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Effects of Mitomycin C on Macromolecular Synthesis in *Escherichia coli*

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ABSTRACT

When cells of *Escherichia coli* B growing in a glucose-synthetic medium were treated with mitomycin C, the effects produced by the antibiotic varied, depending on the concentration. When the concentration was reduced to less than 0.1 μg/ml, the action of the antibiotic was bacteriostatic; cell elongation resulted, but no effect on the synthesis of cellular macromolecules was apparent. At higher levels (more than 5 μg/ml), mitomycin C was highly bactericidal and inhibited deoxyribonucleic acid synthesis almost completely. The exposure of growing cells to a bactericidal level of mitomycin C resulted also in a delayed inhibition of the synthesis of ribonucleic acid (RNA) and protein. The capacity of the treated cells to synthesize β-galactosidase inducibly in a medium free from a carbon source remained constant for the first 30 min and then was destroyed progressively with time. Prolonged incubation with the bactericidal level of mitomycin C caused a degradation of cellular nucleic acids, particularly RNA. The degraded nucleic acid components were eventually released into the medium.

It has been reported that the antibiotic mitomycin C (MC) has a specific effect on cellular deoxyribonucleic acid (DNA), but has little or no effect on either ribonucleic acid (RNA) or protein formation in *Escherichia coli* (19). The primary action of MC is believed to be associated with either the inhibition of DNA biosynthesis (18) or the breakdown of the nuclear apparatus (16). Szybalski and Iyer (23) suggested that cell death caused by MC is a result of cross-link formation in cellular DNA by a reduced form of the antibiotic More direct evidence for the alkylation of the nucleic acids with mitomycin was provided by Weissbach and Lisio (24).

According to Weissbach and Lisio (24), soluble RNA (s-RNA) and ribosomes are alkylated with mitomycins to the same extent as DNA. In fact, it has been observed by many workers that, although RNA is apparently less sensitive than DNA to MC, the synthesis of cellular RNA is also significantly inhibited by prolonged exposure to MC, or by treatment with higher levels of MC (18).

Most studies of MC have been focused on its interaction with cellular DNA, and there are comparatively few reports on its effects on cellular RNA. Smith-Kielland (20), using ultracentrifugal analysis, found that the sRNA fraction in the nucleotide pool of *E. coli* increases in cells treated with MC. Fenwick (7) found that the synthesis of ribosomal RNA was markedly inhibited in MC-treated as well as ultraviolet-treated mammalian cells. Kersten et al. (11) found a decrease in the amount of sRNA and an accumulation of small ribosomal particles in cell extracts of MC-treated *E. coli*. More substantial evidence for the decomposition of ribosomes has been obtained by sucrose density gradient analyses of cell extracts of MC-treated *E. coli* (21).

In the present experiments, the effects of MC on macromolecular metabolism of *E. coli* were studied. Pronounced effects on cellular RNA as well as DNA were found.

MATERIALS AND METHODS

Organisms. E. coli strain B and thymine-requiring strains $15T^{-}H^{-}$ and B3 from the collection of the laboratory were used.

Growth of bacteria. A minimal medium of Davis and Mingioli (6), with 0.2% glucose as a carbon source (DM medium), was used throughout the experiments.

The cells, grown overnight in DM medium, were diluted 20-fold with the fresh medium and allowed to grow for an additional 2 hr. The cells in the exponen-

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tial growth phase thus obtained were harvested by centrifugation, washed twice with 0.05 M phosphate buffer (*p*H 7.0), and resuspended in DM medium (usually 100 ml) to give a turbidity at 550 mμ of 0.30, which corresponded to a cell density of about 5×10^8 cells per milliliter. All cultivations were performed at 37 C with aeration. The generation time under these conditions was about 52 min.

The growth, colony-forming units, and total cell numbers were estimated by turbidity readings at 550 mμ, by colony counts obtained by use of a spreading plate method on Nutrient Agar (Difco), and by cell counts with the use of a Petroff-Hausser counting chamber, respectively.

