MEMBRANE-BOUND RIBOSOMES OF MYELOMA CELLS

I. Preparation of Free and Membrane-Bound

Ribosomal Fractions. Assessment of

the Methods and Properties of the Ribosomes

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ABSTRACT

A cell fractionation procedure is described which allowed, by use of MOPC 21 (P3K) mouse plasmocytoma cells in culture, the separation of the cytoplasmic free and membrane-bound ribosomes in fractions devoid of mutual cross-contamination, and in which the polyribosomal structure was entirely preserved. This was achieved by sedimentation on a discontinuous sucrose density gradient in which the two ribosome populations migrate in opposite directions. A variety of controls (electron microscopy, labeling of membrane lipids, further repurification of the isolated fractions) provided no evidence of cross-contamination of these populations. However, when an excess of free 60S or 40S subunits, labeled with a different isotope, was added to the cytoplasmic extract before fractionation, the possibility of a small amount of trapping and/or adsorption of free ribosomal particles by the membrane fraction was detected, especially in the case of the 60S subunits; this could be entirely prevented by the use of sucrose gradients containing 0.15 M KCl.

EDTA treatment of the membrane fraction detached almost all the 40S subunits, and about 70% of the 60S subunits. 0.5 M KCl detached only 10% of the ribosomal particles, which consist of the native 60S subunits and the monoribosomes, i.e. the bound particles inactive in protein synthesis. Analysis in CsCl buoyant density gradients of the free and membrane-bound polyribosomes and of their derived 60S and 40S ribosomal subunits showed that the free and membrane-bound ribosomal particles have similar densities.

In mammalian cells, ribosomes are either attached to the membranes of the endoplasmic reticulum (ER) or free in the cytoplasm (31). Cells synthesizing large amounts of proteins for secretion contain a high proportion of membrane-bound ribosomes and it has been shown that the proteins intended for secretion are synthesized mainly on membrane-bound polyribosomes, whereas proteins remaining in the cell sap are synthesized on free polyribosomes (13, 15, 36, 44). Membrane-bound ribosomes are also found in smaller amounts in most types of cells, in the absence of known secretory function (4, 38), and they may in this case be involved in the synthesis of some membrane (11, 12) and mitochondrial proteins (6). The cellular mechanisms which regulate this segregation of ribosome function are unclear.

The free and membrane-bound ribosomes can

be separated by cell fractionation. During this procedure, the rough endoplasmic reticulum is fragmented into vesicles bearing ribosomes attached to their outer surface, which can be isolated as rough microsomes (32, 33). In vitro studies on microsomes, combined with in vivo observations, have shown that the binding of the ribosome is mediated through the large ribosomal subunits (41) and that during protein synthesis the nascent polypeptide chain growing on the large subunits of the bound ribosome is vectorially discharged into the cisternae of the endoplasmic reticulum (35, 37). The binding of the ribosomes to the ER membranes does not require the presence of nascent polypeptide chains but becomes tighter when the nascent chains, growing on the ribosomes, enter into close contact with the membranes to reach the cisternal space of the ER (1, 40).

The mechanisms by which ribosomes bind to the membranes and assemble into membrane-bound polyribosomes active in protein synthesis are not clearly understood. Three mechanisms have been proposed: (a) large ribosomal subunits bind directly to the membranes, even in the absence of protein synthesis, to form stable structures which can subsequently bind free mRNA-40S subunit complexes (5). In this case, of course, these mRNA-40S complexes should be able to discriminate between free and membrane-bound 60S subunits, to account for the relative selectivity in the type of proteins synthesized, as discussed above; (b) the formation of membrane-bound polyribosomes depends upon the presence of specific mRNA associated with the membranes (20, 21); (c) the recognition mechanism for membrane binding may be provided by the N-terminal segment of the nascent polypeptide chains present on ribosomes initially free in the cytoplasm (14, 28).

There is evidence that membrane-bound ribosomes are homogeneous and similar to free ribosomes in terms of structure and metabolic properties (23, 43), suggesting that a ribosomal cycle exists which involves the subunit exchange between the free and the bound particles (10). On the other hand, evidence also exists that in some types of cells two classes of bound ribosomes can be found, differing in their type of attachment to the membranes (38) and in their structure (39). Some of the membrane-bound ribosomes are released from the membranes after mild treatment of the microsomes with RNase (21, 38) or after in vivo inhibition of protein synthesis by puromycin (38),

NaF (7), or deprivation of amino acids (20). This has led to the concept that there exist two classes of membrane-bound ribosomes, "tightly" and "loosely" bound. This situation, however, does not seem to apply to liver cells, where in vitro RNase or puromycin treatment does not lead to ribosome detachment from the rough microsomes (1, 8, 10).

In the present work, we have studied the free and membrane-bound ribosomes of P3K cells, which are mouse myeloma cells maintained in tissue culture. Myeloma cells secrete large amounts of immunoglobulin molecules, which are made principally on membrane-bound ribosomes (22). A cell fractionation procedure has been developed which allows the analysis of virtually all the free and membrane-bound ribosomes present in the cytoplasm, under conditions where they are devoid of reciprocal contamination. This first paper studies the sensitivity of the binding of membrane-bound ribosomes to various ionic conditions, as well as the buoyant density of the ribosomal particles attached to the membranes compared to those free in the cytoplasm. The following papers (26, 27) will study the kinetics of appearance of newly made ribosomal particles in the membrane fraction and in the free ribosomal fraction, the nature of the membrane-bound ribosomes which are released after mild RNase treatment, and the effect of various inhibitors of protein synthesis on the association of ribosomal particles with the ER membranes.

MATERIALS AND METHODS

Cells and Conditions of Radioactive Labeling

The MOPC 21 (P3K) mouse myeloma lines (16) were a gift of Dr. Zaguri, University of Reims, France. The cells were grown in suspension culture in Dulbecco-modified Eagle's medium supplemented with heat-inactivated 10% horse serum. Cells were kept in exponential growth with a generation time of approximately 16 h. For the analysis of free and membrane-bound ribosomes, cells were obtained from cultures not exceeding 5.0×10^5 cells/ml in order to ensure maximum protein synthetic activity.

For labeling periods of one cell cycle or greater (20–24 h), cells were suspended at a density of 1.5 to 2.0×10^5 cells/ml in the same medium and labeled by the addition of [^{14}C]uridine. For labeling periods up to 3 h, cells were concentrated by centrifugation, resuspended at a density of 2.0×10^6 cells/ml in the same medium containing additional 20 mM HEPES, and labeled according to the protocol of the experiment.

