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Ribosome Biosynthesis in *Escherichia coli.* Concerning the Limiting Step†

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ABSTRACT: By studying the kinetics of labeling of newly formed ribosomes in *Escherichia coli* cells growing at 37° on different media it has been found that the average time required to convert a single 26S precursor particle (p30S) into a 30S subunit increases with the culture doubling time. This process is immediately blocked by the addition of chloramphenicol. The maturation of 17S precursor RNA (p16S) into 16S RNA occurs after 30S ribosomes have been formed. *In vitro* reconstitution experiments from free rRNA and free ribosomal proteins have shown that p16S RNA is incorporated into 30S particles with the same efficiency as 16S RNA. p30S are converted into 30S particles by the simple addition of ribosomal proteins, instantaneously and in the cold. These results are interpreted as an indication that the rate limiting step in 30S ribosome formation which leads to the accumulation of p30S particles is not a spontaneous conformational rearrangement of the same particles, or the maturation of rRNA. Ribosome formation is probably controlled by the availability of ribosomal proteins.

The assembly of a bacterial ribosome is a relatively slow process. It takes 1–2 min to synthesize an entire chain of ribosomal RNA (rRNA) and less than 1 min to synthesize a single ribosomal protein. But to assemble the macromolecules into a complete particle takes a significant fraction of a cell generation time (McCarthy *et al.,* 1962; Mangiarotti *et al.,* 1968; Michaels, 1971). In bacteria growing in a poor medium this can amount to 15–20 min.

The reason why the process is so slow is not clear. Free ribosomal proteins are present in the cell (Schleif, 1969; Gierer and Gierer, 1968; Gupta and Singh, 1972; Gausing, 1974; Marvaldi *et al.,* 1974) and they can probably interact with RNA chains while these are still being synthesized (Mangiarotti *et al.,* 1968). The addition of proteins, however, occurs in a discontinuous way (Sells and Davis, 1970; Marvaldi *et al.,* 1972; Nierhaus *et al.,* 1973), leading to the formation of incomplete particles sedimenting at 21–27 S (p30S), at 32 S ($p_1$50S), and at 43 S ($p_2$50S) (McCarthy *et al.,* 1962; Mangiarotti *et al.,* 1968; Osawa, 1968; Hayes and Hayes, 1971). The conversion of these particles into complete ribosomal subunits is the rate-limiting step in

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ribosome formation.

In vitro studies (Nomura, 1973) have shown that rRNA and ribosomal proteins can assemble spontaneously into a ribosome. During the process intermediate, incomplete (RI) particles form, which must undergo a conformational change before the addition of protein is completed. The RI particles might correspond to the precursor particles observed *in vivo*. A conformational rearrangement of ribosomal precursors might be the rate-limiting event *in vivo*.

However, there are alternative possibilities. One is suggested by the finding that the RNA contained in natural precursors differs from mature RNA used in *in vitro* reconstitution experiments. Immature RNA has extra nucleotide sequences at both 5′ and 3′ ends, and is not methylated (Adesnik and Levinthal, 1969; Hayes *et al.*, 1971; Dahlberg and Peacock, 1971). Conversion of p30S and p50S particles into mature ribosomes therefore includes processing of precursor 16S (p16S) and 23S (p23S) RNA, as well as addition of protein. An obvious possibility is that the binding sites for some ribosomal proteins might become available only after RNA maturation has occurred. Processing of rRNA could then be the rate-limiting step determining the accumulation of ribosomal precursors.

As another alternative, the synthesis of one or a few ribosomal proteins in cells may lag behind the synthesis of rRNA. As *in vitro* studies have shown, certain ribosomal proteins become bound to the RNA only after other specific proteins are bound (Nomura, 1973). If just one of the branch-point proteins were in short supply, largely incomplete particles would accumulate.

