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Induction by Mercuric Ion of Extensive Degradation of Cellular Ribonucleic Acid in *Escherichia coli*

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Low concentrations of $HgCl_2$ were found to induce extensive degradation of ribonucleic acid (RNA) in exponentially growing *Escherichia coli* cells but not in stationary-phase cells. Whereas 80% of cellular RNA was degraded during 90 min of incubation with 10^{-5} M $HgCl_2$ at 37 C, $HgCl_2$ caused only slight degradation in stationary cells, even when present at concentrations higher than 5×10^{-5} M. Inhibition of RNA synthesis occurred at almost the same concentration of $HgCl_2$ as degradation, and the ability of stationary-phase cells to synthesize RNA was also resistant to $HgCl_2$. The transition of cells from complete sensitivity to $HgCl_2$ to a fully insensitive state took place simultaneously with the cessation of growth. *p*-Chloromercuribenzoate was also found to induce remarkable degradation of RNA. In *E. coli* Q13, a mutant deficient for ribonuclease I, no degradation of RNA was evident, even in the exponential growth phase. 3'-Mononucleotides but not 5'-mononucleotides were found among the degradation products of cellular RNA. 2',3'-Cyclic mononucleotides were produced when RNA was degraded by the cell-free extracts of the Hg treated cells. Almost complete unmasking of the latent ribonuclease occurred in the particle fraction containing subribosomal particles of the Hg-treated cells. These data suggest that the incubation of exponentially growing *E. coli* cells with $HgCl_2$ led to the unmasking of ribonuclease I, which resulted in the extensive degradation of cellular RNA. The activation of ribonuclease by $HgCl_2$ in the isolated particulate fraction of *E. coli* K-12 which occurred in vitro suggested the presence of an Hg-sensitive inhibitor for ribonuclease I.

Autodegradation of cellular nucleic acids in microbial cells occurs frequently. Several triggering conditions, such as incubation in buffer solutions (11, 20), treatment with organic solvents such as toluene (6), irradiation with ultraviolet light or X ray (2), treatment with colicin E_2 (13) or other antibiotics (7), and exposure to elevated temperature (1, 14), have been found to induce remarkable degradation of cellular ribonucleic acid (RNA). These observations clearly indicate the existence of a specific repressing mechanism of RNA-degrading systems. However, the chemical nature of the mechanisms controlling the stability of RNA in microbial cells has not been satisfactorily clarified.

During the course of an investigation of nucleic acid degradation induced by colicin E_2, we found that mercuric ion ($HgCl_2$) and *p*-chloromercuribenzoate (PCMB) at very low concentrations induced extensive degradation of cellular RNA in exponentially growing *Escherichia coli* cells. The data indicated that ribonuclease I was a key enzyme involved in this RNA degradation and that the enzyme, originally present in a latent form, was unmasked during incubation of the cells with $HgCl_2$. Although activation of ribonuclease by thiol reagents which inactivate a ribonuclease inhibitor has been reported to occur in extracts of various animal tissues (16-19), the possibility that RNA-degrading systems in microbial cells might be controlled by thiol groups has not been investigated previously. This approach was supported by our observation that cells of *E. coli* became remarkably insensitive to $HgCl_2$ as soon as the stationary phase was reached. This indicated close relationship between the ribonuclease-repressing mechanisms and growth.

Although the marked killing effect of mercuric ion, one of the oldest germicides being used, against living organisms is understood on the basis of a thiol-blocking reaction, the molecular mechanisms by which low concentrations of mercuric ion cause poisoning are not yet estab-

lished. Our finding that mercuric ion induces degradation of RNA in *E. coli* cells may give some suggestion as to the mechanisms of mercury poisoning.

MATERIALS AND METHODS

Bacteria and media. A thymine-requiring mutant of *E. coli* K-12 W2252 (Hfr, met$^-$, λ^s) was isolated in this laboratory. *E. coli* Q13, a derivative of RNase$_{19}^-$ isolated by Gesteland (5; ribonuclease I$^-$, met$^-$, leu$^-$, trp$^-$, tyr$^-$), was generously provided by Y. Ohtaka, Institute of Physical and Chemical Research, Tokyo, Japan. *E. coli* K-12 (λ), *E. coli* B, and the Marburg strain of *Bacillus subtilis* (ATCC 6051) were obtained from the ATU culture collection (Faculty of Agriculture, University of Tokyo). Most experiments were performed on cells grown aerobically at 37 C in a synthetic medium supplemented with the required growth factors. The medium contained 0.4% glucose, 0.1% NH_4Cl, 0.88% $Na_2HPO_4 \cdot 2H_2O$, 0.3% KH_2PO_4, 0.002% $MgSO_4 \cdot 7H_2O$, and 0.1% trace elements solution (containing 880 mg of $ZnSO_4 \cdot 7H_2O$, 970 mg of $FeCl_3 \cdot 6H_2O$, 270 mg of $CuCl_2 \cdot 2H_2O$, 72 mg of $MnCl_2 \cdot 4H_2O$, 37 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 88 mg of $Na_2B_4O^7 \cdot 10H_2O$ in 1 liter) and was supplemented with 50 μg of thymine per ml and 20 μg of the required L-amino acids per ml if necessary. Glucose was sterilized separately and was mixed with the medium after cooling. *B. subtilis* was grown in the same synthetic medium fortified with 0.4 mg of Difco yeast extract and trisodium citrate$\cdot 6H_2O$ per ml. For labeling of cellular RNA, 10 μg of uracil-2-^{14}C per ml (10 μc/μmole) was added to the medium. For the preparation of ribonuclease I, *E. coli* K-12 W2252 thy$^-$ was aerobically grown at 37 C in a medium containing 2% dehydrated nutrient broth (Kyokuto, Tokyo, Japan) and 50 μg of thymine per ml.

Culture conditions. An overnight culture was diluted 1:20 into fresh medium and grown aerobically at 37 C. Growth was monitored by measuring turbidity with a Spectronic-20 colorimeter (Shimazu-Bausch & Lomb, Tokyo, Japan) at 550 nm. In this apparatus, a calculated optical density of 0.200 was equivalent to 3×10^8 cells/ml. RNA degradation in exponentially growing cells was measured by adding mercuric chloride at mid-log phase (about 6×10^8 cells/ml in the case of *E. coli* K-12 W2252 thy$^-$). Challenge with $HgCl_2$ in the early stationary growth phase was carried out as soon as rate of turbidity increase slowed down (about 1.5×10^9 cells/ml).

