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Regulation of Ribonucleic Acid Synthesis in Spheroplasts, Cold-Shocked Cells, and Toluene-Treated Cells of *Escherichia coli*

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The effects on the stringent control of ribosomal ribonucleic acid synthesis of the removal of cell wall, cold-shock treatment of cells, LiCl treatment of toluene-treated cells, and hypotonic treatment of spheroplasts were examined using *Escherichia coli rel*$^+$ cells. Neither the removal of cell wall with penicillin or lysozyme nor the cold-shock treatment of the cells had an effect on the stringent control. The control mechanism, however, disappeared after the LiCl treatment of the toluene-treated cells, with the release of some protein component(s), possibly from the cytoplasmic membrane. The hypotonic and other treatments of spheroplasts, which disrupt the cytoplasmic membrane, also led to the abolishment of the control mechanism. These results suggested that the operation of the stringent control of ribosomal ribonucleic acid synthesis requires the cytoplasmic membrane, in which some proteins labile with LiCl treatment are embedded.

It is well known that stable ribonucleic acid (RNA) synthesis is sharply curtailed when growing cells of a *rel*$^+$ strain of *Escherichia coli* are deprived of an essential amino acid (stringent control of RNA synthesis). In the course of our analyses on this control mechanism, several attempts have been made to reproduce this stringent phenomenon in vitro without success. Once cells were lysed or crushed, the ability to restrict the ribosomal RNA (rRNA) synthesis upon amino acid deprivation was abolished. Given these circumstances, systematic examination seems indicated to determine how the injury of the cells or the removal from the cells of various components outside the cytoplasm affects the stringent control mechanism.

In the present paper, we have examined the effects on the stringent control of rRNA synthesis of the removal of cell wall, cold-shock treatment of the cells, LiCl treatment of toluene-treated cells, and hypotonic treatment of spheroplasts. The removal of cell wall or the injury of the cells with cold-shock or toluene treatment had no effect on the stringent control of rRNA synthesis, whereas the cells lost the control mechanism by removal from the toluene-treated cells of some component(s), possibly from the cytoplasmic membrane. The disruption of the cytoplasmic membrane immediately caused the loss of the control mechanism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The isogenic *rel*$^+$ and *rel*$^-$ pair of *E. coli* strain 10B6 [*arg*$^-$ *valS*(Ts)] (2) was kindly supplied by G. S. Stent. The bacteria were grown at 30°C in tris(hydroxymethyl)aminomethane (Tris) salts-glucose medium supplemented with 0.2% Casamino Acids and 0.2% yeast extract (Difco).

Penicillin spheroplasts. Penicillin spheroplasts were prepared according to Ehrenfeld and Koch (3). Penicillin (398 U/ml) was added to the cells at a density of 40 Klett units in the presence of 0.5 M sucrose and 10 mM $MgSO_4$. After 3 or 4 h of incubation at 30°C, the resulting spheroplasts were used for experiments. The spheroplast formation was always checked under the microscope.

Lysozyme spheroplasts and hypotonic treatment of the spheroplasts. At a density of 65 Klett units, the exponentially growing cells were harvested, washed with Tris-salts-glucose, suspended in 1/25 volume of 10 mM Tris-hydrochloride (pH 8.1 at 37°C)-0.625 M sucrose, and then mixed with 1/100 volume of 1 mg of lysozyme per ml (6× crystallized; Seikagaku-Kogyo Co.) in 10 mM Tris-10 mM ethylenediaminetetraacetic acid. The mixture was incubated for 2 min at 0°C, and the formation of spheroplasts was checked microscopically. The spheroplasts so obtained were used immediately for assay.

For the hypotonic treatment of spheroplasts, the suspension of lysozyme spheroplasts containing 0.5 M sucrose was diluted 20-fold with the reaction mixture without radioisotope (see below) and incubated for 10 min at 0°C. By this treatment, almost all the

spheroplasts were lysed as a result of disruption of the cytoplasmic membrane.

Cold-shock treatment. The method developed by Atherly (1) for cold-shock treatment was used.

