Decomposition of Ribosomal Particles in *Escherichia coli* treated with Mitomycin C

HIROMI SUZUKI¹ AND WENDELL W. KILGORE

Agricultural Toxicology and Residue Research Laboratory, University of California, Davis, California 95616

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Exposure of cells of *Escherichia coli* to mitomycin C (5 $\mu\text{g/ml}$) resulted in a marked change in the sedimentation profiles of the cell-free extracts, indicating a specific decomposition of ribosomal particles. When the extracts were prepared in the presence of 0.01 M Mg^{++} and analyzed by sucrose density gradient centrifugations, the 100S fraction disappeared rapidly from the treated cells. The 70S ribosomes were also degraded, but more slowly, with a concomitant accumulation of a fraction having a sedimentation coefficient of about 50S. However, decomposition of the 70S ribosomes was preceded by an almost complete loss of the 50S ribosomal subunits, as revealed by sedimentation analyses in the presence of 10^{-4} M Mg^{++} . Synthesis of the ribosomes in the treated cells was also suppressed, being demonstrated by a lower incorporation of uracil-2- ^{14}C into the ribosomal fractions. However, the change in the ribosomal profile in the treated cells apparently resulted from the decomposition of pre-existing ribosomes, rather than from the inhibition of the net synthesis of ribosomes. Sedimentation analyses and chromatography of the nucleic acids extracted from the treated cells indicated extensive but delayed degradation of the ribosomal ribonucleic acid (RNA), but not of the soluble RNA or deoxyribonucleic acid fractions. Altered structure of the ribosomes in the treated cells was also indicated by their lower melting temperature, broadened thermal profile, higher electrophoretic mobility, and extreme sensitivity to ribonuclease treatment, compared with normal ribosomes. The synthesis of messenger RNA was inhibited progressively with time in the treated cells.

Mitomycin C (MC), at a bacteriocidal concentration (5 $\mu\text{g/ml}$), suppresses the formation of ribonucleic acid (RNA) and protein and results in almost complete inhibition of deoxyribonucleic acid (DNA) synthesis in *Escherichia coli* B (Suzuki and Kilgore, *Bacteriol. Proc.*, p. 40, 1964). The ability of the cells to induce β -galactosidase is also arrested during the first 30 min of treatment, and is then destroyed exponentially thereafter. Sucrose density gradient analyses of cell-free extracts have further revealed that the ribosomes are markedly affected and eventually disappear after the cells are treated with MC (14). Of the ribosomal particles, the 50S subunits disappear earlier than the 30S component. The time course of this degradation corresponds well with the loss of the capacity of the cells to form β -galactosidase.

Although most studies with MC have been centered on cellular DNA (16), there are a few

reports which deal with its effect on ribosomes. Marked loss of Palade particles was observed by electron microscopy in ascites tumor cells from mice injected with MC (4). Accumulation of small ribosomal particles was also observed in *E. coli* treated with sublethal doses of MC (5). Fenwick (1), on the other hand, found that the synthesis of ribosomal RNA was markedly depressed in MC- as well as ultraviolet (UV)-treated mammalian cells.

In the present study, the nature of the ribosomal particles formed in cells of *E. coli* B treated with 5 μg of MC per ml was characterized.

MATERIALS AND METHODS

Growth of bacteria. The organism used in this study was *E. coli* strain B. Cells were grown as described previously (15).

Preparation of cell-free extracts. Cell-free extracts were prepared by a high-pressure extrusion method. A sample of the culture (usually 100 ml) was chilled rapidly; the cells were harvested by centrifugation and were washed with 0.01 M tris(hydroxymethyl)amino-

¹ Present address: The Salk Institute for Biological Studies, San Diego, Calif. 92109

methane (Tris) chloride buffer (pH 7.3) containing magnesium acetate at a concentration of 10^{-4} to 10^{-2} M as specified. The cell pellet was resuspended in the same buffer used for the washings (5 to 10 ml) and passed through an orifice of a chilled French pressure cell at a pressure of 6,000 to 8,000 psi. Unbroken cells and cell debris were removed by several centrifugations at $20,000 \times g$ for 15 min. The supernatant solution was used as a crude cell extract (referred to as "13K15S" fraction).

Isolation of ribosomes. Ribonucleoprotein particles were isolated by means of differential centrifugations, as described by Tissières et al. (17). A crude extract (13K15S) made in 0.01 M Tris chloride buffer containing 0.01 M Mg^{++} was centrifuged at $97,000 \times g$ for 120 min in a Spinco model L ultracentrifuge. The supernatant solution was gently removed by pipette, and the yellowish brown pellet was resuspended in the same buffer used for the extraction by use of a Teflon homogenizer at 0°C. The suspension was centrifuged at $20,000 \times g$ for 15 min to remove aggregated materials, and then was recentrifuged at $97,000 \times g$ for 120 min. The sedimented particles were washed by resuspending them in the buffer, and the low and high centrifugations were repeated. The final pellet was suspended in 0.01 M Tris chloride buffer (pH 7.3) containing 0.01 M Mg^{++} , and the suspension was centrifuged at $20,000 \times g$ for 15 min. The supernatant solution thus obtained (40K120P) consisted of the 100S and the 70S ribosomes upon sedimentation analysis.