When RNA degradation was measured by use of radioactivity, an overnight culture was diluted 1:10 into the medium containing ^{14}C-uracil and was grown into early log phase. After the cells were washed with medium containing 10 μg of nonradioactive uracil per ml, they were suspended in fresh medium and grown at 37 C for at least 40 min. Mercuric chloride was then added at the appropriate growth phase.

Treatment of exponentially growing *E. coli* cells with ethylenediaminetetraacetate (EDTA) was performed according to the method of Leive (8).

Determination of cellular contents of macromolecules. Samples of cell suspension were mixed with an equal volume of cold 10% trichloroacetic acid, and cells were washed twice with cold 5% trichloroacetic acid. The precipitate was then extracted with 5% trichloroacetic acid for 30 min at 100 C, and the supernatant extract was saved for nucleic acid determination. RNA was determined by the orcinol reaction (4), and deoxyribonucleic acid (DNA), by Burton's method (3). The debris of the hydrolysate was dissolved in 0.3 N KOH and was tested for the amount of protein by the method of Lowry et al. (9), with bovine serum albumin as standard.

For determination of the radioactivity in the cold trichloroacetic acid-insoluble fraction of cells labeled with ^{14}C-uracil, the cells were washed with cold 5% trichloroacetic acid, dissolved in hot 1% sodium dodecylsulfate, and counted in a liquid scintillation counter.

Paper chromatography of degradation products of RNA. *E. coli* K-12 (λ) labeled with ^{14}C-uracil was chased with the fresh medium and then challenged with 2×10^{-5} M $HgCl_2$. After incubation for 2 hr at 37 C, the incubation mixture was cooled and trichloroacetic acid was added to give a final concentration of 5%. After extraction for 30 min at 5 C, the degradation products of RNA in the supernatant fraction were adsorbed on active charcoal (20 mg/ml) and then eluted from charcoal with 50% ethyl alcohol containing 1% NH_4OH. The products thus obtained were chromatographed on Toyo no. 50 filter paper (Toyo-Roshi Co., Tokyo, Japan), ascending, in the following solvent systems: (i) *n*-butyl alcohol-water-formic acid, 77:13:10, v/v (21); (ii) ethyl alcohol-1 M ammonium acetate, 7:3, v/v, *p*H 7.5 (15); and (iii) isopropanol-concentrated HCl-water, 680 ml: 164 ml: 160 ml (21). By use of these systems in appropriate combinations, it was possible to identify 3'-pyrimidine mononucleotides in clear distinction from 5'-mononucleotides.

For the identification of 2',3'-cyclic mononucleotides as reaction products of ribonuclease I, a mixture of the enzyme preparation and ^{32}P-labeled RNA was incubated for 30 min at 37 C and then was directly spotted on filter paper and chromatographed in isopropanol-water (7:3, v/v) under ammonium vapor (10).

Paper chromatograms were scanned for radioactivity in an Aloka paper chromatogram scanner (PCS-2; Nihon-Musen Co., Tokyo, Japan).

Preparation and fractionation of cell-free extract. *E. coli* K-12 W2252 thy$^-$ was harvested immediately before and 45 min after the challenge with 2×10^{-5} M $HgCl_2$ at 37 C and was washed with ice-cold 0.1 M tris(hydroxymethyl)aminomethane (Tris) hydrochloride buffer (*p*H 8.0) containing 5×10^{-4} M $MgSO_4$. The cells were then suspended in the same buffer (20 ml of buffer per g of wet cells) and were subjected to sonic disruption in a Kubota sonic oscillator (10 kc; Kubota-Seisakusho, Tokyo, Japan) for 5.5 min. Cell debris was removed by centrifugation at $10,000 \times g$ for 15 min. The crude supernatant fluid was then fractionated into a supernatant fraction (designated as 10S) and a precipitate (10P) by centrifugation at $100,200 \times g$ for 60 min. The precipitate (10P) was

dispersed in 20 ml of the same buffer by sonic oscillation for 2 min. The supernatant fraction (10S) was further fractionated by centrifugation at 264,500 × *g* for 120 min to yield another supernatant fraction (26S) and precipitate (26P). The 26P precipitate was similarly dispersed by sonic oscillation.

Assay of ribonuclease activity. Enzymatic degradation of RNA in these fractions was measured with ^{32}P-labeled *E. coli* ribosomal RNA as substrate. The reaction mixture contained 150 nmoles (expressed as moles of mononucleotide unit) of ^{32}P-RNA (approximately 10^5 counts per min per μmole), 50 μmoles of Tris hydrochloride buffer (*p*H 8.0), 0.2 μmole of $MgSO_4$, and 0.1 ml of enzyme preparation in a volume of 0.5 ml. After 10 min of incubation at 37 C, 0.2 ml of bovine serum albumin (2 mg/ml) and 0.7 ml of 10% trichloroacetic acid were added. A sample of supernatant solution was neutralized with K_2CO_3 and counted in a Geiger-Müller counter. A unit of enzymatic activity was defined as the amount causing the production of 10 nmoles of acid-soluble ^{32}P per min. To measure latent ribonuclease activity, enzyme preparation was mixed with an equal volume of 8 M urea solution and incubated for 30 min at 37 C before the assay.

Preparation of ribonuclease I. Cells of *E. coli* K-12 W2252 thy$^-$ were harvested at mid-log phase (about 8×10^8 cells/ml) and washed with ice-cold 0.03 M Tris hydrochloride buffer (*p*H 8.0). The washed cells were resuspended in the same buffer containing 20% sucrose and 0.01 M EDTA and then were treated with 50 μg of lysozyme per ml for 30 min at 37 C. After the removal of cells from the incubation mixture by centrifugation, the supernatant fluid was dialyzed against 0.01 M Tris hydrochloride buffer (*p*H 7.4). The dialysate was directly applied on a column of diethylaminoethyl cellulose and chromatographed according to the method of Neu and Heppel (12). The eluted peak containing more than 80% of initial ribonuclease activity was collected and used.

