JOURNAL OF BACTERIOLOGY, June 1972, p. 889-894

Vol. 110, No. 3
Printed in U.S.A.

Ribosome Maturation of *Escherichia coli* Growing at Different Growth Rates

GEORGE A. MICHAELS[1]

University Institute of Microbiology, University of Copenhagen, Copenhagen, Denmark

Received for publication 29 February 1972

The data reported here are consistent with the hypothesis that the rate of ribosome assembly in vivo approximates a constant fraction of the generation time for the four rates studied. This conclusion is indicated by the following. (i) There is an increased lag period before radioisotopically labeled uracil appears in 23 and 16*S* ribosomal ribonucleic acid of 70*S* ribosomes as a function of growth rate. (ii) The time necessary for ^{3}H-uracil in the 43*S* ribonucleoprotein precursor to the 50*S* subunit to assume a position at 50*S* in sucrose gradients is greatly increased inversely to the growth rate.

The determination of the quantities and rates of synthesis of specific classes of macromolecules has helped in understanding the control of molecular events of cell growth. Recently, the rates of transcription (1, 10) and translation (2, 5) were reported. In addition, the relative amounts of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein and the number of ribosomes present in cells growing at different rates have been estimated (8). Based on such observations, dynamic interrelationships of these cell constituents have been suggested.

Over a certain range there appears to be a direct correlation between the number of ribosomes in a cell and the rate of growth and of protein synthesis (2, 8). The mechanism of control of ribosome synthesis and the means by which a cell determines the appropriate number of ribosomes for a particular environment remains obscure. When a bacterial cell is shifted from minimal medium to a rich medium the rate of ribosomal RNA (rRNA) synthesis accelerates immediately (4). The rate of ribosomal protein synthesis also responds very sensitively to changes in the nutritive conditions (4, 15). The rate of ribosome synthesis is affected by the frequency of initiation of nascent ribosomal peptide chains and of nascent chains of rRNA, and messenger RNA (mRNA) for ribosomal proteins, and by the rates of chain elongation of each of these molecular species; all respond in varying degree to changes in the growth rate (2, 5, 10, 14). The rate of assembly of the ribosomal proteins with the appropriate rRNA and with each other offers yet another mode of control. It has been shown (19) that in vitro ribosome assembly rate depends directly upon temperature.

Although other possible means of controlling ribosome synthesis may exist, it has seemed appropriate to determine whether, as suggested by Schlessinger (16), the rate of ribosome assembly varies with growth rate. Recently van Dijk-Salkinoja and Planta (20) reported that the 50*S* ribosomal subunit was assembled at a constant rate independent of growth rate in *Bacillus licheniformis*. Evidence that the rate of ribosome assembly in vivo varies with the growth rate is presented herein. This finding suggests that the number of ribosomes characteristic of a particular growth rate is regulated at least in part by the rate of ribosome assembly.

MATERIALS AND METHODS

Bacterial strain. A mutant strain of *Escherichia coli* 15 T$^-$ requiring thymine, histidine, arginine, and leucine was used for all of the experiments.

Media. Tris(hydroxymethyl)aminomethane (Tris) minimal medium consisted of: 100 mM Tris, 27 mM KCl, 37 mM NH_4Cl, 1 mM Na_2HPO4, 2.8 mM Na_2SO_4, 0.01 mM $FeCl_3$ (adjusted to *p*H 7.6 with HCl) supplemented with 0.2% glucose, 0.4% succinate, 1% proline, and 0.5% vitamin-free Casamino Acids where indicated.

Buffers. TMN buffer contained: 0.01 M Tris-hydrochloride, 0.01 M $MgSO_4$, 0.05 M NH_4Cl, *p*H 7.4.

Chemicals. Chemicals used were: [2-^{14}*C*]uracil (55 mCi/mmole) from Amersham Radiochemical Centre, C-[5-^{3}*H*]uracil (0.250 mCi/0.0034 mmole)

[1] Present address: Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 01545.

from New England Nuclear, egg white lysozyme (three times-crystallized) and pancreatic deoxyribonuclease (electrophoretically purified) from Sigma, and Brij 58 [polyoxyethylene (20) cetyl ether] from Atlas Chemical Industries.