To obtain the ribosomal subunits, the final pellet described above was suspended in 0.01 M Tris chloride buffer (pH 7.3) containing 10^{-4} M Mg^{++} , and was dialyzed overnight against the same buffer in a cold room (4°C), with several changes of buffer. The dialyzed solution was then centrifuged at $20,000 \times g$ for 15 min. The supernatant solution (40K120P-D) contained the 50S and the 30S subunits of the ribosomes.

Extraction of nucleic acids. Nucleic acids were extracted from crude extracts by the phenol-sodium dodecyl sulfate method. To the cell-free extracts (13K15S) in 10^{-4} M Mg^{++} was added sodium dodecyl sulfate to a concentration of 0.5%. The mixture was left for 30 min at room temperature. The nucleic acids were then extracted by shaking the sample with an equal volume of buffer-saturated phenol (previously neutralized with 0.1 N NaOH) at room temperature for 30 min. Layers were separated by centrifugation at $12,000 \times g$ for 10 min, and the phenol layer was carefully removed. To the remaining aqueous layer was again added an equal volume of the buffer-saturated phenol, and the extraction was repeated. The aqueous layer was removed, and after adding 0.1 M NaCl, the nucleic acids were precipitated by the addition of 2 volumes of chilled absolute ethyl alcohol. The precipitate was collected by centrifugation and dissolved in 0.01 M Tris chloride buffer (pH 7.3) containing 10^{-4} M Mg^{++} , 0.1% sodium dodecyl sulfate, and 0.1 M NaCl. The alcohol precipitation step was repeated several times to remove traces of phenol. The nucleic acids were stored at -10°C , as precipitates in 66% ethyl alcohol.

Ribosomal RNA was extracted in the same way

as above from the isolated ribosome preparation (40K120P).

Pulse and chase experiments. Pulse experiments (2) and chase experiments (7) were performed to study the biosynthesis of metabolically stable and unstable RNA during the treatment with MC.

Cells were exposed to a 30-sec pulse with uracil- 2^{14}C ($5 \mu\text{C}/100 \text{ ml}$; specific activity, 23 mc/mole). At the end of the labeling period, 0.01 M sodium azide was added, and the culture was rapidly poured onto crushed ice of 0.01 M Tris chloride buffer (pH 7.3) containing 0.01 M Mg^{++} . The pulse-labeled cells were then harvested, and the nucleic acids were prepared as described above.

For the purpose of chase experiments, a 60-sec pulse with uracil- 2^{14}C ($2.5 \mu\text{C}/100 \text{ ml}$) was followed by a chase with a 400-fold excess of cold uracil ($50 \mu\text{g}/\text{ml}$) for 15 min. The labeled cells were rapidly chilled, and the cell-free extracts (13K15S) were prepared in the presence of 10^{-2} M Mg^{++} in 0.01 M Tris chloride buffer (pH 7.3).

Sedimentation analysis. The sucrose density gradient was prepared in a centrifuge tube by layering sucrose solutions with pipettes (0.75, 1.5, 1.5, and 0.75 ml of 20, 15, 10, and 5% sucrose solutions made in the buffer used to prepare the extract were layered in that order, and the layered solution in a centrifuge tube was allowed to stand overnight in the cold for diffusion to form a linear gradient). A 0.5-ml sample was gently layered over 4.5 ml of 5 to 20% sucrose gradient in the medium as specified. The centrifugation was carried out at $115,000 \times g$ at 4°C in a Spinco model L ultracentrifuge, with a SW 39L rotor. After centrifugation (5 hr for nucleic acids; 75 to 120 min for ribosomes), the contents of the tubes were dripped out from the bottom through a hypodermic needle (gauge 20) into fractions of 10 drops each. The fractions, diluted to 3 ml with distilled water, served for the UV-absorption and the radioactivity determinations.

Chromatographic analysis. Nucleic acids were analyzed by chromatography on methylated albumin kieselguhr, prepared according to a simplified procedure of Mandel and Hershey (8). The columns were prepared as follows. A suspension of 10 g of Hyflo Supercel (the Johns-Manville Co., New York, N.Y.) in 50 ml of 0.1 M NaCl was boiled to expel the air and then rapidly cooled to room temperature. To the suspension was added 2.5 ml of 1% aqueous solution of methylated albumin, and the mixture was stirred for 15 min. An additional 10 ml of 0.1 M NaCl was added, and agitation was continued for 5 min. The suspension was then transferred to a chromatographic tube ($25 \times 400 \text{ mm}$). The column was washed with several 20-ml portions of 0.1 M NaCl.

