Biosynthetic Precursors of 30S and 50S Ribosomal Particles in *Escherichia coli**

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ABSTRACT: The biosynthesis of *Escherichia coli* 30S and 50S ribosomes was studied, making use of exponentially growing fragile cultures. These cultures permit rapid lysis and total recovery of cellular ribonucleic acid (RNA). By analysis of lysates in zonal sedimentation in sucrose gradients, it was found that mature ribosomes appear in polyribosomes and free 30S and 50S ribosomal particles, while the newly formed ribosomal ribonucleic acid (rRNA) *en route* to complete ribosomes does not appear in polyribosomes to a measurable extent for at least 6 min (doubling time, 120 min; Mangiarotti, G., and Schlessinger, D. (1966a), *Nature 211*, 761; (1966b), *J. Mol. Biol. 20*, 123; (1967), *J. Mol. Biol. 29*, 365). To analyze the synthesis of ribosomes, [^{3}H]uracil was added to the cultures, and samples were lysed and fractionated in sucrose gradients to follow the flow of ^{3}H into RNA. The newly synthesized rRNA appears in precursors that sediment more slowly than complete ribosomes. A whole array of material is observed sedimenting less than 26 S; the labeled RNA extracted from this region is identified as incomplete chains of 16S and 23S rRNA, since it sediments slower than 16S, but competes with purified unlabeled 16S and 23S rRNA in deoxyribonucleic acid–ribonucleic acid hybridization. Since RNA from this region sediments more slowly after phenol extraction, proteins are probably attached to the growing rRNA chains. This conjecture is supported by the observation that about one-half the protein moving in this region of the gradients no longer sediments there after brief treatment of lysates with RNase. The proteins released resemble ribosomal proteins in their behavior on elution from carboxymethylcellulose columns. Three well-defined peaks of larger precursors, sedimenting at 26, 32, and 43 S, were also characterized. RNA extracted from the 26S peak contained only 16S RNA, so that it was assigned as a precursor of the 30S ribosome. The 32S and 43S particle were judged to be successive stages in the maturation of the 50S ribosome, since they contained 23S RNA. The kinetics of labeling of complete rRNA chains indicate that they are formed in 2 min (13 nucleotides/sec) in these cultures. To produce the observed numbers of chains, a number of RNA polymerase molecules must be working in tandem on each cistron specific for rRNA. About 0.5% of the total rRNA is in unfinished chains at any instant. Once completed, 16S rRNA chains are arrested as 26S precursors for an average of about 18 min before they mature to 30S ribosomes; the pool of 26S material contains about 3.5% of the total rRNA. The 23S rRNA halts about 1 min as a 32S precursor, in a pool containing about 0.4% of the total cellular rRNA. The 32S particle then becomes a 43S precursor, in a pool containing about 7% of the cellular rRNA, for about 17 min more before maturing to a 50S ribosome. Thus, complete 30S and 50S ribosomes are made in the same over-all time.

McCarthy *et al.* (1962) were the first to investigate the biosynthesis of the ribosomes of *Escherichia coli*. Their experimental procedure was to add a radioactive precursor of RNA, such as [^{14}C]uracil, to a growing culture of *E. coli*, then sample the culture at intervals, extract the cells, and follow the flow of radioactive RNA into complete and unfinished ribosomal particles in sucrose gradients.

Since fragments of mRNA obscured much of the ribosomal precursors in sedimentation profiles (see Midgeley and McCarthy (1962) and Discussion below), the resolution of the analysis was limited, though discrete ribosomal precursors were observed. Recently, we have prepared lysates of fragile, growing *E. coli* (Mangiarotti *et al.*, 1966; Mangiarotti and Schlessinger, 1966a) in which the ribosomal precursors can be extracted by very gentle procedures and separated from mRNA (Mangiarotti and Schlessinger, 1966b, 1967). The extracts were analyzed by zonal sedimentation, DNA–RNA hybridization, and fractionation of ribosomal proteins. A revision and extension of the earlier analysis of ribosomal precursors has thus become possible. The amounts and saturation times of finished rRNA chains and pools of ribosomal precursors have been estimated. Three major precursors are analyzed, one specific for the 30S and two specific for the 50S ribosomal particle, and evidence is presented suggesting that ribosomal proteins add to rRNA even before chains are completed.

Methods and Plan of the Experiment

As previously described (Mangiarotti and Schles-

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TABLE I: Hybridization of ^{14}C-Labeled 23S rRNA of *B. subtilis* or *E. coli* in Presence of Unlabeled 16S or 23S rRNA.[a]

	Hybridized cpm at Each Level of Added rRNA					
Cold rRNA added (μg)	0	0.2	1	2	5	
16 S added	496	462	582	464	442	*B. subtilis*
23 S added	501	310	156	66		
Cold rRNA added (μg)	0	0.5	1	2.5	5	
16 S added	1010	760	520	290	152	*E. coli*
23 S added	1035	525	320	152	92	

[a] The 23S rRNA (0.2 μg) (2150 cpm) prepared from cells of *B. subtilis* uniformly labeled with [^{14}C]uracil was hybridized with 50 μg of homologous DNA in presence of different amounts of unlabeled 16S or 23S rRNA. For comparison, data obtained with 0.5-μg (4100 cpm) samples of *E. coli* 23S rRNA, hybridized with *E. coli* DNA, are reported (from data of Figure 1).

singer, 1966b), cells of *E. coli* strain E_{203} were grown exponentially in fragile form at 37° (mass doubling time 120 min), labeled, harvested with ice, and lysed with buffers containing 10 mM Tris (pH 7.5), 6 mM Mg^{2+}, 40 mM NaCl, 0.3 mM chloramphenicol, and 0.5% sodium deoxycholate. Under these lysing conditions, essentially all of the cellular RNA, including incomplete chains, is released free of DNA templates. To detect preformed stable RNA in lysates, it was labeled with a pulse of [^{14}C]uracil followed by two generations of growth. At 1.5×10^8 cells/ml, [^3H]uracil was added to the culture to detect newly formed RNA. Where indicated, L-[^3H]-alanine was used to label newly formed proteins. [^{14}C]Uracil (0.52 $\mu c/\mu g$), [^3H]uracil (200 $\mu c/\mu g$), and L-[^3H]alanine (100 $\mu c/\mu g$) were all from Schwarz BioResearch, Inc. Zonal sedimentation analysis of lysates after different times of labeling was carried out in 15–30% sucrose gradients in a Beckman SW 25.1 rotor at 4° (see text and Mangiarotti and Schlessinger, 1966b).

For hybridization trials with purified 16S and 23S rRNA, these were prepared from cells uniformly labeled with [^{14}C]uracil (300 μc/g wet wt). Cells were ground with twice their weight of alumina powder and extracted with three volumes of 10 mM Tris–0.1 mM Mg^{2+}. The 30S and 50S subunits were purified by two successive cycles of centrifugation in sucrose gradients, and the corresponding 16S and 23S rRNAs were extracted. Sedimentation analysis of the 16S and 23S rRNA obtained confirmed that they were at least 95% pure. All extractions of RNA and hybridization reactions were carried out by protocols already published (Mangiarotti and Schlessinger, 1967).

Differential Detection of mRNA, tRNA, 16S rRNA and 23S rRNA. Since all the cellular mRNA is localized in polyribosomes and no ribosomal precursors are found in polyribosomes (Mangiarotti and Schlessinger, 1967), mRNA and ribosomal precursors could be separated by zonal sedimentation in sucrose gradients. Furthermore, since a minimum time of 7 min was required for the completion of new ribosomes, during the first 7 min of continuous labeling, essentially all the newly formed (^3H labeled) stable RNA is contained, free of mRNA, in the fractions from the top of the gradient to the 50S ribosome region.

The total ^3H in tRNA can be estimated by multiplying the ratio of (^3H:^{14}C) at the maximum of the 4S peak by the total ^{14}C in the 4S region. The specific activity of the peak increases linearly from about 1 min of labeling up to 30 min. During the first minute, the rate of labeling of tRNA seems slightly higher, probably because some short chains of nascent rRNA that saturate with label in the first minute (see below) sediment at 4 S or less. The amount of ^3H contributed to the 4S region by rRNA was therefore estimated by extrapolating the rate of labeling of tRNA at times later than 1 min back to zero time (see Figure 3).

In the gradient patterns of lysates or of extracted rRNA, finished 23S chains are easy to measure, but the region 4–16 S contains a mixture of unfinished 16S- and 23S-specific chains. To distinguish these, where necessary, we have used DNA–RNA hybridization (essentially according to Nygaard and Hall (1963), with the addition of RNase treatment to eliminate nonspecific DNA–RNA interactions; for detailed protocols, see Mangiarotti and Schlessinger, 1967).

Unlabeled 16S rRNA will compete with 16S rRNA precursors in hybrid formation. Similarly, unlabeled 23S rRNA will compete with labeled 23S rRNA. If the 16S and 23S rRNA chains contained no appreciable homologous sequences, they would not cross-hybridize, and the proportions of 16S- and 23S-specific fragments in a mixture would be straightforward. However, preliminary trials showed that using high RNA to DNA ratios, purified unlabeled 16S rRNA compete for hybridization with labeled 23S rRNA, and *vice versa*. The competition is not caused by "cross-contamination" of the rRNA fractions, since 16S and 23S rRNA isolated from *Bacillus subtilis* by the same procedure showed no cross-hybridization (sample data reported in Table I). Several groups have published data showing that 16S and 23S rRNA of *Bacilli*, purified on methylated albumin columns, show no detectable cross-hybridization (Yankofsky and Spiegelman, 1963; Doi and Igarashi, 1966).

