

# MEMBRANE-BOUND RIBOSOMES OF MYELOMA CELLS

## II. Kinetic Studies on the Entry of Newly Made Ribosomal Subunits into the Free and the Membrane-Bound Ribosomal Particles

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### ABSTRACT

The kinetics of appearance of newly made 60S and 40S ribosomal subunits in the free and membrane-bound ribosomal particles of P3K cells were explored by determining the specific radioactivities of their 18S and 28S RNA after various lengths of [<sup>3</sup>H]uridine pulse. Both 40S and 60S subunits enter free and membrane-bound polyribosomes at comparable rates from the cytoplasmic pool of newly made, free native subunits, the 40S subunits entering the native subunit pool and the polyribosomes slightly earlier than the 60S subunits. At all times, the specific radioactivity of the membrane-bound native 60S subunits was slightly lower than that of the polyribosomal 60S subunits. This indicates that the membrane-bound native 60S subunits are not precursors destined to enter membrane-bound polyribosomes and suggests that they result from the dissociation of ribosomes after chain termination. The results observed also suggest that the membrane-bound native 60S subunits are not reutilized before their release from the membranes, which probably takes place shortly after dissociation from their 40S subunits. The monoribosomes, both free and membrane-bound, had the lowest specific radioactivities in their subunits. Finally, a small amount of newly made native 40S subunits, containing 18S RNA of high specific radioactivity, and apparently also newly made messenger RNA were detected on the membranes. The high turnover of these membrane-bound native 40S subunits suggests that they may represent initiation complexes formed with mRNA which has just reached the membranes and which has not yet given rise to polyribosomes.

This paper describes studies on the rate of entry of newly made ribosomal subunits into the free and membrane-bound ribosomes of P3K cells, using the cell fractionation procedure described in detail in the preceding paper (12).

### MATERIALS AND METHODS

Exponentially growing MOPC 21 (P3K) mouse myeloma cells were uniformly labeled with [<sup>14</sup>C]uridine for 24 h in

suspension cultures as previously described (12). For pulse-labeling experiments, cells were concentrated to  $2 \times 10^6$  cells/ml and pretreated with 1  $\mu$ M ethidium bromide. Pilot experiments showed that this concentration completely inhibits the synthesis of mitochondrial RNA in P3K cells, with no interference in the nuclear synthesis of ribosomal RNA. After 30 min, [<sup>3</sup>H]uridine was added as specified in the figures. Throughout this labeling period, the ethidium bromide was still present. The methods for cell disruption, preparation of cytoplas-

mic extracts, separation of free and membrane-bound ribosomes, and determination of radioactivity have been previously reported in detail (12).

### *Analysis of Ribosome Distribution by Sucrose Gradient Centrifugation*

Two types of sucrose gradients were used. 15–55% linear sucrose gradients in TK<sub>80</sub>M medium (0.05 M Tris-HCl, pH 7.4, 0.08 M KCl, 0.005 MgCl<sub>2</sub>) were used in the conditions previously reported (12), to analyze the ribosomal distribution of the free and membrane-bound ribosomal fractions. For a more accurate analysis of the 60S and 40S subunits, 15–30% sucrose gradients layered over a 4-ml cushion of 69% sucrose (all sucrose dissolved in TK<sub>80</sub>M) were used. Centrifugation was carried out in a Spinco SW 27 (Beckman Instruments, Spinco Div., Palo Alto, Calif.) rotor at 27,000 rpm for 8.5 h. Under these conditions, polyribosomes accumulate in the bottom sucrose layer while 80S ribosomes and ribosomal subunits are well separated on the gradient.

### *Analysis of RNA by Sucrose Gradient Sedimentation*

Extraction and sucrose gradient sedimentation analysis of RNA was performed according to Kolakofsky and Bruschi (10). Sodium dodecyl sulfate (SDS, final concentration 1%) and sodium acetate (pH 5.3, final concentration 0.2 M) were added to the ribosomal fractions. The RNA was precipitated with 2 vol ethanol at –20°C, recovered by centrifugation, dissolved in 0.5% sodium dodecyl sulfate, and sedimented on 5–23% linear sucrose gradients containing 0.1 M LiCl, 0.01 M Tris-HCl, pH 7.4, 0.004 M ethylenediaminetetraacetic acid (EDTA), and 0.1% SDS for 2.5 h (7.5°C) at 55,000 rpm in a Spinco SW 56 rotor.