Materials. Uracil-2-^{14}C was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). Crystalline lysozyme was a gift from the Eizai Pharmaceutical Co. (Tokyo, Japan).

For preparing ^{32}P-labeled RNA, cells of *E. coli* K-12 (λ) grown in a nutrient broth containing 20 μc of ^{32}P-orthophosphate per ml were suspended in 0.01 M Tris hydrochloride buffer (*p*H 8.0) containing 0.01 M $MgSO_4$ and were disrupted in a sonic oscillator. The pellet, containing ribosomes, was collected by centrifugation at 100,200 × *g* for 60 min, and RNA was extracted by the phenol method.

RESULTS

Degradation of RNA induced by $HgCl_2$. The effect of $HgCl_2$ at a concentration of 2×10^{-5} M on exponentially growing cells of *E. coli* K-12 W2252 thy$^-$ is shown in Fig. 1A. As soon as $HgCl_2$ was added, net increase of RNA, DNA, and protein stopped, and degradation of RNA occurred without a detectable decrease in the chemical amounts of DNA and protein in the cells. A limited decrease in RNA content started immediately, reaching a plateau at about 10% degradation; after 30 min rapid degradation resumed, and more than 80% of the cellular RNA was degraded. When the decrease in radioactivity in the trichloroacetic acid-insoluble fraction of the cell prelabeled with ^{14}C-uracil was measured, exactly the same rate of RNA degradation was observed.

On the other hand, RNA degradation due to $HgCl_2$ did not occur in the cells in the stationary phase. As shown in Fig. 1B, cells in the early stationary phase continued to synthesize RNA and DNA without a net increase in protein content. Addition of 2×10^{-5} M $HgCl_2$ did not induce a decrease in the chemical amount or the radioactivity of RNA nor did it inhibit the increase in chemical amounts of RNA and DNA.

Sensitivity of cells to $HgCl_2$ seemed to depend on the growth phase. In the experiments presented in Fig. 2, cultures of *E. coli* K-12 W2252 thy$^-$ were sampled at various growth stages, mixed with an equal volume of prewarmed fresh medium, and immediately challenged with 2.6×10^{-5} M $HgCl_2$. After 80 min of incubation with $HgCl_2$, amounts of residual RNA relative to the initial contents were measured as an index of RNA-degrading ability. Cells were fully sensitive during the exponential growth phase but attained complete insensitivity to $HgCl_2$ as soon as growth ceased.

The extent of RNA degradation in *E. coli* K-12 W2252 thy$^-$ during 90 min of incubation with various concentrations of $HgCl_2$ is shown in Fig. 3. The inhibitory effect of $HgCl_2$ on the incorporation of ^{14}C-uracil into the cold trichloroacetic acid-insoluble fraction during 10 min of incubation was also plotted in the same figure as an index of the inhibition of RNA synthesis. It is observed that both phenomena occurred at an $HgCl_2$ concentration of about 5×10^{-6} M in the exponentially growing cell, whereas in the stationary-phase cells they appeared only at concentrations higher than 2×10^{-5} M, and the extent of RNA degradation was very small even with an $HgCl_2$ concentration of 5×10^{-5} M or higher.

Similar breakdown of RNA was observed in *E. coli* K-12 (λ) and B, but not in *E. coli* Q13, a mutant deficient for ribonuclease I (Fig. 4). The fact that RNA degradation did not occur at all in *E. coli* Q13, even during exponential growth, indicated that ribonuclease I, among several ribonucleases in *E. coli* cells, was responsible for the RNA degradation induced by $HgCl_2$. Although RNA degradation was negligible in this strain, the killing effect was diminished to a lesser

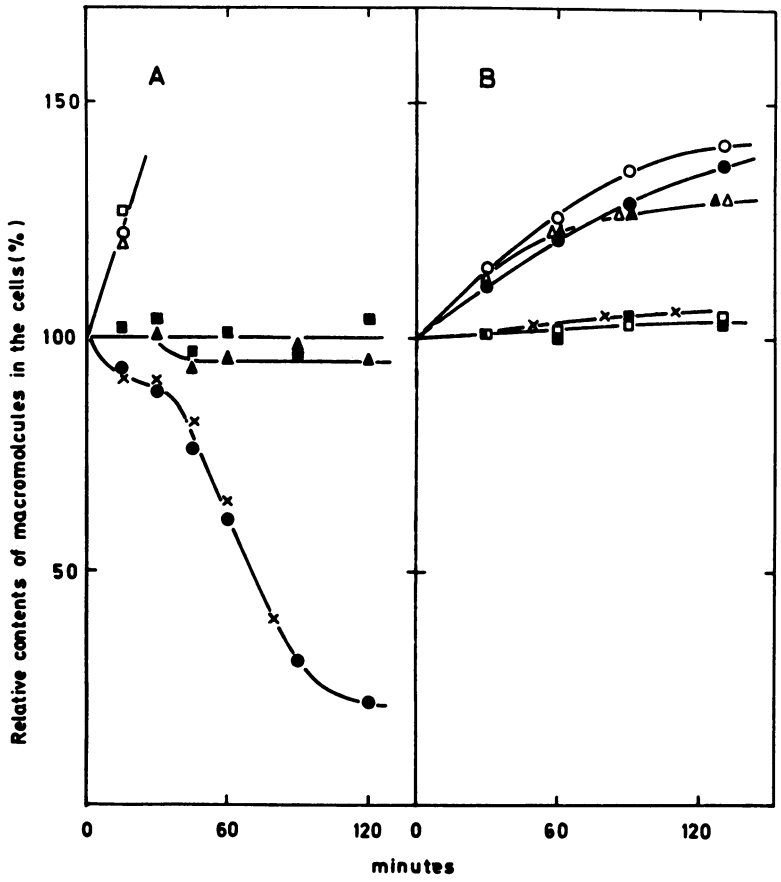

FIG. 1. *Effect of $HgCl_2$ (2×10^{-5} M) on macromolecular synthesis of Escherichia coli K-12 W2252 thy⁻ in exponential growth phase (A) and early stationary phase (B). Symbols: ○, RNA, −Hg; ●, RNA, +Hg; △, DNA, −Hg; ▲, DNA, +Hg; □, protein, −Hg; ■, protein, +Hg; ×, RNA, +Hg, measured by radioactivity. The 100% levels in A were 208 μg of protein per ml, 23.8 μg of RNA per ml, and 7.4 μg of DNA per ml. The corresponding 100% levels for B were, respectively, 496, 59.3, and 18.4.*

extent, which indicated that RNA degradation might not be a primary reason for cell death. Only slight breakdown of RNA took place in the Marburg strain of *B. subtilis*.