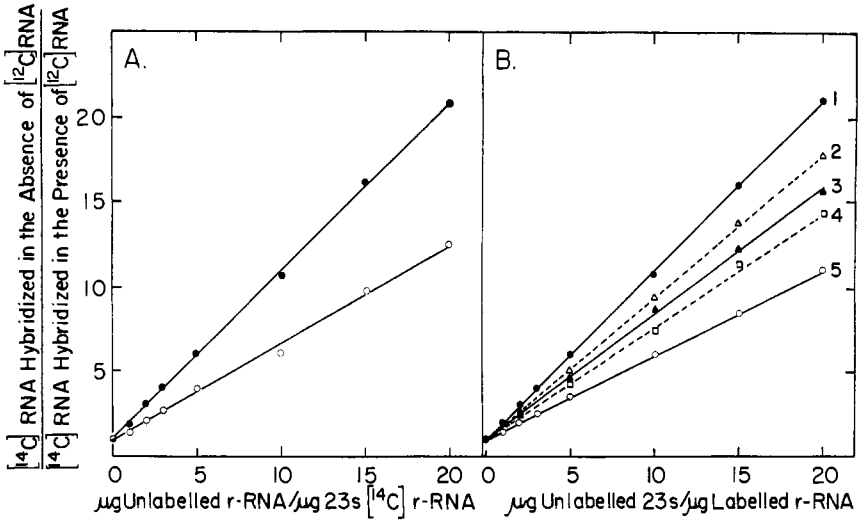


FIGURE 1: Competition in hybridization of 16S and 23S rRNA. (A) Hybridization of labeled 23S rRNA in the presence of cold rRNA. ^{14}C-Labeled 23S rRNA (0.5 μg) (8200 cpm/μg) was hybridized with 75 μg of DNA in the presence of the indicated amounts of 16S (—○—) or 23S (—●—) unlabeled rRNA. The ratio of ^{14}C-labeled RNA hybridized in absence of cold RNA to that hybridized in the presence of cold RNA is plotted against the ratio of unlabeled to labeled RNA. Two straight lines are obtained, with slope 1.0 for competition by homologous RNA (cold 23 S) and approximately 0.5 for competition by heterologous RNA (cold 16 S). (B) Hybridization of mixtures of ^{14}C-labeled 16S and 23S rRNA in presence of unlabeled 23S rRNA. rRNA (0.5 μg) (16 and 23 S of identical specific radioactivity, mixed in different proportions) was hybridized with 75 μg of DNA in the presence of the indicated amounts of cold 23S rRNA. The slope of the curve obtained by plotting the hybridized fraction (defined as in Figure 1A above) against the ratio of unlabeled to labeled RNA varies from 0.5 to 1.0 (see text) for the various mixtures of labeled RNA. The mixtures contained 23S and 16S RNA in the following ratio: 1:0 (curve 1), 2:1 (curve 2), 1:1 (curve 3), 1:2 (curve 4), and 0:1 (curve 5). The observed slopes are 1 (curve 1), 0.85 (curve 2), 0.75 (curve 3), 0.66 (curve 4), and 0.5 (curve 5).

On the other hand, Attardi *et al.* (1965) have shown considerable cross-hybridization of 16S and 23S rRNA of *E. coli* purified by zonal sedimentation. It has not been clear whether differences in technique or species were responsible for these different results. The results reported support the idea that the difference is in the species used, for both sets of published results have been confirmed with 16S and 23S RNA of *E. coli* and *B. subtilis* purified in sucrose gradients.

In spite of the extensive cross-hybridization of *E. coli* 16S and 23S rRNA, the approximate fraction of each in a mixture could be determined, as follows. At RNA: DNA inputs sufficiently high so that all the specific DNA sites are occupied, if we call H and H_{max} the label hybridized in the presence and in the absence of unlabeled competitor RNA, then

$$\frac{H_{max}}{H} = \frac{R + R^*}{R^*} = 1 + \frac{R}{R^*}$$

where R^* and R are the amounts of labeled and unlabeled RNA, respectively. If R is homologous to R^* (*i.e.*, both are 16S rRNA or both are 23S rRNA), the RNA molecules will compete one for one, and a plot of R/R^* *vs.* H_{max}/H will give an ordinate intercept of 1 and a slope of 1 (see upper curve of Figure 1A). If R has only partial homology to R^*, a similar plot will still have an ordinate intercept of 1, but the slope will be less than one. The slope is then a measure of the fractional homology. When such an experiment is carried out with nonradioactive 16S rRNA and radioactive 23S rRNA, the slope obtained is 0.5 (see Figure 1A, lower curve), indicating that nucleotide sequences distributed at intervals in 16S and 23S rRNA overlap enough to permit cross-hybridization with about half the efficiency of homologous hybridization. [It is important to note that these results probably cannot be accounted for by a single sequence within 23S rRNA that is precisely homologous with one in 16S rRNA. If, for example, a 23S rRNA chain contained a length exactly homologous to the complete 16S rRNA chain, and a second unique portion, then, as was observed, 23S rRNA would indeed compete totally with 16S rRNA. However, 16S rRNA would not compete with more than 50% of 23S rRNA, whereas we found that an excess of 16S RNA completely prevented hybridization of the 23S species (Figure 1).]

Artificial mixtures of labeled 23S and 16S rRNA hybridized in presence of increasing amounts of unlabeled 23S rRNA give, as expected, lines of slope intermediate between 0.5 and 1 (Figure 1B); clearly, the slope (S) of the competition curves is linearly related to

the fraction F of labeled 23S rRNA in the mixture

$$S = 0.5\,(1 + F) \qquad (1)$$

By using eq 1 the fraction of 23S rRNA (or unfinished chains thereof) can be measured from the slopes of such competition curves when these chains are found mixed with 16S rRNA (see Results). The assumption is made that regions of homology are distributed in rRNA so that incomplete chains hybridize like complete ones.

Determination of the Specific Radioactivity of New RNA during the Experiment. The processes discussed here are mainly analyzed by *relative* rather than *absolute* rates of incorporation. However, for some determinations (like the rate at which "holdup" ribosomal precursors saturate with label) the observed cpm must be modified to take account of changes in the specific radioactivity of acid-soluble RNA precursors during the experiment. The relative specific radioactivity of these as a function of time is known in these experiments, since it is proportional to the observed rate of uptake of label into new stable RNA (see below and Mangiarotti and Schlessinger, 1967). A correction can thus be derived and applied for the moderate changes in the activity of the nucleotide pool. The specific radioactivity of new RNA is essentially constant for 10–12 min, and then increases by about 30% over the next 6 min to its final value (for the introduction of this correction, see Results section and Figure 8).

Kinetic Analysis. RATE OF SYNTHESIS OF 16S AND 23S rRNA. A fraction of rRNA that sediments between 4 and 16 S saturates with label after about 2 min (Mangiarotti and Schlessinger, 1967), suggesting that it takes of the order of 2 min to synthesize an entire chain of rRNA. To define the transit time more precisely, we analyze here the kinetics of entry of label into finished and unfinished chains of 16S and 23S rRNA, to obtain an independent estimate of the time of synthesis for each.

If L is the time required for the formation of a complete molecule of rRNA, it is convenient to analyze separately the entry of label into rRNA before and after L. Before L (*transient stage*), the chains that are being completed will initially be labeled only in the terminal nucleotides. The rate of labeling will then increase linearly with time (t), until, at time L, the chains that are being completed are maximally labeled, and the rate of labeling will thereafter remain constant (*steady state*). The rate of incorporation of label into finished chains will therefore be, in the two stages

$$\frac{dR^*}{dt} = \frac{C}{L}t \qquad [0 < t < L] \qquad (2)$$

$$\frac{dR^*}{dt} = C \qquad [t > L] \qquad (3)$$

where C is the constant rate of incorporation of radioactivity into the total RNA specific for each class (16 or 23 S), *i.e.*, unfinished as well as finished chains. (C is actually increasing exponentially with time; but over the brief period considered in this analysis, the assumption that C is constant introduces an error of less than 2%.)

Equation 2, in the integral form

$$R^* = \frac{C}{2L}t^2 \qquad [0 < t < L] \qquad (4)$$

predicts that the uptake of label into finished chains will increase with the second power of time *during the transient stage*. At time L, $R^* = CL/2$ (from eq 2). Thereafter, eq 3 is obeyed, so that, for the *steady state*, we have

$$R^* = \frac{CL}{2} + C(t - L) = Ct - \frac{CL}{2} \qquad [t > L] \quad (5)$$

If we set $R^* = 0$, $CL/2 = Ct$, indicating that the linear portion of a plot of R^* *vs.* t should intersect the abscissa at $L/2$. We use these relations to determine the time of synthesis of 16S and 23S rRNA (see Results and Figure 3).

The amount of label in unfinished chains, I^*, will be, at any time, the difference between total label and label in finished chains. During the *transient stage*

$$I^* = Ct - \frac{C}{2L}t^2 = C\left(1 - \frac{t}{2L}\right)t \qquad [0 < t < L] \quad (6)$$

In the *steady state*

$$I^* = Ct - (Ct - CL/2) = +\frac{CL}{2} \qquad [t > L] \quad (7)$$

Thus, if the analysis holds, I^* should remain equal to $CL/2$ from time L onwards (see Results section and curve I of Figure 3).

RATE OF SYNTHESIS OF PRECURSORS OF 30S AND 50S RIBOSOMES. The analysis of Britten and McCarthy (1962) was followed. This is based on the fact that in a series of sequential precursors, the radioactivity in each increases at early times in proportion to t for the first precursor, t^2 for the second, t^3 for the third, etc. On a log–log plot, the slope of the labeling curve for each precursor therefore indicates its position in the series. Furthermore, if the newly synthesized fraction of RNA in each precursor is plotted against time in generations, the percentage of total rRNA in each precursor is given by its saturation value. The relevant procedure, as used in the Discussion, is briefly as follows. (1) The sampling times of Figures 3 and 8 were converted to units of τ (such that $\tau = 1$ when the cells have grown by a factor e) by dividing the time t by the generation time (here equal to the mass doubling time, 120 min, divided by ln 2; *i.e.*, 173 min). (2) In order to show that the data correspond to the theoretical function for the labeling of RNA in the steady state, the ^3H in total rRNA at 30 min was first set equal to $(1 - e^{-30/173}) = 0.1596$. Each of the other points on the rRNA-labeling curve was