### *Materials*

[5-<sup>3</sup>H]Uridine, 25 Ci/mmol; [2-<sup>14</sup>C]uridine, 54 mCi/mmol, were purchased from Amersham Radiochemical Centre. Ethidium bromide was supplied by Sigma Chemical Co., St. Louis, Mo., cycloheximide by Serva, and Brij 58 by Atlas Chemical Co. All other chemicals were of analytical grade.

## RESULTS

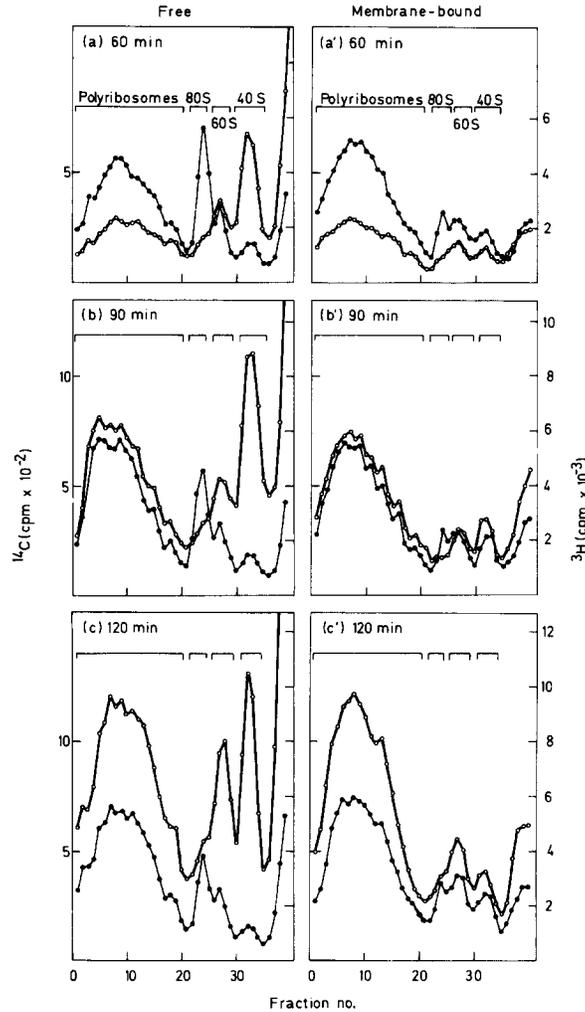
P3K cells were incubated for 24 h in the presence of [<sup>14</sup>C]uridine to obtain uniform labeling of both free and membrane-bound ribosomes, then pulsed with [<sup>3</sup>H]uridine for various periods of time, in the presence of 1 μM ethidium bromide, a concentration which inhibits the synthesis of mitochondrial RNA (see Materials and Methods). After homogenization of the cells, the postnuclear supernate (cytoplasmic extract) was fractionated to yield free ribosomal and membrane fractions on a discontinuous sucrose density gradient containing 0.15 M

KCl, i.e. in conditions which have been found to prevent adsorption of free ribosome particles to the membranes (12). The ribosomes and the RNA of these fractions were analyzed as follows: (a) the distribution of the radioactive ribosomal particles (i.e. polyribosomes, monoribosomes, and native subunits) was determined by sedimentation of the fractions on 15–55% sucrose density gradients (after treatment of the membrane fraction with detergent to release the membrane-bound particles); (b) the RNA of each of the four types of ribosomal particles separated on these 15–55% sucrose gradients (i.e. polyribosomes, monoribosomes, 60S, and 40S subunits) was extracted and analyzed on 5–23% sucrose gradients; (c) finally, the total RNA present in the free ribosomal and membrane fractions was extracted and analyzed on 5–23% sucrose gradients.