Degradation products of cellular RNA. The products of RNA degradation induced by $HgCl_2$ were analyzed by paper chromatography. Cells of *E. coli* K-12 (λ) labeled with ^{14}C-uracil were chased with fresh medium containing cold uracil, and 2×10^{-5} M $HgCl_2$ was added 60 min later. Trichloroacetic acid was added to the incubation mixture 120 min after the $HgCl_2$ challenge so as to give a final concentration of 5%. Degradation products thus obtained were a mixture of the materials excreted from cells into the medium and of those originally present in the metabolic pool of cells. They were analyzed by one- or two-dimensional paper chromatography with the use of several solvent systems, and radioactive spots were identified (Fig. 5). Radioactivity was distributed among several spots which were identified as uracil (9.7%), cytidine (18.2%), 3′-uridine monophosphate (18.8%), 3′-cytidine monophosphate (18.1%), and oligonucleotides (35.2%). The presence of 3′-mononucleotides but not 5′-mononucleotides suggests that the degradation of RNA induced by $HgCl_2$ may occur under the enzymatic action of ribonuclease I.

Degradation of RNA by other thiol reagents. Since $HgCl_2$ is a potent blocking reagent of thiol groups, several other thiol reagents, i.e., PCMB, monoiodoacetamide, *N*-ethylmaleimide, dimercaptopropanol, and arsenite, were tested with some other metabolic inhibitors to determine their ability to induce degradation of RNA in *E. coli*

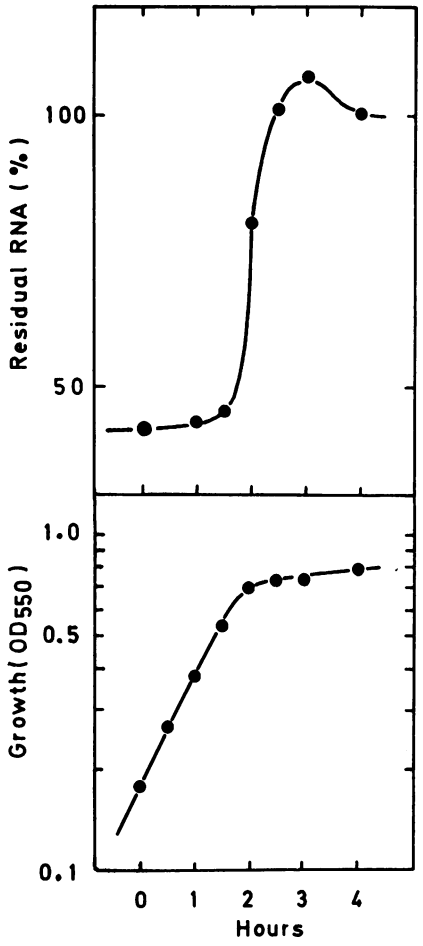

FIG. 2. *Change of Hg sensitivity of cellular RNA with growth phase. The 100% residual level of RNA at 4 hr was 3.18 × 10^{-8} μg per cell. An OD_{550} value of 0.2 corresponds to 3 × 10^{8} cells per ml.*

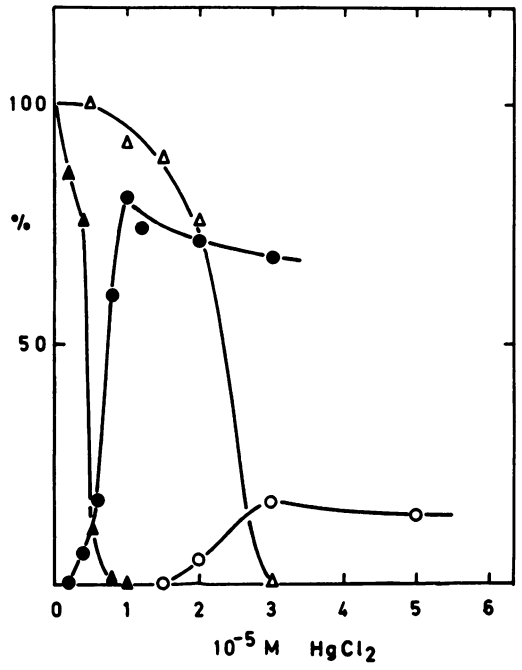

FIG. 3. *Variations of extent of RNA degradation and rate of RNA synthesis with concentration of $HgCl_2$. Symbols: ●, extent of RNA degradation in log-phase cells; ○, extent of RNA degradation in stationary-phase cells; ▲ rate of RNA ynthesis in log-phase cells; △, rate of RNA synth:is in stationary-phase cells. The 100% levels for the ves are, respectively, 3.97 × 10^{-8} μg/cell, 3.48 × 0^{-8} μg/cell, 4.03 × 10^{-8} μg per cell per hr, and (.95 × 10^{-8} μg per cell per hr.*

cells (Table 1). Although these reagents did not stop RNA synthesis completely at the given concentrations, PCMB was found to induce remarkable degradation of prelabeled RNA in intact *E. coli* cells. Furthermore, all the thiol reagents except arsenite induced limited degradation of prelabeled RNA in the cell treated with 10^{-3} M EDTA for 5 min at 37 C and resuspended in the medium. The EDTA treatment alone did not induce degradation of RNA. Although $HgCl_2$ and other thiol reagents inhibited the growth of *E. coli*, growth inhibition or killing of cells alone was not sufficient for induction of RNA degradation. For instance, 10^{-4} M 8-hydroxyquinoline, 10^{-4} M arsenite, 10^{-3} M sodium fluoride, all of which inhibited growth of *E. coli*, did not induce any degradation of RNA.