A sample, containing less than 5 mg of nucleic acids in 10 ml of 0.1 M NaCl, was applied to the column and washed with 50 ml of 0.1 M NaCl. Nucleic acids were then eluted with a linear gradient of NaCl (usually from 0.3 to 1.0 M per 500 ml) at a flow rate of about 2 ml/min, and fractions of 5 ml were collected. All saline solutions were buffered with 0.05 M phosphate buffer (pH 6.7) or with 0.01 M Tris chloride buffer (pH 7.3).

The concentration of NaCl in the eluates was determined with a Buchler-Cotlove Chloridometer.

Starch-block electrophoresis. Cell extracts (13K15S) prepared in the presence of 10^{-4} M Mg^{++} were analyzed by starch block electrophoresis, according to the method of Nomura et al. (9).

A slurry of washed potato starch (Dane's Nutrition, Fresno, Calif.) in 0.03 M Tris chloride buffer (pH 7.8) containing 10^{-4} M Mg^{++} was poured into a plastic tray ($66 \times 390 \times 12$ mm), and was placed in a migration chamber (Research Specialties Co., Richmond, Calif.). The sample in a thick paste of starch was spooned into a slot, cut at 10 cm from the cathode. The developing buffer was 0.03 M Tris chloride buffer (pH 7.8) containing 10^{-4} M Mg^{++} . It was developed at 4 C for 12 hr at 350 v with a current of about 5 ma. After the run, the surface of the starch was blotted with a strip of filter paper. The starch block was then sliced into 0.5- to 1.0-cm sections, depending upon the position relative to the ribosome region. Ribosomes and nucleic acids were extracted from the starch in test tubes by shaking vigorously with 5 ml of the buffer used for the development. The supernatant fluid was decanted after the starch settled, and was used for further analyses.

Measurements of hyperchromicity. Increases in UV absorption at 260 $m\mu$ after alkaline hydrolysis, heating, and urea treatment were compared between the ribosomal preparations from normal cells and from cells treated with MC.

Ribosomal suspensions (40K120P) in 0.01 M Mg^{++} and 0.01 M Tris chloride buffer (pH 7.3) were diluted with the buffer to give an absorbance at 260 $m\mu$ of about 0.4. Alkaline hydrolysis was performed by mixing 2 ml of the sample with 1 ml of 1 N KOH, and incubating the mixture at 37 C for 20 hr. After hydrolysis, the solution was neutralized with 3 N HCl, and filled to 4 ml with the buffer.

Thermal denaturation was carried out by heating a mixture of 2 ml of sample and 2 ml of buffer in a water bath on a heater at a rate of 1 C/min up to 90 C. A thermal transition curve was obtained by plotting the change of absorbance at 260 $m\mu$ at 5 C intervals against the temperature.

For the urea treatment, a 2-ml sample was mixed with 2 ml of 8 M urea, and the mixture was left at room temperature for 30 min.

The hyperchromicity was expressed as a percentage increase of the absorbance at 260 $m\mu$ read against the blank over the control without the treatment. All the absorbance readings were made with a Hitachi-Perkin-Elmer UV-VIS spectrophotometer 139.

The sensitivity of the ribosomes to a mild treatment with ribonuclease was also measured. A sample of 2 ml was mixed with 0.2 ml of 100 μ g/ml ribonuclease (pancreatic ribonuclease, crystalline, five times; Nutritional Biochemicals Corp., Cleveland, Ohio), and incubated for 30 min in an ice bath. Acid-insoluble fractions were precipitated by the addition of 1 ml of cold 2 N perchloric acid and were removed by passage through a membrane filter (type AA; Millipore Corp., Bedford, Mass.) The absorbance at 260 $m\mu$ of the acid-soluble fraction was read against the blank after dilution to 4 ml with buffer.

Measurement of radioactivity. Radioactivity present

in fractions collected by sedimentation analysis, chromatography, or electrophoresis was determined in a scintillation counter. The fractions were acidified by adding cold 70% perchloric acid to a final concentration of 5%, and the precipitated nucleic acids were collected by passing the mixture through a membrane filter (type AA). The filter was air-dried and placed in a counting vial. Approximately 15 ml of scintillating fluid was added to each vial, and the radioactivity was determined with a Packard Tri-Carb liquid scintillation counter. [The scintillation liquid used had the following composition: 2,5-diphenyloxazole (PPO), 0.3 g; 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 0.3 g; naphthalene, 150 g; *p*-dioxane, 1,140 ml; absolute ethyl alcohol, 720 ml; toluene, 1,140 ml (Y. Vadya, *personal communication*).]

