JOURNAL OF BACTERIOLOGY, Jan. 1972, p. 243-249

Vol. 109, No. 1
Printed in U.S.A.

Ribosome Synthesis in Thermally Shocked Cells of *Staphylococcus aureus*[1]

LEONARD J. ROSENTHAL,[2] SCOTT E. MARTIN, MICHAEL W. PARIZA, AND JOHN J. IANDOLO
Division of Biology, Kansas State University, Manhattan, Kansas 66502

Received for publication 27 September 1971

Thermally shocked cells of *Staphylococcus aureus* rapidly synthesized ribonucleic acid (RNA) during the early stages of recovery. During this period, protein synthesis was not observed and occurred only after RNA had reached a maximum level. Even in the absence of coordinated protein synthesis, a large portion of the RNA appeared in newly synthesized ribosomes. Although the 30*S* subunit was specifically destroyed by the heating process, both ribosomal particles were reassembled during recovery. The addition of chloramphenicol did not inhibit the formation of the ribosomal subunits, nor was the presence of immature chloramphenicol particles detected. Extended recovery with highly prelabeled cells showed that the original ribosomal proteins present before heating are conserved and recycled. Furthermore, the data indicate that the 50*S* subunit is turned over and used as a source of protein for new ribosome assembly. Kinetic studies of the assembly process by pulse labeling have not revealed the presence of the normally reported precursor particles. Rather, the data suggest that assembly may occur, in this system, in a manner similar to that reported for in vitro assembly of *Escherichia coli* subunits.

It is generally known that thermally stressed cells of *Staphylococcus aureus* undergo significant changes in their macromolecular makeup. These changes are reflected by a reduction in salt tolerance (4), by alterations in enzymatic activity (1), and by the degradation of ribonucleic acid (RNA) and ribosomes (9, 10). In an earlier paper (9), we reported that 30*S* subunits were specifically destroyed by heating. The mechanism is unknown, although 16*S* RNA was the prime target of degradation, and as a consequence the 30*S* subunit was also destroyed. The status of 23*S* RNA and the 50*S* subunit is also questionable, since 23*S* RNA has been shown to possess an altered secondary structure and possible reduction of functional activity as a result of heating. Although severe, these metabolic alterations are not lethal, and cells in this condition may be revived under the proper conditions of incubation (4, 10). During this incubation, termed "recovery" (4), the ribosomes are regenerated (10) and the cells resume normal activities (1, 4, 7). The purpose of this investigation was to examine the phenomenon of ribosomal regeneration in thermally stressed cells and to evaluate the potential of the system for further studies of ribosome assembly.

MATERIALS AND METHODS

Growth and heating conditions. Cultures of *S. aureus* MF-31 were grown in a semidefined medium (SDM) described elsewhere (7). When it was necessary to have prelabeled cells, the appropriate isotopes (uridine-5-^3H and uniformly labeled ^{3}H-valine and ^{14}C-arginine; Schwarz BioResearch, Inc.) were added to the medium at activity levels of 0.1 μCi/ml. The cells from an 18-hr culture were collected by centrifugation (5,000 $\times$ *g* for 10 min), washed once in 100 mM phosphate buffer at *p*H 7.2, and finally suspended in a minimum volume of phosphate buffer. The washed suspension was then brought up to 50 ml by the addition of phosphate buffer at the proper temperature, and the cells were heated at 55 C for 15 min, as reported earlier (4). After heating, the cells were cooled in an ice bath, collected by centrifugation (5,000 $\times$ *g* for 10 min), and suspended in Trypticase Soy Broth (TSB). When appropriate, the necessary radioisotopes (as stated) were added directly to the recovery medium. Depending upon the type of experiment, the heated cells were incubated at 37 C in the recovery medium for periods up to 6 hr (at which point recovery was complete).

Isotope incorporation by whole cells. Recovering cultures were incubated in TSB containing

[1] Contribution no. 1127, Division of Biology, Kansas Agricultural Experiment Station, Manhattan.