Ribonuclease activated by $HgCl_2$. The fact that *E. coli* Q13 deficient for ribonuclease I did not exhibit RNA degradation upon addition of $HgCl_2$ indicated involvement of the enzyme in this phenomenon. Evidence that ribonuclease I might be activated in the intact cells during incubation with $HgCl_2$ was provided by the results shown in Table 2. When ribonuclease activity was measured with the crude cell-free sonic extracts of *E. coli* K-12 W2252 thy$^-$ incubated with 2 × 10^{-5} M $HgCl_2$ for 45 min at 37 C, the activity was more than three times that in the untreated control. After fractional centrifugation, it was observed that the most remarkable activation occurred in the 26P fraction, which contained subribosomal particles. Since a considerable fraction of *E. coli* ribonuclease was present in a latent form in the sonic extract, total ribonuclease activity was also determined after the treatment of each fraction with 4 M urea for 30 min at 37 C. It was observed that the total activity of latent ribonuclease was not so

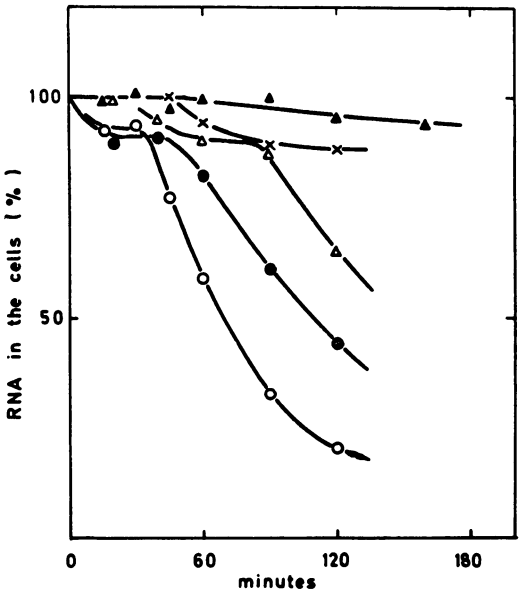

FIG. 4. *RNA degradation induced by $HgCl_2$ (2×10^{-5} M) in various strains of Escherichia coli and Bacillus subtilis. Symbols:* ○, *E. coli K-12 W2252 thy⁻;* ●, *E. coli K-12* (λ); △, *E. coli B;* ▲, *E. coli Q13;* ×, *B. subtilis Marburg. The 100% level in each strain was, respectively, 21.5, 17.0, 12.2, 13.1, and 15.7 μg of RNA per ml.*

much stimulated during incubation with $HgCl_2$. These data showed that latent ribonuclease localized at the 26P fraction in the sonic extracts was almost completely unmasked during the incubation of cells with $HgCl_2$.

In the mixture of the 10S-fraction of the Hg-treated cells and ^{32}P-RNA, 2′,3′-cyclic nucleotides and oligonucleotides were found as the reaction products. As shown in Fig. 6, large amounts of radioactivity were present in oligonucleotides, and cyclic nucleotides accounted for a considerable fraction of the total nucleotides. These facts support the assumption that the enzyme is ribonuclease I which undergoes endolytic hydrolysis of RNA, producing oligonucleotides and 2′,3′-cyclic mononucleotides as an intermediary.

Mechanisms of activation of ribonuclease by $HgCl_2$. Since RNA degradation due to $HgCl_2$ occurred similarly even when glucose was absent from the medium, the activation of the ribonuclease by $HgCl_2$ seemed not to require energy metabolism. In the experiment shown in Fig. 7, 2×10^{-5} M $HgCl_2$ was added to exponentially growing *E. coli* K-12 W2252 thy⁻ cells, and portions of the culture were removed at various incubation times. Cells collected by centrifugation after rapid cooling were disrupted by sonic oscillation, and ribonuclease activities in the crude supernatant fractions were estimated. Activation of ribonuclease progressed gradually, and deprivation of glucose from the incubation medium did not inhibit the activation. Some activation occurred even at 0 C.

Roth (16–19), working with several animal tissues, observed the presence of ribonuclease activity stimulated by PCMB or Pb^{++}. In these tissues, a ribonuclease is present as an inactive complex with a specific inhibitor protein whose thiol groups are essential for combining with the enzyme. The addition of thiol reagents such as PCMB and Pb^{++} results in the inactivation of the inhibitor protein and activation of the ribonuclease. Although such an inhibitor protein had not been found in microorganisms, a similar possibility was carefully tested with the cell-free extracts prepared by sonic oscillation. Activation of ribonuclease could not be observed when the crude supernatant fraction of the sonic extract of *E. coli* K-12 W2252 thy⁻ was incubated with various concentrations of $HgCl_2$ (from 5×10^{-7} to 2×10^{-4} M) for 60 min at 37 C. However, the isolated fresh 26P particles suspended in 0.1 M Tris hydrochloride buffer (*p*H 8.0) containing 5×10^{-4} M $MgSO_4$ showed clear activation of latent ribonuclease after incubation with about 10^{-4} M $HgCl_2$ (Table 3). It seemed possible that the activation occurred as a result of inactivation of a specific ribonuclease inhibitor in the 26P fraction by mercuric ion. If this supposition is true, the inhibitor, but not the enzyme, should be found in extracts of *E. coli* Q13. To confirm the inhibitor activity, ribonuclease I was partially purified from the spheroplast fluid of *E. coli* K-12 W2252 thy⁻ and mixed with various fractions of the disrupted cells of *E. coli* Q13. After incubation for 30 min at 37 C, the enzyme activity of the mixture was measured. It was found that ribonuclease-inhibitory activity was present in 10P and 26P fractions and not in the 26S fraction of *E. coli* Q13 cells. In the experiment shown in Fig. 8, fraction 10P of *E. coli* Q13 was treated with 10^{-4} M $HgCl_2$ in 0.01 M Tris hydrochloride buffer (*p*H 8.0)-0.001 M $MgSO_4$ for 30 min at 37 C, and excess $HgCl_2$ was removed by overnight dialysis. The Hg-treated dialysate had completely lost its ability to inhibit ribonuclease I, but the 10P dialysate without Hg treatment was inhibitory. This result suggests the presence of Hg-sensitive inhibitor for ribonuclease I in the particulate fraction of *E. coli* Q13.

