary strands of the DNA molecule.

Materials and Methods.—Samples of mitomycin C were kindly provided by Dr. J. Lein, Bristol Laboratories, Syracuse, N. Y., by Dr. R. B. Ross, Cancer Chemotherapy National Service Center, NIH, Bethesda, Md., and by the Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. The bacterial strains used included Escherichia coli strain B, Sarcina lutea strain ATCC-272, and the following Bacillus subtilis mutant lines:6-11 wild-type, the indole-requiring, the linked indole- and histidine-deficient (I-, H-), and a prototrophic derivative of the indole-requiring strain 108, the latter bearing in addition a marker (mac-r1) conferring resistance to the macrolide group of antibiotics. Bacteria which were growing exponentially in Difco’s antibiotic medium 3 (Penassay broth) were exposed to MC under the conditions specified, and their survival determined by plating on nutrient agar. To terminate MC exposure the cells were chilled, washed twice with cold SSC (0.15 M NaCl + 0.015 M Na2 citrate), and frozen with 100 μg lysozyme/ml. The procedures used for the isolation of protein- and RNA-free DNA and for the determination of its buoyant density and transforming activity were outlined earlier.11 Thermal transition (“melting”) curves were obtained with a recording thermospectrophotometer.12 Thermal denaturation was carried out by exposing the DNA in 0.015 M NaCl + 0.0015 M trisodium citrate at pH 7.7 (DSC) to 100°C (or other specified temperatures) for 6 min followed by rapid cooling in an ice bath.11

Results.—The exposure of an exponentially growing culture of B. subtilis to an inhibitory concentration of MC resulted in very rapid cell death, colony-forming capacity dropping by several powers of ten in a matter of minutes (Fig. 1, Table 1). Native DNA extracted at this early period from MC-exposed cells and examined...
TABLE 1

<table>
<thead>
<tr>
<th>Exposed to MC (min)</th>
<th>MC concentration (µg/ml)</th>
<th>0.1</th>
<th>0.5</th>
<th>1.0</th>
<th>2.5</th>
<th>12.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (B. subtilis)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>4 × 10⁻⁶</td>
</tr>
<tr>
<td>15 (E. coli)</td>
<td></td>
<td>100</td>
<td>10</td>
<td>0.8</td>
<td>0.6</td>
<td>10⁻²</td>
</tr>
<tr>
<td>15 (% T.A.)</td>
<td></td>
<td>5</td>
<td>-</td>
<td>10⁻²</td>
<td></td>
<td>2 × 10⁻⁷</td>
</tr>
<tr>
<td>30 (B. subtilis)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>4 × 10⁻³</td>
<td></td>
<td>&lt;10⁻⁸</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td>-</td>
<td>-</td>
<td>3 × 10⁻⁴</td>
<td></td>
<td>&lt;10⁻⁸</td>
</tr>
<tr>
<td>60 (% T.A.)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.7</td>
</tr>
<tr>
<td>120 (B. subtilis)</td>
<td></td>
<td>100</td>
<td>-</td>
<td>3 × 10⁻⁶</td>
<td></td>
<td>&lt;10⁻⁸</td>
</tr>
<tr>
<td>300</td>
<td></td>
<td>100</td>
<td>-</td>
<td>10⁻⁴</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* Exponentially growing cells (1–5 × 10⁶ cells/ml, 37°C, Penassay broth) exposed to MC for indicated periods of time. Colony count determined after plating appropriate dilutions on nutrient agar. Transforming activity (indole +) determined for RNA-free DNA extracted from separate aliquots of MC-treated cells.

by the technique of equilibrium density-gradient centrifugation was indistinguishable from DNA extracted from unexposed cells. Its transforming activity was only slightly diminished (Fig. 1, Table 1). However, when the DNA’s from the control and MC-exposed cells were heat-denatured and rapidly cooled, a significant difference in the banding pattern in CsCl and Cs₂SO₄ gradients was revealed. Only a small fraction of the denatured DNA from MC-exposed cells (Figs. 2B and 3B) banded in the position normally occupied by the denatured normal (dN) DNA (Figs. 2A and 3A), while the main band formed at the density characteristic for the native (NN) or renatured state (Figs. 2B and 3B). Such behavior, by analogy with the effects of ultraviolet light¹¹,¹³ and nitrous acid,¹⁴ could be interpreted as resulting from thermostable linking of the complementary strands of the DNA molecules.