As one way to learn more about rate-limiting steps in ribosome formation, we have timed the conversion of precursors into complete 30S ribosomes and the maturation of 16S RNA in different conditions of growth. We have also tested for the incorporation of immature RNA into complete ribosomes *in vitro*. Our results indicate that the assembly of 30S ribosomes is probably not limited *in vivo* by a conformational change of precursor particles, nor by RNA maturation. The most likely limiting factor is the availability of ribosomal proteins.

Materials and Methods

Cells of *Escherichia coli* D10 were grown exponentially at 37° in medium MS9 (Bolle *et al.*, 1968) supplemented with 0.5% casamino acids and 0.2% glucose (doubling time 40 min) or with 0.2% sodium acetate and 0.1% methionine (doubling time 120 min). Cells were harvested by pouring onto crushed ice.

The crude extract (S30) was prepared from alumina ground cells, extracted with 2 vol of TM buffer containing 20 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol. The extract was centrifuged at 30,000*g* for 15 min. The soluble fraction (S100) was prepared by centrifuging the S30 fraction at 100,000*g* for 2 hr.

Native 30S and 50S ribosomes (Green and Hall, 1961) were isolated by zonal sedimentation of a 30S fraction in a 10–30% sucrose gradient in TM buffer containing 40 mM NaCl. Centrifugation was at 40,000 rpm for 6 hr in an SB238 rotor of an International ultracentrifuge. Fractions containing 30S and 50S subunits were separately pooled, dialyzed in TM buffer, and concentrated under reduced pressure.

To label native 30S ribosomes, 1 μCi/ml of [^{14}C]uracil (sp act. 61 mCi/mmol) was added to the culture at an optical density of 0.3 two generations before harvesting. To label p30S particles, 10 μCi/ml of [^{3}H]uracil (29 Ci/mmol) was added to the culture during the last 2 min of growth. Cells were lysed by the procedure of Godson (1967), and the particles isolated by sucrose gradients, as described above.

T4 specific mRNA was prepared as previously described (Mangiarotti *et al.*, 1971).

To dissociate 30S ribosomes into RNA and proteins and reconstitute ribosomes from their components, the procedure of Traub and Nomura (1969) was used with the following modification. The ribosomal preparation was diluted with an equal volume of 8 M urea and 6 M LiCl and kept cold overnight. To avoid loss of material, RNA was not removed from the dissociation mixture. Separation of ribosomal protein and rRNA was verified by showing that centrifugation of control samples at 30,000 rpm for 20 min completely removed RNA leaving proteins in solution. Reassociation was achieved by diluting the dissociation mixture directly with 20 vol of a buffer containing 5 mM potassium phosphate (pH 5.2), 0.3 M KCl, and 6 mM β-mercaptoethanol, prewarmed at 40°. Incubation was continued at 40° for 20 min. The size of reconstituted particles was determined by zonal sedimentation as indicated above.

To detect their incorporation into 70S monosomes and polyribosomes, reconstituted particles were incubated at 37° for 4 min in 0.2 ml of a mixture containing 20 mM Tris-KCl (pH 7.6), 8 mM $MgCl_2$, 6 mM β-mercaptoethanol, 5 mM ATP, 2 mM GTP, 80 mM NH_4Cl, 5 mM each of the 20 amino acids, 20 mM phosphoenolpyruvate, 0.01 mg of pyruvate chinase, 5 mg of polyethylene glycol, 20 μg of T4 RNA, 40 μl of S100, and 200 μg of 50S subunits. Following incubation the mixture was sedimented through a sucrose gradient in 8 mM $MgCl_2$–10 mM Tris-HCl (pH 7.6) for 3 hr at 39,000 rpm to display polyribosomes and free ribosomal particles.

To determine the size of the RNA contained in native and reconstituted 30S ribosomes, and in p30S particles, the RNA was extracted with phenol and subjected to disc gel electrophoresis, according to Bishop *et al.* (1967). Polyacrylamide gels (3.6%) were prepared as previously described (Mangiarotti and Turco, 1973).