It seemed highly possible that the unmasking of latent ribonuclease by $HgCl_2$ observed with the isolated particulates in vitro has a close relation-

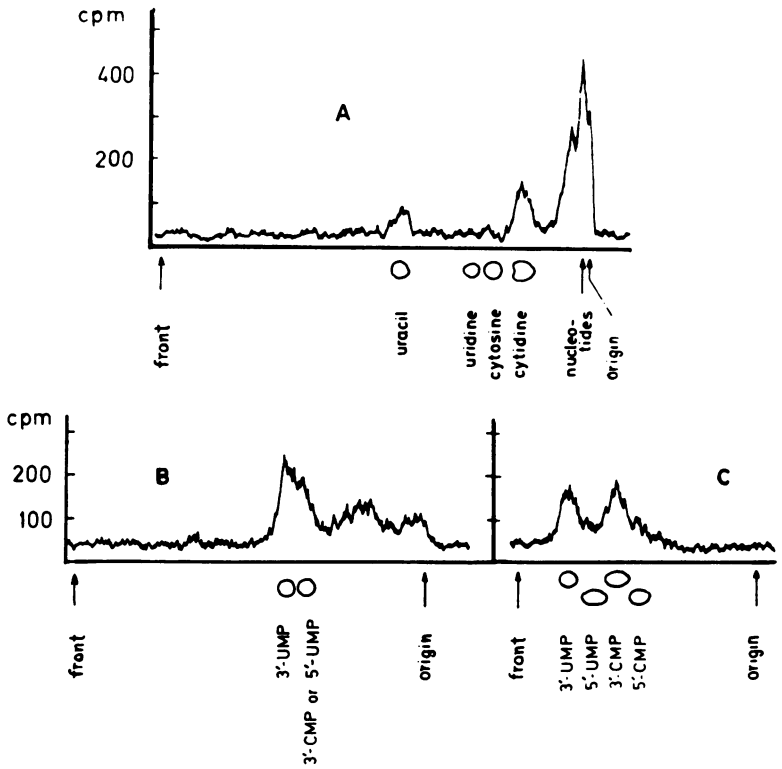

FIG. 5. *Paper chromatograms of degradation products during incubation of Escherichia coli K-12* (λ) *with* $HgCl_2$. *RNA of cells was preliminarily labeled with* ^{14}C*-uracil. (A) Total acid-soluble degradation products; solvent, n-butyl alcohol-water-formic acid. (B) Nucleotide fraction of A; solvent, ethyl alcohol-ammonium acetate. (C) Nucleotide fraction of A; solvent, isopropanol-HCl.*

TABLE 1. *RNA degradation in Escherichia coli K-12 W2252 thy⁻ induced by various thiol reagents*[a]

Reagent	Concn	RNA degradation	
		Intact cells	EDTA-treated cells
	M	%	%
$HgCl_2$	2×10^{-5}	60.2	44.2
PCMB	2×10^{-5}	18.2	36.7
	4×10^{-5}	44.0	69.7
Monoiodoacetamide	1×10^{-3}	4.8	13.0
	3×10^{-3}	6.4	11.2
N-Ethylmaleimide	1×10^{-3}	6.1	16.8
	3×10^{-3}	8.7	22.0
Dimercaptopropanol	1×10^{-2}	12.4	8.7
Arsenite	3×10^{-4}	0.5	−1.2
8-Hydroxyquinoline	1×10^{-4}	−1.6	0.7
NaF	1×10^{-3}		−0.8

[a] Cells prelabeled with ^{14}C-uracil were incubated with various reagents at the indicated concentrations for 60 min at 37 C and were then tested for the degradation of ^{14}C-RNA.

ship to the RNA degradation which occurred in exponentially growing cells. However, the ribonuclease activities in the isolated particulate preparations from the stationary-phase cells, which were almost completely insensitive to $HgCl_2$ in respect to RNA degradation, were activated to a similar extent by incubation with $HgCl_2$ in vitro (Table 4). This suggests that some other factor may control the sensitivity of cells to mercuric ion.

DISCUSSION

The results presented in this paper show that the RNA degradation induced by $HgCl_2$ in *E. coli* cells is due to the activation of ribonuclease I. The evidence is as follows: (i) RNA degradation by $HgCl_2$ did not occur in *E. coli* Q13, which is deficient for ribonuclease I, (ii) 3'-mononucleotides but not 5'-mononucleotides were found among the degradation products, (iii) 2',3'-cyclic mononucleotides were found as the products of the reaction of sonic extracts of the Hg-treated cells with RNA, and (iv) the most remarkable unmasking of ribonuclease took place in the 26P

TABLE 2. *Distribution of "Hg-activated" and latent ribonuclease activities in cells of Escherichia coli K-12 W2252 thy⁻*[a]

Fraction	Hg treatment of cells	Total protein (mg)	Unmasked ribonuclease			Latent ribonuclease		
			Total activity (units)	Specific activity		Total activity (units)	Specific activity	
				Units per mg of protein	+/−[b]		Units per mg of protein	+/−[b]
Crude supernatant	−	40.90	12.75	0.312		87.05	2.130	
	+	35.23	51.90	1.472	4.72	87.03	2.470	1.16
10P	−	14.34	4.27	0.297		86.10	5.990	
	+	20.90	28.50	1.362	4.58	71.55	3.420	0.57
26P	−	16.16	6.52	0.403		85.80	5.312	
	+	11.60	84.20	7.250	17.99	85.70	7.290	1.37
26S	−	19.26	21.39	1.120		6.30	0.327	
	+	15.17	13.54	0.892	0.80	15.97	1.053	3.22

[a] Normal cells were collected from 300 ml of the growing culture when cell density reached about 7×10^8 cells/ml. At the same time, mercuric chloride (2×10^{-5} M) was added to another 300 ml of the same culture, and incubation was continued for 45 min at 37 C. The Hg-treated cells were collected from this incubation mixture.

[b] Ratios of the activation of ribonuclease by incubation of cells with $HgCl_2$ were calculated by dividing the specific activity of each fraction from Hg-treated cells by that of the same fraction from normal cells.