Growth, treatment, and lysis of bacteria. An exponentially growing culture was diluted to an optical density at 450 nm (OD_{450}) of approximately 0.03, and the OD_{450} was followed. At OD_{450} of 0.1, 2,000-ml cultures were given 2 μCi of [2-^{14}C]uracil, and at OD_{450} of 0.5 to 0.6, 200 μCi of C-[5-^{3}H]uracil was added with 5 μg of cold uracil per ml (final concentration) and 200-ml portions of the culture removed at intervals were poured over 200 g of crushed ice containing NaN_3 at a final concentration of 10^{-2} M and cold uracil at 100 μg/ml final concentration. The cells were pelleted at 0 C in a refrigerated Sorvall centrifuge, washed once with TMN buffer, recentrifuged, and frozen at −20 C. The pellet was thawed and resuspended in 2.3 ml of TMN buffer plus 5 μg of deoxyribonuclease per ml at 0 C. The cells were lysed by two 45-sec sonic treatments in an MSE Mullard ultrasonic disintegrator. The lysate was clarified by 5 min of centrifugation at 5,000 × *g* and analyzed immediately.

Sucrose gradient analysis of crude lysate. A 1.5-ml portion of the lysate was layered on the top of a 25-ml linear 15 to 35% sucrose density gradient in TMN buffer, which was centrifuged for 14 hr at 25,000 rev/min at 2 to 4 C in a Spinco model L-2 ultracentrifuge using the SW25.1 rotor. After centrifugation, the centrifuge tube was punctured at the bottom with a hypodermic needle, and the contents were pumped through a Unicam SP800 spectrophotometer equipped with an LKB flow cell with a 1-mm light path; the OD_{260} was monitored continuously.

Counting of radioactive material. Samples (0.7 ml) of the gradient were collected and treated with trichloroacetic acid for 1 hr at 0 C. The precipitate was then collected on Sartorius filters, dried, and counted in 10 ml of a toluene-based scintillation liquid (50 mg of dimethyl 1,4-bis[2-(5-phenyloxazolyl)]-benzene and 4 g of 2,5-diphenyloxazole in 1 liter of toluene).

RNA extraction and analysis. RNA was extracted from ribosomes by collecting 0.7-ml fractions of the above gradients directly into tubes containing 0.1 ml of 10% sodium dodecyl sulfate (SDS; final concentration, 1.25% SDS). For analysis of the RNA of the 70*S* ribosomes, the fractions corresponding to the 70*S* peak (see Fig. 1) were pooled and precipitated in two volumes of 96% ethanol. The precipitated RNA was pelleted and redissolved in 0.01 M sodium acetate, *p*H 4.6, and 1.5-ml portions were layered on 5 to 20% linear sucrose gradients in 0.01 M sodium acetate. The gradients were spun for 17 hr at 25,000 rev/min at 2 to 4 C in a Spinco model L-2 ultracentrifuge using the SW 25.1 rotor. The material was collected and analyzed exactly as described for the gradients with the crude cell lysates.

RESULTS

Ribosome assembly involves the association of ribosomal protein and rRNA in a specific stepwise fashion. Thus one observes the sequential flow of pulse-labeled rRNA to ribonucleoprotein (RNP) subunit precursors of increasing size (13). Since the precursor particles cosediment with mature subunits, kinetic studies of the assembly time of the subunits are difficult. However it is assumed that upon completion of the 50*S* and 30*S* subunits, their transformation to 70*S* ribosomes (in polysomes) occurs more or less instantaneously. Therefore the criterion for the completion of a new ribosome used here is the appearance of labeled 23 and 16*S* rRNA in the 70*S* particles (which can be isolated cleanly, free of any RNA precursors). Thus the first appearance of labeled rRNA in the 70*S* particles is an upper limit of the actual assembly time.

Four growth conditions were studied. For each experiment, cells were harvested at several intervals after the addition of ^{3}H-uracil (0.1 μCi per 5 μg of cold uracil per ml). These cells had been prelabeled more than two generations before with a small amount of ^{14}C-uracil (55 mCi/mmole) which was chased by endogenously synthesized uracil. Thus, at the time of the ^{3}H-uracil addition, all the ^{14}C-uracil was in stable RNA. The harvested cells were sonically treated to convert the polysomes to 70*S* ribosomes, as indicated by sucrose density gradient analysis of clarified sonic extracts (Fig. 1). The resolution of the subunit region was sufficient to permit kinetic analysis of the transformation of the 43*S* precursor to the 50*S* subunit. The portion of the 70*S* peak most distant from the 50*S* peak was isolated, and the RNA was extracted (*see above*). Because such derived 70*S* particles harbor mRNA (18) and transfer RNA (tRNA) in addition to rRNA, it was necessary to display the RNA isolated from 70*S* ribosomes on a sucrose gradient to separate the larger 23 and 16*S* molecules from the tRNA and mRNA fragments (Fig. 2). It was unexpected to find RNA demonstrating the (labeling) kinetic characteristics of mRNA with sedimentation coefficients in the range of 8 to 12*S* (Fig. 2), since it is assumed that the ribosomes would protect only a small portion of the messenger from sonic disruption and ribonucleases.