[2] Present address: John Collins Warren Laboratories, Massachusetts General Hospital, Boston, Mass. 02114.

uracil-5-^3H (1 μCi/ml) and arginine-U-^{14}C (0.1 μCi/ml) to determine the rate and extent of protein and RNA synthesis. At intervals, 1-ml samples were mixed with an equal volume of ice-cold 10% trichloroacetic acid to make a final concentration of 5%. The trichloroacetic acid-treated cells were allowed to stand in the cold for at least 30 min; the precipitates were filtered onto 25-mm fiberglass filters (Reeve Angel Co.) and washed three times with 5-ml volumes of cold 5% trichloroacetic acid. The filters were then dried at 60 C to remove water, and radioactivity was determined.

Preparation and analysis of cell extracts. Cell extracts were prepared by collecting and washing cells in TMK buffer [10 mM tris(hydroxymethyl) aminomethane, 50 mM KCl, *p*H 7.2] containing 10 mM $MgCl_2$ (TMK-10). Washed cell pellets were suspended in 5 ml of TMK-10 containing 60 μg of lysostaphin (Mead Johnson and Co., Evansville, Ind.) and 25 μg of deoxyribonuclease (Worthington Biochemical Corp.). The suspensions were then incubated at 37 C for 10 min, at which time Nonidet NP-40 (Shell Oil Co.) was added to a final concentration of 0.5%. The addition of Nonidet caused complete lysis of the cells by solubilizing membranous materials. However, it could not be added simultaneously with the enzymes as it inhibited the activity of lysostaphin. The lysates were immediately chilled in an ice bath and centrifuged at 5,000 $\times$ *g* to remove cellular debris. The supernatant fluid from the low-speed centrifugation was then centrifuged at 105,000 $\times$ *g* for 2 hr to sediment the ribosomal fraction. Subunits were derived by overnight dialysis of the ribosome pellets against 500 volumes of TMK-1 (TMK containing 1 mM $MgCl_2$) or TMK-0.1 (TMK containing 0.1 mM $MgCl_2$). When necessary, ribosomal RNA was extracted at this point, analyzed by polyacrylamide-gel electrophoresis, and counted as described by Iandolo (3).

Centrifugation to display the ribosomal profiles was carried out by layering samples on 13-ml 5 to 20% sucrose gradients in TMK-1 or on 30-ml 5 to 20% sucrose gradients in TMK-0.1. The gradients were centrifuged for 4.5 hr at 30,000 rev/min in a Beckman SW-36 rotor and for 10 hr at 25,000 rev/min in an SW 25.1 rotor. Samples were dripped out, and the absorbance was monitored at 260 nm with a Gilford spectrophotometer.

Isotope techniques. Labeled preparations of ribosomes were prepared for counting by first adding 100 μg of bovine serum albumin to each fraction as a coprecipitant. The fractions were made up to 5% with cold trichloroacetic acid and allowed to stand in the cold for 30 min. The precipitates were then collected on fiberglass filters, washed, and dried at 60 C. Finally, the dried filters (and those obtained by extracting whole cells) were counted in a scintillation fluid containing 5.5 g of Permablend II [98% 2,5-diphenyloxazole - 2% 1,4 - bis - 2 - (5 - phenyloxazolyl) benzene, Packard Instrument Co., Inc.] per liter of toluene. The samples were counted in a Beckman LS-150 scintillation counter.

Ribosomal preparations containing two isotopes (doubly labeled samples) were counted by use of aqueous techniques. The scintillation cocktail (Tt-21) described by Patterson and Greene (8) was used; it contained 333 ml of Triton X-100 (specially purified for scintillation counting; Packard Instrument Co., Inc.) and 5.5 g of Permablend II made up to 1 liter with toluene. The entire fraction was added to the vial. Quenching was corrected automatically by a Beckman LS-150 liquid scintillation spectrometer.