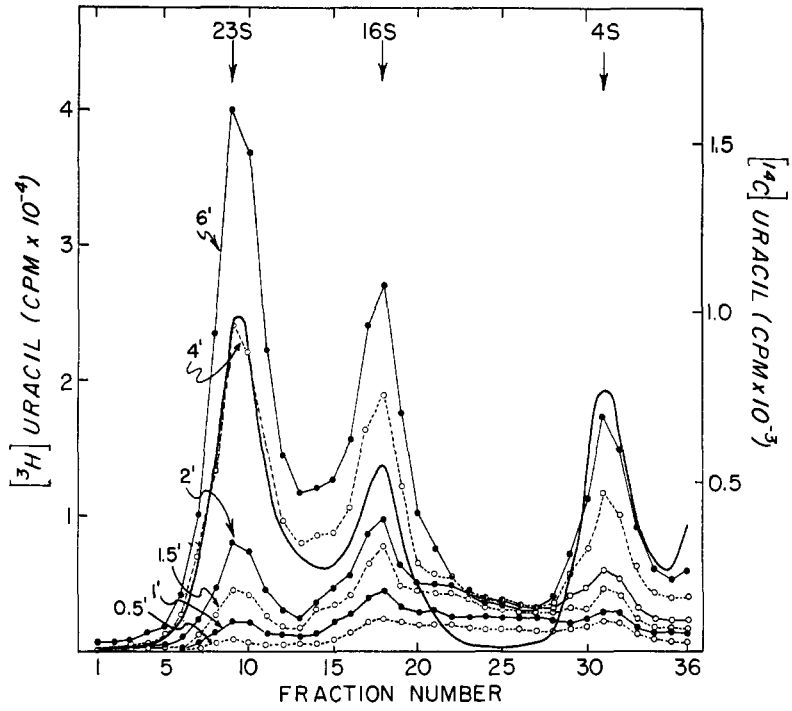


FIGURE 2: Zonal sedimentation analysis of stable RNA labeled with [^{3}H]uracil for various times. A fragile culture (60 ml), prelabeled with [^{14}C]uracil (0.2 μc), was continuously labeled with 50 μc of [^{3}H]uracil from zero time, and analysis of the newly formed stable RNA was carried out as follows. At intervals, 10-ml portions of the culture were centrifuged, cells were lysed, and the lysate was centrifuged in a sucrose gradient for 6 hr at 23,000 rpm (56,000*g*). The position of the 50S peak was determined for each gradient by the ^{14}C content of a portion of each fraction. The fractions from the top of each gradient to the 50S peak inclusive were pooled. After adding 10^{-2} M Versene and 2% sodium dodecyl sulfate, protein was removed by three extractions with phenol at room temperature. The RNA was then precipitated with alcohol and dialyzed against 0.01 M sodium acetate (pH 5.1) containing 0.05 M NaCl. The samples were then reanalyzed in another set of sucrose gradients, containing the acetate–NaCl buffer, for 24 hr at 23,000 rpm. The heavy line indicates the distribution of the stable RNA uniformly labeled with [^{14}C]uracil; the other lines show the ^{3}H-labeled RNA at labeling times indicated. The gradients are all normalized to a [^{14}C]uracil content of 10,000 cpm.

multiplied by the same factor (0.1596 × the number of counts per minute at 30 min), and the data were plotted on a log–log scale as a function of (time in generations). The curve obtained (Figure 11) fits the theoretical function for ϕ_T (which is the newly synthesized fraction of total rRNA; see eq 9 of Britten and McCarthy, 1962), indicating that the precursor analysis is applicable. (3) From the definition of ϕ_X, which is the newly synthesized fraction of total rRNA in any precursor X (eq 9 of Britten and McCarthy (1962)

$$\phi_X = \frac{\mu_X}{\mu}\frac{X}{T} = \frac{X^*}{\mu T}$$

where μ is the specific activity of the tracer, X is the quantity of a precursor, X^* is its content of radioactivity, μ_X is its specific radioactivity, and T is the total amount of rRNA), it follows that

$$\phi_X = \frac{X^*}{T^*}(1 - e^{-\tau}) \qquad (8)$$

For each precursor, the counts per minute at each point in Figure 8 are divided by the counts in total rRNA at that time, then multiplied by $(1 - e^{-\tau})$, and plotted on the log–log scale shown in Figure 11.

Results

A. Rate of Synthesis of 16S and 23S rRNA. In lysates from cells exposed to a continuous label with [^{3}H]uracil, the formation of rRNA can be followed in two ways: (a) by the rate of appearance of complete chains; and (b) by the saturation of precursors. After gradient centrifugation of lysates, the new ^{3}H-labeled stable RNA was extracted with phenol from the region of the gradient extending from the top to the 50S peak. The extracted RNA was then run in another gradient to display tRNA, unfinished rRNA chains, and finished 16S and 23S chains (Figure 2). The increase with time of ^{3}H in each region, with tRNA estimated as in Methods, is plotted in Figure 3.

The experimental curve for labeling of 23S rRNA fits the theoretical curve calculated from eq 4 and 5 (see

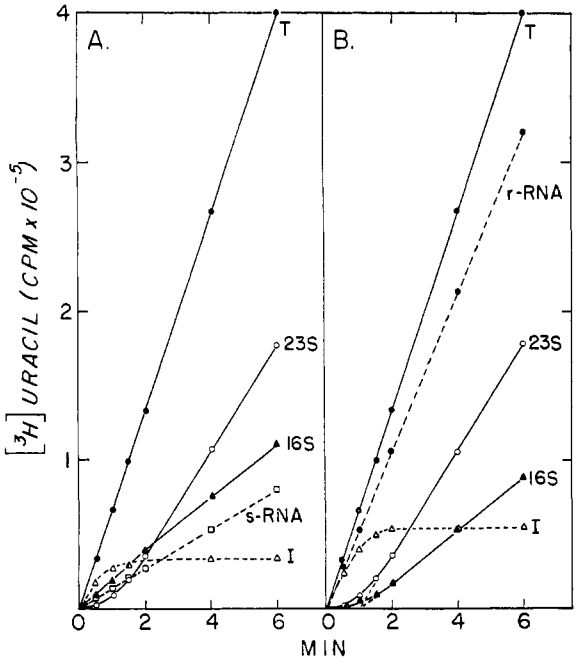


FIGURE 3: Rate of uptake of [^{3}H]uracil into various fractions of stable RNA. (A) From the data of Figure 2, at each time of labeling, the amount of ^{3}H was calculated for each of the following fractions (recognized by the pattern of ^{14}C, long-labeled RNA): 23S rRNA, 16S rRNA, and 4S RNA. The region between the 4S and 16S peaks was designated that of "incomplete chains," I. The amount of ^{3}H in 4S RNA was estimated as in Methods. The curve marked *T* shows the total uptake of [^{3}H]uracil into the cells. (B) The data of Figure 3A replotted with the amount of ^{3}H in 16S and "incomplete chains" corrected on the basis of the hybridization data of Figure 4, so that the amount of ^{3}H in 16S RNA has been reduced to the fraction that hybridizes as 16S rRNA. The remaining ^{3}H in 16 S, which hybridizes as 23S rRNA (but sediments as 16 S) has been added to the fraction of incomplete chains. The curve for rRNA is obtained by subtracting the label in RNA (Figure 3A) from the total (*T*). It is equal to the sum of 23 S, 16 S, and I.

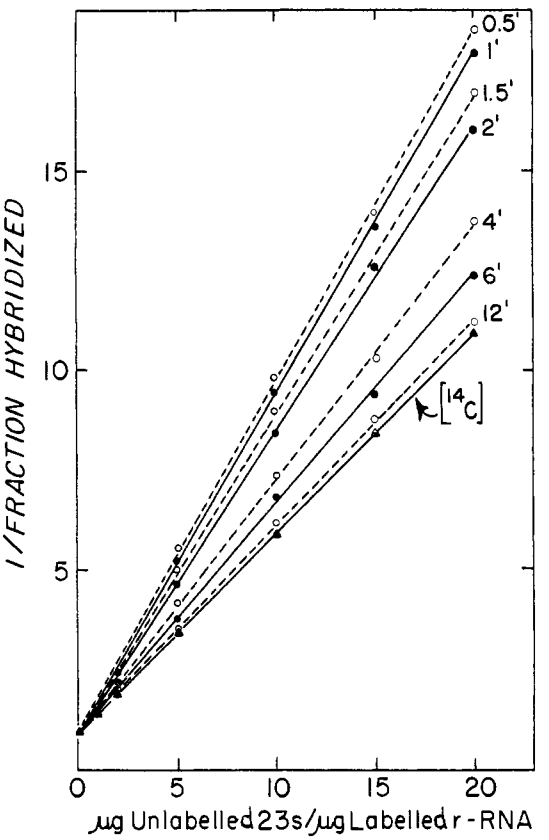
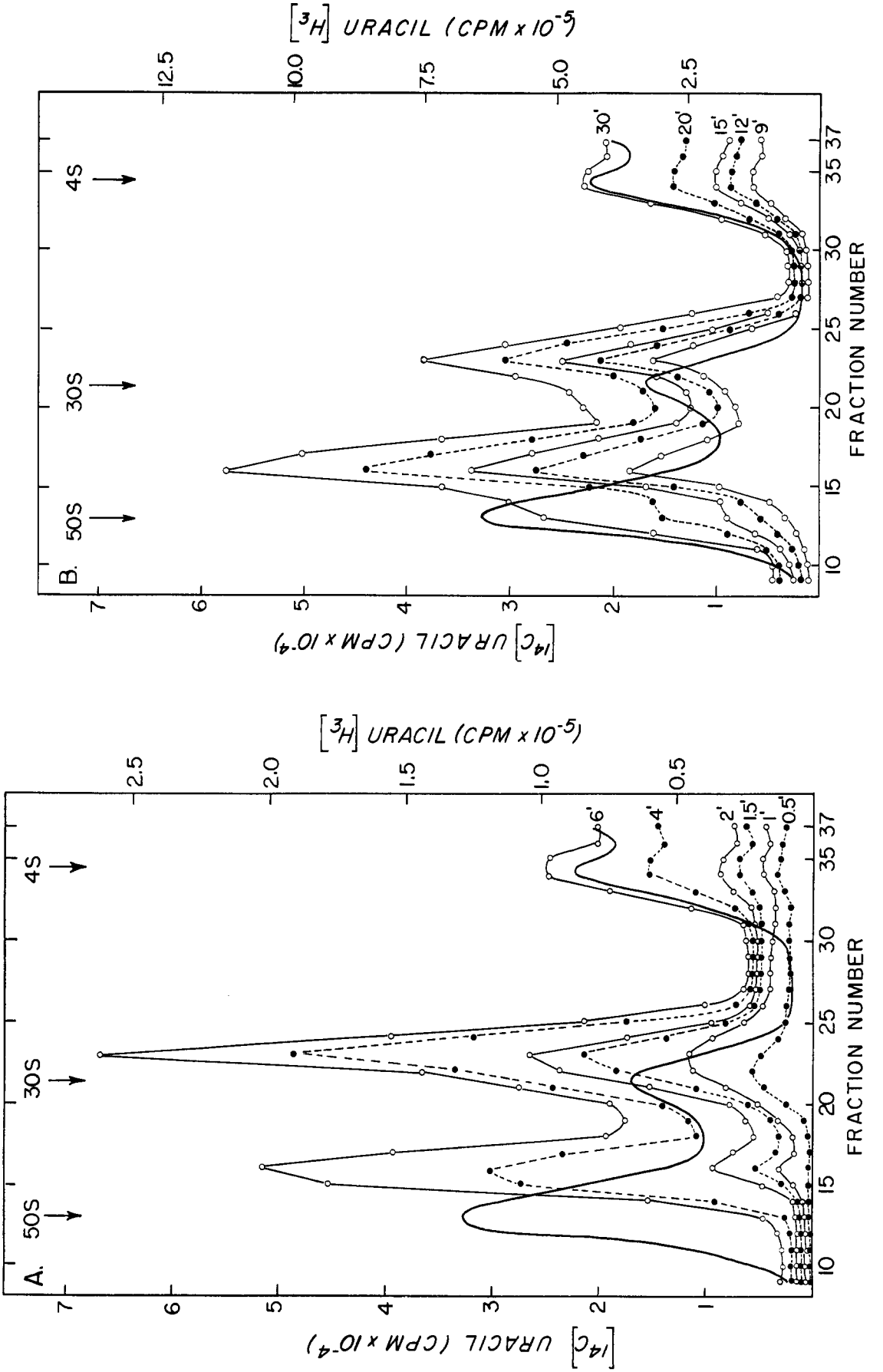