Chemicals. MC was purchased from Sigma Chemical Co., St. Louis, Mo. Uracil- $2^{14}C$ was obtained from Calbiochem, Los Angeles, Calif.; albumin, from Nutritional Biochemical Corp. (bovine serum albumin, fraction V). Reagent-grade or chemically pure grade chemicals were used throughout.

RESULTS AND DISCUSSION

Decomposition of ribosomes. When the cell extracts were prepared in the presence of 0.01 M Mg^{++} and analyzed by sucrose density gradient centrifugations, most of the ribosomal particles in the normal cells were present as 100S particles. Also, a relatively smaller amount of the 70S ribosomes was detected as a shoulder of the 100S peak. However, in cells treated with MC (5 μ g/ml), the 100S particles were degraded after 30 min, and the ratio of 100S to 70S ribosomes decreased progressively with time (Fig. 1). Thus, the 100S peak was hardly detected in the sedimentation profile of the extracts obtained from cells treated for 60 min, whereas the 70S ribosomes were still recognized as a distinct peak. At the same time, there appeared a new peak with a sedimentation coefficient of about 50S. This peak increased progressively as the treatment proceeded, and the 70S peak decreased.

Polyribosomes, clusters of ribosomes held together by RNA strands, are now believed to be the active center of protein synthesis (11). Therefore, the almost complete loss of the larger particles from the cells treated for 60 min with a bactericidal concentration of MC implies the failure of the treated cells to synthesize proteins. Actually, the formation of proteins in the treated cells was found to level off after 45 to 60 min of the treatment and the capacity of the treated cells to induce β -galactosidase was greatly impaired after 30 min.

Accumulation of the 50S fractions was noticed with the advance of treatment. However, it is unlikely that the accumulated particles represent the intact 50S subunits, since there remained only a trace amount of the 50S subunits after 60 min of exposure to MC, as revealed by sedimentation

FIG. 1. Sedimentation analyses of extracts of *Escherichia coli* B treated with MC. A cell suspension in 600 ml of DM medium was incubated in the presence of MC (5 $\mu\text{g}/\text{ml}$). Samples of 90 ml were removed periodically, and the cell extracts were prepared in 5 ml of 0.01 M Tris chloride buffer (pH 7.3), containing 0.01 M Mg^{++} . Samples of 0.5 ml were layered on 4.5 ml of sucrose density gradients made in 0.01 M Tris chloride buffer (pH 7.3), 0.01 M Mg^{++} . The tubes were then centrifuged for 75 min at $115,000 \times g$ at 4 C.

analyses of the cell extracts in the presence of 10^{-4} M Mg^{++} (14). It is also unlikely that the decomposition of ribosomes caused by MC treatment proceeds through a definite intermediary stage, like chloramphenicol particles accumulated in *E. coli* treated with chloramphenicol (10). The particles collected as a sediment at $97,000 \times g$ in the presence of 0.01 M Mg^{++} , which showed the same distribution pattern of the 70S and 50S fractions as with the crude extracts upon sedimentation analysis, had the same RNA-protein ratio as that found with the normal ribosomes. After prolonged incubation with MC (5 $\mu\text{g}/\text{ml}$), all peaks in the sedimentation profile of the extracts merged into one broad peak, implying a heterogeneous population of the particles in sizes.

It is thus clear that the ribosomal particles are decomposed in *E. coli* B treated with a bactericidal concentration of MC. Although the synthesis of ribosomes by the treated cells was almost completely suppressed, after 90 min (14), the apparent loss of the ribosomal particles shown in sedimentation profiles is not simply due to the inhibition of the net synthesis of ribosomes, but seems to be caused as a result of the action of MC on pre-existing ribosomes, probably through the alkylation of the RNA moiety of the ribosomes (18), because in the MC-treated cells the amount of the 50S subunits decreased much below that initially present within 60 min of the treatment (14).

Chase experiments with uracil-2- ^{14}C showed that both the 30S and 50S subunits were equally

labeled in the control cells during the 15-min chasing period, and the sedimentation profiles of the radioactivity and the UV-absorption were parallel, indicating that the synthesis of both particles was taking place at the same rate. However, in the treated cells, the incorporation of uracil into the particles was greatly diminished with the progress of the treatment (14). The amount of ^{14}C incorporated into the 50S subunits was much less than that into the 30S. As the sedimentation profile of the radioactivity was parallel to that of the UV absorption, the lower incorporation of uracil into the 50S particles in the presence of MC might be caused by the decomposition of the particles into the smaller fractions, rather than by the preferential inhibition of the 50S synthesis.