### *Appearance of Newly Made Ribosomal Particles in the Free Ribosomal and Membrane Fractions*

The sedimentation pattern in 15–55% sucrose density gradients of the [<sup>14</sup>C]uridine uniformly labeled ribosomal particles present in the free ribosomes and in the detergent-treated membrane fraction (Fig. 1) has already been described (12). The sedimentation pattern of the newly made ribosomal particles appearing in these fractions after 60, 90, and 120 min of pulse with [<sup>3</sup>H]uridine showed, by comparison, the following features. (a) Newly made ribosomal particles have already entered both free and membrane-bound polyribosomes at 60 min, and accumulate in these two types of polyribosomes at a very similar rate, as judged by their parallel increase in specific radioactivity (<sup>3</sup>H-to-<sup>14</sup>C ratio) (see also below, Fig. 4). (b) In the free ribosome fraction, a high proportion of <sup>3</sup>H-radioactivity sediments in the region of the ribosomal native subunits, with more radioactivity in the 40S than in the 60S subunits at all time points studied. (c) In the membrane fraction, [<sup>3</sup>H]uridine is also detected in the 60S and 40S native subunits region. In these pulse-labeling experiments, the ratio of the <sup>3</sup>H-to-<sup>14</sup>C]uridine radioactivity in native subunits to that in polyribosomes was always higher in the free than in the membrane-bound populations.

The presence in the membrane fraction of a peak of [<sup>3</sup>H]uridine with a sedimentation coefficient of 40S was unexpected. In contrast to the 40S peak of <sup>14</sup>C-radioactivity (see below), this material cannot correspond to newly made mitochondrial



**FIGURE 1** Appearance of newly synthesized ribosomal particles in free ribosomal and membrane fractions.  $9.0 \times 10^7$  P3K cells were labeled for 24 h with  $0.04 \mu\text{Ci/ml}$  [ $^{14}\text{C}$ ]uridine (final cell density was  $5.0 \times 10^6$  cells/ml). Thereafter, the cells were sedimented, resuspended at the density of  $2.0 \times 10^6$  cells/ml in fresh, prewarmed Dulbecco-modified Eagle's medium supplemented with 10% horse serum and 20 mM HEPES, and incubated at  $37^\circ\text{C}$  in an agitated waterbath. Ethidium bromide ( $1 \mu\text{mole/ml}$ ) was added for 30 min, and subsequently, cells were labeled with  $25 \mu\text{Ci/ml}$  [ $^3\text{H}$ ]uridine.  $3.0 \times 10^7$  cells were harvested at 60, 90, and 120 min after the addition of the label. Cytoplasmic extracts were prepared from which free ribosomal and membrane fractions were separated by sedimentation on discontinuous sucrose density gradients containing 0.15 M KCl (see Materials and Methods). Samples of the free and membrane-bound fractions (the latter treated with 0.5% sodium deoxycholate and 0.5% Brij 58) were layered on 15–55%  $\text{TK}_{80}\text{M}$  sucrose density gradients in a Spinco SW 27 rotor and were centrifuged for 8.5 h at 23,000 rpm. Gradients were collected in 40 fractions, and radioactivity from 0.4-ml aliquots was measured. —●—●—,  $^{14}\text{C}$ -Labeled RNA for 24 h; —○—○—,  $^3\text{H}$ -labeled RNA after 60 min (a, a'), 90 min (b, b'), and 120 min (c, c') of incubation in the presence of [ $^3\text{H}$ ]uridine. (a–c) Free ribosomes; (a'–c') membrane-bound ribosomes. The components corresponding to the portions of the sucrose gradient pattern indicated by arrows were utilized for RNA analysis and determination of the specific activity of their 18S and 28S ribosomal RNA (see Fig. 4).

ribosomal subunits, since the synthesis of mitochondrial ribosomes was completely inhibited by the presence of ethidium bromide during the [ $^3\text{H}$ ]uridine pulse (18). There is no binding site known on the microsomal membranes of 40S subunits, except by association to a 60S subunit (16). It seemed possible that the native 40S subunits in the membrane fraction represented a slight degree of artefactual contamination of the membrane fraction by free 40S subunits; free 40S subunits indeed contain a high amount of  $^3\text{H}$ -radioactivity (Fig. 1). It has previously been shown (12) that a small amount of ribosomal subunits can become artefactually associated with the membranes during the fractionation procedure, and that this phenomenon, which is more pronounced for the 60S free subunits, can be completely prevented by increasing to 0.15 M the concentration of KCl in the discontinuous sucrose density

gradient separating the free ribosomes from the membrane fraction.