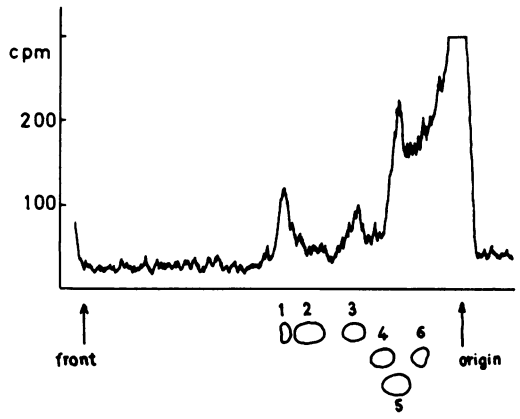

FIG. 6. *Paper chromatograms of degradation products from ^{32}P-RNA and the 10S-fraction of Escherichia coli K-12 W2252 thy⁻ treated with $HgCl_2$. Spot 1, 2',3'-cyclic adenosine monophosphate; spot 2, 2',3'-cyclic uridine and cytidine monophosphates; spot 3, 2',3'-cyclic guanosine monophosphate; spot 4, 3'-adenosine monophosphate; spot 5,3'-uridine and cytidine monophosphates; spot 6,3'-guanosine monophosphate.*

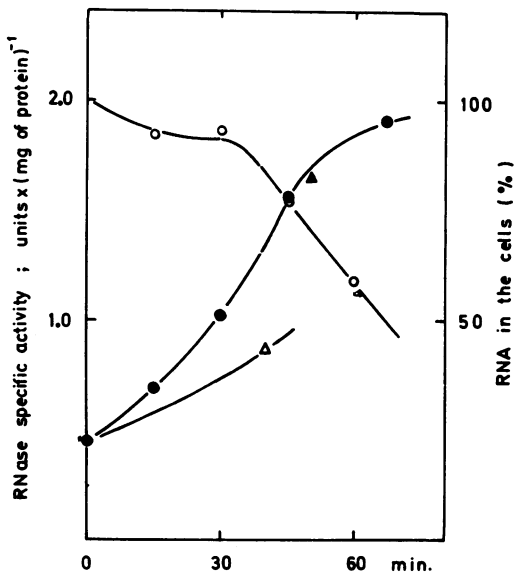

FIG. 7. *Increase in ribonuclease activity in Escherichia coli K-12 W2252 thy⁻ during incubation with $HgCl_2$. Symbols: ●, 37 C; ▲, 37 C in the absence of glucose; △, 0 C; ○, residual RNA at 37 C. At zero time, the RNA level was 3.96×10^{-8} μg per cell.*

fraction, which contained subribosomal particles carrying large amounts of ribonuclease I.

Investigation of *E. coli* ribonuclease (12) suggests that most ribonuclease I is located at the cell surface and is released into the medium when cells are converted to spheroplasts; it is recovered in an inactive bound form (latent ribonuclease) with 30*S* ribosomes in broken-cell preparations, and it is activated by treatment with EDTA or urea. The latency of ribonuclease I suggests that the enzyme in cells is engaged in a strong inter-

TABLE 3. *Activation of ribonuclease in the 26P fraction in vitro by incubation with various concentrations of $HgCl_2$ for 30 min*

Source	$HgCl_2$ concn	Temp	Specific activity (units/mg of protein)
	M	C	
Normal cells	0	0	0.920
	0	37	1.136
	1×10^{-6}	37	1.122
	5×10^{-6}	37	1.765
	1×10^{-5}	37	2.401
	2×10^{-5}	37	3.490
	1×10^{-4}	37	5.055
	2×10^{-4}	37	5.001
	5×10^{-4}	37	2.950
Hg-treated cells (2×10^{-5} M, 37 C, 45 min)	0	0	6.830

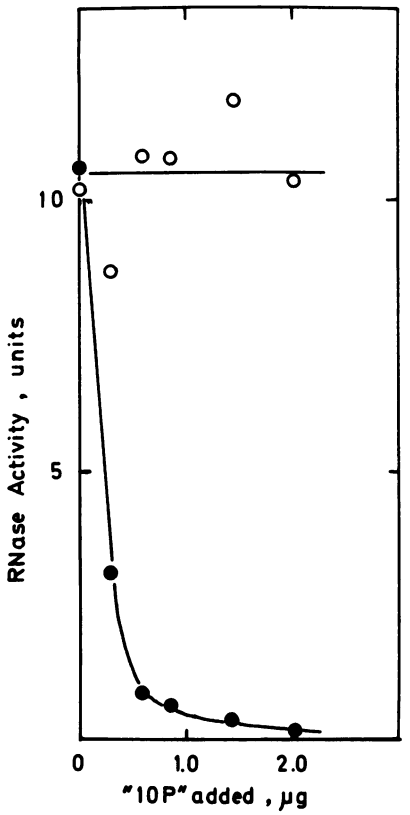

FIG. 8. *Masking of ribonuclease I by fraction 10P of E. coli Q13. Partially purified ribonuclease I (0.2 ml) was incubated with various amounts of 10P in 0.01 M Tris hydrochloride buffer (pH 8.0)-0.001 M $MgSO_4$ (total volume, 0.5 ml) for 30 min at 37 C, and the residual ribonuclease activity was measured as described in the text. Symbols: ○, Hg-treated and dialyzed 10P; ●, dialyzed 10P without Hg treatment.*

TABLE 4. *Unmasking of ribonuclease in vitro in various fractions of disrupted cells obtained from exponential and stationary growth phase*[a]

Fraction and growth phase	Total ribonuclease activity (units)		B/A
	(A) Before treatment with $HgCl_2$	(B) 30 min after incubation with $HgCl_2$	
Crude supernatant			
Exponential	57.2	38.7	0.68
Stationary	62.5	48.9	0.78
Ribosome (10P)			
Exponential	1.5	47.3	32.8
Stationary	1.9	58.0	31.4
Subribosome (26P)			
Exponential	0.7	95.1	144.4
Stationary	1.4	94.7	67.7
Supernatant (26S)			
Exponential	76.0	16.9	0.22
Stationary	68.3	28.8	0.42