Sedimentation analyses of the extracts prepared in the presence of 0.01 M Mg^{++} from treated cells labeled with uracil- $2\text{-}^{14}\text{C}$ also showed the low but parallel distribution of UV absorption and radioactivity.

Degradation of nucleic acids. As shown in the previous section, ribosomal particles in the MC-treated cells are degraded progressively with time and are eventually lost from the cells. By comparing the time course of the degradation of the particles shown between the sedimentation analyses in the presence of 10^{-4} and 0.01 M Mg^{++} , it was noticed that, after 60 min of treatment, there was only a trace amount of the 50S subunits detected in the presence of 10^{-4} M Mg^{++} , whereas substantial amounts of the 70S ribosomes still existed in the sedimentation profile in 0.01 M Mg^{++} . As the degradation of the subunits should result in the degradation of the ribosomes themselves, the selective degradation of the 50S subunits is not adequate to explain the degradation of the ribosomal particles during the treatment with MC. Another possibility is that MC causes some structural distortion of the particles, probably by alkylating the RNA moiety, resulting in a loosened structure. Thus, the particles sediment more slowly than the intact particles in the low Mg^{++} concentration, but, in the presence of high concentration of Mg^{++} , the compact structure of the modified particles can be preserved to some extent because of the high ionic strength (19). Experiments were therefore carried out to study the effects of MC on the structure of RNA in the ribosomal particles.

Nucleic acids extracted by the phenol method consisted mainly of ribosomal RNA, soluble RNA, and DNA. The ribosomal RNA was separated by sedimentation analysis into two components, 23S and 16S RNA, in the ratio of about 2:1. However, in cells treated with MC ($5\text{ }\mu\text{g/ml}$), the ratio decreased progressively as

the treatment proceeded, and the peak having a sedimentation coefficient of about 8S increased (Fig. 2). At the same time, the separation of the two original peaks became more difficult, and, after 2 hr of the treatment, all peaks in the sedimentation profile were intermingled to form one broad peak, suggesting a polydisperse distribution of the sizes of nucleic acids. Thus, ribosomal RNA was probably randomly fragmented after prolonged incubation with MC. However, the degradation of ribosomal RNA during MC treatment did not proceed as fast as was observed with the ribosomal subunits. After 60 min, when almost no 50S peak was present, there were still two distinct peaks of the ribosomal RNA in the sedimentation profile of nucleic acids from the treated cells. The 23S RNA is known to originate exclusively from the 50S subunits (6). Therefore, the presence of 23S RNA in the treated cells after 60 min of the treatment indicates that the apparent loss of the 50S subunits from the treated cells does not represent a complete breakdown of the particles.

Nucleic acids were also fractionated by column chromatography, with the use of methylated albumin kieselguhr. Results of the chromatography of nucleic acids extracted from the cells grown for 90 min with and without MC are shown in Fig. 3. The elution pattern of the nucleic acids from the treated cells was essentially the same as that of the control, though the amounts under each peak were markedly reduced. Soluble RNA and DNA fractions of the treated cells formed peaks as sharp as those of the control, indicating no extensive degradation of the molecules by the treatment with MC. The most marked effect of MC on the chromatographic behavior of nucleic acids was observed with the ribosomal RNA fractions. These were eluted at nearly the same range of NaCl concentrations as those of the control cells, but the peaks became significantly widened and flattened. This was more clearly shown when the RNA extracted from the ribosome preparations was subjected to chromatography, eluted by a narrower range of NaCl (0.6 to 0.9 M). Ribosomal RNA from the control cells formed two distinct peaks, whereas those from the treated cells were eluted by the lower concentration of NaCl and were distributed throughout the wider range of NaCl concentration compared with the RNA from the control. The wide elution pattern of the RNA probably indicates that the RNA molecules are randomly fragmented during the treatment with MC. However, as there were no peaks other than the normally present 16S and 23S peaks, it is unlikely that the molecules were degraded to subunits or to small molecules with a definite size.

FIG. 2. Sedimentation analyses of nucleic acids prepared from *Escherichia coli* B treated with MC. A cell suspension in 600 ml of DM medium was incubated in the presence of MC (5 μ g/ml). Portions of 100 ml were removed periodically from the culture, and the cell extracts were prepared in 5 ml of 0.01 M Tris chloride buffer (pH 7.3), containing 0.01 M Mg^{++} . Nucleic acids were then extracted by the phenol method. The final alcohol precipitates of the nucleic acids were dissolved in 2 ml of 0.01 M acetate buffer (pH 5.1) containing 0.1 M NaCl. Samples of 0.2 ml were layered on 4.8 ml of sucrose density gradients made in 0.01 M acetate buffer (pH 5.1) and 0.1 M NaCl. The tubes were then centrifuged for 5 hr. at $115,000 \times g$ at 4 C.