Toluene-treated cells and LiCl treatment. Toluene-treated cells were prepared according to Peterson et al. (9) with slight modifications. The exponentially growing cells were harvested and suspended in 0.10 volume of 50 mM Tris-hydrochloride (pH 7.4 at 37°C) buffer containing 10 mM $MgCl_2$. Toluene (0.75%) was then added to the cell suspensions, which were stirred in an ice bath for 5 min. The cells were centrifuged down and washed to remove residual toluene.

For LiCl treatment, the toluene-treated cells were resuspended in the same buffer containing various concentrations of LiCl. After 10 min of incubation at 30°C, the cells were centrifuged and washed to remove the residual LiCl. The LiCl-treated cells were used immediately for assay.

The amount of protein extracted from toluene- and LiCl-treated cells was measured according to the method of Lowry et al. (7).

RNA and protein synthesis in culture medium. To the culture of penicillin spheroplasts (3×10^8 cells/ml), 0.1 μCi of [^{14}C]uracil (22 mCi/mM) or 0.2 μCi of [^{14}C]phenylalanine (522 mCi/mM) per ml was added. At intervals, a 0.5-ml portion was taken out and mixed with 0.5 ml of 20% cold trichloroacetic acid. For measurement of protein synthesis, the trichloroacetic acid precipitates were heated at 90°C for 15 min, collected on glass-fiber filters, and dried. The radioactivity was counted in a liquid scintillation spectrometer as described previously (6).

RNA and protein synthesis in the reaction mixture. A typical reaction mixture contained: 50 mM Tris-hydrochloride (pH 7.8 at 37°C); 50 mM KCl; 10 mM $MgCl_2$; 0.25 mM each adenosine 5'-triphosphate, guanosine 5'-triphosphate, and cytidine 5'-triphosphate; 5 μM phosphoenol pyruvate; 40 μg of pyruvate kinase per ml. A total of 2 μCi of [^{3}H]-uridine 5'-triphosphate (UTP; 10.5 Ci/mmol) and 0.2 μCi of [^{14}C]phenylalanine (522 mCi/mmol) per ml was added for the measurement of RNA synthesis and protein synthesis, respectively. Eighteen amino acids (2 mM) (minus phenylalanine and arginine) and/or arginine were added when necessary. The concentrations of the cold-shock cells, toluene-treated cells, and lysates used in the above experiments were equivalent to 4×10^8, 8×10^8, and 8×10^8 cells/ml, respectively. When lysozyme spheroplasts (3×10^8 cells/ml) were incubated in the above mixture, sucrose was added at a final concentration of 0.5 M. The reaction mixture was incubated at 30°C. A 0.1-ml portion was taken out at time intervals and poured into 0.5 ml of 10% trichloroacetic acid, and the radioactivity was counted.

The RNA synthesis in the spheroplasts was about 70% of the intact cells. The synthesis in the cold-shock cells, toluene-treated cells, LiCl- and toluene-treated cells, and spheroplast lysates was less than 10% of that of the intact cells. However, the stringent control of rRNA synthesis as seen with [^{3}H]UTP incorporation in the cold-shock cells and toluene-treated cells was not due to the contamination of the intact cells, since (i) no incorporation of [^{14}C]uracil or [^{3}H]uridine (5 Ci/mmol) was observed with these cells, (ii) the incorporation of [^{3}H]UTP into nontreated cells was less than 0.1% of the treated cells, and (iii) the stringent control was completely abolished by treating toluene-treated cells with 3 M LiCl.

Deoxyribonucleic acid (DNA)-RNA hybridization. The RNA for the hybridization experiments was labeled as described in the last two sections, except that the concentrations of [^{14}C]uracil (22 mCi/mmol) and [^{3}H]UTP (10.5 Ci/mmol) added in the medium were 1 and 20 μCi/ml, respectively. Each incubation mixture was preincubated for 10 min without isotope and then labeled with the isotope for 5 min in the case of penicillin spheroplasts. The labeling time of other experiments was 7.5 min. After the incubation, the mixture was subjected to freezing and thawing in the presence of lysozyme (100 μg/ml) and deoxyribonuclease (100 μg/ml). The RNA was then extracted by using the sodium dodecyl sulfate-phenol method.