The radioisotopes used in this study were obtained from Schwarz BioResearch, Inc., at the following activities: uridine-5-^3H, 8 Ci/mmole; uracil-5-^3H, 21.6 Ci/mmole; valine-U-^3H, 5 Ci/mmole; arginine-U-^{14}C, 316 mCi/mmole.

RESULTS

It was shown earlier that recovery from heat injury involved macromolecular synthesis that was devoted almost exclusively to RNA synthesis. Furthermore, a larger portion of the RNA produced appeared in new ribosomes that were assembled without the benefit of de novo protein synthesis (4, 10). To verify this situation in the present system, the incorporation of isotopically labeled uracil and arginine was examined (Fig. 1). Heated, washed cells were incubated in TSB containing both arginine-U-^{14}C and uracil-5-^3H. Samples were withdrawn at intervals, and the trichloroacetic acid-precipitable label was measured. These data showed a rapid and immediate initiation of RNA synthesis that continued in a linear manner for 75 min and finally plateaued at approximately 90 min of incubation. In contrast, the uptake of arginine into protein did not occur until after RNA synthesis had ceased (90 min), whereupon arginine incorporation was rapid and continued to increase throughout the remainder of the experiment. Plate counts showed that recovery was complete at 120 min.

The lack of protein synthesis during the first 2 hr was enigmatic, especially since previous reports indicated that ribosome degradation occurred as a result of heat treatment (2, 9, 10) and presumably their resynthesis was an obligatory step in the recovery process.

On the other hand, these data suggested that, since only RNA and not protein was resynthesized, new ribosome formation seemed unlikely. To resolve this problem, the following experiment was performed. Ribosomes from cells recovered for 90 min in TSB containing 1 μCi of uracil-5-^3H/ml were co-centrifuged with a large excess of ribosomes from heated unlabeled cells (Fig. 2). The absorbance trace shows that 30*S* subunits were specifically destroyed, as we reported in an earlier publication (9). But these data demonstrated that, even though protein synthesis did not occur,

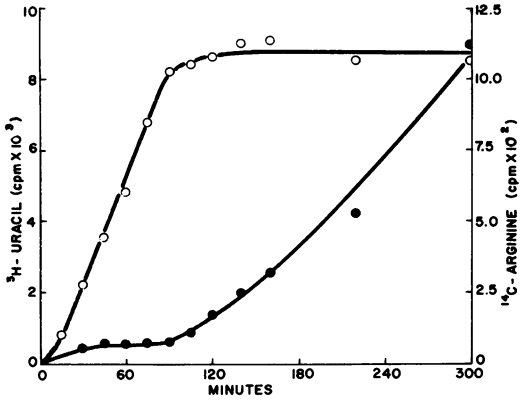

FIG. 1. *Synthesis of RNA and protein by recovering cells of S. aureus. Heated cells were incubated at 37 C in Trypticase Soy Broth containing 0.1 μCi of uracil-5-^{3}H/ml and 0.1 μCi of arginine-U-^{14}C/ml. Samples were withdrawn, mixed with an equal amount of 10% trichloroacetic acid, and allowed to stand for at least 30 min in the cold. The precipitates were recovered by filtration, dried, and counted. Symbols: O, uracil counts; ●, arginine counts.*