A comparison was therefore made of the ribosomal particle sedimentation profiles obtained from the free ribosomal and membrane fractions separated on a discontinuous sucrose density gradient containing either 0.025 M or 0.15 M KCl. In the membrane fraction, the amount of [ $^3\text{H}$ ]uridine radioactivity sedimenting as 60S subunit was lower (and slightly higher in the free ribosomal fraction) when the separation was performed in the presence of 0.15 M KCl; in the 40S region, however, the  $^3\text{H}$ -radioactivity of the membrane fraction was unchanged (Fig. 2). Moreover, when the membrane fraction was centrifuged a second time on a discontinuous sucrose gradient, there was no release of [ $^3\text{H}$ ]uridine-labeled 40S subunits or of any type of ribosomal particle (results not shown). It seemed likely, therefore, that native 40S subunits

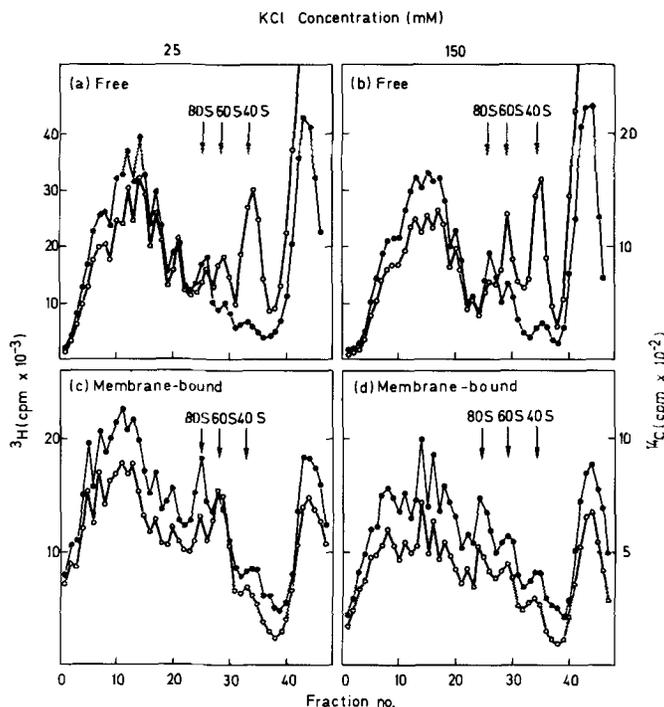


FIGURE 2 Effect of KCl concentration on the distribution of ribosomal particles during separation of free ribosomal and membrane fractions.  $3.0 \times 10^7$  P3K cells were uniformly labeled with  $0.05 \mu\text{Ci/ml}$  [ $^{14}\text{C}$ ]uridine, then pulse labeled for 90 min with  $30 \mu\text{Ci/ml}$  [ $^3\text{H}$ ]uridine in the presence of ethidium bromide ( $1 \mu\text{mol/ml}$ ) as described in Fig. 1. A cytoplasmic extract was prepared and divided into two parts. The KCl concentration was readjusted to 0.025 M in one half and to 0.15 M in the other. A free ribosomal fraction and a membrane fraction were separated by sedimentation on discontinuous sucrose density gradients containing the same KCl concentration as the sample and, thereafter, analyzed on 15–55% sucrose density gradients in  $\text{TK}_{80}\text{M}$ . —●—●—,  $^{14}\text{C}$ -labeled RNA; —○—○—,  $^3\text{H}$ -labeled RNA; (a, b) free ribosomes; (c, d) membrane-bound ribosomes separated in presence of (a, c) 0.025 M or (b, d) 0.15 M KCl.

were probably truly bound to membranes in the cell, and did not result from contamination of the membranes by native 40S subunits free in the cytoplasm.

### Sucrose Gradient Analysis of the RNA of Ribosomes Obtained from the Free Ribosomal and Membrane Fractions

These experiments were performed to explore two main points: (a) the nature of the radioactive RNA present in the ribosomal particles sedimenting in the region of the native subunits, in both the free ribosomal and the membrane fractions (Fig. 3); (b) the specific radioactivity of the newly made 28S and 18S RNA present in the different ribosomal particles observed after 60, 90, and 120 min of pulse with [<sup>3</sup>H]uridine in the gradients shown in Fig. 1.

The <sup>14</sup>C- and <sup>3</sup>H-labeled native 60S and 40S subunits of free ribosomes were found to contain exclusively 28S and 18S RNA (Fig. 3 a, b).