[a] Cells in the exponential growth phase were harvested at cell density of about 7×10^8 cells/ml, and those in stationary phase were harvested 1 hr after the cessation of turbidity increase (cell density, about 3×10^9 cells/ml). Amounts of 1.0 g (wet weight) of both types of cells were disrupted and fractionated.

action with some other cell constituents which results in masking of the enzyme activity. However, the chemical entity responsible for this masking has not been satisfactorily clarified. In addition to EDTA and urea, $HgCl_2$ was found to induce unmasking of latent ribonuclease in the isolated particulate preparations, a result which suggested the possibility that the latency of ribonuclease I bound to subribosomal particles of *E. coli* was also governed by a specific inhibitor protein susceptible to thiol reagents such as that found in the animal tissues (16–19). Although solubilization and purification of the presumed ribonuclease inhibitor from the particulate had not been achieved, the finding that the particulate fraction of the disrupted *E. coli* Q13 cells showed masking of ribonuclease I and that the masking effect was inactivated by treatment with 10^{-4} M $HgCl_2$ suggests the presence of the inhibitor.

As shown in Fig. 2, the rapid transition of cells from the Hg-sensitive state to the insensitive state occurred with the cessation of growth. Furthermore, the transition from the insensitive to the sensitive state also began concomitantly with the initiation of growth when the stationary-phase culture was diluted with fresh medium. These facts suggest that a mechanism to suppress ribonuclease I has a close relationship with cellular growth. Although the latency of ribonuclease I

and its reversal by $HgCl_2$ observed with the isolated particulate preparations might be one of the basic factors involved in the RNA degradation induced by $HgCl_2$, the difference between the Hg sensitivity of exponentially growing cells and stationary-phase cells could not be attributed to the nature of ribosome and subribosomal particles, since these particles obtained from both types of cells showed similar sensitivity to mercuric ion. This suggests that some factors controlling the Hg sensitivity may be concerned with the transition phenomenon mentioned above. In this regard, several phenomena suggesting alteration of the cell envelope (for instance, the decrease in colicin E_2 sensitivity) were found to occur concomitantly with the cessation of growth at the stationary phase. It is also known that about 90% of the ribonuclease I in exponentially growing *E. coli* cells is released during formation of spheroplasts by EDTA-lysozyme in sucrose solution, but less than 50% of the enzyme is released from stationary-phase cells (12). It seems possible that the alteration in the envelope (wall or membrane) induces a change of enzyme location from the cell surface into the cytoplasm or a change in the tightness of enzyme-cell surface bonding, which makes the cell resistant to the destabilizing effects of $HgCl_2$.

LITERATURE CITED

1. Alwood, M. C., and A. D. Russell. 1968. Thermally induced ribonucleic acid degradation and leakage of substances from the metabolic pool in *Staphylococcus aureus*. J. Bacteriol. 95:345–349.
2. Billen, D. 1957. Modification of the release of cellular constituents by irradiated *Escherichia coli*. Arch. Biochem. Biophys. 67:333–340.
3. Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315–323.
4. Dische, A. 1955. Color reactions of nucleic acid components, p. 285–305. *In* E. Chargaff and J. N. Davidson (ed.), The nucleic acids: chemistry and biology, vol. 1. Academic Press Inc., New York.
5. Gesteland, R. F. 1966. Isolation and characterization of ribonuclease I mutants of *Escherichia coli*. J. Mol. Biol. 16:67–84.
6. Jackson, R. W., and J. A. DeMoss. 1965. Effects of toluene on *Escherichia coli*. J. Bacteriol. 90:1420–1425.
7. Kinsky, S. C. 1961. Alterations in the permeability of *Neurospora crassa* due to polyene antibiotics. J. Bacteriol. 82: 889–897.
8. Leive, L. 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. J. Biol. Chem. 243:2373–2380.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
10. Markham, R., and J. D. Smith. 1952. The structure of ribonucleic acids. 2. The smaller products of ribonuclease digestion. Biochem. J. 52:558–565.
11. Nakao, Y., A. Imada, T. Wada, and K. Ogata. 1964. Degradation of nucleic acids and their related compounds by microbial enzymes. XVI. Excretion of mononucleotides by yeasts. Agr. Biol. Chem. 28:151–159.
12. Neu, H. C., and L. A. Heppel. 1964. The release of ribonuclease into the medium when *Escherichia coli* cells are converted to spheroplasts. J. Biol. Chem. 239:3893–3900.
13. Nose, K., D. Mizuno, and H. Ozeki. 1966. Degradation of ribosomal RNA from *Escherichia coli* induced by colicine E_2. Biochim. Biophys. Acta 119:636–638.
14. Nozawa, R., T. Horiuchi, and D. Mizuno. 1967. Degradation of ribosomal RNA in a temperature-sensitive *Escherichia coli*. Arch. Biochem. Biophys. 118:402–409.
15. Ogata, K., A. Imada, and Y. Nakao. 1962. Excretion of 5'-nucleotides by bacteria. I. Accumulation of 5'-nucleotides in the culture fluid of a *Bacillus* during growth. Agr. Biol. Chem. 26:586–595.
16. Roth, J. D. 1956. Ribonuclease. V. Studies on the properties and distribution of ribonuclease inhibitor in the rat. Biochim. Biophys. Acta 21:34–43.
17. Roth, J. D. 1958. Ribonuclease. VII. Partial purification and characterization of a ribonuclease inhibitor in rat liver supernatant fraction. J. Biol. Chem. 231:1085–1095.
18. Roth, J. D. 1958. Ribonuclease. VIII. Studies on the inactive ribonuclease in the supernatant fraction of rat liver. J. Biol. Chem. 231:1097–1105.
19. Roth, J. D. 1964. Ribonuclease. IX. Further studies on ribonuclease inhibitor. Biochim. Biophys. Acta 61:903–915.
20. Wade, H. E. 1961. The autodegradation of ribonucleoprotein in *Escherichia coli*. Biochem. J. 78:457–472.
21. Wyatt, G. R. 1955. Separation of nucleic acid components by chromatography on filter paper, p. 243–265. In E. Chargaff and J. N. Davidson (ed.), The nucleic acids: chemistry and biology, vol. 1. Academic Press Inc., New York.