FIGURE 4: Hybridization of newly labeled 16S rRNA in the presence of unlabeled 23S rRNA. From an experiment similar to that reported in Figure 2, the fractions in the 16S region of the gradients (16–19) were pooled, dialyzed against 0.01 M Tris (pH 7.3)–0.01 M sodium acetate (pH 6.3), and then tested for hybridization in presence of cold 23S rRNA. Labeled RNA (0.5 μg) (containing 4000 cpm of [^{3}H]uracil), 75 μg of DNA, and the indicated amount of cold 23S RNA were used for each sample. Hybridization curves of ^{3}H-labeled RNA are shown for samples labeled for different times. Data are calculated and plotted as in Figure 1. In all the series of trials, the ^{14}C-labeled RNA is competed for as pure 16S rRNA (slope, 0.5); only one of the essentially superimposable curves is shown (—▲—). The slopes obtained reflect the relative amounts of 23S-like RNA sedimenting in the 16S region at each time point (see Table I).

Methods) with L = 2 min. As expected (see Methods), the curve reaches its maximum slope at about 2 min, and the final slope extrapolates back to an intercept of $L/2$ = 1 min on the abscissa. We conclude that it takes 2 min to synthesize an entire chain of 23S rRNA.

The rate of formation of 16S rRNA and RNA precursors is not as immediately clear. This is because, as we show below, many unfinished chains of 23 S sediment in the 16S region and slower (I region). As a result, at early times, the rate of labeling of unfinished chains in the I region was lower, while the apparent rate of formation of 16S rRNA chains (Figure 3A) was higher than the true one, even higher than the maximal rate observed when the precursor pool of incomplete chains is saturated.

To find out how much 23S-specific precursor was present in the 16S region of the gradients, we have used the cross-hybridization test (described in Methods). The authentic 16S species of rRNA in the peak was uniformly labeled with [^{14}C]uracil. At each time point, as expected, the ^{14}C was competed for by unlabeled 23S rRNA with a slope of 0.5 (Figure 4). The ^{3}H-labeled rRNA was competed for to an extent dependent on its relative content of 16S and 23S rRNA. The percentage of 23S rRNA in the 16S peak at different times was determined from the slopes of the inhibition curves (Figure 4) and eq 1 (see Methods). The values are listed in Table


[3H] URACIL (CPM x 10-5)
12.5
10.0
7.5
5.0
2.5
30'
20'
15'
12'
9'
4S
30S
50S
B.
35 37
30
25
20
15
10
FRACTION NUMBER
[14C] URACIL (CPM x 10-4)
7
6
5
4
3
2
1
[3H] URACIL (CPM x 10-5)
2.5
2.0
1.5
1.0
0.5
6'
4'
2'
1.5'
1'
0.5'
4S
30S
50S
A.
35 37
30
25
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10
FRACTION NUMBER
[14C] URACIL (CPM x 10-4)
7
6
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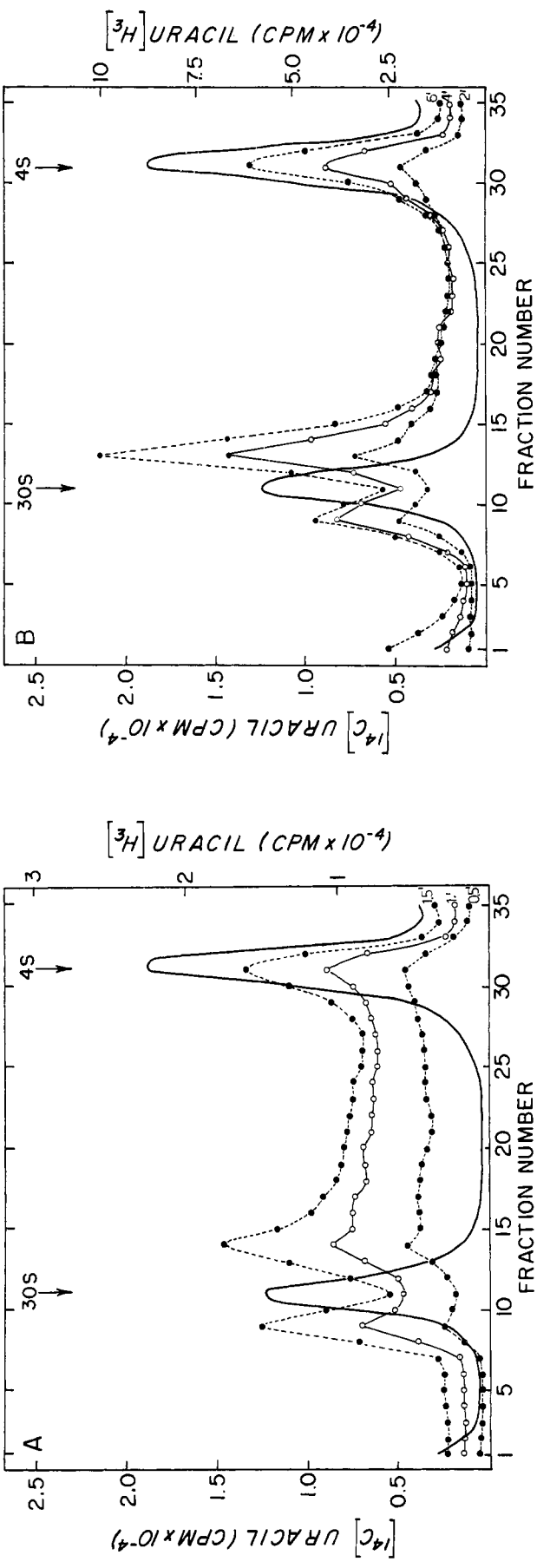

FIGURE 5: Rate of uptake of [^{3}H]uracil into ribosomes and ribosomal precursors. The RNA of a culture of fragile cells was first labeled with a pulse of 0.6 μg/ml of [^{14}C]uracil (0.33 μc) followed by two generations of growth. At 1.5 $\times$ 10^8 cells/ml, [^{3}H]uracil was added. At intervals, 10-ml samples were harvested, centrifuged, and lysed. Equivalent portions of samples were then run on sucrose gradients at 4° at 23,000 rpm for 10 hr to sediment most of the polyribosomes to the bottom of the gradient and display free 30 and 50 S. In order to increase the counts measured at early times without using exorbitant quantities of [^{3}H]uracil to permit long-term labeling, the experiment was run in two flasks. To one (A), 0.15 μg/ml (10 μc) was added for one set of points, from 30 sec to 6 min; to the other (B) 0.75 μg/ml (10 μc) was added for points from 6 to 30 min. The incorporation of [^{3}H]uracil in the two parts of the experiment was then normalized by multiplying the amount of ^{3}H in each sample from 6 to 30 min by the ratio of specific activities of exogenous uracil in the two series of points (0.75/0.15 = 5). The two patterns at 6 min thereby become superimposable. The fractions close to the bottom of the tubes, containing polyribosomes, are not shown. The heavy line indicates the amounts and positions of stable ^{14}C-labeled RNA; the other lines show the pattern of ^{3}H-labeled RNA at the times indicated.

FIGURE 6: Resolution of the peaks of 26S and 32S precursors. Portions of the lysates of the experiment reported in Figure 5 were centrifuged in sucrose gradients for 22 hr instead of 12 hr, in order to resolve ^{3}H-labeled peaks moving at 26 and 32 S. The heavy line indicates the position of the ^{14}C-labeled RNA. The other lines show the distribution of the ^{3}H-labeled RNA after 0.5, 1.0, and 1.5 min of labeling (Figure 6A) and after 2, 4, and 6 min (Figure 6B).

TABLE II: Detection by Hydridization of 16S-like and 23S-like rRNA Sedimenting at 16 S.[a]

Time of Labeling (min)	Slope	% of Component 16 S like	% of Component 23 S like
0.5	0.88	25	75
1	0.85	30	70
1.5	0.80	40	60
2	0.76	50	50
4	0.64	75	25
6	0.57	85	15
12	0.51	>95	<5

[a] From the data of Figure 4 (see legend, Figure 1), the slopes of the curves were determined and the fraction of 16S- and 23S-like RNA were computed from eq 1 (see Methods and Figure 1B).

II, and declined from 75% at 30 sec to less than 5% at 12 min. These values were then used to correct the data of Figure 3A. The fraction of 23S-specific RNA sedimenting in the 16S region is shifted to the unfinished chains (I); the corrected curves for 16S rRNA and I are drawn in Figure 3B.

With this correction made, it became clear (Figure 3B) that the labeling of 16S rRNA has the same kinetics as that of 23S rRNA. The experimental curves for 23S and 16S rRNA fit precisely eq 4 and 5 (see Methods) for the specified time intervals. The corrected fraction of unfinished chains is exactly equal to a 1 min ($L/2$) equivalent of total rRNA synthesis, as predicted from eq 5. Also, as expected from the ratio of 23S rRNA to 16S rRNA, known to be 2:1 at all times in the cell, it can easily be verified that labeling curves constructed by adding two-thirds of the unfinished chains to 23S and one-third to 16S rRNA are straight lines with slopes in the ratio 2:1. We conclude that in our cultures it takes about 2 min to make a chain of 23S or 16S rRNA.