The relative amount of rRNA in the labeled RNA was estimated by the DNA-RNA hybridization-competition technique. Nitrocellulose filters (HAWP; pore size, 0.45 μm) to which 100 μg of *E. coli* denatured DNA was attached were incubated with ^{3}H- or ^{14}C-labeled RNA (0.5 μg) in the presence and absence of unlabeled rRNA (50 μg) for 20 h at 66°C in 0.75 ml of 6× SSC containing 0.05% sodium dodecyl sulfate. The filters were then soaked for 1 h at 25°C in 2× SSC containing 20 μg of pancreatic ribonuclease A per ml, washed six times with 2× SSC, dried, and counted in a scintillation spectrometer. The difference between competed and uncompeted radioactivities was taken to represent the rRNA in the transcript.

RESULTS

Stringent control in spheroplasts. The first experiment was designed to examine whether spheroplasts that have lost cell wall and are still impermeable to nucleoside triphosphates retain the mechanism for stringent control of RNA synthesis. Penicillin-induced spheroplasts derived from 10B6 cells were prepared, and their RNA synthesis was examined at 30°C (permissive) and 42°C (nonpermissive temperature for inducing valyl-transfer RNA deprivation). Figure 1 shows the incorporation of [^{14}C]uracil into RNA in *rel*$^+$ and *rel*$^-$ spheroplasts. At 42°C, the RNA synthesis in *rel*$^+$ spheroplasts occurred only slightly during the first 5 min and stopped thereafter, whereas that in *rel*$^-$ spheroplasts continued for more than 30 min.

To examine the relative amount of rRNA in the total RNA synthesized in the spheroplasts at 30 and 42°C, the RNA-DNA hybridization-competition technique was used. The spheroplasts were incubated at 30 or at 42°C for 10 min and then pulse-labeled with [^{14}C]uracil for 5

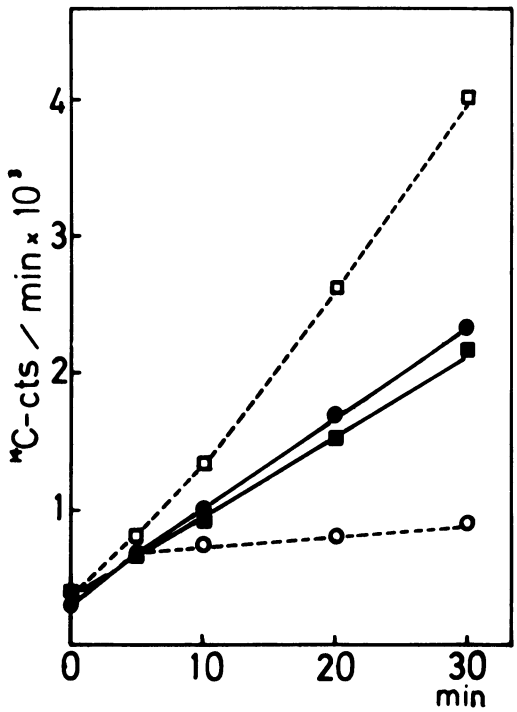

FIG. 1. *RNA synthesis in rel$^+$ and rel$^-$ penicillin spheroplasts. The culture containing penicillin spheroplasts was incubated at 30 and 42°C in the presence of [^{14}C]uracil (0.1 μCi/ml). Symbols: (●) rel$^+$ at 30°C; (○) rel$^+$ at 42°C; (■) rel$^-$ at 30°C; (□) rel$^-$ at 42°C.*

min. The labeled RNA was prepared and hybridized with denatured *E. coli* total DNA in the presence and absence of nonlabeled rRNA (a mixture of 23S, 16S, and 5S rRNA). Forty-six percent of ^{14}C-labeled RNA made in *rel$^+$* spheroplasts at 30°C was competed by the excess amounts of nonlabeled rRNA, whereas only 18% of RNA labeled at 42°C was competed (Table 1, lines 1 and 2). On the other hand, the relative amount of rRNA synthesized in the *rel$^-$* spheroplasts was about 40% both at 30 and 42°C (Table 1, lines 3 and 4).

Protein synthesis of the spheroplasts was also examined and found to be inhibited at 42°C both in *rel$^+$* and *rel$^-$* spheroplasts.

These situations are essentially the same as those in intact 10B6 cells, indicating that the removal of cell wall by penicillin does not affect the stringent control of rRNA synthesis.