Similar elution patterns were obtained with nucleic acids from the cells treated for 60 or 120 min.

By utilizing this type of column chromatography, it was possible to estimate the amounts of each nucleic acid fraction by measuring the area under the respective peaks. Figure 4 shows the increase of each fraction in the culture during the treatment with 5 μ g of MC per ml. The amount of nucleic acids was measured by absorbance at 260 m μ (phenol-extracted fractions). The amounts estimated above and those obtained by chemical analyses of the cultures showed a good correla-

tion, regardless of the treatment with MC. The yield of the treated nucleic acids after the phenol extraction was the same as that of the control.

The total amount of nucleic acids increased progressively for the first 40 min and then gradually leveled off. The amount of DNA remained almost constant throughout the 2-hr treatment. The soluble RNA fraction was less sensitive to the action of MC, at least with regard to its chromatographic behavior, than were the other nucleic acid components.

The total amount of the ribosomal RNA fraction in the treated cells increased in parallel with

FIG. 3. Chromatographic profiles of nucleic acids prepared from *Escherichia coli* B incubated in the absence and presence of MC. A cell suspension in 200 ml of DM medium was divided into two portions, to one of which was added MC at 5 μ g/ml. The cultures were incubated for 90 min. Cell extracts were prepared in 10 ml of 0.01 M Tris chloride buffer (pH 7.3) containing 10^{-4} M Mg^{++} , and nucleic acids were extracted by the phenol method. The final alcohol precipitates of the nucleic acids were dissolved in 10 ml of 0.1 M NaCl. A portion of 5 ml (as RNA, 4.4 mg in the control, and 2.1 mg in the treated) was subjected to column chromatography on methylated albumin kieselguhr. The concentration gradient of NaCl was from 0.4 to 1.1 M per 500 ml. All saline solutions were buffered by 0.05 M phosphate buffer (pH 6.7).

the growth for the first 60 min and then began to decrease. It should be noted that the amount of ribosomal RNA shown in Fig. 4 was the total amount under the peak after the chromatography, and was not the amount of the intact molecules. As shown by the spread profile of Fig. 3, it is clear that, although the macromolecular nature is still conserved, the RNA molecules are qualitatively altered from those in the control cells. The decrease in the amount of the ribosomal RNA indicates the extensive breakdown of the RNA molecules.

FIG. 4. Effects of MC on nucleic acid components of *Escherichia coli* B. The samples of nucleic acids used in experiments for Fig. 2 were reprecipitated with ethyl alcohol, and the precipitates were dissolved in 5 ml of 0.1 M NaCl. The absorbancies of each sample were measured at 260 mμ with 0.1 ml of the samples diluted to 5 ml with water. The remaining volume of each sample was then subjected to column chromatography on methylated albumin kieselguhr. The columns were eluted with a concentration gradient of NaCl from 0.3 to 0.9 M per 500 ml. All saline solutions were buffered with 0.01 M Tris chloride buffer (pH 7.3). The amount of each fraction of nucleic acids was estimated as a sum of the absorbances at 260 mμ in the fraction and was expressed as a ratio to the initial amount. The growth of the treated culture was measured as turbidity at 550 mμ.

It was also noted that the recovery of the nucleic acids from the treated cells was significantly lowered with the progress of the MC treatment. About 20% of the nucleic acids applied to the column, obtained from the cells treated for 2 hr with MC (5 μ g/ml), could not be recovered from the column even after repeated washings with 1 M NaCl. This lowered recovery may partly explain the decrease in the amount of ribosomal RNA after 90 min of the treatment.

Properties of ribosomal particles from treated cells. When cell-free extracts from 90-min cultures with and without MC were subjected to starch-block electrophoresis, ribosomal particles of the treated cells were found to migrate slightly faster than those of the control cells, whereas free nucleic acids in both cultures showed the same electrophoretic mobility (Fig. 5). RNA in the treated cells was heavily labeled by incubating the cells in the presence of MC and uracil- $2^{14}C$ for 90 min. Cell-free extracts of the treated cells were then mixed with those of the unlabeled control

FIG. 5. Starch-block electrophoresis of cell extracts. A cell suspension of *Escherichia coli* B in 200 ml of DM medium was divided into two portions, to one of which was added MC at 5 μ g/ml and uracil-2- ^{14} C (5 μ g/100 ml; 22.7 mc/mmole). The cultures were then incubated for 90 min, and the cell extracts (13K15S) were prepared in 5 ml of 0.01 M Tris chloride buffer (pH 7.3) containing 10^{-4} M Mg^{++} . A mixture of 1 ml of the control and 0.2 ml of the treated extracts was subjected to starch-block electrophoresis as described in Materials and Methods. Measurements of UV absorption and radioactivity in sectioned fractions are described in Materials and Methods.