Analytical procedures. Nucleic acid and protein determinations were made on samples of the culture, usually 10 ml, removed at various intervals. The samples were chilled rapidly, and the cells were harvested by centrifugation and washed twice with 0.05 M phosphate buffer (*p*H 7.0). The washed cells were suspended in 3.0 ml of 0.5 N perchloric acid and left for 25 min in an ice bath. Insoluble materials were collected in a refrigerated centrifuge, and the supernatant solution was retained as the acid-soluble fraction. The precipitate was resuspended in 2.5 ml of 0.5 N perchloric acid, and the nucleic acids were extracted by heating the suspension at 80 C for 25 min and were separated from the insoluble materials by centrifugation.

Nucleotides in the acid-soluble fractions were estimated by ultraviolet absorption at 260 mμ. DNA and RNA in the acid-insoluble fractions were estimated colorimetrically with diphenylamine (2) and with orcinol (1), respectively. Protein was determined with the residue suspended in 1 N NaOH by a modified Folin-Ciocalteau method (12).

Assay of β-galactosidase. Cells (5 ml) were harvested from a growing culture at various intervals and washed twice with 0.05 M phosphate buffer (*p*H 7.0). The cells were then resuspended in 10 ml of DM medium free from a carbon source, and β-galactosidase was induced with 5×10^{-4} M thiomethyl β-galactoside by incubating the mixture with aeration for 30 min at 37 C. A 2-ml sample was removed, and the activity of β-galactosidase was determined by the method of Kuby and Lardy (9).

Measurement of radioactivity. C^{14}-labeled chemicals, having specific activities in the range of 10 to 20 mc/mmole, were obtained from Calbiochem, Los Angeles, Calif.

Measurements of the radioactivity in perchloric acid extracts were carried out with a thin-window gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Samples of 10 to 50 μliters were spread on aluminum planchets and dried under an infrared lamp.

To prelabel cellular components, actively growing cells were incubated for 90 min with radioactive precursors (1 to 2.5 μc/10 ml of the culture). The cells were washed thoroughly and served for the further treatments.

Chemicals. MC was purchased from Sigma Chemical Co., St. Louis, Mo. The phenol-reagent for protein determinations was obtained from Van Waters and Rogers, Inc., San Francisco, Calif. Reagent-grade chemicals were used throughout.

RESULTS

Effects of growth of E. coli B. The overall effects of various concentrations of MC on the growth and macromolecular synthesis of *E. coli* B are shown in Table 1. DNA was the most sensitive, but there were no marked imbalances in the amounts of DNA, RNA, or protein in the cells after 90 min of treatment with the antibiotic at any level. By increasing the concentration of MC, the formation of RNA and protein, as well as of DNA, was greatly suppressed. The amount of acid-soluble nucleotides decreased as the concentration of MC was elevated, whereas that of

TABLE 1. *Effects of various concentrations of mitomycin C on growth and macromolecular synthesis of Escherichia coli B*[a]

Concn of mitomycin C	Turbidity[b]	Filaments	Viability[c]	Ultraviolet absorption at 260 mμ		Protein	RNA	DNA
				In medium	In cold PCA extracts			
μg/ml								
0	100	−	3.27	0.77	100	100	100	100
0.0	100	+	2.09	0.76	128	98	100	95
0.0	95	+	1.59	0.65	120	96	96	88
0.1	94	++	1.00	0.77	106	96	94	71
0.5	89	+++	0.17	0.89	89	86	94	65
1.0	78	+++	1.7×10^{-2}	1.04	62	79	75	48
5.0	42	++	2.1×10^{-5}	1.74	−22	45	44	21

[a] Results are expressed as increments relative to those of the control culture after 90 min of incubation. The increase of the control culture is represented by 100.

[b] Optical density at 550 mμ.

[c] Expressed as multiples of the initial amounts (1.0).

ultraviolet-absorbing materials in the medium increased significantly. In the presence of 0.05 to 0.1 μg/ml of MC, growth of the cells, when measured by turbimetric procedures, was only slightly affected, but the cells became filamentous and the viability remained almost constant.

Similar results were obtained with other strains of *E. coli* (K-12, 15T⁻H⁻, and B3), though the sensitivity to MC varied slightly with each strain.