Spheroplasts were also prepared by ethylenediaminetetraacetic acid-lysozyme treatment of the cells and shown to retain the same stringent response as in the case of penicillin treatment (data not shown).

Cold-shock treatment. The 10B6 *rel$^+$* and *rel$^-$* cells were subjected to cold-shock treatment as described by Atherly (1). With this treatment, cells became permeable to nucleoside triphosphates. We then examined whether this treatment exerts any effect on the stringent control mechanism. Figure 2 shows the incorporation of [^{3}H]UTP into RNA in the cold-shocked *rel$^+$* and *rel$^-$* cells at permissive and nonpermissive temperatures. At 42°C (nonpermissive temperature), RNA synthesis in *rel$^+$* cells occurred only during the first 5 min of incubation and stopped thereafter. On the other hand, RNA synthesis in *rel$^-$* cells continued for more than 30 min. The relative amounts of rRNA synthesized in the *rel$^+$* and *rel$^-$* cells at 30 and 42°C were almost the same as those observed in the case of the penicillin spheroplasts (Table 1, lines 4 to 8).

The result indicated that the stringent control of rRNA synthesis was not affected by cold-shock treatment under our experimental conditions. This is in contrast to the result obtained by Atherly (1), who reported that the cold-shocked *rel$^+$* cells did not restrict the rRNA synthesis upon amino acid deprivation. The discrepancy between his result and ours may be due to some unknown difference in the experimental conditions.

Toluene-treated cells. Toluene has been known to attack the lipid layer of the cytoplasmic membrane so that the cells become permeable to nucleoside triphosphates (8). The 10B6 *rel$^+$* and *rel$^-$* cells were treated with toluene as described in Materials and Methods. During the treatment, no appreciable morphological change of the cells was observed under the microscope. Figure 3a and b shows the incorporation of [^{3}H]UTP into RNA in the toluene-treated *rel$^+$* and *rel$^-$* cells. In the *rel$^+$* cells, RNA synthesis occurred at least for 30 min at 30°C in the presence of amino acids. The rate of synthesis was faster during the first 10 min than thereafter. Without amino acids, the RNA synthesis occurred only during the first 10 min of incubation and stopped completely afterwards. The deprivation of arginine alone delayed the cessation of RNA synthesis. On the other hand, [^{3}H]UTP incorporation into the toluene-treated *rel$^-$* cells proceeded for over 30 min irrespective of the presence or absence of amino acids. The DNA-RNA hybridization-competition experiments show that the relative amount of rRNA synthesized in the *rel$^+$* toluene-treated cells was about 40% in the presence of amino acids and about 20% in the absence of amino acids, whereas that in the *rel$^-$* toluene-treated cells was about 40 to 50% either in the presence or absence of amino acids (Table 1, lines 9 to 12).

The results indicated that the toluene-

TABLE 1. *Hybridization-competition of labeled RNA*

Cells	Strain	Condition of RNA synthesis[a]	Input (cpm)	^3H- or ^{14}C-labeled RNA hybridized (cpm)[b]		% rRNA in total RNA formed
				Uncompeted	Competed	
Spheroplasts	*rel*$^+$	30°C	1,070	222	121	46 ± 4
		42°C	1,320	230	189	18 ± 3
	rel$^-$	30°C	1,576	262	152	42 ± 6
		42°C	1,836	300	185	38 ± 2
Cold-shock cells	*rel*$^+$	30°C	1,174	206	112	45 ± 5
		42°C	1,142	200	166	17 ± 3
	rel$^-$	30°C	2,954	670	403	40 ± 2
		42°C	1,272	312	204	35 ± 4
Toluene-treated cells	*rel*$^+$	+a.a	2,802	496	290	42 ± 3
		−a.a	2,668	435	352	19 ± 3
	rel$^-$	+a.a	1,882	665	371	44 ± 4
		−a.a	1,570	495	246	50 ± 2
Toluene- and LiCl-treated cells	*rel*$^+$	+a.a	2,376	406	261	36 ± 2
		−a.a	1,430	258	155	40 ± 6
	rel$^-$	+a.a	2,016	353	232	34 ± 4
		−a.a	1,422	266	166	38 ± 3
Hypotonic treated spheroplasts	*rel*$^+$	+a.a	2,516	503	302	40 ± 5
		−a.a	2,540	456	231	49 ± 3
	rel$^-$	+a.a	1,640	335	198	41 ± 5
		−a.a	1,566	382	199	48 ± 4
Control (purified ^{14}C-labeled rRNA)			2,210	438	407	93 ± 2

[a] a.a, Mixture of 20 amino acids.
[b] Mean of four measurements; in the case of control rRNA, mean of eight measurements.

treated cells still maintained the mechanism for stringent control of rRNA synthesis.