Preparation of a Cytoplasmic Supernatant Fraction and Separation of Free and Membrane-bound Ribosomes

After labeling, P3K cells were rapidly chilled by the addition of ice-cold Earle's saline solution, harvested by centrifugation, washed once in Earle's saline solution, and resuspended at a concentration of 10^8 cells/ml in ice-cold hypotonic buffer medium RSB (0.01 M-KCl, 0.0015 M-MgCl$_2$, 0.01 M Tris-HCl, pH 7.4) pretreated with 0.1% diethylpyrocarbonate. The following operations were carried out at 4°C. Cells were allowed to swell for 5 min and were then ruptured mechanically with 10 strokes of a tight-fitting (B) Dounce glass homogenizer (Kontes Glass Co., Vineland, N.J.); complete cell breakage was ascertained by phase-contrast microscopy. The homogenate was centrifuged at 1,000 g for 45 s to sediment the nuclei, the supernate retrieved, and the nuclear pellet resuspended with RSB in half the volume of the homogenate and centrifuged as described above. The two postnuclear supernates were pooled, and this fraction, called the "cytoplasmic extract," was further used for the separation of free and membrane-bound ribosomes. The washed nuclear pellet was resuspended in an RSB solution containing 1% Nonidet P40 (Shell Oil Co.) (30), and sedimented as described above to yield a "detergent-treated nuclear pellet." The amount of radioactivity present in the supernate of the detergent-treated pellet was routinely measured and compared to the radioactivity in the cytoplasmic extract.

For the separation of the free ribosomal and of the membrane fractions, 0.35 ml of the cytoplasmic extract was mixed with 1.85 ml of 2.5 M sucrose made up in D$_2$O-TK$_x$M buffer (0.05 M Tris-HCl, pH 7.4, 0.025 to 0.15 M KCl, x being the KCl concentration indicated in mM, which remained constant for all the sucrose solutions of the same gradient, 0.005 M MgCl$_2$), and layered over 1.5 ml of 2.5 M sucrose in D$_2$O-TK$_x$M. (The use of D$_2$O, allowing to reach, without significant increase in viscosity, a density higher than the highest density reported for any membrane-bound structure, was found to be convenient, although not absolutely essential). Two layers of sucrose solutions in H$_2$O-TK$_x$M were successively added, first 6.0 ml of 2.05 M sucrose, then 2.3 ml of 1.3 M sucrose. These gradients were centrifuged for 5 h (4°C) in a Spinco SW 41 rotor at 25,000 rpm. The bottom of the tube was punctured and 20-drop fractions were collected into glass tubes. Total cold 10% trichloroacetic acid-precipitable radioactivity was determined either on the total fractions or on 50-µl aliquots.

For the analysis of the sedimentation characteristics of the ribosomal particles contained in the free ribosomal and membrane fractions, 15–55% (wt/vol) linear sucrose density gradients in TK$_{80}$M were used. In order to dissolve the membranes, 0.5% sodium deoxycholate and 0.5% Brij 58 were added to the membrane fraction before loading on the 15–55% sucrose gradient. Centrifugation was carried out for 8.5 h at 4°C in a Spinco SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 23,000 rpm. The gradients were fractionated with a peristaltic pump (Vario-Perpex, Werner Meyer, Luzern, Switzerland). Fractions, usually 0.8 ml, were collected and treated with an equal volume of cold 20% trichloroacetic acid with 0.1 mg RNA as carrier. The precipitates were collected by filtration on glass fiber filters GF/C (Whatman), washed twice with 5% ice-cold trichloroacetic acid, once with ethanol, and dried.

Preparation of Ribosomal Subunits

10^8 P3K cells labeled for 24 h with [^{14}C]uridine (0.05 µCi/ml) were concentrated to a density of 10^7 cells/ml. 4.5×10^{-4} M puromycin was added and the cells were incubated for 10 min, then centrifuged and resuspended in 1.0 ml RSB. A cytoplasmic extract was prepared as indicated above and KCl was added up to a final concentration of 0.5 M. The ribosomal subunits were then isolated as previously described (25). When subunits were to be prepared from the isolated free and membrane fractions, [^{14}C]uridine-labeled ribosome fractions were diluted to a final concentration of 15% sucrose (wt/vol) with TK$_{80}$M buffer and were preincubated with 10^{-3} M puromycin and 0.4×10^{-3} M GTP for 15 min at 37°C. At the end of the incubation, KCl was added to a final concentration of 0.5 M and the membrane-bound ribosomes were released from the membranes by addition of sodium deoxycholate and Brij 58 at final concentrations of 0.5%. Samples of both ribosome fractions were centrifuged as described above except that the Tris-HCl concentration was reduced to 10 mM for experiments in which particles were later fixed with glutaraldehyde.

Analysis of the Density of Ribosomal Particles on Cesium Chloride Gradients

Appropriate samples from the sucrose gradient fractions (buffered at pH 7.4 with Tris-HCl or with cacodylate) were fixed with glutaraldehyde and sedimented on CsCl density gradients according to the method of Huang and Baltimore (17). 0.8-ml samples were fixed with 0.2 ml of 25% glutaraldehyde adjusted to pH 7.4 with 1 M NaHCO$_3$ just before fixation. The samples were layered onto preformed CsCl gradients (1.35–1.70 g/cm³) and centrifuged for 18 h in a Spinco SW 50.1 rotor at 35,000 rpm. The bottoms of the tubes were punctured and 0.1-ml fractions collected. Densities were obtained by determinations of refractive index. Each fraction was further plated on glass fiber filters GF/C (Whatman). The filters were dried, batch washed twice with 5% ice-cold trichloroacetic acid, washed once with ethanol, and dried for counting.

Radioactive Counting

Dried glass fiber filters GF/C (Whatman) were placed in plastic scintillation vials, and 10 ml of 2,5-diphenylox-

azole (PPO), dimethyl 1,4-bis[2-(5-phenyloxazolyl)]ben-zene (POPOP) and toluene base scintillation fluid were added. The radioactivity was measured in a Beckman liquid scintillation spectrometer and corrected for back-ground and overlap of ^{14}C and ^{3}H.

Fixation and Staining for Electron Microscopy

The free ribosome and membrane fractions obtained from the discontinuous sucrose density gradient were diluted to approximately 0.3 M sucrose and centrifuged in a Spinco SW 50.1 rotor for 5 h (4°C) at 40,000 rpm. These pellets, as well as nuclear pellets, were fixed by layering 4 ml of Karnovsky's fixative (18) diluted 1:1 with distilled H_2O on top of the pellet for 2 h at 4°C, postfixed in 2% OsO_4 for 1.5 h, and embedded in Epon. Ultrathin sections at different levels of the pellet were stained with uranyl acetate and lead citrate and examined with a Philips EM 300 electron microscope.

Materials

Commercial sources of chemicals were as follows: Amersham Radiochemical Centre, [9, 10-^{3}H]oleic acid, 2 Ci/mmol, [5-^{3}H]uridine, 25 Ci/mmol, [2-^{14}C]uridine, 54 mCi/mmol; Worthington Biochemicals Corp., Freehold, N.J., bovine pancreatic ribonuclease A; Sigma Chemical Co., St. Louis, Mo., GTP and ethidium bromide; Serva Co., Ltd., Heidelberg, W. Germany, cycloheximide; ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio, puro-mycin; Atlas Chemical Co., Brij 58. Actinomycin D

was a gift from Merck, Sharpe & Dohme, and diethyl-pyrocarbonate was kindly provided by Bayer Div., Sterling Drug, Inc., New York. All other chemicals were of analytical grade.