Results

Rate of Conversion of p30S into 30S Particles Parallels the Cell Growth Rate. Figures 1 and 2 show the flow of newly formed, ^{3}H-labeled RNA into p30S and 30S ribosomes in two cultures of *E. coli* D10 growing with doubling times of 40 and 120 min. Cells were prelabeled with [^{14}C]uracil to distinguish precursor from mature products. Samples were taken at intervals proportional to the culture doubling time (after 2, 3, 5, and 7 min, and after 6, 9, 15, and 21 min of labeling with [^{3}H]uracil, respectively). 30S ribosomes were separated from p30S by two successive runs of zonal sedimentation on sucrose gradients (Figures 1A and 1B, panels a, b, d, e, g, h, l, and m). The amount of ^{3}H label in each fraction was calculated as described in the legend to Figure 2, and plotted against the labeling time, expressed as a fraction of the culture doubling time.

The patterns obtained with the two cultures are similar. In both cases a single major kinetic hold up was seen, corresponding to the state of 26–27S particles. The relative amounts of ^{3}H label in p30S and 30S ribosomes in corresponding samples of the two cultures are identical (Figure 2a,b). This indicates that the average time required to convert a single p30S into a 30S particle is roughly proportion-

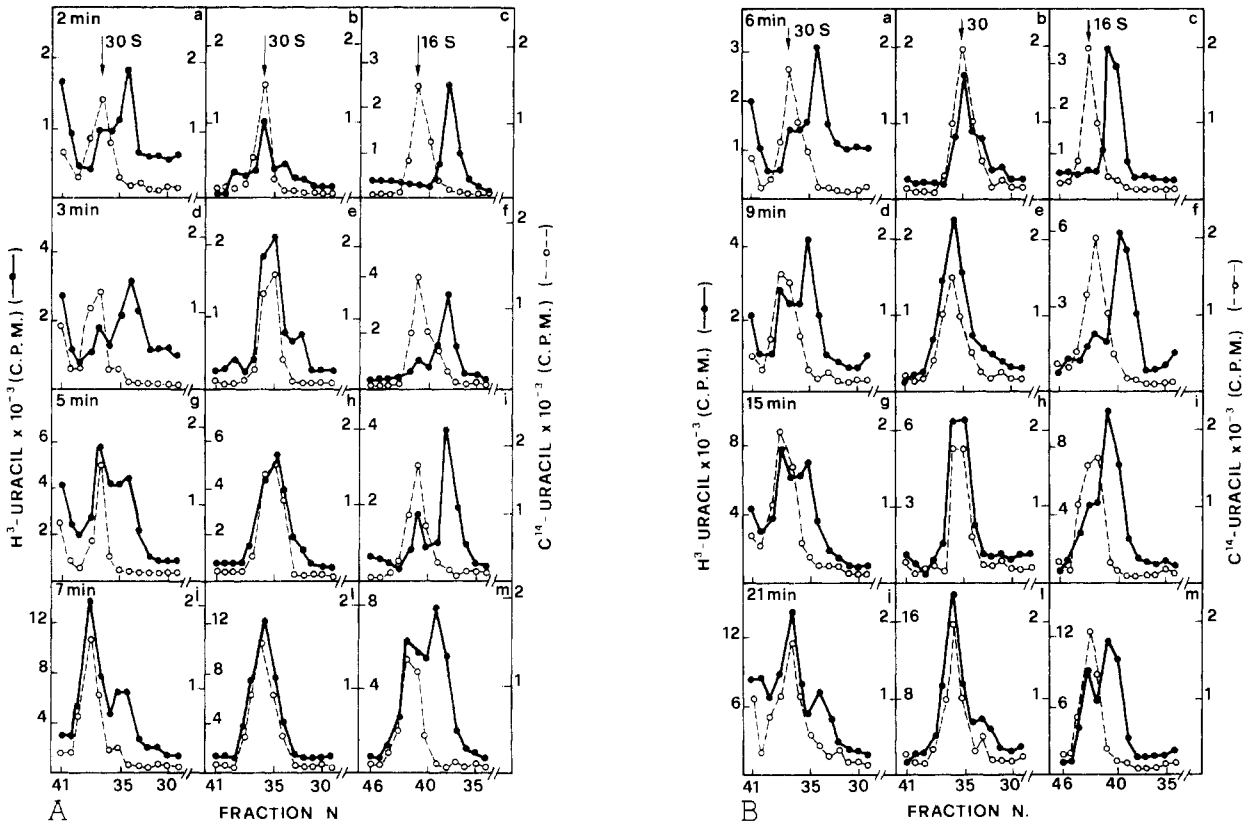

FIGURE 1: [^{3}H]Uracil uptake in ribosomal RNA and ribosomal particles. *E. coli* D10 cells were grown in medium MS9 (Bolle *et al.*, 1968) supplemented with 0.5% casamino acids and 0.2% glucose (doubling time 40 min) (A) or with 0.2% sodium acetate and 0.1% methionine (doubling time 120 min) (B). The cultures received [^{14}C]uracil (0.1 μCi/ml; sp act. 61 Ci/mmol) at an optical density at 420 nm of 0.3. After two more generations, [^{3}H]uracil was added (5 μCi/ml; 29 Ci/mmol). At indicated times, samples were rapidly chilled and lysed. A portion of each lysate was centrifuged through a 10–30% sucrose gradient in 10^{-4} M $MgCl_2$–10^{-2} M Tris-HCl (pH 7.8) at 40,000 rpm for 8 hr to display 30S and p30S particles (panels a, d, g, and j). The peak fraction of the 30S region of each gradient was diluted with three volumes of 10^{-4} M $MgCl_2$ and centrifuged through a second sucrose gradient under the same conditions (panels b, e, h, and k). Another portion of each lysate was treated with phenol and sodium dodecyl sulfate to extract the RNA. After ethanol precipitation, the RNA was electrophoresed for 6 hr at 5 mA/tube on 3.6% polyacrylamide gel (panels c, f, i, and l): (○) [^{3}H]uracil; (●) [^{14}C]uracil.

al to the doubling time of a culture.

Maturation of 16S RNA Occurs in 30S Particles. To follow its maturation, ribosomal RNA was extracted from portions of the same lysate which had been analyzed by zonal sedimentation in the experiment described in Figure 1. p16S and mature 16S (m16S) RNA were separated by electrophoresis on polyacrylamide gels (Figures 1A and 1B, panels c, f, i, and n). The amount of ^{3}H label in the two fractions is plotted in Figure 2.

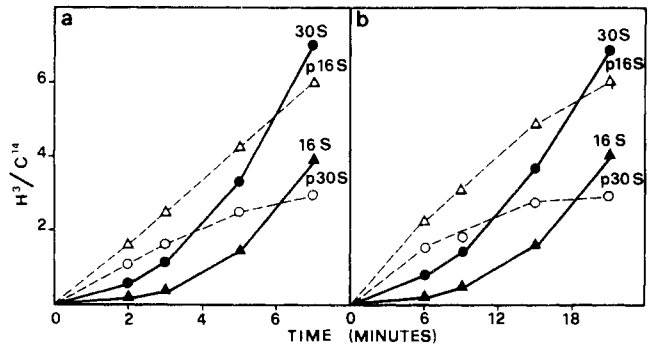

FIGURE 2: Distribution of ^{3}H in ribosomal RNA and particles as a function of time of labeling. The amount of label in the different fractions reported in a and b was calculated from the data of Figure 1A (fast growing cells) and 1B (slow growing cells), respectively. The amount of label in 30S ribosomes and in mature 16S RNA has been estimated from the ^{3}H/^{14}C ratio in the left-side fractions of the ^{14}C peaks shown in the second and third columns of Figures 1A and 1B. The amount of ^{3}H in p16S RNA and in p30S particles has been calculated by subtracting from the label in the ^{3}H peaks of the first and third columns of Figures 1A and 1B the amount accounted for by the overlap of mature RNA and ribosomes, estimated from the ^{14}C pattern. For all fractions, the amount of ^{3}H label is given per unit of ^{14}C label present in mature RNA and ribosomes.