The effects of MC on the growth and macromolecular synthesis of *E. coli* B during the period of treatment are shown in Fig. 1 and 2. MC at a level of 5 μg/ml was highly bactericidal, and the viability of the culture decreased exponentially with time. The increase in the turbidity also leveled off gradually after 30 min. At this time, filamentous cells appeared and predominated over the normal short rods. DNA formation was almost completely inhibited from the outset of the incubation, whereas RNA and protein, like the turbidity, increased normally for the first 30 min and then gradually leveled off. A slight decrease in RNA content was always observed after prolonged incubation. Ultraviolet-absorbing materials in the acid-soluble fraction decreased with time, whereas those in the medium increased slightly. This was probably due to partial lysis or damage to the cell wall of the treated cells, but gross lysis of the cells was unlikely, since the total

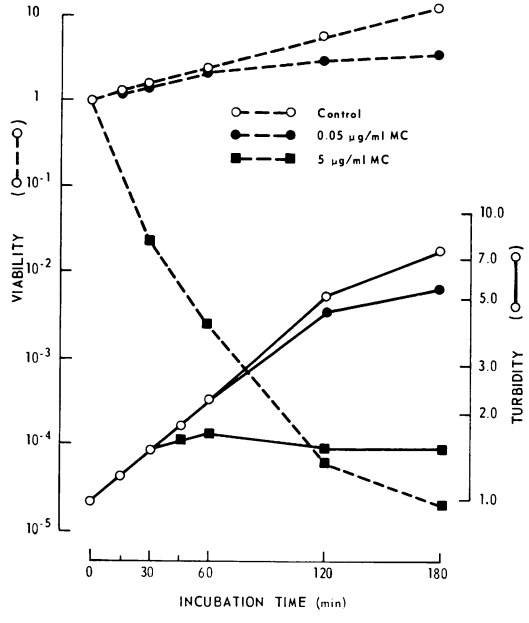

FIG. 1. *Changes in colony-forming units and turbidity of Escherichia coli B cultures during treatment with MC. The values are given as ratios to those of the initial stage.*

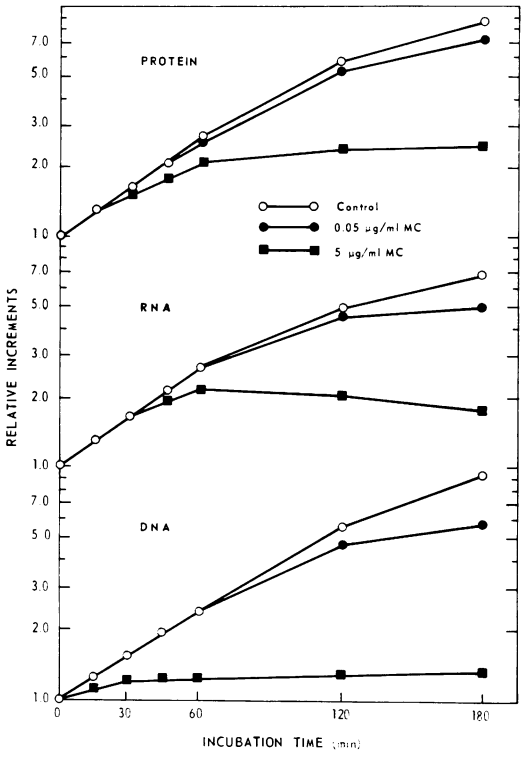

FIG. 2. *Synthesis of protein and nucleic acids in Escherichia coli B during treatment with MC. The values are expressed as relative increments per unit volume of culture to the initial amounts.*

cell counts remained constant during the treatment.

In the presence of 0.05 μg/ml of MC, the turbidity of the culture continued to increase, but at a slightly lower rate than that of the control. Also, filamentous forms became obvious after 90 min. The increase in viability was significantly suppressed, but death of the cells apparently did not occur. Thus, MC at 0.05 μg/ml was apparently bacteriostatic. The only distinct effects observed were filament formation and suppression of viability.

The bactericidal action of MC at 5 μg/ml was irreversible. Cells treated for 15 min could not resume their normal growth after the removal of the antibiotic, and the viability of the culture neither increased nor decreased further during postincubation in the absence of the antibiotic (Fig. 3). After the 30-min pretreatment, the killing action of MC was still evident during the postincubation period.