Protein synthesis of toluene-treated *rel*$^+$ cells was also examined by measuring [^{14}C]phenylalanine incorporation into protein. In the presence of 19 amino acids, protein synthesis continued at least for 20 min. However, the rate of the synthesis was less than 10% as compared with intact cells. In the absence of amino acids, protein synthesis occurred only during the first 10 min and then stopped completely. This initial synthesis seems therefore to be a residual one, which continues until the amino acid pool in the cells is exhausted. The delay of the exhaustion of amino acids may be due to the much slower protein synthesis in this system. The initial occurrence of the RNA synthesis during the first 10 min in the toluene-treated *rel*$^+$ cells would have been supported by the residual amino acids present during this period.

LiCl treatment of toluene-treated cells. The toluene-treated cells were incubated with 0 to 3 M LiCl at 30°C for 10 min to release some components without disrupting the cytoplasmic membrane. The cells so treated were washed and submitted to the assay for RNA synthesis in the presence or absence of amino acids. Using higher concentrations of LiCl, more proteins were recovered in the LiCl wash fraction, whereas the optical density at 260 nm in this fraction did not increase with any concentrations of LiCl used. These data together with the microscopic examination of the treated cells indicated that LiCl treatment removed some proteins, but not nucleic acids, without disrupting the cytoplasmic membrane. Furthermore, protein-synthesizing activity was not significantly decreased by LiCl treatment up to a 3 M concentration.

Figure 4 shows RNA synthesis in the toluene-treated, 3 M LiCl-treated *rel*$^+$ cells. Either in the presence or absence of amino acids at 30°C, the RNA synthesis as measured by [^3H]UTP incorporation into RNA continued at least for 30 min. In either case, about 35 to 40% of the RNA synthesized was rRNA (Table 1, lines 13 to 16). This result indicated that stringent control of rRNA synthesis did not operate in the toluene- and LiCl-treated cells. Since the

control operated in the toluene-treated, LiCl-untreated cells, some substance, possibly proteins, removed by the 3 M LiCl treatment would be responsible for the maintenance of stringent control of rRNA synthesis in the toluene-treated cells. Readdition of the LiCl wash fraction to the toluene-treated, LiCl-treated cells has so far been unsuccessful in recovering the control mechanism.

Hypotonic treatment of spheroplasts. The next experiment was performed to determine the effect on the stringent control of rRNA syn-

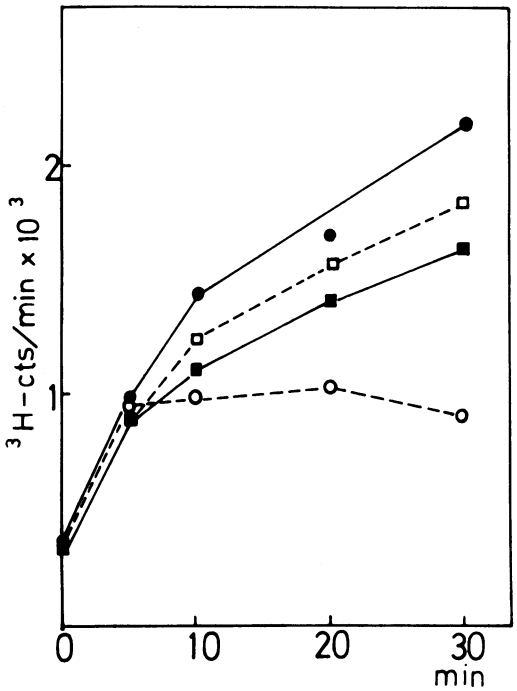

FIG. 2. *RNA synthesis in cold-shocked rel$^+$ and rel$^-$ cells. The reaction mixture containing cold-shocked cells was incubated at 30 and 42°C in the presence of [^{3}H]UTP (2 μCi/ml). Symbols: (●) rel$^+$ at 30°C; (○) rel$^+$ at 42°C; (■) rel$^-$ at 30°C; (□) rel$^-$ at 42°C.*