As in the case of p30S and 30S particles, the rate of conversion of p16S into m16S RNA is related to the culture growth rate. However, formation of 30S ribosomes and maturation of rRNA do not appear as immediately linked processes. Both in fast and in slow growing cells the ^{3}H label enters p16S RNA and 30S particles at the same time. This is shown by the fact that the sum of the label found in p16S and in 16S RNA equals the sum of the label found in p30S and 30S ribosomes. In both cultures, however, there is a delay of several minutes in the labeling of 16S RNA compared to 30S ribosomes.

This finding suggested that maturation of rRNA occurs after, not during the formation of 30S particles. The inference was confirmed by isolating newly formed, ^{3}H-labeled 30S particles and analyzing their RNA. They contained p16S RNA (Mangiarotti *et al.*, 1974).

Conversion of p30S into 30S Particles is Blocked by Chloramphenicol. Figure 3 shows the effect of chloramphenicol on the conversion of preformed p30S particles into 30S subunits. Cells growing with a doubling time of 100 min and prelabeled with [^{14}C]uracil received [^{3}H]uracil at zero time, followed 3 min later by an excess of cold uracil. After 2 more min of incubation the culture was split into

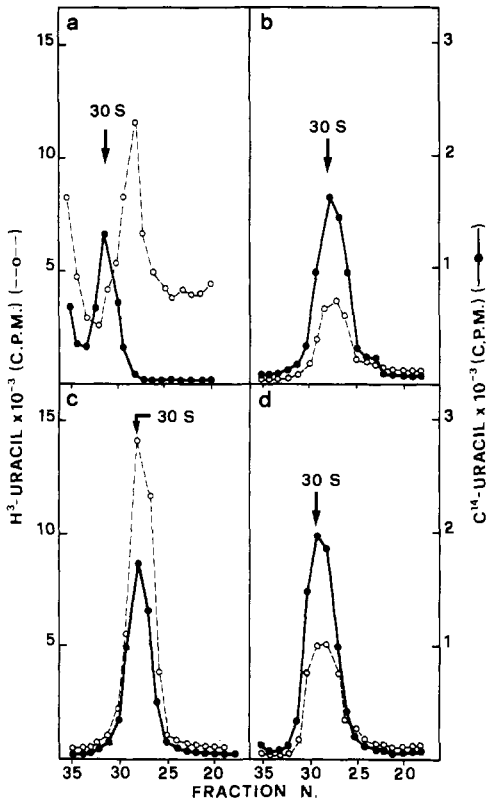

FIGURE 3: Effect of chloramphenicol on the maturation of preformed p30S particles. Cells growing with a doubling time of 100 min and prelabeled with [^{14}C]uracil received [^{3}H]uracil (1 μCi/ml) at zero time, followed 2 min later by cold uracil (20μg/ml). After 2 more min of incubation the culture was split into three samples: one was poured onto crushed ice (sample 1), the other two were transferred into separate flasks, one of which did not (sample 2) and the other did (sample 3) contain chloramphenicol (final concentration 0.2 mg/ml). Incubation of the last two samples was continued for 6 min before harvesting. The cell lysates were centrifuged on sucrose gradients to display 30S and p30S particles. 30S subunits were purified by a second centrifugation as described in Figure 1: (a) pattern of the first gradient obtained from sample 1; (b) 30S subunit from sample 1; (c) 30S subunit from sample 2; (d) 30S subunit from sample 3.