B. Synthesis of Ribosomal Subunits. Figure 5 shows the regions of 50S and slower moving material in gradient centrifugations of cells sampled during the continuous label with [^{3}H]uracil (complete gradients from a similar experiment were presented in Figure 2 of Mangiarotti and Schlessinger, 1967). Two ^{3}H-labeled peaks that overlap but do not coincide with the complete ^{14}C-labeled 30S and 50S ribosomes are evident. One moves in the 30S region; the other at about 43 S. The peaks accounted for most of the label incorporated at early times, but progressively decreased with time relative to the label in finished subunits. Addition of a large excess of unlabeled uracil to the medium chased the label into complete subunits (unpublished data). These observations are in agreement with the conclusion of earlier analyses that these regions contain precursors of ribosomal subunits (McCarthy *et al.*, 1962; Osawa, 1965).

To distinguish label in precursors from that in finished subunits, the subunits were purified by further gradient analysis (Mangiarotti and Schlessinger, 1967), and their content of ^{3}H:^{14}C was determined as a function of time. The ^{3}H in completed subunits was then subtracted from the gradient patterns to yield estimates of the amounts in precursors.

The breadth of the ^{3}H peak in the 30S region and the sudden increase in its radioactivity between 2 and 4 min (see Figure 5) made us suspect that the peak contained more than one component. In order to resolve it into components, longer centrifugation times were used to sediment the ^{14}C-labeled 30S ribosomes nearly to the bottom of the tube (Figure 6). Two ^{3}H-labeled precursor peaks, a small one moving faster (32 S) and a larger one moving slower (26 S) than the 30S ribosomes, were now resolved at early times.

To identify the particles in the 26S, 32S, and 43S peaks as 30S or 50S ribosome precursors, RNA was extracted from them and analyzed in sucrose gradients (Figure 7). The 43S precursor, in agreement with previous reports (Osawa, 1965), contains exclusively 23S rRNA (Figure 7C), and is therefore assigned to the 50S ribosome which contains only 23S rRNA (Kurland, 1960; Morimoto, 1966). The 32S peak, which also contains only 23S rRNA (Figure 7C), is thus also designated a 50S precursor.

The 26S peak contained 16S rRNA and also some slower material, which built up for about 2 min (Figure 7A). At 30 sec, the slower moving material accounted for 80% of the label, but this declined to only 20% at 6 min, with a concomitant progressive sharpening of the peak (Figure 7B). We therefore assume that the 26S ribosome precursor peak contains, in addition to completed 16S rRNA molecules, unfinished chains of rRNA; these saturate with label after 2 min (see above). However, the bulk of the material in the 26S peak is a precursor containing 16S rRNA, and is therefore identified as a 30S precursor.

Combining the data of Figures 5–7, the labeling patterns of Figure 8 are obtained. To avoid complexity in further analysis (see Discussion), a small correction is here introduced for the variation in relative specific activity of new rRNA from 0.7 to 1.0 during the experiment (see Methods). The points for each time have been corrected by the factor required to raise the experimental values for labeling of total RNA (Figure 3B) to a constant specific activity throughout the experiment.

Precursors of ribosomes, unlike the finished particles, are highly sensitive to degradation by added RNase (Sypherd, 1965). Their sensitivity to RNase permits the direct demonstration that they contain protein as well as RNA, for destruction of precursors labeled with [^{14}C]uracil (Figure 9) leads to the release into the soluble fraction of a corresponding fraction of protein, pulse labeled with L-[^{3}H]alanine, from the precursor region of the gradient. In the conditions of RNase treatment used, we have verified that no radioactive protein is released from purified 30S or 50S ribosomes labeled with [^{3}H]-alanine.

C. rRNA Associates with Protein as the RNA is Formed. In earlier studies, it was observed that all of the

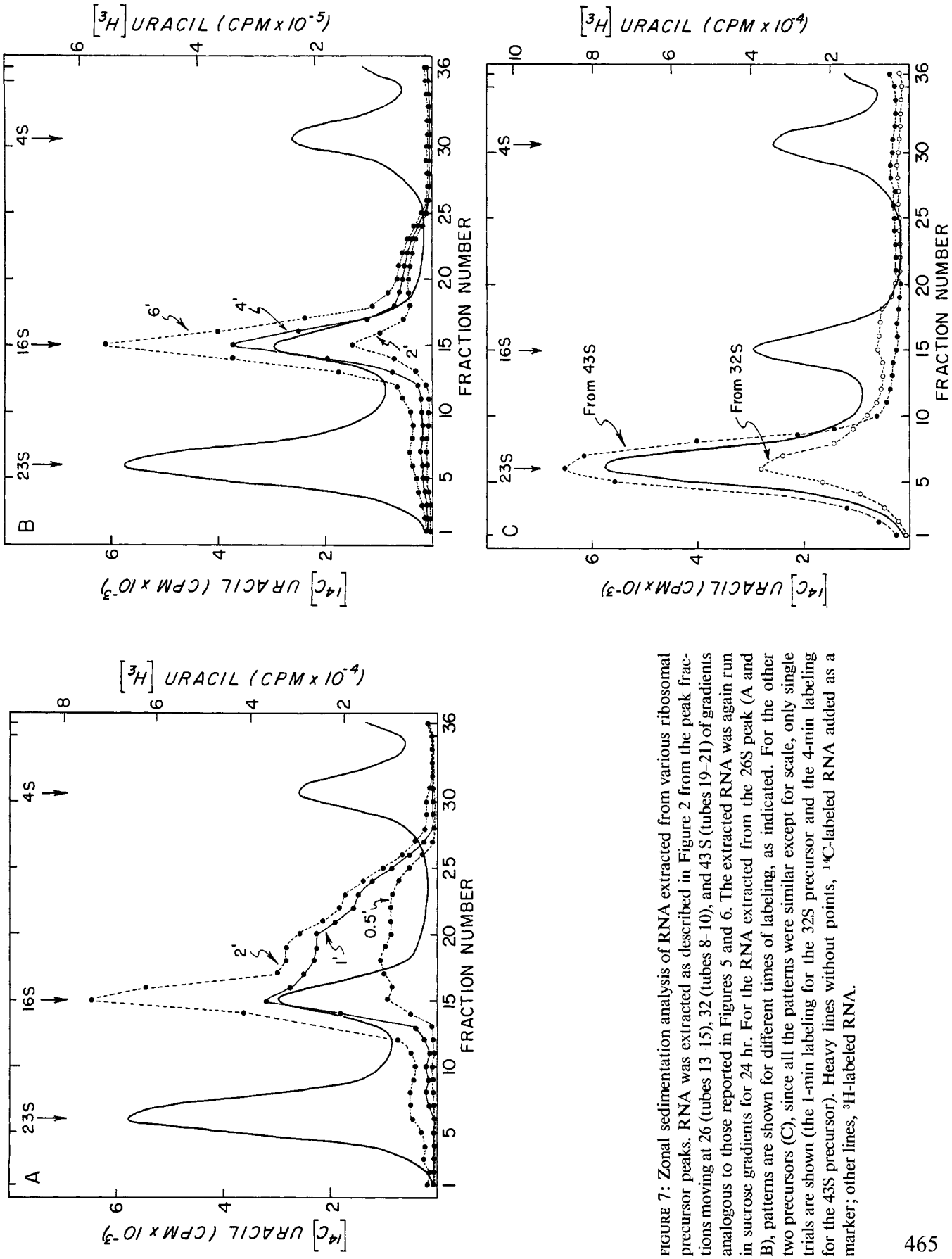

FIGURE 7: Zonal sedimentation analysis of RNA extracted from various ribosomal precursor peaks. RNA was extracted as described in Figure 2 from the peak fractions moving at 26 (tubes 13–15), 32 (tubes 8–10), and 43 S (tubes 19–21) of gradients analogous to those reported in Figures 5 and 6. The extracted RNA was again run in sucrose gradients for 24 hr. For the RNA extracted from the 26S peak (A and B), patterns are shown for different times of labeling, as indicated. For the other two precursors (C), since all the patterns were similar except for scale, only single trials are shown (the 1-min labeling for the 32S precursor and the 4-min labeling for the 43S precursor). Heavy lines without points, ^{14}C-labeled RNA added as a marker; other lines, ^{3}H-labeled RNA.

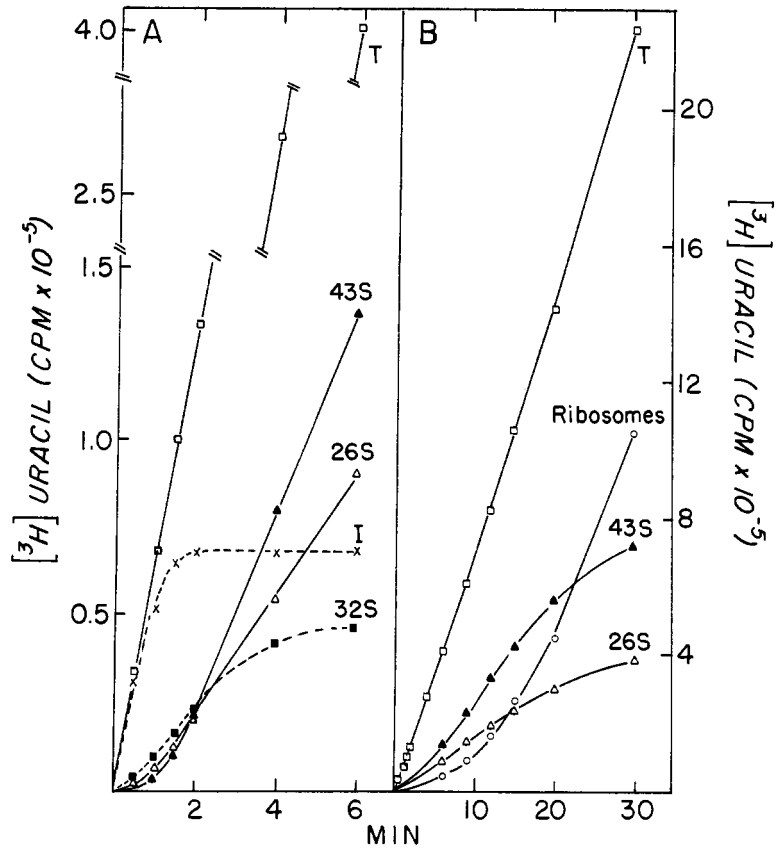

FIGURE 8: Distribution of ^3H in ribosomes and ribosomal precursors as a function of time of labeling. The amount of label in different RNA fractions was calculated from the data of Figures 5–7, as discussed in the text (see legend, Figure 5, for definition of *T*, I, etc.). The amount of label in complete 30S and 50S particles has been estimated as reported previously (Mangiarotti and Schlessinger, 1967) by repurification of the particles through sucrose gradients (not shown here; see, *e.g.*, Figures 2 and 3 of Mangiarotti and Schlessinger, 1967). The amount of ^3H in the precursors has been calculated by subtracting from the label in each peak the amount accounted for by the overlap of finished ribosomal particles, determined from the ^{14}C patterns and the ^3H:14C ratio of the purified ribosomes. (B) Shows the over-all patterns during 30 min of labeling. (A) Gives further detail about the distributions at early times.

rRNA extracted with phenol or sodium dodecyl sulfate from material (ribosomal precursors) moving at less than 26 S in a sucrose gradient moved slower than 16 S upon recentrifugation after the extraction (see Figure 6, Mangiarotti and Schlessinger, 1967). This shift in sedimentation rate suggested that incomplete chains of rRNA are already associated with some protein.