The ^{3}H content of 23 and 16*S* RNA was determined for each gradient. To correct for variations in time and efficiency of extraction, the samples in a particular experiment were all normalized to a constant content of ^{14}C-stable RNA. Figure 3 shows the increase of ^{3}H-uracil in the rRNA isolated from purified 70*S* ribosomes with the increase in cell mass. This is shown for several different growth rates. At faster rates (glucose-Casamino Acids cells),

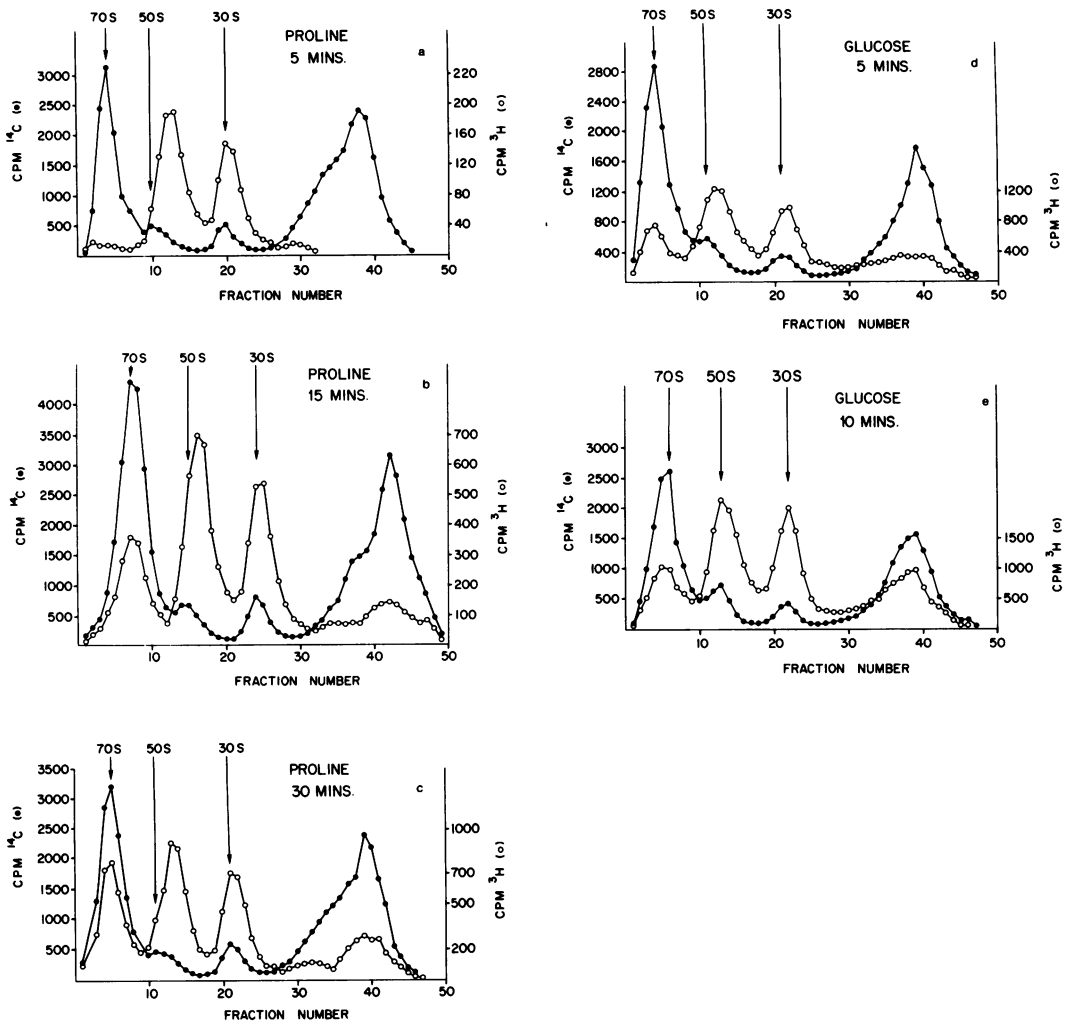

FIG. 1. *Counts per minute in ribosome profiles of cells grown on 0.2% glucose or 1% proline. The cells were labeled with 2 μCi of ^{14}C-uracil at OD_{450} 0.1 and grown to OD_{450} 0.5 to 0.6. Then ^{3}H-uracil, to a final concentration of 0.1 μCi per 5 μg of uracil per ml, was administered. The cells were harvested at several intervals after ^{3}H-uracil addition by combining 200-ml portions with distilled water ice plus 0.01 M sodium azide and 100 μg of cold uracil per ml. After centrifugation the cells were washed in TMN buffer and frozen at −20C. Upon thawing, the cells were diluted to 2.3 ml with the TMN buffer containing 5 μg of deoxyribonuclease and sonically treated twice for 45 sec. The sonic extract was centrifuged at 5,000 rev/min for 5 min in a refrigerated Sorvall centrifuge and layered on a 15 to 35% sucrose density gradient in TMN. It was then centrifuged for 14 hr at 25,000 rev/min at 0 C in the L2-65B Spinco ultracentrifuge. The spun gradients were monitored at 260_{nm} on a Unicam SP800 spectrophotometer equipped with a flow-through cuvette. Fractions (0.7 ml) were collected and treated with 10% trichloroacetic acid and the precipitate was collected on Sartorious filters and counted in a toluene-based scintillation fluid.*