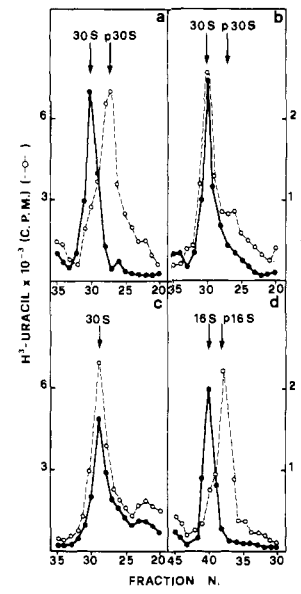

FIGURE 4: *In vitro* conversion of p30S into 30S particles. Native 30S ribosomes and p30S particles, labeled with [^{14}C]uracil and [^{3}H]uracil, respectively, were prepared as described under Material and Methods. They were mixed together and diluted into the reconstitution buffer (see Material and Methods) in the presence of an excess of unlabeled native 30S ribosomes. The ^{3}H- and ^{14}C-labeled particles could be resolved almost completely by centrifugation on a sucrose gradient (a). If the unlabeled ribosomes had been treated with urea and LiCl before being added to the labeled particles, most of the ^{3}H label was found to cosediment with the ^{14}C label at 30S (c). Incubation of the mixture at 40° for 20 min before gradient analysis did not improve the yield of ^{3}H-labeled 30S ribosomes. Incorporation of most of the ^{3}H-labeled RNA into 30S ribosomes could be obtained also by mixing labeled and unlabeled particles in the presence of urea and LiCl, and then diluting the mixture into reconstitution buffer and incubating at 40° for 20 min (b). Fractions 29–31 of the gradient shown in b were pooled. The RNA was extracted with phenol and analyzed by gel electrophoresis (d).

three samples: one was poured onto crushed ice, the other two were transferred into separate flasks, one of which contained chloramphenicol. Incubation of the last two samples was continued for 6 min before harvesting. Cell lysates were analyzed by sucrose gradients to display 30S and p30S particles. As shown in Figure 3a, at the time of addition of chloramphenicol most of the ^{3}H-labeled rRNA was in the form of precursor particles. To see whether chloramphenicol effected the transfer of this label into 30S particles, these were isolated by a second run on sucrose gradients as described in Figure 1. During the final 6 min of incubation the ^{3}H/^{14}C ratio in 30S particles increased over the initial value (Figure 3b) by a factor of 1.2–1.5 in the presence of chloramphenicol (Figure 3d) and by a factor of 5 in its absence (Figure 3c). Both in the presence and in the absence of chloramphenicol the ^{3}H/^{14}C ratio in total acid precipitable material increased only by a factor of 1.2 indicating that very little exogenous [^{3}H]uracil was incorporated into RNA. It is still possible that some of the ^{3}H label originally present in messenger RNA molecules has been transferred during the chase into newly synthesized rRNA molecules contributing to the rise in the ^{3}H/^{14}C ratio in 30S particles. Chloramphenicol could interfere with this transfer, for example by stabilizing messenger RNA or by other mechanisms. However, the increase of the ^{3}H/^{14}C ratio in 30S particles during the last 6 min of incubation can be accounted for completely by the maturation of preexisting precursor particles and is clearly largely due to this process. Thus it would appear that this step in the formation of ribosomes is strongly inhibited by chloramphenicol. This interpretation is supported by the fact that the addition of chloramphenicol sharply repressed the maturation of 30S ribosomes even in the presence of rifampycin, when all RNA synthesis was shut off. A more detailed analysis of the effect of protein synthesis inhibitors on the formation of ribosomes and on the maturation of rRNA will be published elsewhere.

In Vitro Incorporation of p16S RNA into 30S Particles. The assembly of 30S particles from p16S RNA and ribosomal proteins was tested in the *in vitro* system described by Traub and Nomura (1969). p30S particles containing ^{3}H-labeled p16S RNA were isolated by sucrose gradient sedimentation and mixed with native 30S ribosomes containing ^{14}C-labeled 16S RNA, in the presence of 4 M urea and 3 M LiCl. The mixture was diluted in 20 vol of reconstitution buffer and incubated at 40° for 20 min. The size of reconstituted particles was determined by zonal sedimentation. As seen in Figure 4b, ^{3}H- and ^{14}C-labeled RNAs were incorporated to the same extent into particles sedimenting as 30S. The ^{3}H-labeled RNA extracted from reconstituted particles and analyzed by gel electrophoresis still migrated as p16S (Figure 4d).

