# AN IMPROVED CELL FRACTIONATION PROCEDURE FOR THE PREPARATION OF RAT LIVER MEMBRANE-BOUND RIBOSOMES

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ABSTRACT

A cell fractionation procedure is described which allows the preparation from rat liver of a rough microsome population containing almost 50% of the membrane-bound ribosomes of the tissue. The fraction is not contaminated with free ribosomes or smooth microsomes, and, by various other criteria, is suitable for studies of ribosome-membrane interaction.

## INTRODUCTION

It has long been realized that, to a large extent, the microsome fraction prepared from rat liver (1, 2) represents the vesiculated remains of the endoplasmic reticulum (ER)[1] of the intact hepatocyte (3, 4). Interest in the role of the ER in cell function has spurred numerous analytical and enzymatic studies on the membranes of microsomes (5–7). As is the case in many other mammalian tissues which are active in the synthesis of proteins for export (4, 8), the membranes of the ER of rat liver provide binding sites for a large proportion of the cytoplasmic ribosomes. Studies on isolated ribosome-studded, or rough, microsomes (RM) have increased our understanding of the functional significance of ribosome-membrane interaction (9, 10), and it has become apparent that, while bound ribosomes synthesize secretory products, free ribosomes manufacture proteins which remain in the cell sap (11–14).

While some structural features of ribosome-membrane interaction are known (15, 16), a detailed understanding of the binding mechanism will require more refined in vitro studies on isolated RM. Such studies are hampered, however, because most commonly employed cell fractionation procedures give very low yields of RM. Analysis of our own RM preparations (17, 18), as well as computations based on published data (see reviews 5–7), indicate that in those cases where purified RM fractions have been isolated, they contain no more than 5–10% of the total bound ribosome population Since such a small sample of the total RM may be a nonrepresentative one, we have attempted to improve on the existing cell fractionation schemes.

Most fractionation procedures involve the preparation of a postmitochondrial supernatant (PMS) from which smooth (SM) and rough microsomes and free ribosomes are prepared using discontinuous sucrose density gradients. It is

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; IS, inhibitory supernatant, PCA, perchloric acid; PLP, phospholipid, PMS, postmitochondrial supernatant; poly U, polyuridylic acid, RM, rough microsome; SM, smooth microsome; STKM, TKM containing sucrose; TCA, trichloroacetic acid; TKM, 50 mM Tris·HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$.

known that the RNA of membrane-bound ribosomes amounts to ~60% of the total RNA of rat liver (19). Usually, 50% or more of the total RNA is sedimented with the nuclei and mitochondria, and most of this reflects the loss of RM (19–21) Furthermore, the bound ribosomes in the PMS are only partially recovered in the purified RM fraction Such losses of RM elements during cell fractionation should be avoidable, however It is known that mitochondria can be washed relatively free of contaminating RM if simple sucrose solutions are used (22, 23), but that the addition of mono- or divalent cations must be avoided since these cause clumping and aggregation of membranous organelles (3, 24). Nuclei, on the other hand, cannot be washed extensively in salt-free media, since they tend to swell and produce a nucleoprotein gel (25, 26) Rather pure nuclei can be separated from total homogenates, however, by sedimentation through dense sucrose solutions (26, 27) Guided, in part, by the above considerations, we have devised an improved fractionation scheme which yields RM preparations representing nearly 50% of the membrane-bound ribosomes of rat liver. Such preparations have been used in studies leading to nondestructive disassembly of the rough microsome (28). A preliminary account of this work has been given (29).

## MATERIALS AND METHODS

### *Sources*

Trizma (Tris base), sodium borohydride, cytochrome *c* (horse heart, type III), dithiothreitol, adenosine triphosphate (ATP), and guanosine triphosphate were obtained from Sigma Chemical Co (St. Louis, Mo.); enzyme-grade sucrose and sodium deoxycholate, from Mann Research Labs, Inc., (New York); bovine plasma albumin (crystalline), from Armour Pharmaceutical Co. (Chicago, Ill.), deoxyadenosine, from P-L Biochemicals, Inc. (Milwaukee, Wis.); Triton X-100 from Rohm and Haas Co (Philadelphia, Pa.); [$^3$H]leucine (58 0 Ci/mmol) and [$^3$H]orotic acid (14.1 Ci/mmol), from Schwarz/Mann Div , Becton, Dickinson & Co. (Orangeburg, N.Y.); [$^{14}$C]phenylalanine (0.362 Ci/mmol) and Liquifluor, from New England Nuclear (Boston, Mass ), phosphoenolpyruvate and phosphoenolpyruvate kinase, from Calbiochem (San Diego, Calif.), and polyuridylic acid (poly U) from Miles Laboratories, Inc. (Kankakee, Ill.). All other reagents were analytical grade.

### *General*

All solutions were prepared using deionized distilled water, were Millipore filtered (0.45 $\mu$m for most, 1.2 $\mu$m for concentrated sucrose stock solutions), and were stored in the cold. All operations, unless otherwise specified, were carried out in an IEC B-60 centrifuge (International Equipment Co., Needham Heights, Mass ) The notation "30 min-44K-A211 (200,000)" is used to denote a 30-min centrifugation at 44,000 rpm in the A211 rotor under which conditions $g$ max $\sim$ 200,000. Rotors A211 and SB110 are roughly comparable to Spinco (Spinco Div., Beckman Instruments, Inc , Palo Alto, Calif ) rotors 50.1 and SW27, respectively. All pH's are those measured at room temperature using a Radiometer model No. 4 pH meter (London Co., Cleveland, Ohio). Visible and UV absorption measurements were made in 1-cm path length cuvettes using a Zeiss PMQII (Carl Zeiss, Inc., New York) or Cary model No 14 (Cary Instruments, Monrovia, Calif ) spectrophotometer.