RESULTS

Cell Fractionation Procedure

PREPARATION OF A POSTNUCLEAR SUPERNATE

The first aim of the cell fractionation procedure was to obtain, in a simple step, a cytoplasmic extract containing most, if not all, of the ribosomes present in the cytoplasm of the cells. P3K cells which had been cultured for 24 h in the presence of [^{14}C]uridine were homogenized as described in Materials and Methods, and the cell lysate was treated as described on Fig. 1; an aliquot was taken to determine the acid-insoluble ^{14}C-radioactivity present in the whole lysate. The percentage of radioactivity recovered in the various cell fractions usually amounted to more than 95% of the radio-activity present in the whole lysate (Fig. 1).

It was important to determine what proportion of the cytoplasmic ribosomes remained in the washed (second) nuclear pellet. Electron micro-scope examination of this pellet showed nuclei surrounded by a nuclear membrane bearing at-tached ribosomes, as well as a few microsomal vesicles (Fig. 3 a). After treatment of the nuclear pellet with NP 40, which dissolves the cytoplasmic
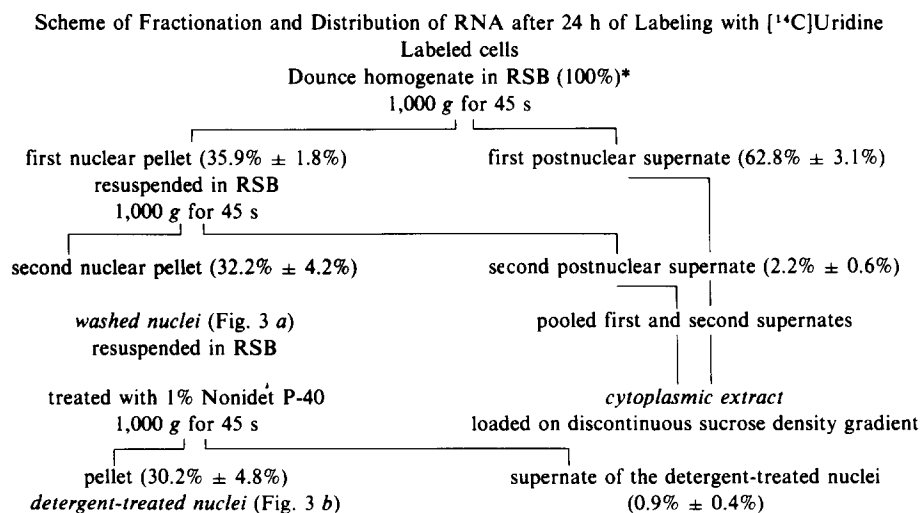
Scheme of Fractionation and Distribution of RNA after 24 h of Labeling with [^{14}C]Uridine

Labeled cells

Dounce homogenate in RSB (100%)*

1,000 g for 45 s

first nuclear pellet (35.9% ± 1.8%)   first postnuclear supernate (62.8% ± 3.1%)

resuspended in RSB

1,000 g for 45 s

second nuclear pellet (32.2% ± 4.2%)   second postnuclear supernate (2.2% ± 0.6%)

washed nuclei (Fig. 3 a)   pooled first and second supernates

resuspended in RSB

treated with 1% Nonidet P-40   cytoplasmic extract

1,000 g for 45 s   loaded on discontinuous sucrose density gradient

pellet (30.2% ± 4.8%)   supernate of the detergent-treated nuclei

detergent-treated nuclei (Fig. 3 b)   (0.9% ± 0.4%)

FIGURE 1 Flow diagram showing (*) percent distribution of ^{14}C-labeled RNA in subcellular fractions. The amount of each fraction is expressed as a percent of the RNA content of the homogenate. The cells were labeled for 24 h with [^{14}C]uridine. The [^{14}C]RNA present in the cytoplasmic extract (about 65% of the total radioactivity), consisted of about 75% of sedimentable RNA as measured by sucrose density gradient sedimentation of the free and membrane-bound labeled fractions (see Fig. 4 c, d).
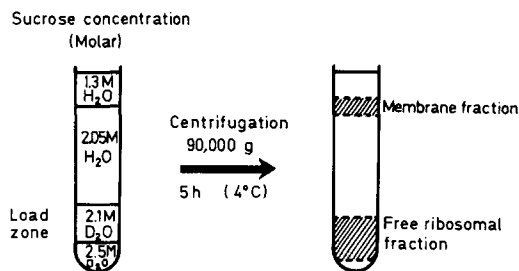
Sucrose concentration
(Molar)

FIGURE 2 Gradient separation scheme of free and membrane-bound ribosomes.

membranes and leaves the nuclei intact, a small amount of ^{14}C-radioactivity was liberated to the supernate (Fig. 1), and electron microscope examination showed that the nuclear pellet was free of microsomal and ribosomal contamination (Fig. 3 b). The ribosomal nature of most of the radioactivity released from the nuclear pellet was confirmed by sucrose gradient sedimentation (not shown). It can be calculated from the distribution of radioactivity given in Fig. 1 that the loss of cytoplasmic ribosomes to the nuclear pellet was very small and that the postnuclear supernate contained about 98% of the cytoplasmic ribosomes. It is possible that a large part of the radioactivity remaining with the detergent-treated nuclei (Fig. 3 b) was incorporated into DNA as suggested by Plagemann (34). More than 80% of this nuclear radioactivity was resistant to mild alkaline digestion (0.6 N KOH for 1 h at 37°C), while close to 100% of the cytoplasmic radioactivity became acid soluble in these conditions.

SEPARATION OF FREE AND MEMBRANE-BOUND RIBOSOMES

The postnuclear supernate was brought to a final concentration of 2.1 M sucrose and 70% heavy water (see Materials and Methods) and then used to make a discontinuous sucrose density gradient as described on Fig. 2. The density of the different solutions was chosen so as to achieve the best resolution between the free ribosomes, whose high density makes them sediment during the centrifugation, and all the membrane-containing cell organelles, including the membrane-bound ribosomes which, because of the low density of the membranes, float above the 2.05 M sucrose layer. The separation achieved after 5h of centrifugation at 90,000 g is seen in Fig. 4 a, which shows the distribution of the [^{14}C]uridine radioactivity into the two peaks. Longer centrifugation times did not substantially modify this pattern.