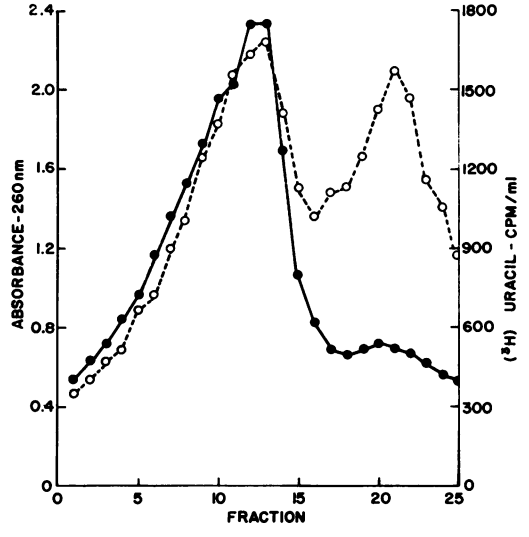

FIG. 2. *Comparison of ribosomal profiles from cells recovered for 90 min with those from heated cells. A large excess of unlabeled ribosomes (30 A_{260}) from heated cells were co-centrifuged (25,000 rev/min for 10 hr; SW 25.1) with ribosomes from cells that were recovered for 90 min in Trypticase Soy Broth containing 1 μCi of uracil-5-^{3}H/ml. Absorbance was monitored at 260 nm, and radioactivity was assayed as described. Symbols: ●, absorbance of ribosomes from heated, unlabeled cells; O, ribosomes from recovered cells (counts/min, uracil-5-^{3}H).*

the regeneration of 30S particles occurred and, in addition, new 50S subunits were also constructed. Examination of the RNA from these ribosomes by polyacrylamide-gel electrophoresis (Fig. 3) demonstrated that both 23S and 16S RNA were synthesized during recovery. Although not presented, additional data indicated that 5S RNA is also synthesized.

The question still remained, however, regarding the disposition of the preexisting protein and the source of protein required for new ribosome synthesis. Although these experiments were carried out under conditions which have been shown to be independent of protein synthesis, the possibility that small amounts of protein, almost entirely ribosomal in nature, were synthesized and appearing in 30S and 50S subunit peaks still remained as a viable hypothesis. Therefore, to test this possibility, recovery of injured cells was carried out in TSB that contained 100 μg of chloramphenicol/ml. This concentration was 50 times greater than necessary not only for complete inhibition of the organism but also for total suppression of label into the trichloroacetic acid-precipitable fraction of normal or heated cells. These conditions were used in the following two experiments. Cells were prelabeled for 16 hr with 0.03 μCi of arginine-*U*-^{14}*C* per ml and then heated and cooled as described in Materials and Methods. The suspension was then

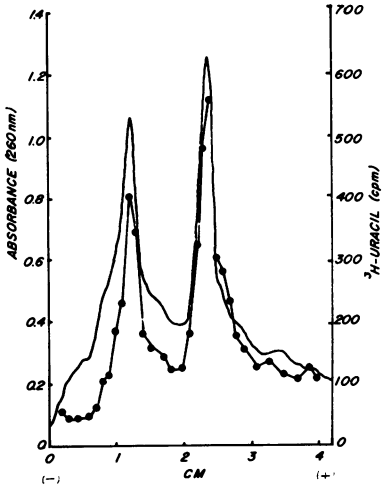

FIG. 3. *Polyacrylamide-gel electrophoresis of RNA from ribosomes of cells recovered for 90 min at 37 C. Approximately 3 A_{260} of phenol-extracted ribosomal RNA was layered on top of 8-cm, 2.4% polyacrylamide gels, and electrophoresis was carried out for 2 hr at 5 ma/gel. The gels were analyzed as described. Solid line, absorbance trace; closed circles, uracil-5-^{3}H (counts/min).*

divided into two portions, and both were recovered in TSB containing chloramphenicol. The first portion of TSB contained uridine-5-^3H (1 μCi/ml) to indicate RNA synthesis and to mark the 30S region of the gradient; the second portion contained valine-U-^3H (1 μCi/ml) to determine protein synthesis. Recovery was carried out for 90 min, and the cells were removed and analyzed. As shown in Fig. 4, ribosomal RNA was synthesized and ribosomes were assembled, as indicated by the uridine counts. However, protein synthesis was not observed, as noted by the absence of any significant valine incorporation. Furthermore, it appeared as though the original ribosomal protein would have to be recycled rather than turned over for the observed patterns. Turnover would necessitate de novo synthesis, which was not possible in this experiment because of the presence of chloramphenicol. A last possibility, that of a protein synthetic mechanism that is resistant to chloramphenicol action, hardly seems likely, because such a mechanism would have resulted in the incorporation of valine. Therefore, the only source of 30S proteins were the original subunits prelabeled with ^{14}C-arginine, and any new particles formed would contain those proteins. Although a slight ^{14}C-arginine peak existed at the 30S region, it was far from convincing.