there is a more rapid flow of ^{3}H-uracil into the rRNA compared to the more slowly growing cells. This indicates a definite difference in the rate of ribosome synthesis under the various growth conditions. An extrapolation of these curves (curves consistent with late points in the experiment which are independent of pool differences) back to the abscissa should give an approximation of the lag (ribosome synthesis) time. The numbers achieved by this process closely match the time actually observed for the first label detected in rRNA; they are listed in Table 1. If one takes the lower values (Table 1), it appears that the time for labeled rRNA to reach 70*S* ribosomes approximates a constant fraction of the generation time for all four growth conditions. It is particularly noteworthy that the lags observed for the Casa-

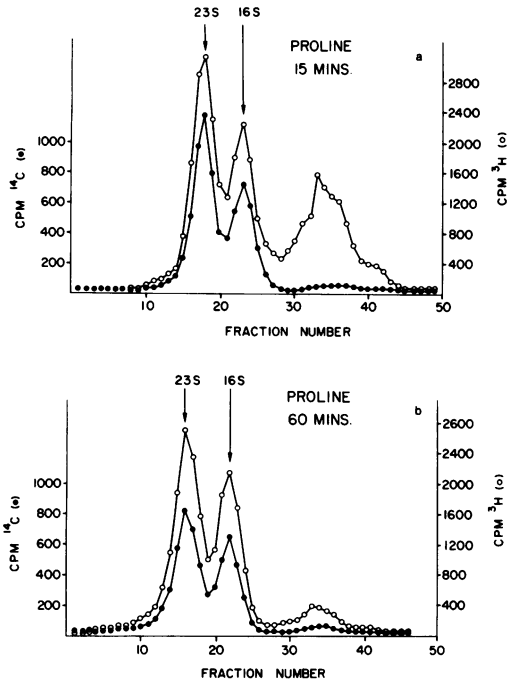

FIG. 2. *Profiles of RNA isolated from 70S ribosomes. Ribosomes were prepared as those in Fig. 1 and centrifuged on sucrose density gradients. Appropriate fractions were pooled and treated with sodium dodecyl sulfate and ethanol. The resulting precipitate was spun down and washed once in 0.01 M sodium acetate buffer, pH 4.6. The redissolved RNA was then layered on a 5 to 20% sucrose density gradient in the acetate buffer and centrifuged for 19 hr at 25,000 rev/min at 0 C in a Spinco centrifuge. The* OD_{260} *and the* ^{3}H*-uracil counts are shown.*

mino Acids-plus-glucose culture and the glucose culture differ by a factor of two. Cultures growing at these rates do not show appreciable differences in the rate of peptide bond formation (2) or nucleotide addition (1, 10).