The presence of some 18S RNA in the native 60S subunit region and of some 28S RNA in the native 40S subunit region reflects some degree of overlap between the native subunits and between the 80S ribosomes and the 60S subunits. In the membrane fraction, the 60S native subunit region was found to contain <sup>14</sup>C as well as <sup>3</sup>H 28S RNA (Fig. 3 c). In the 40S subunit region, the [<sup>3</sup>H]RNA sedimented mainly as an 18S peak and also contained RNA molecules with smaller, heterogeneous sedimentation coefficients; on the other hand, the [<sup>14</sup>C]RNA was lighter than 18S, with two discrete peaks corresponding to 16S and 12S RNA (Fig. 3 d), i.e. the two RNA species found in mitochondrial ribosomes (1). Thus, in uniformly labeled cells cultured in the absence of ethidium

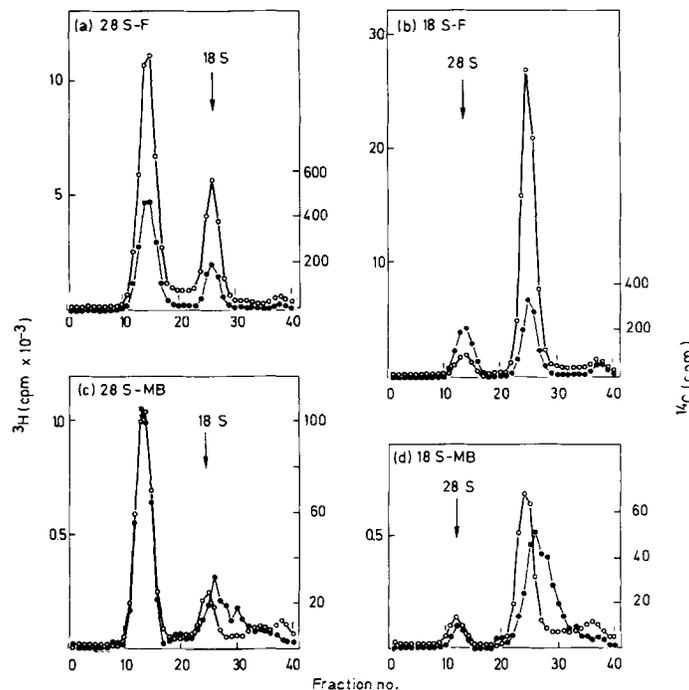


FIGURE 3 Sedimentation pattern of the RNA contained in the free and the membrane-bound native 60S and 40S ribosomal subunits.  $3.0 \times 10^7$  P3K cells were uniformly labeled with  $0.05 \mu\text{Ci/ml}$  [<sup>14</sup>C]uridine and pulse labeled with  $67 \mu\text{Ci/ml}$  [<sup>3</sup>H]uridine for 2 h in the presence of ethidium bromide ( $1 \mu\text{mol/ml}$ ). A free ribosomal and a membrane fraction were separated on a discontinuous sucrose gradient containing  $0.15 \text{ M KCl}$ , and native 60S and 40S ribosomal subunits were prepared and their RNA was analyzed as described in Materials and Methods. —●—●—, <sup>14</sup>C-labeled RNA for 24 h; —○—○—, <sup>3</sup>H-labeled RNA for 2 h. Native ribosomal subunits: (a) free 60S; (b) free 40S; (c) membrane-bound 60S and (d) membrane-bound 40S.

bromide, the ribosomal particles from the membrane fraction sedimenting in the 40S subunit region are mostly of mitochondrial origin. What is particularly interesting is that this fraction also contains a significant amount of newly made, nonmitochondrial native 40S subunits, as well as some newly made heterogeneous RNA, which may well be mRNA. These newly made native 40S subunits must have a relatively high rate of turnover, since they do not accumulate in large enough amounts to be clearly detectable in uniformly labeled cells.

To evaluate the rapidity of entry and accumulation of 28S and 18S RNA in the different ribosomal particles shown on Fig. 1, the RNA extracted from each was analyzed by sucrose gradient sedimentation, and the specific radioactivity ( $^3\text{H}$ -to- $^{14}\text{C}$  ratio) of the 18S and 28S RNA were determined. Fig. 4 shows such data after 60–120 min pulse with [ $^3\text{H}$ ]uridine.