The extracted rRNA all sedimented at the same relatively slow rate even in the presence of 0.01 M Mg^{2+}, 0.04 M NaCl, or after it was added to lysing medium containing a fresh lot of unlabeled fragile cells (unpublished data). Therefore, its rapid sedimentation in a lysate before extraction is unlikely to be caused by aggregation in high Mg^{2+} concentrations, or by nonspecific binding to another component in the lysates.

That the unfinished chains of rRNA are indeed bound to protein is supported by more direct tests. The experiments (Figure 10 and Table III) are based on the sensitivity of unfinished ribosomes to RNase (see above). If protein were bound to unfinished chains of rRNA, it should be released from them after RNase treatment.

The results of such a trial appear in Figure 10. Protein was specifically labeled with L-[^3H]alanine of high specific activity during a 2-min pulse. Portions of cell lysates, treated or untreated with RNase, were then analyzed in sucrose gradients (Figure 10A,B). After treatment with RNase to destroy precursors, nearly all the [^3H]uracil in the region of gradients between 5 and 26 S is converted into acid-soluble material, and about 50% of the protein label disappears from this region.

An attempt was made to lower the background of contaminating protein by a second gradient analysis, but the same degree of contamination was still observed. The material not removed by RNase probably consists of large proteins and aggregates, such as those reported for succinic dehydrogenase (Koike *et al.*, 1963), α-ketoglutarate dehydrogenase (Mukherjee *et al.*, 1965), and RNA polymerase (Richardson, 1966).

To find out whether or not the protein released from the precursor region by RNase action resembles ribosomal proteins, a rough column chromatographic analysis was carried out, following the technique of Williams (1967). Carrier ribosomes labeled with L-[^{14}C]alanine were added to the pooled precursor region of the gradient (labeled with L-[^3H]alanine), and ribosomal protein was extracted with LiCl–urea and fractionated on carboxymethylcellulose (see Table III). The analysis was carried out before and after RNase treatment. Batchwise elution permits the resolution of a nonribosomal fraction (F_1) and four gross fractions of ribosomal proteins

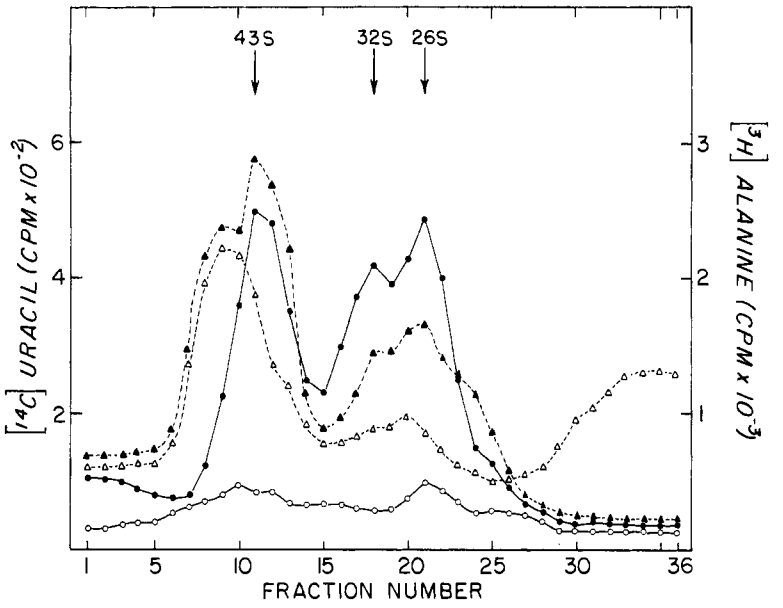

FIGURE 9: Uptake of [^{3}H]alanine into protein sedimenting with 23S, 32S, and 43S precursors of ribosomes. A fragile culture (50 ml) was labeled with [^{14}C]uracil (0.1 μc) for 4 min and with L-[^{3}H]alanine (25 μc) for 2 min before harvest of the cells. After centrifugation of the lysate in a sucrose gradient at 23,500 rpm for 16 hr, fractions corresponding to the region of the ribosomal precursors (from about 25 to 45 S; see, *e.g.*, Figure 5) were pooled, dialyzed against 0.01 M Tris (pH 7.3)–0.01 M $MgSO_4$–0.04 M NaCl, and concentrated by pervaporation. After a second dialysis against the same buffer, the sample was divided in two, and one half was treated with RNase (1 μg/ml) for 10 min at 25°. Both portions were then centrifuged in sucrose gradients for 20 hr at 23,500 rpm. Portions of each resultant gradient fraction were counted after plating and washing in ice-cold 5% trichloroacetic acid. Replicate portions were counted to measure acid-insoluble labeled protein after RNA was hydrolyzed by 20 min of boiling in hot acid. (—●—) [^{14}C]Uracil (RNA), no RNase treatment; (—○—) [^{14}C]uracil (RNA) after RNase treatment; (—▲—) [^{3}H]alanine (protein), no RNase treatment; (—△—) [^{3}H]alanine (protein) after RNase.

(F_2–F_4). While the untreated material revealed proteins resembling the carrier ribosomal proteins added, the ^{3}H remaining after RNase treatment behaved differently from the carrier protein. Most of it moved in F_1 and F_4, while F_2, F_5, and F_3 were greatly reduced. The material in F_4 is probably not composed of ribosomal proteins, since comparable amounts of contaminating ^{3}H-labeled protein elute with "F_4" from complete 50S particles unless the ribosomes are washed with salt solutions of high ionic strength (Kurland, 1966; Spirin *et al.*, 1963) before protein extraction. Here treatment with high salt was not carried out, in order to avoid losses of precursor protein. The true F_4 in these analyses is therefore masked; but it is clear that most of the ^{3}H in F_2 and F_5 was selectively removed from the precursor region by RNase treatment.

Discussion

A scheme of ribosome biosynthesis based on these results is depicted in Figure 12. As indicated there, 2 min are required to make a complete chain of 16S or 23S rRNA. While the chains are forming, ribosomal proteins are added to them. The completed chains then appear in holdup precursors *en route* to complete ribosomes; the 16S rRNA moves for some time as a 26S particle that then matures to the 30S ribosome; the 23S rRNA moves as a 32 S, and then as a 43S holdup precursor particle that then matures to a 50S particle. Here we present a precis of the kinetic analysis and discuss some of its implications, especially those concerning the rate of synthesis of rRNA.

How Are 16S and 23S rRNA Formed in the Same Time? The results indicate that both 16S and 23S rRNA (see Results) are made in the same time length, even though the 23S rRNA is twice as long as the 16S rRNA (Kurland, 1960). One possibility is that the rate of movement of RNA polymerase could be twice as fast on a 23S cistron. On the other hand, the rate of polymerase movement might be the same, but the production of a 23S rRNA chain might take place by covalent linkage of two different 16S chains. In this case, twice as many molecules of RNA polymerase would be active in the synthesis of 23S rRNA, and there would be two classes of 16S cistrons in the cell, one specific for the 30S ribosome, and the other for the 50S ribosome, all transcribed at about the same rate.

If 23S rRNA is indeed formed by joining two 16S molecules, then extracted 23S-specific RNA would appear only at 23 S and in chains smaller than 16 S. A suggestion that there may indeed be no rRNA that sediments between 17 and 22 S is given by the gradient