To evaluate the ribosome content of the lower F (high density) and upper MB (low density) fractions obtained from this discontinuous sucrose density gradient, aliquots of these fractions were diluted with buffer to a final sucrose concentration of 15% (wt/vol), and analyzed on 15–55% sucrose gradients (Fig. 4 c, d). The lower (F) layer showed the presence of polyribosomes, and of a small amount of monoribosomes and of native 60S and 40S subunits (Fig. 4 c); in addition, it contained a large amount of slowly sedimenting RNA, presumably tRNA, since sucrose gradient analysis of the RNA extracted from this lower layer showed a large peak of 4S RNA (not shown). The upper (MB) layer showed no sedimentation profile corresponding to ribosomal particles, most of the radioactivity being found in a single large peak probably representing microsomes and other membrane-containing structures of large mass (not shown). Indeed, when the upper layer fraction was pretreated with detergent in order to dissolve the membranes and release the membrane-bound ribosomal particles, the sedimentation profile (Fig. 4 d) showed the presence of polyribosomes and some monoribosomes and 60S subunits. The sensitivity of the polyribosomal patterns to mild ribonuclease treatment is described elsewhere (27). The small amount of radioactivity sedimenting in the 40S region was of mitochondrial origin, since analysis of its RNA content showed that it consists of 16S and 12S RNA (26), while the 60S subunit region contains essentially 28S RNA. Finally, this upper layer also contained some slowly sedimenting RNA, which was found to consist mainly of 4S RNA. It thus appears that the discontinuous sucrose gradient fractionation of the postnuclear supernate results in the separation of free ribosomes, which sediment, and of a membrane fraction containing membrane-bound ribosomes, including mitochondrial ribosomes, which float. The membrane fraction obtained from uniformly labeled cultures contained very consistently 18–20% of the [^{14}C]uridine radioactivity present in the cytoplasmic extract, whether the cells were exponentially growing or had reached a stationary phase. However, the polyribosome profiles shown on Fig. 4 c, d were observed only when exponentially growing cells at a concentration below 5 × 10^5 cells/ml were used; at higher cell concentrations, the proportion of monoribosomes increased at the expense of the polyribosomes, in both the free ribosomal and membrane fractions. The amount of mitochondrial 16S and 12S ribosomal
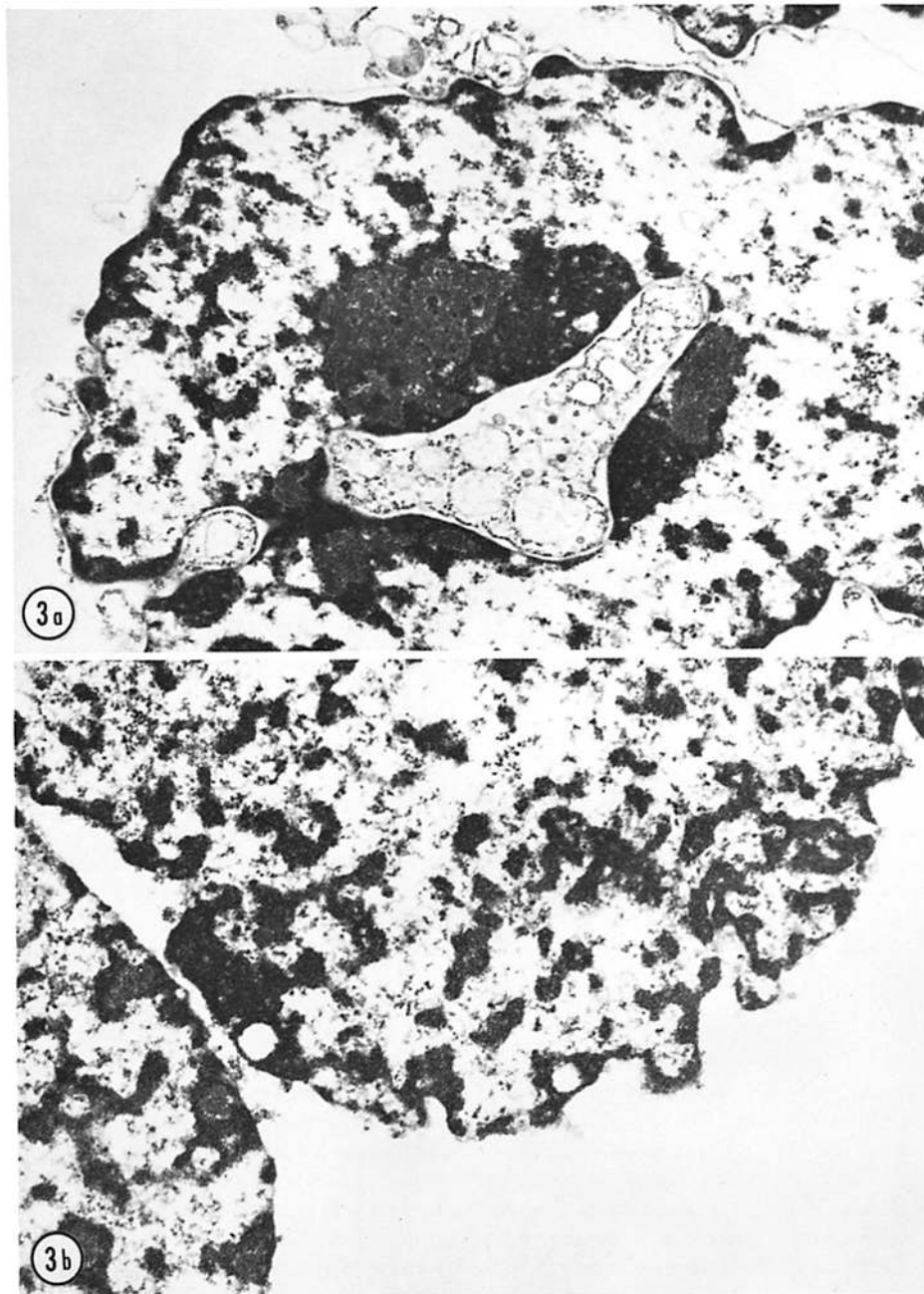
FIGURE 3 (*a*) Electron micrograph of the "washed nuclei" (see Fig. 1). Portions of nucleus are seen, showing ribosomes attached to fragments of the perinuclear membranes. In addition, microsomes are found trapped within nuclear invaginations. × 14,500. (*b*) Electron micrograph of the "detergent-treated nuclei" (see Fig. 1). The nuclear membrane has disappeared and there is no contamination by fragments of rough microsomes. × 14,500.

RNA (3) present in the membrane fraction was estimated by sucrose gradient sedimentation of the RNA extracted from the membrane fraction after treatment with EDTA and 0.5 M KCl, which releases almost all the membrane-bound, nonmitochondrial ribosomes (see below and legend of Table I). The 16S and 12S RNA represented 5.4–8.0% of the ribosomal RNA present in the
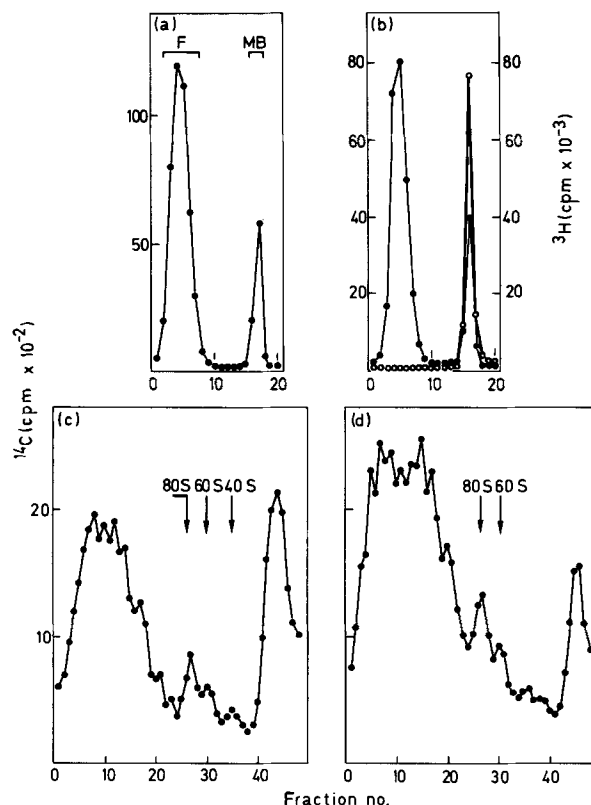
FIGURE 4 Separation and analysis of the free ribosomal and membrane fractions of the cytoplasmic extract. (a) Separation on the discontinuous sucrose density gradient (Fig. 2) of the free ribosomal (F) and membrane fractions (MB) obtained from 3×10^7 P3K cells labeled with [^{14}C]uridine (0.05 μCi/ml) for 24 h. Radioactivity was measured on 0.05-ml aliquots. (b) Separation on the discontinuous sucrose density gradient of the free ribosomal and membrane fractions obtained from 3×10^7 P3K cells labeled with [^{14}C]uridine (0.05 μCi/ml) for 24 h, and pulse labeled with [^{3}H]oleic acid (0.5 μCi/ml) for 2 h. (●——●), ^{14}C Radioactivity: (○——○), ^{3}H Radioactivity. (c) and (d) Sedimentation profiles on 15-55% sucrose density gradients in TK$_{30}$M of the ^{14}C radioactivity of the free ribosomal (c) and membrane (d) fractions from the experiment shown in Fig. 3 a. The membrane fraction was treated with detergent (see Materials and Methods). Centrifugation was carried out for 8.5 h at 23,000 rpm in a Spinco SW 27 rotor at 4°C.