In a second type of experiment, a mixture of ^{3}H-labeled

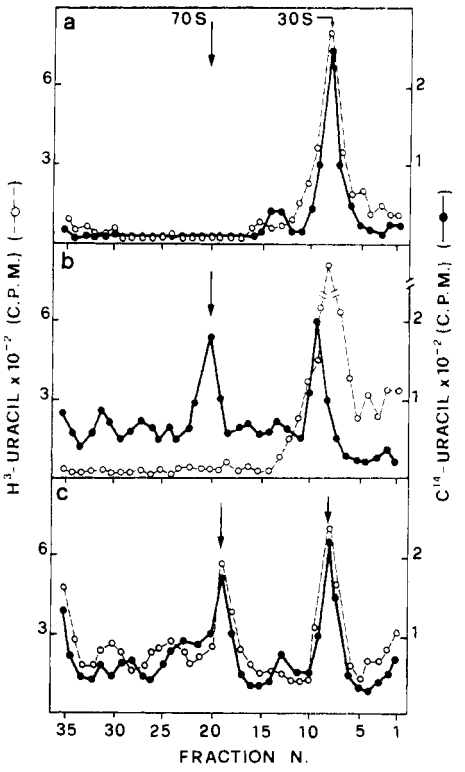

FIGURE 5: *In vitro* response of 30S particles to T4 specific mRNA. ^{14}C-Labeled native 30S ribosomes and ^{3}H-labeled p30S particles were prepared as described under Materials and Methods. p30S particles were converted into 30S particles as described in Figure 4b. ^{14}C- and ^{3}H-labeled 30S particles were mixed together and incubated under conditions of protein synthesis (see Materials and Methods) in the presence (c) and in the absence (a) of T4 RNA. The mixtures were analyzed by zonal sedimentation to detect the formation of labeled 70S and polyribosomes. Figure 5b shows the gradient pattern obtained from a mixture of ^{14}C-labeled 30S and of ^{3}H-labeled p30S particles incubated in the presence of T4 RNA. In this case p30S were tested for polyribosome formation directly, without previous conversion into 30S particles.

p30S and of ^{14}C-labeled 30S was diluted directly into the reconstitution buffer, together with unlabeled native 30S ribosomes that had been exposed to urea and LiCl overnight. In this case the ^{3}H-labeled RNA was found to cosediment with the ^{14}C marker at 30 S after an incubation of only 5 min at 0–2° (Figure 4c). Incubation at high temperature, necessary to mature RI particle (Nomura, 1973) is therefore not required to convert a p30S into a 30S particle *in vitro*.

30S Particles Assembled from p16S RNA Respond to T4-Specific mRNA. The above results have shown that p16S and m16S RNA are equally competent in forming 30S particles *in vitro*. Accordingly p30S could be converted into 30S particles by the simple addition of ribosomal protein, without requiring maturation of rRNA. However, since p30S particles could not be obtained completely free of native 30S particles, we could not test whether p16S containing 30S particles formed *in vitro* have a full complement of ribosomal proteins and are competent in protein synthesis.

The two kinds of particles (p16S containing 30S formed *in vitro* and m16S containing native 30S), however, could be labeled differentially with [^{3}H]- and [^{14}C]uracil. We could therefore compare their ability to respond to natural mRNA by measuring the formation of labeled 70S monosomes and polyribosomes in conditions of protein synthesis. Figure 5 shows the efficiency and the specificity of the assay. When ^{14}C-labeled native 30S ribosomes were incubated with 50S subunits and T4 specific mRNA, about 50% of the label moved as 70S particles and polyribosomes on a sucrose gradient (Figure 5b). No label was found in the same region of the gradient when T4 RNA or 50S subunits were omitted (Figure 5a). ^{3}H-Labeled p30S, incubated in the complete mixture, did not show any comparable complex formation with T4 RNA (Figure 5c).

^{3}H-Labeled 30S particles, assembled *in vitro* from free p16S RNA or from p30S particles, entered the region of 70S monosomes and polyribosomes at the same extent as ^{14}C-labeled native 30S particles (Figure 5b). By this criterion, 30S particles assembled *in vitro* from p16S behave as normal ribosomes. The same criterion was shown to hold for 30S particles containing p16S RNA which were isolated from growing cells (Mangiarotti *et al.*, 1974).