cells in the ratio of 2:10, so that the contribution of the extracts of the treated cells to the UV-absorption of the mixture was less than 8%. The peaks of the radioactivity and of the UV absorption, therefore, represent those of the treated cells and of the control, respectively. Both peaks at the nucleic acid region were at the identical position, but the peak of the radioactivity at the ribosome region was slightly shifted to anode. When radioactively labeled extracts of the control cells were mixed with unlabeled extracts of the control and subjected to the electrophoresis in the same way, the profiles of the radioactivity and of the UV absorption were identical. Therefore, it is unlikely that the shift is due to an isotope effect. The faster migration rate of the ribosomal particles in the treated cells implies that they are structurally altered and charged more negatively than the normal ones.

It has been reported (12) that ribosomes show hypochromic effects due to the presence of the partial helical structure in the RNA moiety. Any

alterations in the RNA structure of ribosomes will, therefore, result in changes of the hyperchromicity shown by the ribosomes. Thus, increases of the UV absorption at 260 $m\mu$ of ribosome suspensions (40K120P) after various treatments were measured with samples prepared from the control and the MC-treated cells. The increases in UV absorption of these preparations after urea, alkali, and heat treatments are shown in Table 1. The degrees of the hyperchromicity of the preparation from the treated cells after these treatments did not deviate significantly from those of the control ribosomes. An increase of the UV absorption of ribosomes in a high concentration of urea, which replaces hydrogen bonds, is due to the breakdown of hydrogen bonds in ribosomal particles. The hyperchromicity after alkaline hydrolysis and after slow heating to 90 C, which activates the latent ribonucleases, is due to the complete hydrolysis of the RNA molecules, in addition to the breakdown of hydrogen bonds. Therefore, the same hyperchromicity shown by the ribosomal preparations from the control and treated cells indicates that the number of hydrogen bonds and the degree of polymerization of

TABLE 1. Effects of MC on hyperchromicity of ribosomes^a

Treatment sample	Alkaline hydrolysis	Urea, 4 M	Heating to 90 C	T_m	Ribonuclease
	%	%	%	C	%
40K120P: control.....	67.0	24.0	60.0	70.5	17.7
40K120P: treated.....	53.5	22.7	59.0	62.5	78.5

^a A cell suspension of *Escherichia coli* B in 200 ml of DM medium was divided into two portions, to one of which MC was added at 5 μ g/ml. The cultures were incubated for 90 min, and ribosomes (40K120P) were isolated from the cultures in 5 ml of 0.01 M Tris chloride buffer (pH 7.3) containing 0.01 M Mg^{++} . The preparations were diluted with the same buffer to give absorbances at 260 $m\mu$ of about 0.4. Absorbances of the samples were measured after alkali, urea, and heat treatments, performed as described in Materials and Methods, and expressed as per cent increases over the absorbances of the controls (the same samples diluted twofold with the buffer). The melting temperature (T_m) was deduced from Fig. 6. Ribonuclease treatment of the samples was carried out as described in Materials and Methods, and the absorbancies at 260 $m\mu$ of the acid-soluble fractions were read and expressed as a percentage of the absorbancies of the same samples after alkaline hydrolysis.

nucleotides in the RNA moiety are of about the same order of magnitude.

However, there was a significant difference detected in the melting temperatures of ribosomes (Fig. 6). When a ribosomal suspension of the control cells was subjected to slow heating and the increase of the UV absorption at 260 $m\mu$ was plotted against the temperature, the absorbance increased abruptly at 60 C and reached a maximum at 80 C. The melting temperature was about 70 C. However, with a ribosomal preparation from the treated cells, the thermal profile did not form the typical sigmoidal curve shown with the normal ribosomes. The UV absorption began to increase gradually from a much lower temperature, and the melting temperature was significantly lower (about 62 C) than that shown by the normal ribosomes. This suggests that the hydrogen bonds present in the ribosomal particles in the treated cells are more labile and dissociate more easily than those in the normal ribosomes.