Effects of formaldehyde (HCHO) and hydrodynamic shear on the denaturation process: A scheme involving MC-induced linking of the complementary DNA strands poses two questions: (1) Do the linked strands fail to separate, even partially, when heated, or is the renaturation process very fast and efficient? (2) How frequent are the cross links? Firstly, when renaturation of the heat-treated MC-linked DNA was partially prevented by HCHO¹¹ (see Fig. 2C), no band was formed at the native DNA density, indicating that the apparent heat stability of MC-linked DNA can be attributed to its spontaneous renaturability in the absence of HCHO. Secondly, if the links are infrequent (one or only a few per molecule), shearing of

![Fig. 1.—Survival of B. subtilis cells (●) (colony-forming ability) grown in Penassay broth and exposed for 15 min (37°C) to the indicated concentrations of mitomycin C (0, 0.1, 0.5, 2.5, 12 µg/ml), and of the specific transforming activity (C) (macrolide resistance marker) determined for denaturated and RNA-free DNA extracted from the above cells. Other markers gave similar results (47, 74, and 48% survival at 2.5 µg MC/ml, and 16, 31, and 22% survival at 12.5 µg MC/ml for indole, histidine, and linked indole-histidine markers, respectively). Transforming activity for each marker was determined at DNA concentrations of 0.01, 0.1, 1, and 10 µg/ml. For each series of MC concentrations tested, the slopes of the resulting titration curves (DNA concentration versus number of transformant colonies) were essentially parallel.](image-url)
The native cross-linked DNA should result in separation of the linked from the non-linked DNA fragments and a decrease in the relative amount of native-like "molecules" upon subsequent denaturation (Fig. 7, third line), as revealed by density-gradient centrifugation. This prediction was verified in the experiment illustrated in Figures 2B and 2D. When shearing reduced the sedimentation constant of the DNA from $S_{20W}^{0} = 30-40$ to $15-20$ S, the amount of cross-linked molecules decreased from 85 to 35 per cent.

**Dependence of the cross-linking on MC concentration, period of exposure, and temperature:** When exponentially growing *B. subtilis* cells were exposed to a concentration of 0.1 $\mu$g/ml MC at 37°C, multiplication of the cells was arrested, but there
was no observable reduction in viability during the 20-hr period of observation (Table 1). By the experimental criteria employed, DNA extracted from these cells behaved like normal DNA. The MC-imposed anomaly in the banding pattern of the denatured DNA became apparent at higher concentrations of MC. The relative amount of the DNA that resists denaturation (shaded areas) increased with increasing concentrations of MC (1-12 µg MC/ml; Figs. 3G and 3B or 3C), or increasing periods of exposure (5-120 min; Figs. 3G and 3H); it was also a function of the temperature (7°C versus 37°C; Figs. 3D and 3C). For intermediate degrees of reaction with MC, the heated and rapidly cooled DNA comprised two distinct molecular classes, one of which had the buoyant density of denatured normal molecules, and the other (spontaneously renatured cross-linked molecules; shaded areas in Figs. 2 and 3) banding at a buoyant density slightly greater than that of the native normal molecules. On further reaction, the fraction banding in the renatured region increased until there was no detectable banding in the denatured region, unless hydrolytic or mechanical breakdown of the DNA intervened. Spreading of the bands (Fig. 3E) and a decrease in the alcohol precipitability of the DNA heralded the onset of its enzymatic destruction.

Cross-linking in the absence of DNA and protein synthesis, and lack of in vitro interaction between purified DNA and MC: The question of whether the changes induced by MC in DNA molecules in vivo are dependent on active synthesis of DNA or protein was examined by studying the effect of MC (12 µg/ml, 15 min, 37°C) under conditions of inhibition of these syntheses by 5-fluorodeoxyuridine (10 µg/ml added 5 min ahead of MC) and chloramphenicol (50 µg/ml added 15 min ahead of MC), respectively. Notwithstanding the previous exposure to and continued presence of these inhibitors, MC induced the molecular changes in the DNA to the same degree as in the absence of the inhibitors.