membrane fraction. The amount of viral RNA in the membrane fraction (see below) after 24 h of [^{14}C]uridine labeling was estimated by tartrate gradient sedimentation according to Watson et al. (45). The viral particles which sedimented at a density of 1.29–1.32 g/cm^3 (45) represented, at most, 4–5% of the RNA of the membrane fraction.

EXPLORATION OF POSSIBLE CROSS CONTAMINATION OF THE FREE AND MEMBRANE-BOUND RIBOSOMES

The possibility exists of substantial contamination of each of the two fractions by ribosomes from the other fraction. During the fractionation procedure, a small amount of free ribosomes might become trapped in the membrane fraction, and conversely, some membrane-bound ribosome could be artefactually detached from the membranes and thus contaminate the free ribosomes fraction. The following experiments were performed to explore these possibilities.

LABELING OF THE MEMBRANE LIPIDS: Cells were labeled for 24 h with [^{14}C]uridine and for 2 h before homogenization with [^{3}H]oleic acid or [^{3}H]choline. The distribution of the radioactivity after fractionation of the postnuclear supernate on the discontinuous sucrose gradient is shown in Fig. 4 b. ^{3}H-radioactivity was present only in the membrane fraction, indicating the absence of contamination of the free ribosome fraction by membrane fragments.

ELECTRON MICROSCOPE EXAMINATION OF THE TWO FRACTIONS: The two fractions

separated by the discontinuous sucrose gradient were diluted with buffer and centrifuged to obtain small pellets which were fixed for electron microscope examination. Sections at different levels of the free ribosomal pellets revealed only free ribosomes (Fig. 5 a); no membrane fragments were detected. The pellet obtained from the membrane fraction contained microsomal vesicles studded

with ribosomes, mitochondria, and various types of membrane-limited structures (Fig. 5 b). The microsomal vesicles frequently contained murine leukemia virus particles (45). These viral particles are numerous in P3K cells, especially within the smooth endoplasmic reticulum.

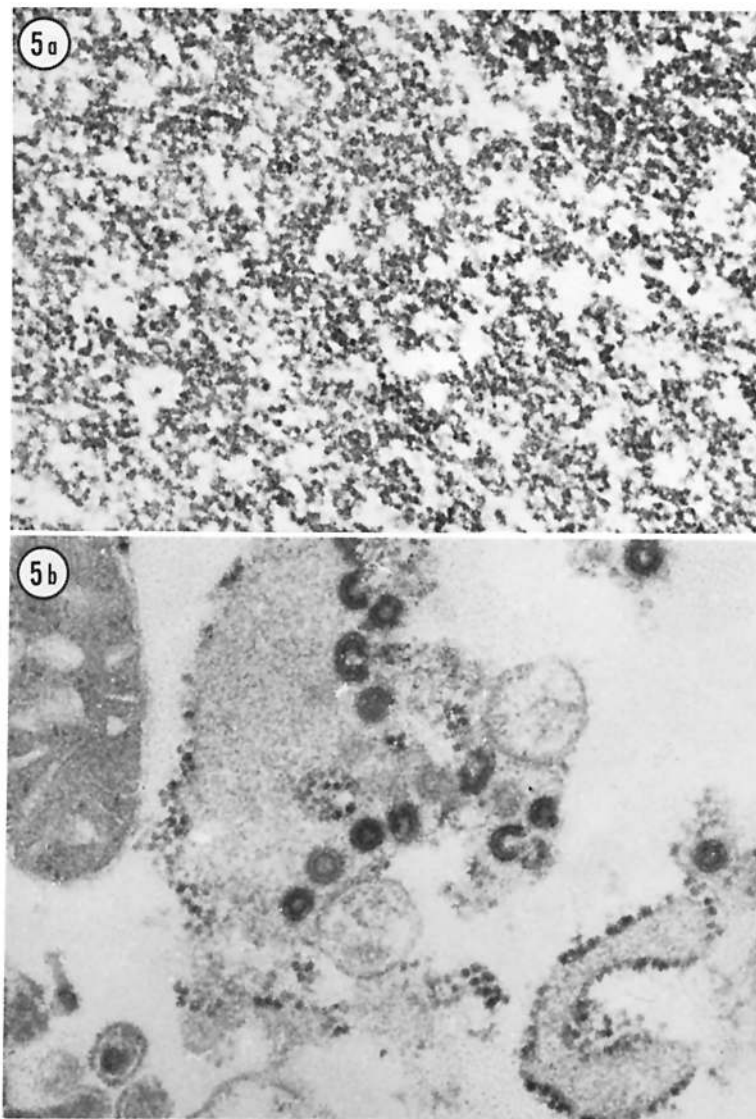REPEATED PURIFICATION OF THE FREE RIBOSOMAL AND MEMBRANE FRACTIONS ON

FIGURE 5 Electron microscopy of the free ribosomal and membrane fractions isolated on the discontinuous sucrose density gradient. (a) Free ribosomal fraction. This fraction consists only of ribosomes; no membrane structures are visible. × 44,000. (b) Membrane fraction. This electron micrograph shows a representative sampling of the structures observed in this fraction. Cisternae of rough microsomes often containing murine leukemia virus particles, a mitochondrion on the left, and a few unidentified membranous structures devoid of attached ribosomes. × 44,000.