Discussion

Nature of the Rate-Limiting Step. Biogenesis of bacterial ribosomes has been known for some time to be a stepwise process. The most obvious explanation of this fact has been that a cellular component (ribosomal or not) is made in a limiting amount.

More recently, reconstitution of 30S subunits from free RNA and ribosomal proteins has been shown to occur spontaneously *in vitro*, through the formation of an intermediate particle. This has given support to an alternative explanation, namely that the *in vivo* process merely reflects an intrinsic feature of the assembly mechanism. Even in the presence of all necessary components, ribosomes would assemble in several steps, due to the need for the initial RNA-protein complex to assume a specific configuration before the assembly can proceed.

The distinction between these two possibilities is important, because it may have relevance to the mechanism by which the cell controls ribosome formation. If the rate-limiting step is a conformational change of the precursors, which occurs spontaneously and is not influenced by nonribosomal components, the entire process of ribosome formation must be controlled at the very initial step, *i.e.*, at the level of synthesis of the first interacting molecules. If the event which leads to the accumulation of precursors is instead their interaction with a cellular component, the rate of assembly would depend also on the availability or the activity of this component. Therefore, multiple levels of control would be available.

The data reported here contain two lines of evidence which are against the first alternative. If a spontaneous conformational change in an intermediate precursor were the rate-limiting step, the average time required to complete the assembly of a single ribosome should remain constant at a given temperature, independently of the concentration of precursor particles or of any other cellular component. Our data show instead that the rate of 30S ribosome assembly is roughly proportional to the cell growth rate, and thus can vary over a large range.

The second relevant finding is that p30S particles, isolated from growing cells, can bind additional proteins *in vitro* and be converted into 30S particles readily, even in the cold. The conformational change which limits the reconstitution *in vitro* from free 16S RNA and free ribosomal proteins has a high energy of activation, and occurs only at high temperature (Nomura, 1973). We therefore presume that no such change could occur during or after extraction of

cells at low temperature. If a similar event occurs *in vivo*, most p30S particles must have already undergone this change. Therefore, any such change would not be rate limiting *in vivo*.

Our results are rather consistent with the hypothesis that the rate of ribosome assembly is determined by the concentration of one or more cellular components. The limiting factor might be an enzyme involved in rRNA processing, some other molecule that is not incorporated into complete ribosomes, or a ribosomal protein.

The first possibility is made unlikely by the finding that *in vivo* p16S RNA is converted into mature 16S not before, but after 30S particles have been assembled. This observation has also been reported by Lindhal (1973). Maturation of rRNA may in fact be completed only after newly formed ribosomes have interacted with mRNA, and entered polyribosomes (Mangiarotti *et al.*, 1974). This indication is reinforced by the fact that conversion of p30S into 30S particles can be obtained *in vitro* by the simple addition of ribosomal proteins, and that it is not accompanied by the transformation of p16S into m16S RNA. The same experiment makes it unlikely that a nonribosomal factor controls the formation of ribosomes *in vivo*. However, we have not yet fully characterized the RNA molecules contained in p30S and in 30S particles. While they have similar electrophoretic mobility, they may not be identical. Therefore, we cannot exclude the possibility that RNA processing plays a role in ribosome assembly. Similarly the fact that 30S ribosomes self-assemble *in vitro* does not prove that this happens *in vivo*.

On the other hand, the possibility that at least one ribosomal protein is available in a very limited amount is supported by the finding that chloramphenicol blocks the conversion of p30S into 30S ribosomes almost immediately. Though other interpretations of this result are possible, the simplest way to explain it is to suggest that maturation of p30S particles depends on the continuous supply of ribosomal protein. Thus, though other possibilities are not conclusively ruled out, our data do indicate that the availability of ribosomal protein is the most likely rate-limiting factor in ribosome assembly.

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