The implication that no concomitant DNA or protein synthesis is necessary for the MC-induced linking to occur was further strengthened by the observation that MC effectively cross-links purified B. subtilis DNA in the presence of either homologous or heterologous (Sarcina lutea) cell-free lysates prepared by lysozyme treatment and osmotic rupture (cf. Fig. 3F and legend). Nevertheless, MC failed to induce such changes in deproteinized DNA or DNA plus RNA extracts even after prolonged periods of in vitro exposure to 100 µg MC/ml (banding profile identical to that in Fig. 3A). Likewise, the transforming activity of purified DNA was unaffected by MC in vitro.

Stability of the effect: The continued presence of MC in the culture was not a requirement for persistence of the observed effects on the DNA. In fact, 2 hr incubation (37°C) of MC-treated (15 min, 12 µg MC/ml), washed cells in the absence of the antibiotic failed to reverse the MC effects.

Generality of the effect: In the light of recent reports\textsuperscript{11, 12} that a fraction of the DNA molecules of B. subtilis normally fails to undergo strand separation on thermal denaturation, it was important to determine whether the observed effects of MC were peculiar to this bacterial species or could be extended to others. DNA extracted from Escherichia coli strain B cells exposed to MC under similar conditions behaved in a manner analogous to the B. subtilis DNA on heating and rapid cooling. A mutant strain of E. coli, partially resistant to MC, required 10 times higher MC concentrations to exhibit comparable bactericidal and DNA cross-linking effects.
In preliminary studies, we have also observed similar effects of MC on the DNA of cultured human cells and of replicating coliphage T4 or B. subtilis phage PBS 2, although 5-10 times higher MC concentrations were required.

The thermal transition characteristics of "MC-linked" DNA: The changes observed in the absorbance of E. coli DNA, both normal and MC-linked, slowly heated in 7.2 M NaClO₄, indicate that most of the hydrogen bonds "melted out" (Fig. 4, heavy lines). Differences were observed only in subsequent cooling and heating cycles, which were carried out in the thermospectrophotometer cuvette compartment. The temperature-absorbance profiles indicate that a high proportion of the MC-linked DNA returns to the native-like (hypochromic) state (Fig. 4, thin solid line) unlike the normal DNA (thin dotted line). Analogous results were obtained with B. subtilis DNA.

Transforming activity of "MC-linked" DNA: While MC-induced loss of cell viability and DNA cross-linking are rapid processes, the transforming activity of the DNA, tested for three different characters, is only gradually lost (Fig. 1). Even when cross-linking was quite extensive (Figs. 2B and 3B), as much as 20 per cent of the original transforming activity was still retained by the molecules. Under these conditions, the ability of two genetically linked markers (I⁺, H⁺) to be cotransferred was also retained, suggesting that the region concerned had not yet suffered any damage. Furthermore, the transforming activity of MC-linked DNA, unlike the activity of normal DNA, was not critically and abruptly destroyed over a narrow temperature range (Fig. 5). This remarkable heat stability of DNA isolated from MC-treated cells indicates that its residual transforming activity is primarily associated with the cross-linked DNA molecules.
On prolonged exposure to MC (over 2 hr), the ability of the I+H+ region to be cotransferred to the doubly deficient strain fell more rapidly than that of the component markers. A similar genetic dissociation of associated markers under the influence of MC has recently been reported by Yuki in the Escherichia coli K12 system. Prolonged exposure to MC resulted in eventual loss of all detectable transforming activity and recoverability of polymerized DNA.

Discussion.—The most obvious interpretation of the foregoing results is the formation of rare links between complementary DNA strands by metabolically activated MC. The high chemical reactivity of this antibiotic could be inferred from its structure (Fig. 6): aziridine ring 1, 2, 1a, methylurethane at position 9, quinone structure 5, 8, and substitutions at positions 7 and 9a are all highly reactive groups. The polycyclic structure might favor intercalation of this compound into DNA, as a step prior to selective cross-linking. The density shift in the presence of HCHO (Fig. 2C) and the increase in absorbance of the MC-linked DNA (Fig. 4, solid heavy line) attest to almost complete collapse of the hydrogen-bonded structure during thermal denaturation, as schematically indicated in Figure 7 (column B), although the MC-induced link does not permit complete separation of the complementary strands. This link, maintaining the original alignment, permits very rapid return of the DNA molecule to the original native, hydrogen-bonded structure, a process kinetically improbable for denatured normal DNA (column C). Assuming a Poisson's distribution of the links, and further, that one of these links is sufficient to permit the spontaneous renaturation of the molecule, it is possible to make a very rough estimate of the average number of cross-links per molecule, \( m = \ln (1/P_0) \) where \( P_0 \) is the proportion of the molecules unlinked. Based on the numerical data derived from Figures 2B and 2C, the average frequency of MC-induced links corresponds to one per 20,000 nucleotide pairs, i.e., per mol. wt. \( 12 \times 10^6 \) (1.8 links \( B \) per mol. wt. \( 23 \times 10^6 \), or 0.43 link \( D \) per mol. wt. \( 5 \times 10^6 \)). Similar estimates for various conditions of MC treatment are listed in the legend of Figure 3.