The following features can be noted: (a) the specific radioactivities of 18S and 28S RNA in polyribosomes are at all times the same, regardless of their free or membrane-bound origin. The same is true for monoribosomes; (b) in the polyribosomes and the monoribosomes, the specific radioactivity of the 18S RNA is at all times slightly higher than that of the 28S RNA; (c) the specific radioactivities of the 18S and 28S RNA present in the polyribosomes are always higher than those of the corresponding RNA species present in the monoribosomes regardless of source; (d) in the case of native 60S subunits, the specific radioactivity of the 28S RNA is higher in the free than in the membrane-bound subunits and, when compared to the specific radioactivity of the polyribosomes, is higher in the free native subunits than in the polyribosomes, but slightly lower in the case of the membrane-bound native subunits; (e) in the native free 40S subunits, the specific radioactivity of the 18S RNA is much higher than that of the polyribosomal 18S RNA and is increased considerably up to 120 min. The possibility was considered that the free native 40S subunits had such a high specific radioactivity because part of them were initiation complexes carrying newly made mRNA. The sharp, symmetrical nature of the peak of  $^3\text{H}$ -labeled 18S RNA extracted from these subunits (Fig. 3 b) was, however, not in favor of this possibility. In the case of the membrane-bound 40S subunits, it is difficult to calculate the specific radioactivity of their 18S RNA with accuracy since, as already mentioned (Fig. 3 d), the amount

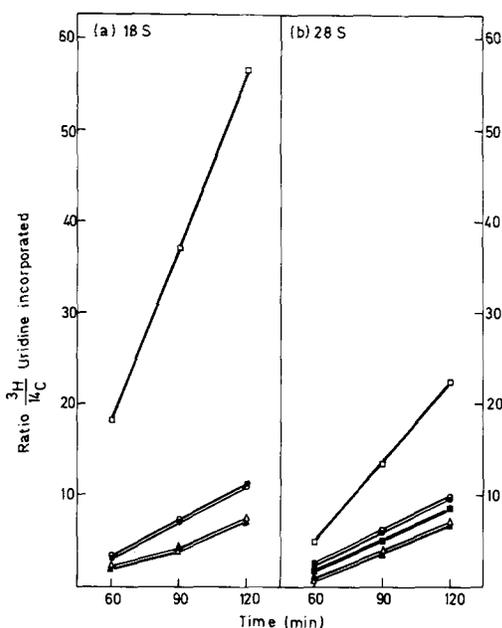


FIGURE 4 Rate of entry of newly made 18S and 28S ribosomal RNA into polyribosomes, 80S monoribosomes, and native 60S and 40S ribosomal subunits. The specific activity ( $^3\text{H}$  to  $^{14}\text{C}$  ratio) of the 18S and 28S ribosomal RNA was determined for the fractions defined in Fig. 1. The specific activity of the 18S ribosomal RNA, present in the membrane-bound 40S ribosomal subunits, was not calculated since it is difficult to resolve  $^{14}\text{C}$ -labeled 18S RNA from the  $^{14}\text{C}$ -labeled mitochondrial RNA as shown in Fig. 3 d. Specific activity of (a) 18S ribosomal RNA and (b) 28S ribosomal RNA in: —○—○—, free polyribosomes; —●—●—, membrane-bound polyribosomes; —△—△—, free monoribosomes; —▲—▲—, membrane-bound monoribosomes; —□—□—, free native ribosomal subunits and —■—■—, membrane-bound native ribosomal subunits.

of  $^{14}\text{C}$ -uniformly labeled 18S RNA present in these particles is very low and difficult to determine because of the presence of  $^{14}\text{C}$  16S mitochondrial RNA. Nevertheless, it is clear that these native 40S bound subunits have a high specific radioactivity, probably in the range of that of the native 40S free subunits.