However, when more highly prelabeled cells (0.3 μCi of arginine-U-^{14}C/ml) were heated and

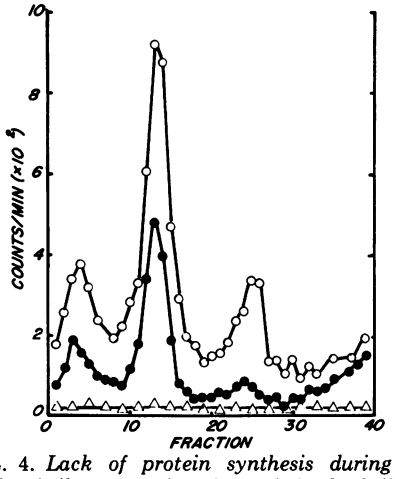

FIG. 4. *Lack of protein synthesis during reassembly of ribosomes after thermal shock. Cells were prelabeled with arginine-U-^{14}C (●), heated, and recovered in two portions. The first contained uridine-5-^3H plus 100 μg of chloramphenicol/ml (○); the second contained valine-U-^3H plus 100 μg of chloramphenicol/ml (△). Ribosomes were isolated and centrifuged through 5 to 20% sucrose gradients in TMK-1 for 4.5 hr.*

then recovered for 60 min in TSB with 100 μg of chloramphenicol/ml and one-tenth the amount of uridine-5-^3H/ml (0.1 μCi/ml), the problem was resolved (Fig. 5). The left-hand panel of Fig. 5 is the ribosomal profile from heated cells. A large prominent 50S peak is present, but the 30S region (arrow at fraction 16) is totally lacking. When recovery had taken place (right-hand panel), 30S subunits were present, indicating that new 16S RNA was synthesized and the prelabeled 30S proteins were recycled; this was shown by the reappearance of ^{14}C counts in the 30S region. Several replications of this experiment have confirmed these data, but in each case the recycled 30S protein peak is small in comparison with the 50S peak. In attempting to reconcile this discrepancy, we examined the stability of the 50S subunit of heated cells. That is, we supposed that the original 50S particle (prelabeled 50S) was largely inactive and served as a source of protein for subunit assembly with newly synthesized 23S RNA. This was suggested in an earlier paper (9) and, although these data were consistent with this idea, experimental corroboration was necessary. Therefore, cells were doubly prelabeled with arginine-U-^{14}C and uridine-5-^3H prior to heating. The cells were processed and recovered in nonradioactive medium, and the specific activity of the 50S and 30S peaks was monitored. If 50S ribosomes were turning over, the specific activity of the particle would decrease. On the other hand, if the protein were recycled, only the specific activity of the RNA would decrease, as new RNA would be synthesized from unlabeled precursors. When the data were examined (Table 1), it was found that the specific activity of the protein was relatively constant whereas RNA turnover was extensive. At 90 min, 20% of the prelabeled 23S RNA and 60% of the prelabeled 16S RNA were turned over. This is also reflected in the ratios of protein to RNA calculated from these data. For the 50S particle, the ratio changes from an initial value of 0.268 to 0.286 at 90 min, and the ratios for the 30S particle increased from 0.300 at the start of the experiment to 0.550 at 90 min.

The resynthesis of new RNA species and the recycling of protein led us to question the kinetics of assembly and whether the process was similar or dissimilar to normal ribosome biogenesis. Injured cells were suspended in TSB and incubated at 37 C. After 30 min, uridine-5-^3H was added to the medium, and samples were removed at 2, 5, and 10 min. The cells were collected and washed on membrane filters to remove the label rapidly. The cells