The action of MC at 0.05 μg/ml was, however, partially reversed by the removal of the drug. After the 15-min pretreatment at this level, the

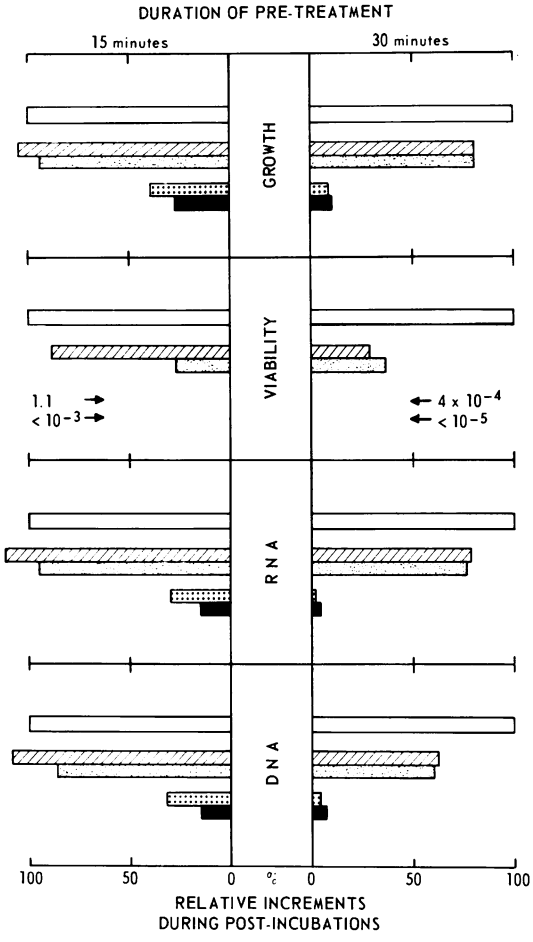

FIG. 3. *Recovery of the growth and nucleic acid synthesis in Escherichia coli B after removal of MC. Actively growing cells in DM medium were incubated in the presence of MC for 15 min (left side) and for 30 min (right side), respectively. The cells were then washed by centrifugation to remove the antibiotic, and resuspended in an equal volume of fresh medium. Each suspension was divided into two portions, one with MC at the same level as used for the pretreatment, and the other without MC. The duration of the postincubation was 120 and 180 min, respectively. Controls were carried out similarly without the pretreatment with MC. The values are shown in percentage of the control. The open bar represents the control. The first pair of bars below the control represents treatment with MC at 0.05 μg/ml, and the lowest pair of bars represents treatment with MC at 5 μg/ml. In each of these pairs, the upper bar shows the effect when MC was present during preincubation but not during postincubation, and the lower bar shows the effect when MC was present both pre- and postincubation.*

cells were able to resume normal growth, judging from the increase in colony-forming units during postincubation without the antibiotic. The cells did not recover, however, from the damage caused during the 30-min pretreatment period, and the viability remained constant for at least 3 hr during the postincubation in the absence of MC.

These results suggest that there is an apparent shift in the mode of action of MC in *E. coli* B, depending upon the concentration of the antibiotic. At a level of less than 0.1 μg/ml, the action of MC was bacteriostatic and caused only filament formation or division inhibition. At a level higher than 5 μg/ml, its action was highly bactericidal and caused the inhibition not only of DNA but also, though to a lesser extent, of RNA and protein formation. Decrease of the cellular contents after prolonged treatment with the higher level of MC was obvious with RNA, but not with DNA.

There are many substances reported to have a counteracting effect on the action of MC, including yeast extract (19), protamine sulfate (14), and certain cations (8). The effects of the following compounds on the action of MC were tested by adding them to cultures of *E. coli* B in the presence of MC: yeast extract (Difco), deoxyguanosine, a mixture of deoxyribonucleosides, ethylenediaminetetraacetate, reduced glutathione, thiosulfate, and pantoyl lactone. None of these compounds showed any effects on the action of MC, as revealed by microscopic observations or chemical analysis. Also, the growth phase of the cultures had no significant effect on the action of MC.

When actively growing cells in a glucose DM medium were transferred to a DM medium containing lactose as a sole source of carbon, there was a lag of about 45 min, after which the cells began to grow exponentially at a rate slightly lower than that in glucose. In the presence of 0.05 μg/ml of MC, growth and macromolecular synthesis continued to increase at a slightly lower rate than in the control, but the viability of the culture remained constant throughout the 2-hr incubation and the cells became filamentous. These effects were the same as observed with cells treated in glucose. A marked difference was, however, observed when the concentration of MC was raised to 5 μg/ml. The formation of RNA as well as DNA was completely arrested from the beginning, and protein synthesis was also greatly suppressed. The amount of β-galactosidase formed in the cells was very low compared with that in the control. The cells observed under the microscope appeared normal, and no long filaments were observed. The total cell counts re-

mained constant, but the viability of the culture was lost rapidly. Thus, the bactericidal action of MC was not necessarily followed by filament formation. The amount of cellular RNA decreased slightly after prolonged exposure to MC, as it did in the culture treated in glucose medium. Quite similar results were obtained with cultures treated with 5 μg/ml of MC in a medium free from carbon source for 30 min when glucose was added to initiate growth.