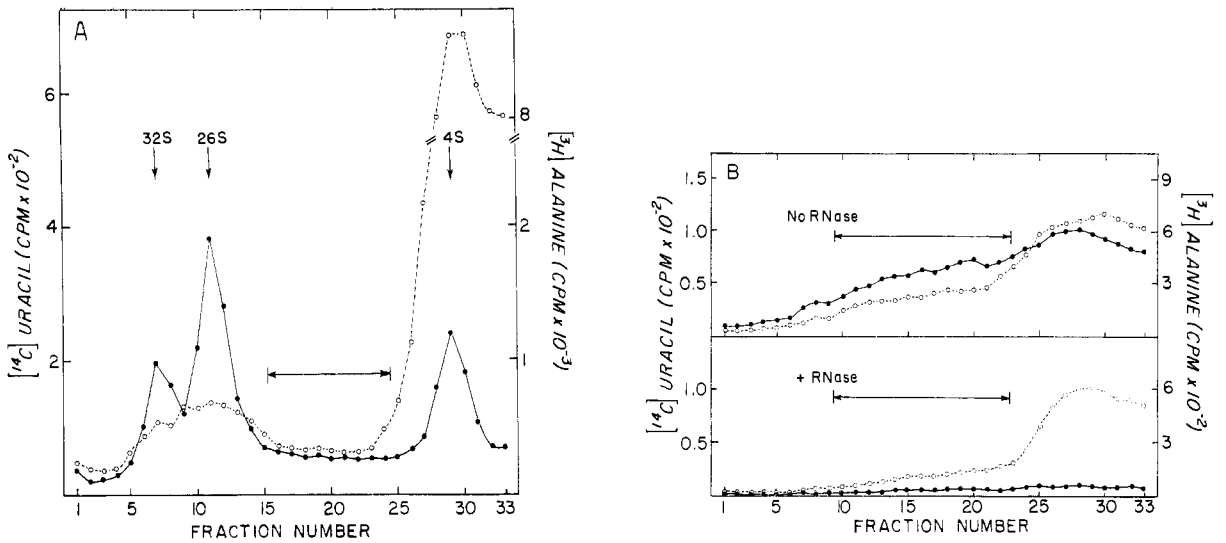

FIGURE 10: Uptake of [^{3}H]alanine into proteins sedimenting with incomplete rRNA chains. A culture growing in fragile form (50 ml) was labeled with [^{14}C]uracil (0.2 μc) for 4 min, and with [3H]alanine (50 μc) for 2 min before harvesting. The lysate was centrifuged on a sucrose gradient for 28 hr at 23,500 rpm in order to display the region of small precursors and incomplete chains of rRNA. Aliquots of each gradient fraction were plated and counted, with and without 20-min boiling in acid, to give the patterns of radioactivity in RNA and protein shown in part A (—●—, [^{14}C]uracil; —○—, L-[^{3}H]alanine). The fractions indicated by the arrows (tubes 16–24) were pooled, dialyzed against 0.01 M Tris (pH 7.5)–0.01 M $MgSO_4$–0.04 M NaCl, and concentrated by pervaporization. After a second dialysis against the same buffer, the sample was divided into two portions, one of which was treated with RNase (1 μg/ml) for 10 min at 25°. Both aliquots were then run in sucrose gradients at 23,500 rpm for 36 hr, and portions of each fraction precipitated and were counted to give the patterns shown in part B (—●—, [^{14}C]uracil; —○—, L-[^{3}H]alanine). The fractions indicated by the arrows (tubes 9–23) were utilized for the experiment reported in Table III.

analyses of Figure 2. At early times of labeling with [^{3}H]uracil, all chains are labeled to an approximately equal extent. If there are all intermediate sizes of both 16S- and 23S-like rRNA chains, one would expect a flat distribution of label across the gradient. Instead, the region between the 16S and 23S peaks contains a relative deficit of label compared with the region between 4 and 16 S. However, these data are only suggestive, and lack the resolution required to settle the problem.

Whichever mechanism is employed to ensure the synthesis of 16S and 23S rRNA chains in equal times, the process is evidently different from that in mammalian cells. There, a large molecule of 45S rRNA forms and is later split to yield molecules of the different rRNA chains (Scherrer *et al.*, 1963).

Rate of Movement and Amount of RNA Polymerase on Each Gene. The formation of a chain of 16S RNA in 2 min means that the rate of rRNA propagation in these cultures is about 1600/120 = 13 nucleotides/sec. In order to produce the observed amount of rRNA from the known numbers of gene copies, many RNA polymerase molecules must function on a cistron at the same time. There are about four 16S and four 23S rRNA gene copies [0.2% (Yankofsky and Spiegelman, 1963) in a chromosome of molecular weight about 2×10^9 (Cairns, 1963)]; each must make about 30 rRNA copies/min to account for the increase in cellular ribosomes (20,000/cell; see Mangiarotti and Schlessinger, 1967) during exponential growth. Since it takes 2 min to form a complete chain, at least 60 molecules of RNA polymerase must be at work at the same time on each cistron that specifies rRNA.

An analogous argument can be made for the time to synthesize the average mRNA chain (see Figure 5 and Discussion, Mangiarotti and Schlessinger, 1967). More indirect evidence has been adduced that, for specific mRNA molecules, including those for the lactose (Alpers and Tomkins, 1965; Leive, 1965) and histidine (Goldberger and Berberich, 1965) operons, each cistronic equivalent of mRNA takes 1–2 min to form. For the tryptophan operon, the synthesis of the corresponding molecule of a very large mRNA again takes place at about one to two gene equivalents per minute after the start of derepression (Imamoto *et al.*, 1965). A number of copies of each cistron are probably made in that time (Attardi *et al.*, 1963). Therefore, in the case of mRNA as well, each gene can probably be copied by a number of polymerase molecules in tandem at the same time.

While all the data are consistent with the notion that the rate of movement of RNA polymerase in a cell may not vary greatly from one cistron to another, it is difficult to extrapolate the observed rate to more rapidly growing cultures. For example, in rich medium, cells grow as much as five times faster and must duplicate their ribosome complement, which is about fivefold that in very slow-growing cells (Maaløe and Kjeldgaard,

TABLE III: Effect of RNase on ^{3}H-Labeled Protein Bound to Unfinished Chains of rRNA.[a]

Column Fraction	RNase		^{14}C Labeled Carrier
	Untreated Sample	Treated Sample	
F_1	103	186	30
F_2	206	30	140
F_5	200	55	124
F_3	269	121	48
F_4	275	273	48

[a] Cells labeled with L-[^{3}H]alanine for 2 min were processed in the experiment described in the legend of Figure 10. Thus, pooled samples from the 8S to 20S precursor region of the initial gradient, treated or untreated with RNase, were reanalyzed by zonal sedimentation in sucrose gradients to display the unfinished chains of rRNA (Figure 10B). Fractions 10–23 (Figure 10B) of each gradient were pooled and dialyzed against 10 mM Tris (pH 7.5)–10 mM $MgSO_4$. Ribosomes (1 mg) uniformly labeled with a small amount of L-[^{14}C]alanine were added to each sample as a carrier, and the mixture was lyophilized. The dry powder was redissolved in 0.5 ml of 6 M urea–3 M LiCl, stirred for 5 hr, and then centrifuged at 30000*g* for 10 min to obtain the supernatant phase, containing more than 85% of the starting amount of labeled protein. The extraction and the fractionation of the ribosomal proteins were carried out according to the technique of Williams (1967). Subfractions of ribosomal protein were obtained by chromatography on carboxymethylcellulose columns (0.5 × 16 cm). Eluents (6 ml each) were, in the order used, 0.1 M sodium formate (pH 2.7) (F_1), 0.3 M $BaCl_2$ in 0.02 M sodium formate (pH 2.7) (F_2), formic acid (pH 2.0) (F_5), formic acid (pH 1.7) (F_3), and 40% formic acid (F_4). Fractions (1 ml) were collected, precipitated with 5% trichloroacetic acid, heated to 90° for 25 min, and plated to measure acid-insoluble radioactivity. The counts per minute listed represent the total observed in the various column fractions. For both the columns (with and without RNase) the recovery of ^{14}C-labeled carrier protein was the same in each fraction (±15%), and 90% of the applied radioactivity was recovered.

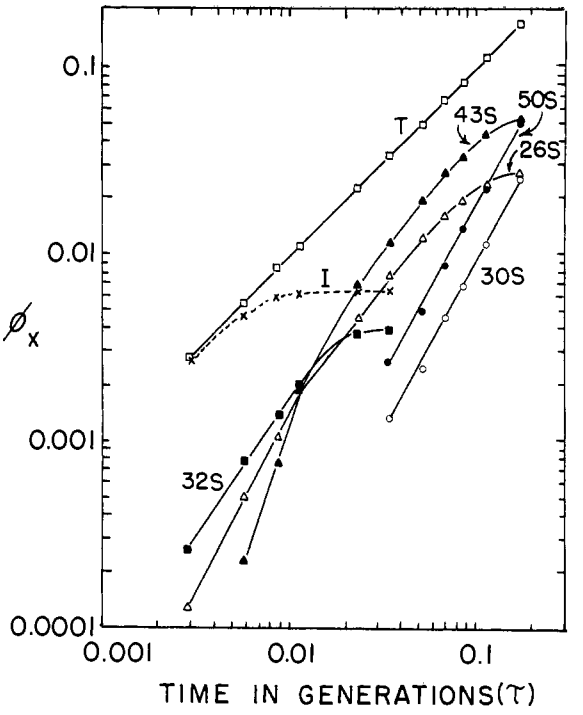

FIGURE 11: Log–log plot of the newly synthesized fraction of RNA present in ribosomal precursors and ribosomes as a function of time, ϕ_X is the fraction in a component (X) at each time, and has been calculated from the data summarized in Figure 8, according to the analysis given in Methods (eq 8). See legend, Figure 5, for the definition of *T*, I, etc.

1966), in one-fifth the time. If each chain of rRNA were still to take 2 min to form in the rapidly growing culture, each cistron specific for rRNA would bear more than 300 molecules of RNA polymerase at a time. Each enzyme molecule could then extend over only a few nucleotides of DNA, a dimension much smaller than the protein (Fuchs *et al.*, 1964). Such a concentration of enzyme is very unlikely, and we therefore raise the alternative possibility that the rate of RNA propagation increases as a function of growth rate.

Ribosomal Precursors. Most previous studies of the assembly of protein and rRNA chains into ribosomes have been done on cultures whose growth was interrupted by one of a number of inhibitors. Under these conditions a wide variety of ribonucleoprotein particles accumulate; however, it is not clear whether any of the species of accumulated particles represents a form of ribosome precursor that naturally exists in the cell in small amounts, or whether they are only anomalous by-products that may or may not find their way into finished ribosomes when growth resumes (for a review, see Raskas, 1967).

The more direct studies of the natural precursors have provided most of the available information about ribosome formation (McCarthy *et al.*, 1962; Kono and Osawa, 1964). The kinetic analysis of McCarthy *et al.*, as well as sedimentation measurements on labeled rRNA extracted from sucrose gradients fractions (Osawa, 1965), and a direct pulse-chase experiment in a mutant that accumulated large amounts of 43S precursor (Brownstein and Lewandowski, 1966), have already shown the 43S precursor to be 50S specific. Britten *et al.* (1962) also presented evidence that the 43S precursor contains protein. Several other precursors were also suggested, but the analysis was not complete.