THE DISCONTINUOUS SUCROSE GRADIENT: When the free ribosome fraction sucrose was introduced into a second discontinuous sucrose density gradient, its sedimentation remained identical (not shown). When the membrane fraction, brought to the appropriate concentration of sucrose in heavy water, was recentrifuged on the same type of gradient (Fig. 6), almost all the ^{14}C-radioactivity sedimented again in the membrane fraction, while a very small amount of radioactivity remained in the loading zone. Analysis of this latter material by sedimentation on a 15-55% sucrose density gradient showed that it consisted almost exclusively of slowly sedimentable, nonribosomal RNA, presumably mainly tRNA. Thus, these experiments provided no evidence that the membrane fraction contains a significant amount of trapped free ribosomes which could be liberated by a second separation on the discontinuous sucrose density gradient, nor that a detectable number of bound ribosomes
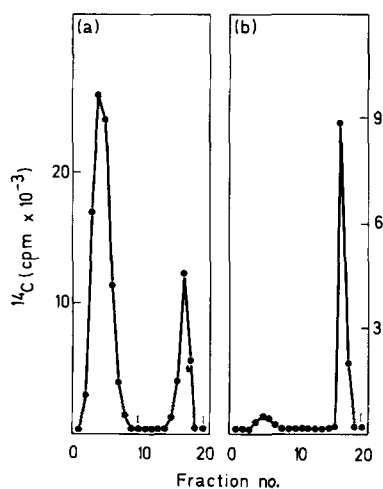

FIGURE 6 Repeated fractionation of the cytoplasmic extract membrane fraction. (a) Pattern of sedimentation on the discontinuous sucrose density gradient (Fig. 2) of a cytoplasmic extract from P3K cells labeled for 24 h with [^{14}C]uridine. Radioactivity contained in 0.05 ml aliquots of each fraction (0.5 ml) collected was measured. (b) 0.8 ml of the membrane fraction shown in (a) (tubes 17 and 18) was resedimented on the same type of discontinuous sucrose density gradient, and the radioactivity contained in 0.05-ml aliquots of each fraction determined. The small amount of radioactive material found in the region of the free ribosomal fraction was analyzed by sedimentation on a 15-55% sucrose $TK_{80}M$ density gradient, as described in Fig. 4 c. This material sedimented as small molecular weight RNA; polyribosomes and ribosomal subunits were not detected.

could be progressively detached during this separation.

ADDITION OF FREE ^{14}C-LABELED RIBOSOMAL PARTICLES TO THE HOMOGENATE OF [^{3}H]URIDINE-LABELED CELLS, FOLLOWED BY THE SEPARATION OF THE RIBOSOMAL AND MEMBRANE FRACTIONS: The possibility of a contamination of membrane fraction by a small amount of free native subunits was particularly important to explore, in view of the finding of native subunits in the membrane fraction, especially of 40S newly made native subunits, as will be shown and discussed elsewhere (26). [^{14}]Uridine-labeled 60S and 40S KCl-derived ribosomal subunits (see Materials and Methods) were added either before homogenization, just after homogenization, or directly to the postnuclear supernate of cells cultured for 24 h in the presence of [^{3}H]uridine. The added ^{14}C-labeled free subunits were in large excess, i.e. in an amount corresponding to about five times that of the native subunits present in the postnuclear supernate of the ^{3}H-labeled cells. A high concentration of cycloheximide (1 mg/ml) was added to the cell homogenate before the addition of ^{14}C subunits, in order to prevent the possibility of subunit exchange due to the persistence of low-rate protein synthesis during the cell fractionation. Separation on the discontinuous sucrose gradient showed that about 10% of the ^{14}C-free 60S subunits (Fig. 7 a) and 2.5% of the ^{14}C-free 40S subunits (Fig. 7 c) were recovered in the membrane fraction, indicating that, in these conditions of cell fractionation, and with the type of derived subunits used, some free subunits could be artefactually bound to the membrane fraction. Even though it seemed that the normal cytoplasmic content of free native subunits would make such a contamination actually very small, the possibility of eliminating all detectable contamination of the membrane fraction by free subunits was explored by progressively increasing the salt concentration (from 0.025 M to 0.15 M KCl) in all the sucrose solutions used in the discontinuous sucrose gradient. The contamination of the membrane fraction by the ^{14}C-free subunits was found to decrease progressively with the increase in KCl concentration, and was totally suppressed for both 60S and 40S subunits at 0.15 M KCl (Fig. 7 b and d). In one experiment, ^{14}C-labeled free polyribosomes were added to the postnuclear supernate of [^{3}H]uridine-labeled cells, and the cell fractionation was explored by using sucrose gradients containing either 0.025 M or

0.15 M KCl; it was also observed that the higher salt concentration was useful, since a slight degree of trapping of ¹⁴C-free polyribosomes could be observed after fractionation on gradients containing 0.025 M KCl.

In conclusion, no evidence was found indicating the possibility of significant contamination of the free by the membrane-bound ribosomes or vice versa. When 0.15 M KCl is present during the fractionation procedure, the possibility of even a very small contamination of the membrane fraction by native subunits which were originally free in the cytoplasm seems to be ruled out.

Effects of Salt on the Attachment of Ribosomes to the Membranes

It is known that membrane-bound ribosomes are attached to the membranes through their 60S subunits (41). Evidence has been presented, however, that all the ribosomes do not bear the same relationship to the membranes. It has been observed in HeLa cells that when a cell fraction rich in membrane-bound ribosomes is treated in vitro with ethylenediaminetetraacetic acid (EDTA), ribonuclease, or high salt concentration (0.5 M KCl), or when cells are treated in vivo with puromycin, about half the ribosomes present in this fraction are released (38). It has therefore been proposed by Rosbash and Penman (38) that two

classes of membrane-bound ribosomes exist: the "loose" ribosomes, easily detached in the conditions mentioned above, and the "tight" ribosomes, whose association to the membranes is more stable. The effects of EDTA and 0.5 M KCl treatment on the membrane fraction isolated as described above were consequently explored; those of ribonuclease and puromycin treatment will be reported in an accompanying paper (27). EDTA treatment in 0.15 M KCl released from the membranes virtually all the 40S subunits and the vast majority of the 60S subunits, as shown in Table I.

Treatment of the membrane fraction in 0.5 M KCl without EDTA, on the other hand, released only a small proportion of the ribosomal particles (Table I). Analysis of the ribosomes remaining in the membrane fraction showed that monoribosomes and the native 60S subunits, but not polyribosomes, had been released (Fig. 8 b); the released ribosomal particles were recovered as 60S and 40S subunits (Fig. 8 c). It appears that 0.5 M KCl treatment of the membrane fraction releases and dissociates only the ribosomes (and 60S subunits) lacking nascent polypeptide chains, since nascent chains are absent from the membrane-bound monoribosomes and 60S subunits (27). Combination of EDTA with 0.5 M KCl treatment released virtually all the ribosomes and subunits from the membranes (Table I).
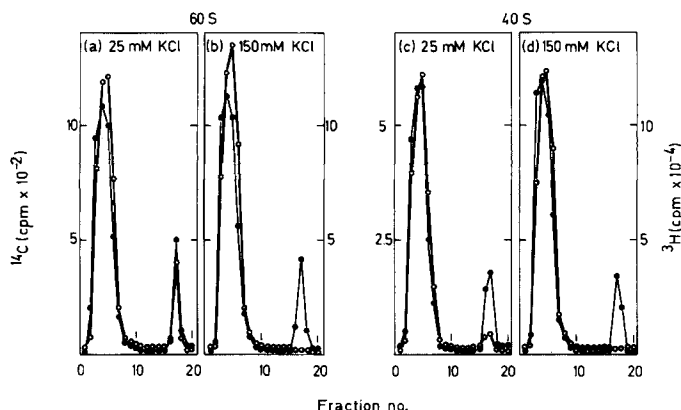

Fraction no.