Further substantiation of the idea that ribosome assembly time varies with growth rate can be gained by comparing the rate at which ^{3}H-uracil in the larger RNP subunit precursor (43*S*) moves into the 50*S* region under different growth conditions (Fig. 1). In the glucose culture, the newly synthesized rRNA (^{3}H) sediments at 50*S* by 10 min, whereas the proline culture has most of the ^{3}H-labeled material still at 43*S* even 30 min after the ^{3}H-uracil labeling has begun. The rate of the 50*S* subunit assembly in the proline culture is definitely slower than that of the glucose culture. The bulk of the ^{3}H-labeled 30*S* material (a more difficult region to interpret due to its complex composition) sediments at exactly 30*S* from the outset under both conditions, due probably to (i) the ^{3}H-labeling of mRNA in 30*S* mRNA initiation complexes and (ii) very early precursors to both the 50*S* and 30*S* subunits which sediment just ahead and just behind the 30*S* subunit. The appearance of disproportionately larger amounts of native 30*S* subunits at slower growth (12) would support the idea of a decreased rate of 50*S* subunit assembly. The study of Forget and Varricchio (3) indicates that the 43*S* to 50*S* step seems to be a bottleneck in the pathway of ribosome synthesis in K-12 strains as well.

DISCUSSION

It has been shown in bacteria (8) that the number of ribosomes per cell depends upon the growth rate. Uncertainty exists as to the scheme of regulation invoked by the cells to achieve such variation. Several possibilities exist, none of which excludes the others. (i) The rates of chain elongation vary with the growth rate for rRNA and ribosomal proteins. (ii) The number of chain initiations of rRNA and mRNA for ribosomal proteins increase with growth rate, thus providing the observed increase in ribosome number. It has been shown that there is a bias toward the synthesis of stable RNA in cells growing at faster rates (14). Because of the low levels of free ribosomal proteins detected in growing cells (15), it is assumed that the synthesis of rRNA and the messages for ribosomal proteins are tightly coupled and respond coordinately to relevant perturbations. (iii) A third and rather unlikely possibility is that the rate at which ribosomes are destroyed varies inversely with growth rate. (iv) Although all the above mechanisms affect the number of ribosomes in a cell and the rate at which ribosomes are synthesized, it has been shown here that the rate of the assembly process itself is a function of growth rate; i.e., there is a facilitation of assembly by some hypothetical factor in the assembly process which is present at reduced amount or inactive condition at slow growth.

The rates of peptide addition (2) and nucleotide chain elongation (1) have been shown to be constant at growth rates faster than one generation per hour. Thus the factor of two difference in the time necessary for labeled rRNA to appear in the 70*S* ribosome between glucose-grown cells (50-min generation time) and glucose-Casamino Acids cells (30-min generation time) must be the result of something other than the variations in steptime of the ribosomal peptides and ribonucleotide addition. Other studies with slow-growing cells are