![Figure 6](image_url)

Fig. 6.—Structure of mitomycin C according to Webb et al.17

![Figure 7](image_url)

Fig. 7.—Molecular events following the denaturation (A to B) and rapid cooling (B to C) of normal and MC-linked DNA, the latter before and after subjection to hydrodynamic shear. The possibility of hydrolytic breakage of the phosphate ester bonds and its consequences are indicated for MC-linked DNA (middle row).
From our present findings and those of others, it is suggested that the "cross-links" imposed by MC (approximately 50 per bacterial DNA complement for 5 min exposure to 1 μg MC/ml; Fig. 3F) interfere with DNA replication; (2) rapidly lead to cell death; (3) nevertheless have little effect on the ability of the DNA to transform for a given marker or direct the synthesis of one or a few proteins, since these two activities reflect a function of a relatively small fraction of the total DNA complement. The reported partial resistance of viral DNA replication to the action of MC remains to be explained, but may reflect the relatively smaller size of the viral DNA molecules and/or their fragmentation during the replication process.

The observation that some natural bacterial and viral DNA's appear to be cross-linked together with the results here obtained with mitomycin, which is a natural product, suggests some biological role for this cross-linking process. If reversible, this process would permit turning on or off DNA synthesis, and thus would assume a regulatory significance. Although the determination of the exact chemical nature of the cross-links might be difficult because of their low frequency, the presently acquired knowledge of the mechanism of MC action permits application of this antibiotic in a variety of experiments, both with intact cells and transforming DNA.

Summary.—Exposure of Bacillus subtilis or Escherichia coli cells to inhibitory concentrations of mitomycin C for periods of 1–60 min results in covalent linking of the complementary DNA strands. This cross-linked DNA denatures upon thermal treatment, but renatures spontaneously even with rapid chilling, as revealed by its return to the native-like buoyant density in CsCl or Cs2SO4 gradients and by the reversibility of the optical density changes during heating and cooling cycles. Hydrodynamic shearing of the cross-linked DNA permits separation of the linked and nonlinked regions.

Only 10−4 per cent of B. subtilis cells survive 15 min exposure to 12 μg of mitomycin per ml. Transforming DNA isolated from cells so treated (one cross-link per mol. wt. of 10−12 million) retains 20–30 per cent of its activity, which becomes resistant to thermal denaturation. Higher concentrations of mitomycin are required to effect a comparable degree of cross-linking of vegetative T4 or PBS 2 phage DNA and the DNA of human cells or mitomycin-resistant bacterial mutants. Mitomycin has no in vitro effect on purified DNA unless a cell extract is added.

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† The following abbreviations are used in this paper: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; RNase, ribonuclease; MC, mitomycin C; UV, ultraviolet light.

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Goldusche, E. P., these PROCEEDINGS, 47, 950 (1961), and J. Mol. Biol., 4, 467 (1962).


Yuki, S., Biken’s J., 5, 47 (1962).


The degree of cross-linking observed for short-term MC exposures might be somewhat exaggerated, since cell-absorbed MC could have been active during cell washing (4°C) and during subsequent lysozyme treatment (37°C). This delayed effect of MC might explain the high viability of cells exposed for 5 min to 1 μg MC/ml (Table 1), although on the average their DNA showed one cross-link per mol. wt. 135 X 10⁶ (Fig. 3F).

The effect of subinhibitory concentrations of MC on pneumococcal receptor cells was reported by G. Balassa, Ann. inst. Pasteur, 102, 547 (1962).


The rapid and presumably selective effect on DNA exhibited by MC might suggest the use of this agent in antineoplastic perfusion techniques (Cohen, D. H., Med. J. Australia, 2, 807 (1960)), where other less selective cross-linking agents, e.g., nitrogen mustards, are now principally employed. See Research in Radiotherapy, ed. E. T. Krementz, NAS-NRC Publ. 888 (1961), p. 220.