The most marked difference in the properties of ribosomes from the treated cells compared with the normal ribosomes was noticed in their behavior toward a mild ribonuclease treatment. Column 5 of Table 1 shows the percentage of UV-absorbing materials made acid-soluble after the ribonuclease treatment. Normal ribosomes were rather resistant to the action of ribonuclease, and only 18% of the total UV absorption of a ribosome suspension of the control cells became acid-soluble after 30 min of incubation with 10 μ g

of ribonuclease per ml at 0 C. A sedimentation analysis of the preparation after the ribonuclease treatment showed that about one-half of the originally present 100S fraction was transformed into the 70S ribosomes, but there was a very small accumulation of nonsedimentable fractions detected in the profile after the treatment. On the other hand, with ribosomal particles obtained from the MC-treated cells, a substantial portion of the UV-absorbing materials (nearly 80%) in the preparation became acid-soluble under the same conditions. A sedimentation analysis revealed that a fraction sedimenting around the 70S region diminished greatly and a fraction around the 50S region disappeared almost completely after the ribonuclease treatment. Concomitantly, a large fraction of the nonsedimentable materials were accumulated in the preparation. It is thus clear that the ribosomal particles in the MC-treated cells were structurally modified so that they became more vulnerable to the action of ribonuclease than the normal ribosomes. There was no detectable shift in the UV-absorption spectra of ribosome preparations of the control and MC-treated cultures.

Effects on the synthesis of RNA. All of the effects of MC on ribosomes shown above are apparently due to the interaction of MC with preformed ribosomal particles, and are not due to the inhibition of their synthesis by MC. However, as shown by chase experiments, the synthesis of ribosomes also appears to be greatly suppressed during the treatment.

It is now believed that all the cellular RNA and proteins are formed by transcribing genetic codes imprinted on DNA. Therefore, if MC forms complexes covalently with DNA molecules, either monofunctionally or bifunctionally (16, 18), it is very likely that the transcription of DNA to RNA is blocked at a point of the attachment with MC, resulting in the inhibition of RNA synthesis. To study the effects of MC on the synthesis of RNA, pulse experiments with uracil- 2^{14} C were carried out.

Exponentially growing control cultures incorporated uracil- 2^{14} C into acid-insoluble fractions very rapidly in their early growth phase. The rate was then diminished after 90 min, probably because of the slow-down in the synthesis of RNA and proteins in the later phase of growth. The rate of uracil uptake by the MC-treated cells was comparable to that of the control cells for the first 20 min of treatment, but, thereafter, the rate decreased markedly, and only a trace of the radioactivity was taken up by the cells treated for 2 hr. Incorporation of uracil into the acid-soluble fractions was also suppressed significantly after 40 min.

FIG. 6. Thermal profile of ribosomes. Samples of the ribosome preparations [in 0.01 M Tris chloride buffer (pH 7.3) containing 0.01 M Mg^{++}] used in the hyperchromicity measurements were subjected to slow heating to 90 C. The absorbance at 260 $m\mu$ was read at 5 C intervals, and the ratio of the absorbance to that at 30 C was plotted against the temperature.

Sedimentation analyses of the rapidly labeled RNA of *E. coli* B grown with and without MC are shown in Fig. 7. Most of the pulse-labeled RNA sedimented between the 16S RNA and DNA regions. Sedimentation profiles of pulse-labeled RNA from the MC-treated cells were similar to those of the control cells. However, the amounts of labels incorporated diminished greatly with the progress of MC-treatment. Thus, the synthesis of RNA took place normally for the first 30 min of the treatment with 5 μ g of MC per ml, but it was gradually suppressed, and after 90 min the synthesis in the treated cells was almost completely inhibited.

Rapidly labeled RNA can be fractionated

roughly into four fractions by column chromatography on methylated albumin kieselguhr (3). The elution pattern of the pulse-labeled RNA, prepared from the 60-min cultures grown with and without MC, was not particularly affected by the MC treatment, and there was still noticeable incorporation into four fractions of rapidly labeled RNA, though the amounts incorporated in each fraction were significantly lowered.

As a conclusion, a bactericidal level of MC (5 μ g/ml) caused a delayed decomposition of ribosomes, as well as the inhibition of the net synthesis of DNA and also RNA. This decomposition was probably caused by some conformational changes, like unfolding or unpacking, of

FIG. 7. Sedimentation analyses of rapidly labeled RNA. A cell suspension of *Escherichia coli* B in 600 ml of DM medium was divided into two portions, one as a control without MC and the other for the treatment with MC (5 μ g/ml). After a given time of incubation, portions of 100 ml were removed and given a 60-sec pulse with uracil-2- ^{14} C. The nucleic acids were prepared as described in Materials and Methods and dissolved in 5 ml of 0.01 M acetate buffer (pH 5.1) containing 0.1 M NaCl. Sedimentation analyses were carried out as in Fig. 4. For radioactivity measurements, fractions were placed directly into vials, and the radioactivity was measured by a scintillation counter. Profiles of UV absorptions were obtained with fractions from different runs of the same samples.

the particles. As a result of these changes, the particle became susceptible to further degradation, and the ribosomes were eventually degraded in the treated cells.

All of the results reported herein are consistent with the assumption that MC exerts its effects by alkylating cellular nucleic acids, thereby making them labile. Aberrant chromatographic behavior of ribosomes from MC-treated *E. coli* cells was recently reported by Smith-Kielland (13).

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