#### Sucrose Gradient Analysis of the Total RNA Extracted from the Free Ribosomal and Membrane Fractions

These experiments analyzed by sucrose density gradient sedimentation the totality of the RNA extracted from the free ribosomal and membrane

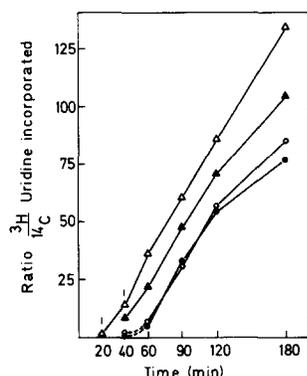


FIGURE 5 Rate of entry of newly made 18S and 28S ribosomal RNA into free ribosomal membrane fractions.  $1.8 \times 10^8$  P3K cells, labeled for 24 h with  $0.01 \mu\text{Ci/ml}$  [ $^{14}\text{C}$ ]uridine, were pulse labeled with  $6 \mu\text{Ci/ml}$  [ $^3\text{H}$ ]uridine in the presence of ethidium bromide ( $1 \mu\text{mol/ml}$ ) as described in Fig. 1. At various intervals after initiation of the pulse, aliquots containing  $3 \times 10^7$  P3K cells were withdrawn and RNA from the separated free ribosomal and membrane fractions was extracted and analyzed as described (see Materials and Methods). The specific activity ( $^3\text{H}$ -to- $^{14}\text{C}$  ratio) of the 18S and 28S ribosomal RNA was calculated from the radioactivity present in the sucrose LiCl-SDS density gradient fractions containing these RNA species. Specific activity of: —△—△—, free, and —▲—▲—, membrane-bound 18S ribosomal RNA; —○—○—, free, and —●—●—, membrane-bound 28S ribosomal RNA.

fractions of cells uniformly labeled with [ $^{14}\text{H}$ ]uridine and pulsed with [ $^3\text{H}$ ]uridine for 20–180 min in the presence of ethidium bromide. The distribution of the  $^3\text{H}$ -radioactivity between the free ribosomal and the membrane fractions was always closely comparable to that of the  $^{14}\text{C}$ -radioactivity (17–20% of the total postnuclear radioactivity being in the membrane fractions), except after 20 min of [ $^3\text{H}$ ]uridine pulse, (where about 10% only of the postnuclear  $^3\text{H}$ -radioactivity was membrane bound).

The RNA of each fraction showed three peaks of  $^3\text{H}$ - and  $^{14}\text{C}$ -radioactivity with sedimentation values of 28S, 18S, and 4S, except in the 20-min [ $^3\text{H}$ ]uridine pulse, where the only distinct  $^3\text{H}$  peak was at 4S in both fractions. The plots of the specific radioactivity ( $^3\text{H}$ -to- $^{14}\text{C}$ ) of the 18S and 28S peaks after different times of pulse (Fig. 5) show the following features: (a) the newly made 18S RNA enters the cytoplasm before the newly made 28S RNA and has at all times a higher specific radioactivity; (b) the specific radioactivity

of the newly made 18S RNA is always slightly higher in the free ribosomal than in the membrane fraction. This probably results from the presence of a larger pool of native 40S subunits with high specific activity in the free ribosomal fraction (Figs. 1 and 4); (c) the specific radioactivity of the newly made 28S RNA is closely comparable in the free ribosomal and membrane fractions. These results are in complete agreement with the observations made in the experiments analyzing the specific radioactivities of the different ribosomal particles.

## DISCUSSION

It is known that the newly made 40S subunits enter the cytoplasm earlier than the 60S subunits (3, 5–9, 14), and indeed, we found that the specific radioactivity of the 18S RNA in the cytoplasmic pool of free native subunits was higher than that of the 28S RNA at all times explored. Apart from the membrane-bound native 40S subunits, which will be discussed later, the first ribosomal structures into which the newly made subunits entered were the polyribosomes, both free and membrane bound. It is striking that, at all times, the specific radioactivities of the 18S and 28S RNAs are very similar in the free and the membrane-bound polyribosomes, the specific radioactivity of the 18S RNA being always slightly higher than that of the 28S RNA. This is consistent with other observations (2, 11, 17) and indicates that the newly made subunits enter the membrane-bound and free polyribosomes at the same rate, the 40S subunits appearing slightly earlier than the 60S subunits. There was no evidence for a heterogeneous labeling kinetics within either population of polyribosomes.