FIGURE 7   Effect of KCl concentration on the distribution in the free ribosomal and membrane fractions of [¹⁴C]uridine labeled 60S or 40S ribosomal subunits added to a [³H]uridine labeled cell homogenate. Puromycin-KCl-derived ribosomal subunits labeled with [¹⁴C]uridine (see Materials and Methods; 21.550 cpm/μg RNA) were added to whole cytoplasmic extracts from cells labeled with 10 μC [³H]uridine for 24 h, in the following amounts: 20 μg of ¹⁴C-labeled 60S subunits in (a) and (b), and 0.8 μg of ¹⁴C-labeled 40S subunits in (c) and (d). The mixtures were centrifuged in discontinuous sucrose density gradients, whose KCl concentration was as indicated on the graphs: (—O—O—), ¹⁴C radioactivity; (—●—●—), ³H radioactivity.

TABLE I

Ribosomal RNA Remaining on the Membrane Fraction after Treatment with EDTA and KCl

| | Ribosomal RNA | |
	28S	18S*
Untreated membrane fraction	1.0	1.0
Treated membrane fractions		
EDTA and 0.05 M KCl	0.29	0.14
EDTA and 0.15 M KCl	0.13	0.05
EDTA and 0.50 M KCl	0.09	0.0
0.50 M KCl	0.88	0.92

P3K cells were uniformly labeled with 0.1 μCi/ml [^{14}C]uridine. The membrane fraction was prepared by sedimentation on a discontinuous sucrose density gradient containing 0.15 M KCl. Aliquots of the membrane fraction were adjusted to the indicated KCl concentrations, with or without 10 mM EDTA, then refractionated on a discontinuous sucrose density gradient containing the same KCl concentration, and in the case of EDTA treatment, no Mg^{++}. The RNA present in the membrane fraction was extracted with 1% sodium dodecyl sulfate (SDS), isolated by ethanol precipitation, and analyzed on 5-23% sucrose gradients containing 0.1 M LiCl, 0.004 M EDTA, 0.01 M Tris-HCl, pH 7.6, and 0.1% SDS according to Kolakofsky and Bruschi (19). The amount of radioactivity under the 28S and 18S ribosomal RNA (rRNA) components was measured and the values were compared to those obtained for the control.

* Values for the 18S rRNA were corrected for the estimated contamination by 16S mitochondrial RNA (mRNA) since the membrane fraction contains mitochondria (see text). In the case of the membrane fraction treated with EDTA and 0.50 M KCl, all the radioactivity sedimenting more slowly than 28S appeared to be represented only by 16S and 12S mRNA.

Analysis of the Free and Membrane-Bound Ribosomal Particles in CsCl Buoyant Density Gradients

Rosbash and Penman (39) found that two classes of membrane-bound ribosomes could be separated according to their density: a heavy class, with a density of 1.55 g/cm³, which appears to correspond to the "loose" ribosomes, and a heterogeneous light class, with a mean density of 1.49 g/cm³, probably corresponding to the "tight" ribosomes. However, analysis in CsCl density gradients of polyribosomes prepared from free ribosomal and membrane fractions showed that these polyribosomes have identical distribution (Fig. 9), as well as their 60S and 40S subunits obtained after incubation with puromycin followed

by separation in high salt concentration (see Materials and Methods).

DISCUSSION

The major pitfalls in the study of the relationship between free and membrane-bound ribosomes are methodological: available procedures are not quantitative and little attention has been given to mutual cross-contamination. Differential centrifugation procedures are unsatisfactory because there is a large amount of overlap between the sedimentation coefficients of the smaller microsomal vesicles and the larger free polyribosomes (2). "Isopycnic" fractionation procedures based on differences in density (9) usually involve pelleting the free ribosomes through a density interface at which the microsomes are retained. Free ribosomes contaminate such microsomal fractions probably because the microsome layer at the density interface retains some of the free ribosomes which, because of their smaller sedimentation coefficient, reach this interface more slowly (24). Furthermore, membranes can sediment through such interfaces (29).

The procedure of cell fractionation used in the present experiments was developed in order to make possible kinetic studies of the assembly and interrelationship between free and membrane-bound ribosomal populations. Thus, this procedure had to satisfy the following criteria.

High Recovery of Cytoplasmic Ribosomes

The cytoplasmic extract obtained includes about 98% of the ribosomes present in the cytoplasm, and about 90% of the membrane-bound ribosomes.

Preparation of Free and Membrane-Bound Ribosomes Devoid of Reciprocal Contamination

To this end, in the discontinuous gradient used, the free ribosomes and the membranes sediment in opposite directions. A variety of controls (electron microscopy, labeling of membrane lipids, further repurification of the isolated fractions) did not provide evidence of cross-contamination. However, when the possibility of trapping and/or adsorption of small amounts of free ribosomal particles in the membrane fraction was analyzed by adding to the cytoplasmic extract [^{14}C]uridine-labeled free polyribosomes or derived 60S or 40S subunits, it was found that this contamination could exist, especially in the case of the 60S
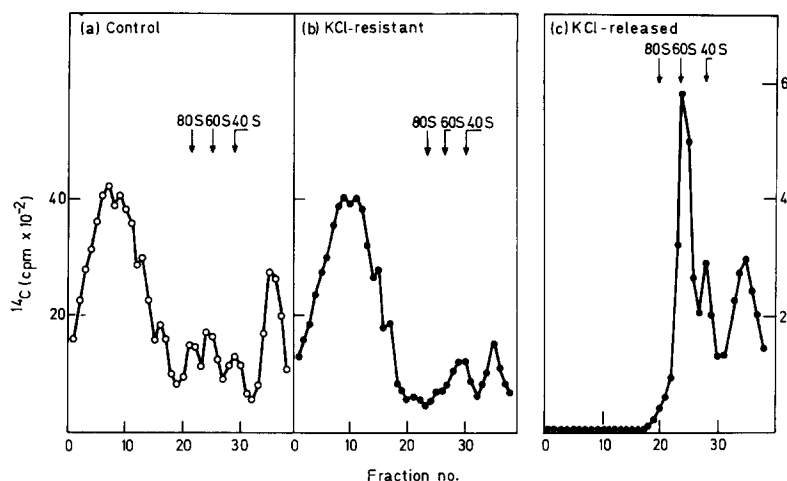
FIGURE 8 Analysis of the ribosome distribution on the membranes and in the released fraction after treatment with 0.5 M KCl. 3 × 10⁷ P3K cells were uniformly labeled for 24 h with [¹⁴C]uridine (0.1 µCi/ml). A membrane fraction was obtained. One half was treated with 0.5 M KCl and fractionated on a second discontinuous sucrose density gradient (see legend, Table I); the other half was kept as a control. The control and KCl-treated membrane fractions, dissolved with 0.5% sodium deoxycholate and 0.5% Brij 58, and half of the ribosomes released from the KCl-treated fraction were centrifuged on 15–55% sucrose gradients as in Fig. 3, containing either 0.08 M (*a*) or 0.3 M KCl (*b*, *c*). (*a*) Control, total membrane-bound ribosomes. (*b*) KCl-resistant ribosomes associated with the membranes. (*c*) KCl-released ribosomes recovered in the free ribosome region of the discontinuous sucrose density gradient.

subunits, and could be entirely prevented only when the salt concentration reached 0.15 M KCl. Furthermore, there was no evidence that the fractionation procedure on discontinuous gradients containing either 0.025 M or 0.15 M KCl might lead to the detachment of membrane-bound ribosomes.