Instability of macromolecules. When actively growing cells of *E. coli* B were grown in the presence of thymidine-2-C^{14}, the thymidine was incorporated progressively for the first 30 min. But after this period almost no incorporation occurred, probably because thymidine phosphorylase induced in the cells converted thymidine to thymine (15). MC at 0.1 μg/ml had no effect on the incorporation of thymidine into the cells. However, in the presence of 5 μg/ml of MC, the amount of thymidine incorporated into the acid-insoluble fraction was low and it did not increase at all after 30 min of the treatment. When *E. coli* B, whose DNA was prelabeled by incubation with thymidine-2-C^{14}, were reincubated in the DM medium with 0.1 μg/ml of MC, the radioactivity in the acid-insoluble fraction remained constant, as in the control culture. However, in the presence of 5 μg/ml of MC, the radioactivity in the acid-insoluble fraction decreased to the extent of 60% of the initial amount. Within 2 hr, the radioactivity of the acid-soluble fraction also diminished progressively. Thus, cells treated with a bactericidal level of MC lost some of their DNA into the surrounding medium after prolonged incubation.

Effects of MC on the uptake of uracil-2-C^{14} by *E. coli* B are shown in Fig. 4a. The rate of the incorporation in the presence of 5 μg/ml of MC gradually leveled off from 30 to 60 min, and after 2 hr the amount of uracil-2-C^{14} in the acid-insoluble fraction decreased. The radioactivity incorporated into the acid-soluble fraction also decreased progressively.

The degradation of RNA in the presence of 5 μg/ml of MC is shown in Fig. 4b. More than 50% of the radioactivity originally present in the acid-insoluble fraction was lost to the medium during the 2-hr exposure period. However, the radioactivity in the control cells remained almost constant throughout the 2-hr incubation period.

Protein metabolism was less sensitive to MC than was DNA or RNA. In the presence of 5 μg/ml of MC, incorporation of histidine-2-C^{14} proceeded normally for 60 min and then leveled off.

These results correspond closely to those obtained by chemical analyses of cell components, and indicate that the synthesis of not only DNA

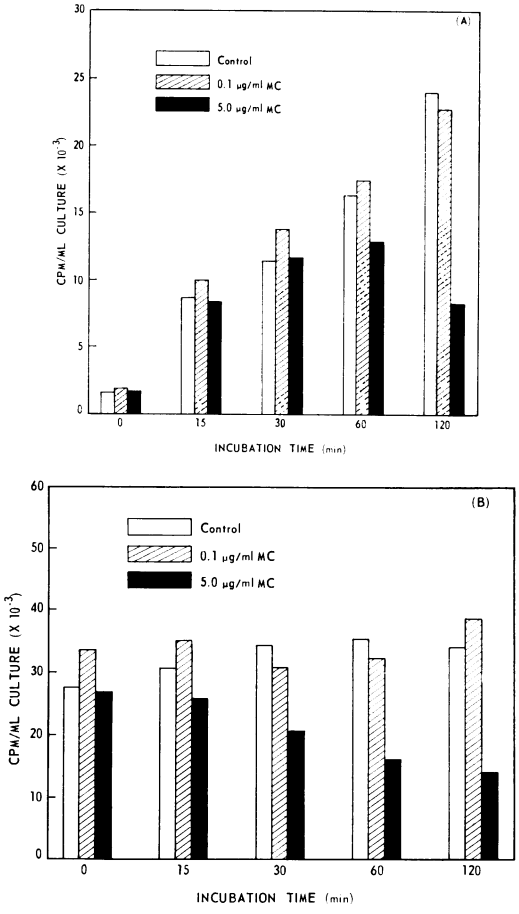

FIG. 4. *Effects of MC on the synthesis and the stability of RNA in Escherichia coli B. (a) Incorporation of uracil-2-C^{14} into RNA. (b) Release of radioactive uracil from RNA prelabeled with uracil-2-C^{14}.*

but also RNA and even protein is inhibited by the bactericidal level of MC, and that nucleic acids (both DNA and RNA) become labile or are degraded during the treatment with the antibiotic.