Evaluation of the specific radioactivity of the membrane-bound native subunits requires especially well-controlled conditions of cell fractionation, since even a small contamination of the membranes by free native subunits can easily lead to erroneous conclusions, because of the high specific radioactivity of these latter particles. We previously showed that 60S subunits are especially prone to trapping in or binding to the membranes during the fractionation procedure (12). The ribosomal content of the membrane fraction probably does not reflect the conditions existing within the cell, unless the fractionation is performed in medium containing 0.15 M KCl. Contamination of the membrane fraction by native 60S subunits of

high specific radioactivity might become especially critical when the centrifugation procedure does not provide a sharp discrimination between the membrane and the free ribosome fractions (see, for instance, discussion of reference 45 in the preceding paper) (12). Our finding that membrane-bound native 60S subunits have at all times a slightly but significantly lower specific radioactivity than 60S subunits present in polyribosomes indicates that they cannot be precursors destined to enter the membrane-bound polyribosomes, as was suggested by Baglioni et al. (2) on the basis of fractionation procedures which may easily lead, for the reasons discussed above and elsewhere (12), to inaccurate separation from the membrane fraction of the free 60S native subunits of high specific radioactivity. It is more likely that these native subunits result from dissociation of ribosomes present in membrane polyribosomes after chain termination. Since these 60S particles do not accumulate as such, they probably leave the membranes shortly after dissociation from their 40S subunits. There is indeed no evidence that they are reutilized before their release from the membranes. If there were such reutilization, the specific radioactivity of the 60S of these bound polyribosomes would not increase in parallel with the 40S, as was observed. Thus, the formation of active monomers must occur at a distance from the membrane, as will be more extensively discussed in the next paper (13).

The monoribosomes, both free and membrane bound, are the ribosomal particles with the lowest specific radioactivities in their subunits. The slow kinetics of labeling of the free monoribosomes has been noticed in a variety of cells (3, 6, 7, 9, 14). There is evidence that they represent the product of a side reaction, and do not participate in the ribosomal cycle which accompanies protein synthesis (4). It is likely that the same situation applies to the bound monoribosomes, which seem to carry few or no nascent polypeptide chains.

The most unexpected finding of the present experiments was the detection in the membrane fraction of labeled native 40S subunits after short exposure of cells to [<sup>3</sup>H]uridine. These subunits were labeled in the presence of ethidium bromide and, therefore, are not of mitochondrial origin. They contain 18S RNA and some heterogeneous RNA, probably mRNA. In contrast, the cosedimenting <sup>14</sup>C-radioactivity, synthesized during a uniform labeling of RNA in the absence of ethidium bromide, contains mainly mitochondrial 16S and 12S RNA. The possibility that these newly

made native 40S subunits resulted from a contamination of the membrane fraction by free subunits was rendered unlikely by cell fractionation at various KCl concentrations (see Results and reference 12). The specific radioactivity of these bound native 40S subunits is certainly high, but could not be calculated with accuracy, since no 18S peak of <sup>14</sup>C-radioactivity is clearly detectable in this fraction, due to the overlap of <sup>14</sup>C 16S mitochondrial RNA. Furthermore, the newly made native 40S subunits do not accumulate on the membranes, as is the case for the newly made polyribosomal 40S particles, since after 60 min of labeling they represent 15–20% of the total <sup>3</sup>H-labeled membrane-bound 40S subunits, and after 120 min, only 5–10%. Experiments reported in the following paper (13) are consistent with the possibility that these particles are initiation complexes bound to the membranes by mRNA molecules, which have just reached the membrane fraction and not yet formed polyribosomes. The lack of accumulation, or high turnover, of these particles might reflect the rapid transformation of initiation complexes into polyribosomes (13).

In summary, these kinetic experiments indicate that, from the pool of newly made subunits free in the cytoplasm, both 40S and 60S subunits enter at comparable rates free and membrane-bound polyribosomes. This is compatible with the idea that the main factor directing the entry of new subunits into the polyribosomes, whether free or membrane bound, is the possibility of forming new initiation complexes at the 5' end of the mRNA molecules linking the ribosomes into polyribosomes. Upon chain completion, the membrane-bound ribosomes appear to dissociate, with 60S subunits remaining on the membranes for a short period.

Finally, it must be pointed out that the increase in specific radioactivities of the native subunits free in the cytoplasm showed a steeper slope than those of the polyribosomal subunits; this was especially marked for the native free 40S subunits, and did not appear to result from the association of newly made mRNA with these particles. Thus, the native subunits may require secondary maturation steps before becoming eligible to enter the polyribosomes, as suggested by Perry and Kelley (15). Alternatively, their entry into polyribosomes may depend upon the availability of mRNA, or upon their rate of diffusion through the cytoplasm.

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