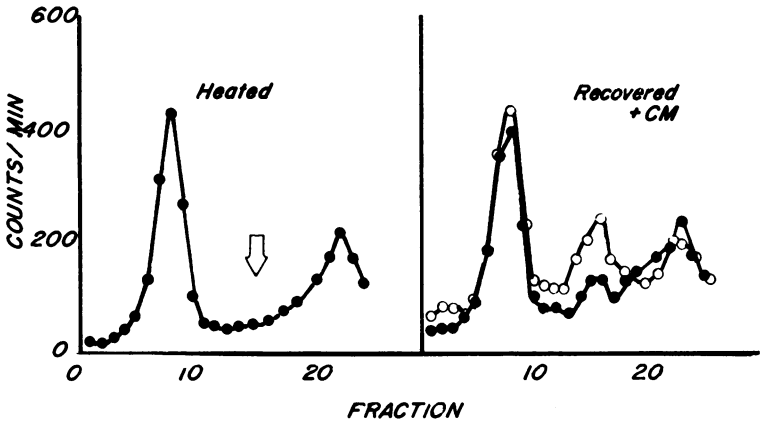

FIG. 5. *Recylcing of ribosomal protein by recovering cells of heat-shocked S. aureus. Cells were prelabeled with arginine-U-^{14}C (0.3 μCi/ml) and heated. A portion was kept as a control, and the remaining cells were recovered in Trypticase Soy Broth containing 0.1 μCi of uridine-5-^{3}H/ml and 100 μg of chloramphenicol (CM)/ml for 120 min. Ribosomes were extracted and displayed as described in Materials and Methods. Symbols: ●, arginine-U-^{14}C; ○, uridine-5-^{3}H; the arrow is placed in the heated panel to mark the 30S region.*

were then processed, and the ribosomal profiles were examined (Fig. 6). After a 2-min pulse, what appears to be the uncoordinated synthesis of 30*S* particles was observed. However, as the pulse time was extended to 5 min, both 50*S* and 30*S* subunits were observed, and, after 10 min, amplification of both subunits was evident along with the formation of the larger 70*S* ribosome. It is significant to note that conventional precursor particles seen in nonheated preparations were not found. Refinement and replication of this experiment have yielded results consistent with these data regardless of whether the uridine pulse was shortened or lengthened.

DISCUSSION

The data presented in this communication have demonstrated that the regeneration of ribosomes in thermally shocked staphylococci occurs independently of protein synthesis. This occurrence is in marked distinction to the repair of injury in the gram-negative bacterium *Salmonella typhimurium*, wherein protein synthesis must begin before new ribosomes are generated (11). Furthermore, subunit degradation in this organism is generalized, and recovery may therefore represent only a specialized case of ribosome biogenesis. However, the staphylococcal system described here appears to be yet another special case wherein assembly is carried out with preexisting proteins and de novo synthesis is limited solely to RNA.

Even when protein synthesis is inhibited by chloramphenicol, mature ribosomes are found rather than the immature particles that accumulate in the presence of this antibiotic. This is unexpected if the conclusion of Yoshida and Osawa (13) is accepted, which allows the formation of chloramphenicol particles as a result of the nonspecific binding of intracellular protein to nascent RNA. However, in the case of thermally shocked staphylococci, because of 30*S* subunit degradation, a concentrated pool of basic ribosomal protein exists, which presumably has a higher binding efficiency than nonribosomal protein and is thereby able to displace it on RNA. The result of this would be the formation of normal particles.

The conservation of ribosomal protein is not unique to this system. Lefkovits and Di-Girolamo (5) have shown that ribosomal protein recycling occurs in Mg^{++}-starved *Escherichia coli* in the presence of chloramphenicol. Their data, like the results presented here, suggest that the ribosomal proteins are recycled through the pool. Although they did not

TABLE 1. *Specific activity[a] of ribosomal subunits during recovery in non-radioactive Trypticase Soy Broth*

Time (min)	50*S* subunit		30*S* subunit	
	RNA	Protein	RNA	Protein
0	820	220	956	287
30	824	217	585	248
60	685	191	490	254
90	679	194	378	207

[a] Expressed as counts per minute per unit of absorbancy at 260 nm.