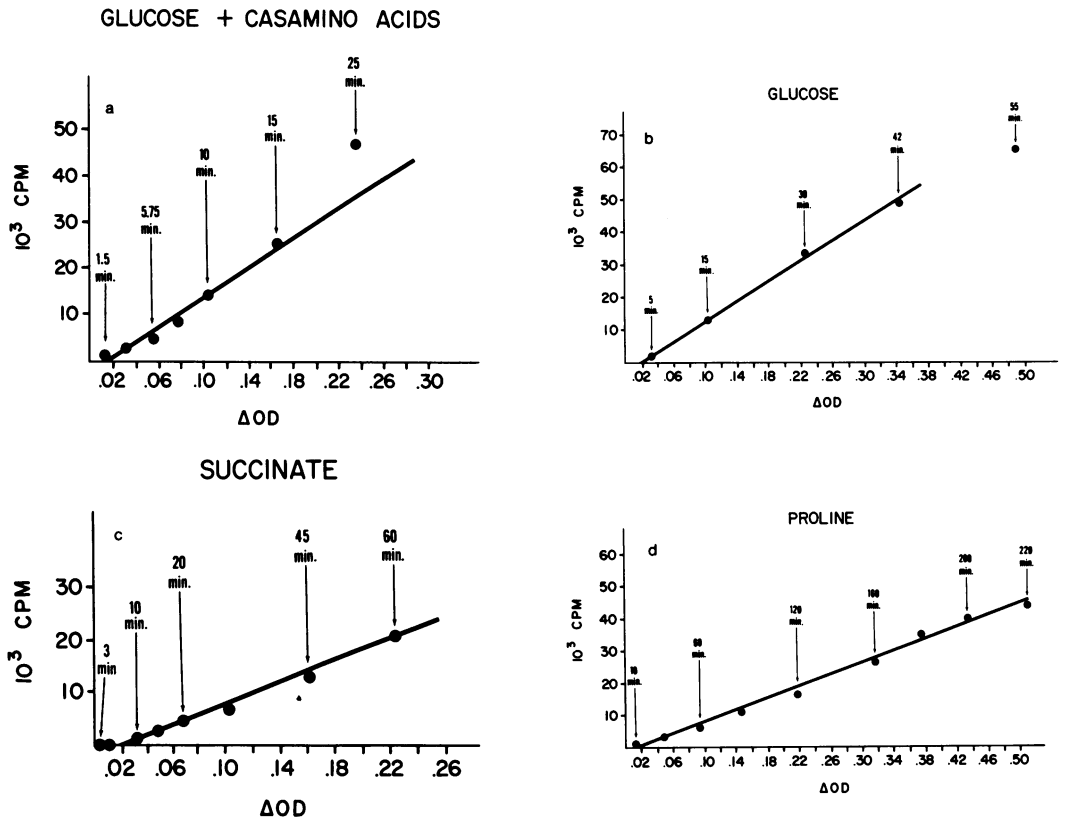

FIG. 3. *The totaled ^3H counts per minute in sucrose density gradient purified rRNA isolated from 70S ribosomes at several times after administration of ^3H-uracil graphed against the increase in mass (OD_{450}) of the culture. The amount of time between the beginning of the labeling and the time of harvest is indicated where space allows. The growth rates of the various cultures were the following: a, 0.2% glucose plus 0.5% Casamino Acids, doublings/hour (μ) = 2; b, 0.2% glucose, μ = 1.25; c, 0.4% succinate, μ = 0.6; d, 1% proline, μ = 0.35.*

TABLE 1. *Lag time necessary for ^3H-uracil to appear in purified rRNA isolated from 70S ribosomes*

Growth rate (doublings/hr)	Lag time[a] (min)	Lag time/generation time
2	1.5	0.05
1.25	3-3.5	0.062-0.073
0.66	6-7	0.06-0.077
0.35	9-10	0.053-0.058

[a] Time necessary for ^3H-uracil to appear in rRNA of 70S ribosomes.

consistent with the findings reported herein. Sells and Davis (17) observed a reduced rate of 50S subunit synthesis when the growth rate was slowed by manipulation of temperature. This is consistent with the in vitro studies of Nomura on the assembly of the 30S particle (13). It is possible that, in *B. licheniformis*, where a constant assembly time for the 50S particle at several growth rates is reported (20), the rate of ribosome assembly may be controlled in a different manner. Since several factors affect the rate of ribosome assembly, it may be that various species emphasize different mechanisms of control of ribosome synthesis. It is possible that the different observations may stem from the differences in experimental design.

Further clarification of the ribosome assembly process as well as its control can be gained through the study of ribosome synthesis in the transport mutants described by von Meyenburg (21). Their growth rate in batch cultures can be manipulated by varying the concentration of a single substrate. Thus cells growing at different rates contain the same cell machinery (the identical array of degradative enzymes, the same fraction of messenger RNA, i.e., the same degree of genomic induction) quite in contrast to the cells in the study reported herein. Such slow-growing cells could

contain more "available" RNA polymerase than cells growing at comparable rates produced by a poor carbon source such as proline. They also offer potentially better resolution of the control mechanisms for the synthesis of ribosomal proteins.

Since the rate of 43*S* to 50*S* transformation is independent of RNA steptime, uracil uptake, or pool size, the great disparity in the time necessary for the formation of the 50*S* subunit from the 43*S* precursor in the proline culture (generation time, 170 min) supports the notion that the ribosome assembly time increases with decreasing growth rate, due, for instance, to a regulatory factor integral either to the ribosome itself or to its assembly apparatus. The decreased rate of synthesis of the 50*S* subunit may be the result of production of at least one of the late-added 50*S* proteins which may act as an important control factor. Study of the proportions of the newly synthesized 50*S* proteins under different growth conditions should clarify this point. Reported ribosomal mutants often have blocks or constrictions of the 43*S* to 50*S* step (3, 9). Studies with low concentrations of chloramphenicol (13) also report accumulation of 43*S* precursors, supporting the idea that the addition of the final (outer, or split) proteins (11) is a complicated and perhaps critical step in the control of ribosome assembly.

ACKNOWLEDGMENTS

I am very grateful to O. Maaløe for his guidance and discussions and to M. Schaechter for helpful criticism and discussions.

This investigator was supported by a Postdoctoral Fellowship from the Damon Runyon Memorial Fund for Cancer Research, which he gratefully acknowledges.

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