Effects on β-galactosidase induction. Samples of a unit volume were removed periodically from an actively growing culture (in glucose) of *E. coli* B, and β-galactosidase was induced as described in Materials and Methods. The capacity of the culture to synthesize β-galactosidase was found to increase proportionally to the increase in the turbidity of the culture during the 2-hr incubation period; in other words, the differential rate of enzyme synthesis remained constant for this period (Fig. 5). (The "capacity" to synthesize β-galactosidase was defined as the amount of the enzyme formed per unit volume of noninduced

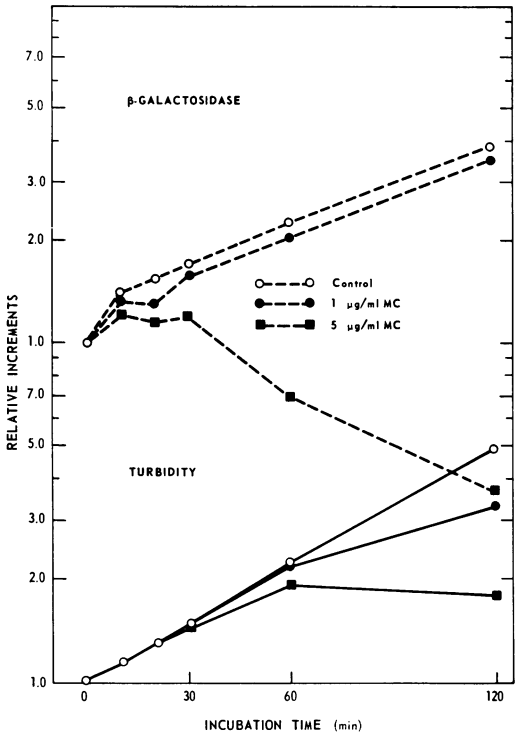

FIG. 5. *Changes in turbidity and capacity to synthesize β-galactosidase by Escherichia coli B during treatment with MC. Induction and measurement of β-galactosidase were performed as described in Materials and Methods.*

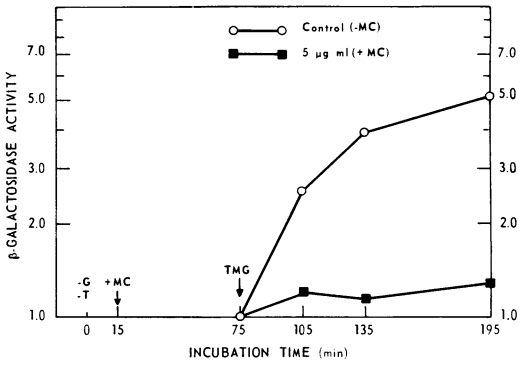

FIG. 6. *Capacity for β-galactosidase induction of thymine-starved Escherichia coli 15T⁻H⁻ under nongrowing conditions. Washed cells in 100 ml of DM medium free from carbon source, supplemented with 156 μg/ml of histidine were divided into two portions and incubated. After 15 min, 5 μg/ml of MC was added to one of the cultures, and the incubation was continued. After 75 min, 10^{-5} M thiomethyl β-galactoside was added to induce β-galactosidase.*

cultures during 30 min of incubation with thiomethyl β-galactoside under nongrowing conditions.) However, in a culture treated with 5 μg/ml of MC, the capacity remained at the same level for the first 30 min, and then was lost exponentially. The cells treated for 60 min could synthesize only a trace amount of the enzyme. MC at 1 μg/ml had little effect on the capacity to synthesize β-galactosidase.

This loss of the capacity of the MC-treated cells to form β-galactosidase was apparently not a consequence of the inhibition of DNA synthesis, since cells subjected to thymineless death still retained the capacity to synthesize β-galactosidase (13.) As show in Fig. 6, when β-galactosidase was induced in cultures of thymine-starved *E. coli* 15T⁻H⁻ in DM medium free from a carbon source, the culture without MC synthesized β-galactosidase continuously, whereas the one with 5 μg/ml of MC could not form the enzyme at all. Under these conditions, no net synthesis of DNA took place in either culture.