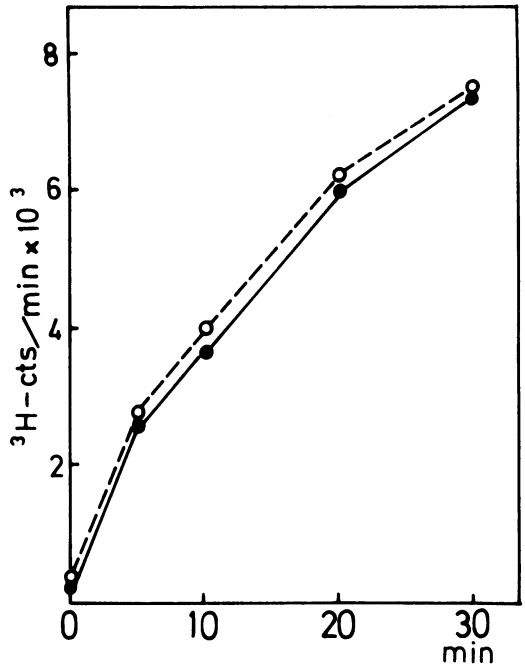

FIG. 4. *RNA synthesis in toluene-treated, 3 M LiCl-treated cells. The reaction mixture containing LiCl-treated rel$^+$ cells was incubated at 30°C with (●) and without (○) amino acids in the presence of [^{3}H]UTP (2 μCi/ml).*

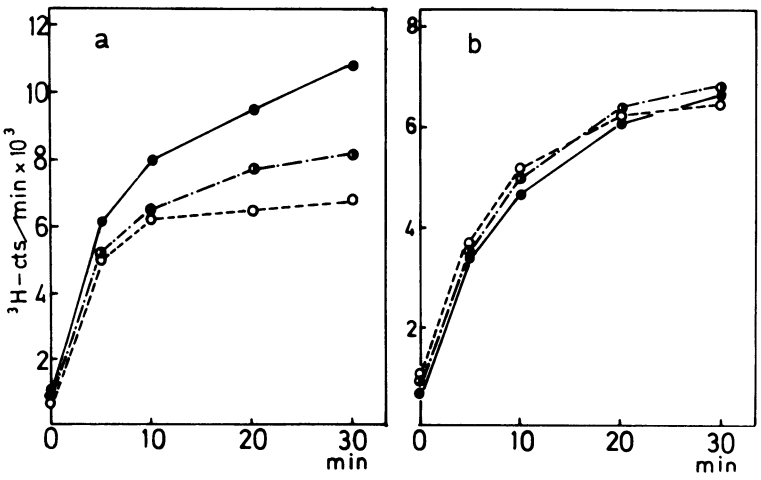

FIG. 3. *RNA synthesis in toluene-treated (a) rel$^+$ and (b) rel$^-$ cells. The reaction mixture containing toluene-treated cells was incubated at 30°C with amino acids (●), with amino acids minus arginine (◑), and without amino acids (○) in the presence of [^{3}H]UTP (2 μCi/ml).*

thesis of the disruption of the cytoplasmic membrane of spheroplasts by hypotonic treatment. Spheroplasts were prepared by lysozyme treatment of the washed 10B6 (*rel*$^+$) cells in the presence of 0.5 M sucrose. The concentration of sucrose was then gradually reduced to 0.025 M by dilution to disrupt the cytoplasmic membrane. As a result, the spheroplasts were lysed and nucleoside triphosphates were used for RNA synthesis by these lysates. This process was followed by microscopic examinations. The RNA synthesis in the spheroplast lysates was measured in the presence or absence of required amino acids by [^{3}H]UTP incorporation into RNA. The RNA synthesis continued at least for 30 min even in the absence of amino acids (Fig. 5). Under these conditions, about 40% of the RNA synthesized was rRNA (Table 1, lines 17 to 20), showing that spheroplast lysates had lost their ability to restrict rRNA synthesis upon amino acid deprivation. The experiment using *rel*$^-$ cells showed the same result as that using *rel*$^+$ cells.