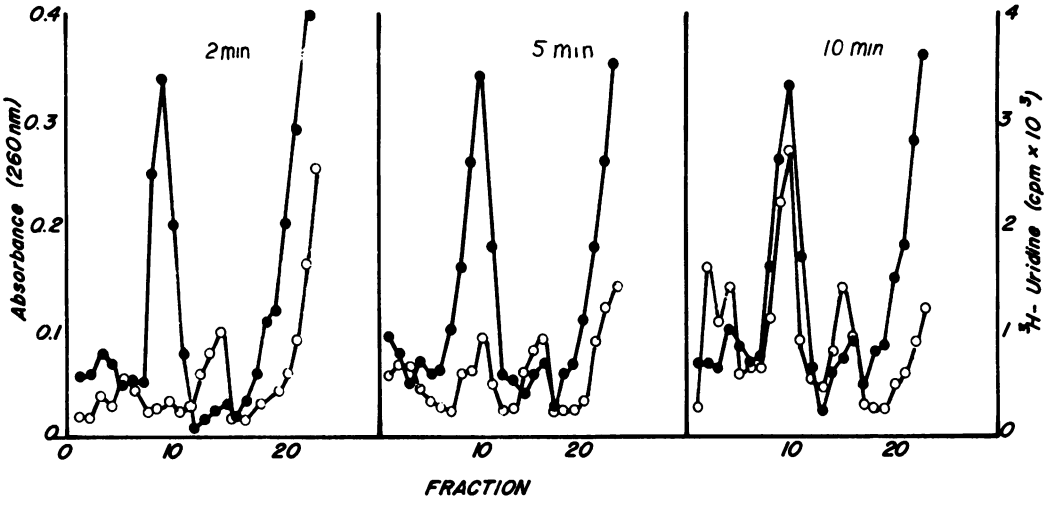

FIG. 6. *Kinetics of appearance of newly synthesized ribosomes. Heated prelabeled cells (arginine-U-*^{14}C, ●) *were incubated in Trypticase Soy Broth containing 100 μg of chloramphenicol/ml. After 30 min, enough uridine-5-*^{3}H *to make 1 μCi/ml was added, and samples were removed and rapidly filtered at 2, 5, and 10 min. Ribosomes were extracted and displayed as given in Materials and Methods. Symbols: ●, arginine-U-*^{14}C*; ○, uridine-5-*^{3}H.

directly show the reappearance of original protein as was done here, this fact was inferred from their results. They further implied that the recycling process was complete. However, even in the absence of chloramphenicol we have not observed 100% reutilization of the original protein. This is due, we assume, to the resumption of protein synthesis, resulting in new ribosomal protein which dilutes out the system. On the other hand, even when protein synthesis is inhibited, incomplete recycling occurs. In this case, not only is new synthesis inhibited but, in addition, the rate of recycling is depressed and suggestive of the fact that the antibiotic may affect the intermolecular binding of protein to RNA.

The inability to demonstrate precursor particles by pulse labeling has been problematic. One possible explanation may be that reassembly of 30*S* subunits proceeds in vivo as described by Traub and Nomura (12) for *E. coli* in vitro, and, as observed by Nashimoto and Nomura (6), 50*S* assembly may occur as a consequence of this system. Preliminary experiments involving low-temperature recovery support this contention and will be reported in a subsequent publication. These data may also explain the apparent uncoordination of 16*S* and 23*S* RNA synthesis. For example, although 16*S* and 23*S* RNA continue to be synthesized in a normal coordinated manner, ribosome regeneration may depend upon the fact that the original 50*S* subunits must be turned over to liberate 50*S* protein, in effect limiting the rate at which new RNA appears in the 50*S* peak. The pulse data from later times showing the rapid appearance of 50*S* particles once assembly has begun, and the fact that the association of RNA and protein occurs quite rapidly and without the formation of precursor at the temperature used in this study (12), would support this conclusion.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant FD-00160 from the Food and Drug Administration and by Biomedical Sciences Support grant FR-7036.

We thank Richard A. Consigli for reading and commenting on the manuscript.

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