DISCUSSION

It is well known that many alkylating agents, at low concentrations, suppress cell mitosis (cytostatic effect), whereas at high levels irreparable damage occurs and the cells are killed (cytoxic effect; 17). The same thing seems to be true in *E. coli* after treatment with MC. There is an apparent shift in the effects of MC on *E. coli* from bacteriostatic to bactericidal, depending on the concentration of the antibiotic. In the case of *E. coli* B, the threshold concentration of MC is about 0.1 μg/ml.

In *E. coli* B exposed to a bacteriostatic level (less than 0.1 μg/ml) of MC, the growth and macromolecular syntheses continue at nearly the same rate as in untreated control cells, but cell division is inhibited, resulting in filament formation. Increase in the colony-forming units of the culture is greatly suppressed. Protein synthesis, measured by β-galactosidase induction, is not inhibited at all by the treatment, and, as shown in the following paper (23), nuclei of the filaments remain normal in appearance. The effects are partially reversible, and the cells can recover from damage after 15 min of treatment. Thus, the only apparent effect of MC at this low level is filament formation.

On the other hand, in the presence of a high level (5 μg/ml) of MC, DNA is apparently the most sensitive component of *E. coli* B, and its net synthesis is almost completely inhibited from the outset of the treatment. However, the effects are not restricted to DNA, and formation of RNA

and even protein is greatly suppressed after 30 to 60 min. The effect on RNA synthesis is manifested when cultures transferred from a glucose medium into a lactose medium are then treated with the antibiotic. In this case, RNA and DNA synthesis are both completely inhibited. This is probably owing to an initial lag period during which MC acts on RNA metabolism.

Degradation of nucleic acids during the treatment with a bactericidal level of MC was observed, as reported by Reich et al. (26), and degraded materials were excreted mostly into the medium. However, the degradation of nucleic acids occurs in the later phase of the treatment, and may result secondarily from alkylation and labilization of the nucleic acids with MC (35). The degradation of nucleic acids by MC is not restricted to DNA and is more conspicuously observed with RNA rather than DNA. Decrease of cellular contents detected by chemical analyses is obvious only with RNA, not with DNA. This might be due to the degradation of ribosomal particles (33). According to Kersten et al. (14, 15), MC acts primarily by degrading RNA, probably sRNA, whose degradation will in turn result in the activation of deoxyribonucleases, followed by the breakdown of DNA. It is now difficult to determine whether the degradation of RNA or of DNA is caused first by MC. The degradation of both components becomes apparent only after a certain lapse of the treatment, and the possibility cannot be excluded that nucleic acid molecules become labile after being extensively alkylated with mitomycin C, rather than by enzymatic attack.

Concomitantly with the degradation of nucleic acids, the capacity of the treated cells to synthesize β-galactosidase diminishes progressively, reflecting impairment in protein synthesis. Shiba et al. (19) concluded from their O_2-uptake studies that MC had no effect on β-galactosidase synthesis. On the other hand, the rate of β-galactosidase formation by cells pretreated with MC was reported to decrease with increased duration of the treatment (3). According to Cummings (5), net synthesis of β-galactosidase by *E. coli* B/r was stopped about 20 min after the addition of MC. Similarly, penicillinase induction in *Staphylococcus aureus* was inhibited abruptly after 40 min of nearly normal synthesis in the presence of MC (4). Thus, synthesis of inducible enzymes is inhibited after about 30 min of treatment with MC. It is not clear why the inhibition is delayed for 30 min, but considering the fact that nucleic acids are degraded at about the same time, it can be suggested that these two phenomena are closely related. Ribosomes in the treated cells were found to be degraded after 30 min of treatment (21).

In summary, bacteriostatic concentrations of MC cause filament formation and suppress the increase of colony-forming units without any apparent effects on nucleic acid metabolism. On the other hand, the action of MC at its bactericidal concentration is not specific for DNA, as previously believed. RNA and protein synthesis are also greatly affected during the treatment. It takes about 30 min before the effects on RNA and β-galactosidase synthesis in actively growing cells are manifested. Degradation of nucleic acids also becomes apparent 30 min after the addition of the antibiotic. The degradation is most obvious with ribosomes. Studies on the decomposition of ribosomes by MC will be published elsewhere.

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