DISCUSSION

The effects of a series of treatments of *rel*$^+$ cells on the stringent control of rRNA synthesis have been examined. The removal from the cells of cell wall with penicillin or lysozyme produces spheroplasts that are still capable of restricting rRNA synthesis upon amino acid starvation. This implies that the cell wall is not required for the control mechanism.

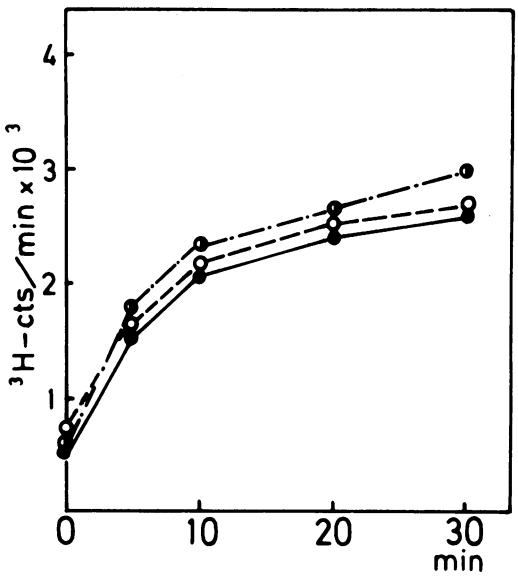

FIG. 5. *RNA synthesis in spheroplast lysates. The lysates were prepared by the hypotonic treatment of rel*$^+$ *spheroplasts. The reaction mixture containing spheroplast lysates was incubated at 30°C with amino acids (●), with amino acids minus arginine (◑), and without amino acids (○) in the presence of [^{3}H]UTP (2 μCi/ml).*

Cold-shocked cells or toluene-treated cells, which are now permeable to nucleoside triphosphates, also retain the stringent control mechanism. These results indicate that the conversion of the cells to the state permeable to nucleoside triphosphates is not enough to destroy the control mechanism. The additional LiCl treatment of the toluene-treated cells leads to the abolishment of the control mechanism without affecting the protein synthesis. During the LiCl treatment, no detectable disruption of the cytoplasmic membrane can be seen microscopically, and yet some proteins are released from the cells. It has been reported that in *Bacillus megaterium* some membrane-bound proteins are removed with LiCl from the toluene-treated cells (11). Autoradiographic and electron microscopic examinations also suggested that proteins are extracted from the cytoplasmic membrane through cell wall (4). Thus, in our case also, it is highly possible that the proteins released from the toluene-treated cells with LiCl are some cytoplasmic membrane proteins, at least part of which could play an important role in the stringent control of rRNA synthesis.

The hypotonic treatment of the spheroplasts, which disrupts the cytoplasmic membrane, also abolishes the ability to restrict rRNA synthesis upon amino acid starvation. Other treatments such as cold-shock or detergent treatments of spheroplasts, which also cause the disruption of the cytoplasmic membrane, immediately destroy the control mechanism. Gallant and Cashel (5) reported that uracil or uridine 5′-monophosphate incorporation into the plasmolyzed *rel*$^+$ cells showed amino acid dependency whereas UTP incorporation showed only a weak dependency. One might then argue that the abolishment of stringent control in the LiCl-treated, toluene-treated cells or in the lysates would be simply due to the use of UTP as the isotope. However, this is not likely since the operation of stringent control can be demonstrated with the cold-shocked and toluene-treated cells by the use of [^{3}H]UTP.

It has been established that the stringent factor is somehow involved in the stringent control of rRNA synthesis (10). The identity of the LiCl-labile component with the stringent factor is not likely, because the former may be present in the cytoplasmic membrane and the latter mainly in combination with ribosomes.

From the considerations mentioned above, we suggest that the operation of the stringent

control mechanism of rRNA synthesis in *rel*$^+$ cells requires, besides the presence of the stringent factor in the cells, maintenance of the state of the cytoplasmic mass enclosed by the cytoplasmic membrane, in which some LiCl-labile proteins are organized.

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