The limitations of the analysis of Britten *et al.* (1962)

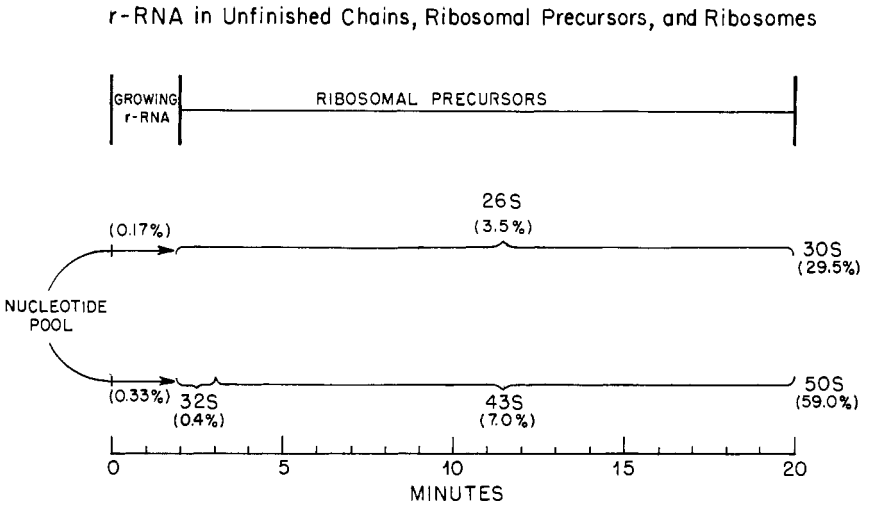

FIGURE 12: The time course of assembly of 30S and 50S ribosomes. Along the time scale (bottom) is indicated the average period spent by a newly forming ribosome in each precursor stage in *E. coli* E 203 (growing with a mass doubling time of 120 min). In addition, the numbers in parentheses give the percentage of the total cellular rRNA in each of these precursor stages. Thus, unfinished chains of 16S and 23S rRNA (at left), grow to complete chains in about 2 min. Protein chains probably bind to the growing RNA chains (see text), so that as soon as the chains are complete, they appear, respectively, as 26S and 32S precursor particles. The 26S particle matures to a 30S ribosome after about 18 min. After a brief (1 min) halt in the 32S pool, the nascent 50S ribosome moves to a 43S form for about 17 min before completing its maturation.

were imposed by the resolution obtained in their sucrose gradient analyses. The gradients did not distinguish between label in some of the precursors and complete ribosomes or other precursors, and several fractions were contaminated by fragments of mRNA. The precursors suggested included the "eosome," a broad peak sedimenting at about 14 S. In addition to the eosome and the 43S precursor of 50S particles, two precursors, one specific for the 30S ribosome and one for the 50S ribosome, both moving in the 30S region, were suggested, but not resolved. Also, a direct flow of some radioactivity from the eosome to the 43S precursor, without passage through the putative precursor in the 30S region, was hypothesized.

The eosome turned out on subsequent analysis to be a mixture of two-thirds rRNA and one-third mRNA (Midgeley and McCarthy, 1962). In the analyses reported here, in which all the mRNA is confined in the polyribosomes, no eosome-like peak that can be identified as a precursor is observed: all the heterogeneously distributed label in the 14S region is found by sedimentation and hybridization properties to consist of incomplete chains of rRNA.

The existence of two precursors in the 30S region is confirmed by our results (Figures 6 and 7, and below); but there seems to remain no basis for the direct movement of material into the 43S particle without prior passage through the 32S stage. Similarly, we have detected no trace of the 17S peak that Kono and Osawa (1964) observed in cells transferred from one growth medium to a more fortified one, and which they interpreted as free 16S rRNA, an early ribosomal precursor. Instead, the first complete chains of 16S and 23S rRNA appear in these analyses in ribonucleoprotein particles moving, respectively, at 26 and 32 S.

All the kinetic data and our more direct experiments (Figure 10 and Table III) are consistent with the interpretation that proteins are adding onto rRNA chains as they are formed. For example, only chains smaller than 16 S are found between the 4S and 28S peaks in gradient analyses of cell lysates. Furthermore, at early times, the labeling of the 28S and 32S peaks is superimposable with the respective labeling of complete 16S and 23S rRNA chains (Figures 3B and 5–8); *i.e.*, the precursors contain all, or very nearly all, the complete rRNA chains synthesized during the first minutes of labeling.

In order to determine the order of synthesis and the fraction of rRNA present at any time in each ribosomal precursor, the data of Figures 3 and 8 were replotted, essentially according to McCarthy *et al.* (1962); see Methods. According to their analysis, a log–log plot of the radioactivity of a given precursor against time (expressed in units of τ; see Methods), will indicate by its *initial* slope the serial position occupied by that precursor in the biosynthetic sequence, and by its horizontal asymptote the amount of precursor in the steady state. Plots of this kind for all the relevant ribosome precursor fractions are shown in Figure 11.

Considering first the 30S ribosomes, we note that during the first few minutes of labeling, the pool of unfinished rRNA chains is saturated with label. A completed chain then appears in a 26S precursor, in a pool

containing about 3.5% of the total rRNA, for an average of about 18 min (about 10% of a generation) before it matures to a 30S ribosome. During this time, the radioactivity in the 26S and 30S particles rises as a function, respectively, of about τ and τ^2. This is expected, in keeping with the idea that finished chains of 16S rRNA appear in the 26S precursor as the only holdup precursor leading to 30S particles.

Considering next the 50S ribosomes, the completed chain of 23S rRNA apparently spends about 1 min in a pool of 32S precursors that contains about 0.4% of the total rRNA. It then is found as a 43S precursor for about 17 min, in a pool containing about 7% of the total rRNA, before it becomes a completed 50S ribosome. Accordingly, the label in 43S particles increases as a function of τ^3 during the first 2 min of labeling (Figure 11), when new rRNA is delayed in two states (growing chains and 32S particles). The final product, 50S ribosomes, is synthesized in the same average time as are 30S ribosomes (Figure 12). They should increase in their content of label at very early times as a function of τ^4. However, the amount of label in 50S particles is too low to measure accurately until 4–6 min have elapsed. At that time, the radioactivity in 50S particles is already increasing as a function of τ^2, since most of the precursor pools are saturated with radioactivity.

An internal check on the over-all consistency of the analysis can be made, using the additional knowledge that the weight ratio of RNA in 50S and 30S particles in the cells is always 2:1. If one plots, as a function of time, the total amount of radioactivity present in the 50S ribosome and its precursors (two-thirds of the label in unfinished rRNA chains, plus the label in 32S and 43S precursors), a straight line is obtained. A similar plot of the total radioactivity present in the 30S ribosome and its precursors (one-third of the label in unfinished rRNA chains, plus the label in the 26S precursor) also yields a straight line. The ratio of the slopes of the two lines is 2:1, as predicted. About 0.5% of the total rRNA is in unfinished chains at any instant, with a total amount of 10–12% of the rRNA in precursors. This value is in good agreement with the estimate of 10% in McCarthy *et al.* (1962).

The Origin of the Pools of Precursors. Unfinished chains of rRNA, unlike unfinished mRNA molecules, do not bind to ribosomes or enter polyribosomes (Mangiarotti and Schlessinger, 1967). Instead, they apparently associate with ribosomal proteins, so that they are transformed into the first holdup precursors as soon as they are finished. The sharpness of the 26S, 32S, and 43S peaks, and the fact that they saturate exponentially with label, indicates that each is homogeneous, and not a family of precursors containing different amounts of protein but all sedimenting at about the same rate. Each precursor is therefore a major holdup point in the synthesis of the ribosomal particles. Such precursors could arise if, for instance, the synthesis of a rare component, such as 5S rRNA (Rossett *et al.*, 1964) or an infrequent protein species, becomes rate limiting; or if the accretion of the next element in biosynthesis requires a rare, spontaneous transition of the precursor from one configuration to another. The recognition of rRNA, during its formation and afterwards, by ribosomal protein awaits further structural studies.

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The Incorporation of Mevalonic Acid into the N^6-(Δ^2-Isopentenyl)adenosine of Transfer Ribonucleic Acid in *Lactobacillus acidophilus**

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ABSTRACT: *Lactobacillus acidophilus*, which can utilize mevalonic acid as a growth requirement, will assimilate this compound into transfer ribonucleic acid (tRNA) but not ribosomal ribonucleic acid (rRNA). The level of incorporation is consistent with the occurrence of one mevalonic acid derivative in approximately ten tRNA chains. An N^6-(Δ^2-isopentenyl)adenosine nucleotide in the transfer ribonucleic acid was identified as the compound derived from radioactive mevalonic acid.

During the course of the elegant determination of the sequence of bases in the serine tRNA of yeast, Zachau *et al.* (1966) showed the presence therein of 1 mole of N^6-(Δ^2-isopentenyl)adenosine (iPA)[1] (Figure 1). Simultaneously, Hall *et al.* (1966) isolated this compound from digests of unfractionated yeast tRNA. The recent studies of Madison *et al.* (1967) indicate that this isoprenoid nucleotide occurs once in the structure of yeast tyrosine tRNA. The cytokinin, N^6-(Δ^2-isopentenyl)-adenine, has been isolated from *Corynebacterium fascians* (Klämbt *et al.*, 1966) and identified (Helgeson and Leonard, 1966).

Mevalonic acid (MVA) (Figure 2) has been shown to be the precursor of sterols and other compounds built up from isoprene units (Bloch, 1965). The isoprene structure of the alkyl substituent of iPA suggested that it, too, might have its origin in MVA. Although neither sterols nor tRNA containing iPA had been described in bacteria, the advantages of experimentation with such systems prompted a search for an involvement of MVA in iPA synthesis in microorganisms. Although no evidence could be obtained for formation of iPA from MVA in yeast or *Escherichia coli*, such a precursor–product relationship was demonstrated in *Lactobacillus acidophilus*, which requires MVA for growth.

The studies of Thorne and Kodicek (1962b) have demonstrated that about two-thirds of the mevalonic acid incorporated by *Lactobacilli* is lipid material. More recently, these same investigators (Thorne and Kodicek, 1966) have shown the most abundant lipid to be a C_{55}-polyisoprenoid alcohol, which they have named bactoprenol. The current studies of Wright *et al.* (1967) and Higashi *et al.* (1967) on a lipid intermediate in cell wall synthesis suggest a bactoprenol structure for these compounds. The suggestion was made (Higashi *et al.*, 1967) that the nutritional requirement for MVA in some *Lactobacilli* may be explained by its necessity for bactoprenol synthesis. The work described herein shows that in *Lactobacilli* another probably essential macromolecule, tRNA, requires MVA for its biosynthesis. Thus,

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[1] Abbreviations used: MVA, mevalonic acid; iPA, N^6-(Δ^2-isopentenyl)adenosine; [^{14}C]iPA-*t*RNA, amino acid acceptor RNA in which the bound N^6-(Δ^2-isopentenyl)adenosine was labeled with ^{14}C; ATP, adenosine triphosphate; TCA, trichloroacetic acid; A_{260}, an amount of material in a volume of 1 ml with an optical density of one when measured in a 1-cm light path at 260 mμ.