The membrane fraction also contained mitochondria and viruses. The mitochondrial RNA accounted for less than 10% of the membrane fraction RNA; furthermore, in cells incubated for short periods with [³H]uridine, mitochondrial RNA synthesis can be completely prevented by ethidium bromide (47). The RNA of the murine leukemia viral particles represented at most 4–5% of the RNA of the membrane fraction after 24 h of [¹⁴C]uridine labeling.

Preservation of the Integrity of Polyribosomal Structure

This was satisfactorily achieved, as seen in Fig. 4 *c* and *d*, so long as the cells were chilled rapidly after labeling.

EDTA treatment in the presence of 0.025 M KCl of the purified membrane fraction released almost all the 40S subunits, as was initially observed with liver microsomes by Sabatini et al. (41), and about 70% of the 60S subunits (Table I).

This latter value is similar to that observed by some authors (4), but somewhat higher than the 50% release reported by others (38, 46). When EDTA was used in association with increasing concentration of KCl, virtually all the membrane-bound ribosomes were released. In contrast, 0.5 M KCl alone removes from the membranes only the monoribosomes and the 60S native subunits, which represent about 10% of all the membrane-bound ribosomes. These salt-releasable membrane-bound ribosomal particles do not carry nascent chains (27), thus, this observation is in agreement with the experiments of Adelman et al. (1) who showed that, when not anchored to the membranee through nascent chains, ribosomes are released by high molarity salt. This low detachment of membrane-bound ribosomes in 0.5 M KCl contrasts with reports by others also using P3K cells. Zauderer et al. (46) found that, in addition to monoribosomes and 60S subunits, about 35% of the polyribosomes present in the membrane fraction were released. Since these polyribosomes were also released by repeated centrifugations through sucrose without high molarity KCl, they probably represented, as point out by the authors themselves, free polyribosome contamination of the membrane fraction. Harrison et al. (14) observed that 40–45% of the bound ribosomes were released
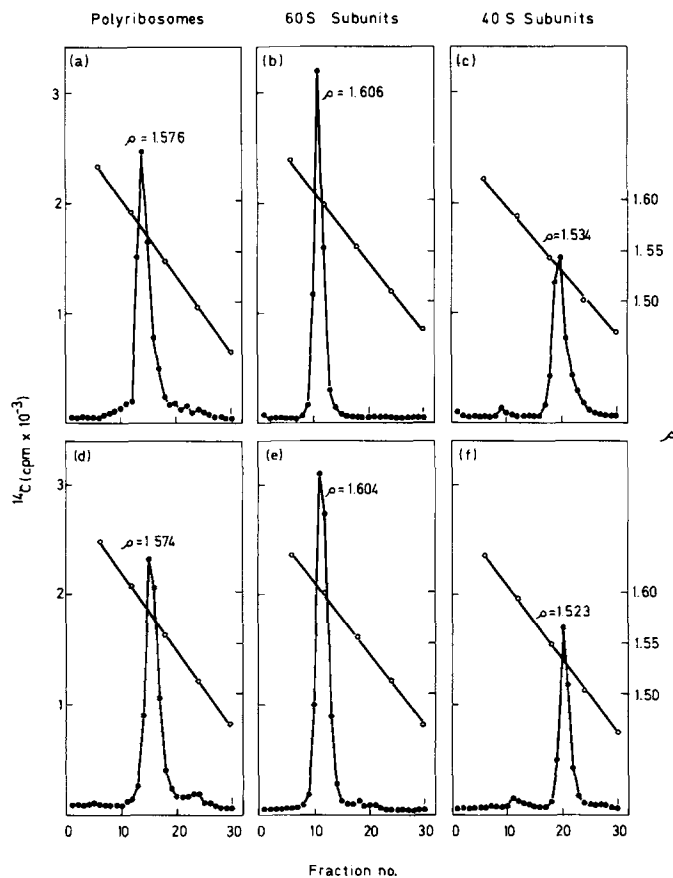
Polyribosomes   60S Subunits   40S Subunits

FIGURE 9   CsCl equilibrium centrifugation analysis of free and membrane-bound ribosomes. P3K cells were labeled with [^{14}C]uridine for 24 h. Free ribosomal and membrane fractions were separated and the fractions divided into two portions. Half of the material was used for the isolation of polyribosomes by sedimentation on 15–55% sucrose $T_{10}K_{50}M$ density gradients after treatment with detergents (0.5% sodium deoxycholate and 0.5% Brij 58) as described in Fig. 4 c and d. Ribosomal subunits were dissociated and released from the second portion, after incubation with puromycin and detergent treatment, by adjusting the KCl concentration to 0.5 M KCl. The ribosomal subunits were isolated on 15–30% sucrose $T_{10}K_{300}M$ density gradients (see Materials and Methods). Polyribosomes, 60S and 40S ribosomal subunits were fixed with glutaraldehyde, layered onto preformed CsCl gradients (1.35–1.70 g/cm^3) and centrifuged 18 h in a Spinco SW 50.1 rotor at 35,000 rpm. Fractions were collected, densities obtained from refractive index measurements, and radioactivity was determined. (a) Free polyribosomes; (b) free KCl-derived 60S ribosomal subunits; (c) free KCl-derived 40S ribosomal subunits; (d) membrane-bound polyribosomes; (e) membrane-bound KCl-derived 60S ribosomal subunits; (f) membrane-bound KCl-derived 40S ribosomal subunits.

by 0.5 M KCl and consisted of monoribosomes present in the membrane fraction; since such a high content of monoribosomes was never observed in the present experiments, these results are difficult to interpret.

Using cesium chloride equilibrium sedimentation analysis of the polyribosomes of HeLa cells obtained by detergent treatment of a membrane fraction, Rosbash and Penman (39) observed two classes of membrane-bound ribosomes of different density. The light class (density 1.49 g/cm^3 with a heterogeneous distribution) was presumed to correspond to "tightly bound" ribosomes, and the heavy class (density 1.55 g/cm^3) to "loosely bound" ribosomes. The observation of these two classes of membrane-bound ribosomes of different density was used as evidence for the existence of two separate classes of membrane-bound polyribosomes. Our experiments, however, did not reveal differences in density among the membrane-bound

ribosomes, or between the free and membrane-bound ribosomes or their subunits. The heterogeneous "light" membrane-bound ribosomes observed by Rosbash and Penman (39) may have resulted from an incomplete membrane solubilization and ribosomal washing.

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