### *Analytical*

Fractions in pellet form (e.g , nuclei) were suspended in either 0.25 M sucrose (if enzyme assays were to be done), or in 0.25 M sucrose, 50 mM Tris·HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$ (0.25 M STKM), and final volumes were noted Cytochrome oxidase was assayed essentially according to Smith (30), with cytochrome *c* reduced according to Martin et al. (31). Conditions were chosen so that the rate (30) was proportional to protein concentration. 1 U of cytochrome oxidase is here defined as $10^3$ times the change in (log OD 550 nm at time $X$ − log OD 550 nm for the fully oxidized sample) per minute Catalase and acid phosphatase were assayed essentially according to the automated procedures described by Leighton et al. (32), and the results are presented in terms of activity units as defined there. We are most grateful to Dr. Brian Poole for his help in carrying out these measurements.

For analysis of protein, RNA, DNA, and phospholipid (PLP) phosphorus, samples of the various fractions were diluted with water to a convenient volume (usually 1 ml) and precipitated with an equal volume of 20% trichloroacetic acid (TCA). The precipitates were collected by centrifugation (IEC Universal Model UV, ~1000 $g$), and were washed twice with 0.2 N perchloric acid (PCA). Protein was analyzed essentially according to Lowry et al. (33). The standard curve was constructed using bovine plasma albumin dissolved in water to a concentration determined from UV absorption, assuming $\epsilon^{1\%}_{1cm}$ at 279 nm = 6.67 (34). RNA was measured according to Blobel and Potter (35). The procedure represents several minor modifications of that of Fleck and Munro (36). DNA was measured

using the residues left from the RNA analyses, essentially according to the procedure of Burton (37), with modifications suggested by other reports (38, 39) The residues were hydrolyzed twice for 20 min at 70°C 1 ml of the pooled hydrolyzates was mixed with 2 ml of diphenylamine reagent (1 g diphenylamine plus 40 ml glacial acetic acid plus 1 ml concentrated sulfuric acid, brought to 50 ml final volume), and to this was added 0.1 ml of 0.2% (vol/vol) acetaldehyde (in water) Samples were left at room temperature for 24 h and the OD 600 nm was determined The standard was a solution of deoxyadenosine whose concentration was determined spectrophotometrically It was assumed that half the bases in DNA were reactive (37), and a mean residue weight of 311 g/mol was taken for computations. Phospholipid extracts were prepared essentially according to Folch et al. (40). Samples were taken to dryness under an $N_2$ stream and analyzed for phosphorus (41). The phosphate standard was a solution of ATP, the concentration of which was determined spectrophotometrically, and which was assumed to contain 3 mol P/mol ATP. It was assumed that 25 mg phospholipid contains 1 mg phospholipid phosphorus

In vitro amino acid incorporation studies were carried out essentially according to Blobel and Sabatini (17), except that the high-speed supernatant was passed over Sephadex G-25, rather than G-100. Filter disks (Whatman 3 MM), containing radioactive samples, were processed by standard procedures (42), placed in toluene-Liquifluor, and counted in a Beckman model LS-250 scintillation counter (Beckman Instruments, Inc, Fullerton, Calif.) In vivo amino acid incorporation was examined by injecting ether-anesthetized rats via the portal vein with ~100 μCi [$^3$H]leucine 2 min before sacrifice. Appropriately diluted cell fractions were processed and counted as for in vitro incorporations. For labeling of RNA, animals were injected (intraperitoneally) with ~100 μCi [$^3$H]orotic acid ~36 h before sacrifice (35) Samples of the RNA hydrolyzates were placed in Bray's solution (43) and counted.

## *Electron Microscopy*

Microsomes suspended in 0 25 M sucrose were fixed by addition of 1 vol of 4% glutaraldehyde in 0 2 M sodium cacodylate, pH 7.2, and kept at 0°C for 60 min. After centrifugation (25 min-39K-SW39 [~170,000]), pellets were postfixed for 2 h in the cold with 2% $OsO_4$ in 0.1 M sodium cacodylate, pH 7.2. Blocks were washed in 0.90% NaCl and stained with 0.5% magnesium uranyl acetate for 30 min at room temperature, dehydrated, and embedded in Epon. Sections were stained with lead citrate and uranyl acetate For random sampling, resuspended fractions were collected on Millipore filters and processed essentially according to Baudhuin et al. (44)

## *Fractionation*

Male Sprague-Dawley rats (~120–150 g) were used for all experiments Animals were starved for about 18 h before sacrifice and, between 9:00 and 10:00 a.m, were decapitated using a guillotine (Harvard Apparatus Co, Inc., Millis, Mass ). The livers were quickly excised into ice-cold 0 25 M sucrose and cut into three to five large pieces. For cell fractionation studies, four to five livers were obtained, whereas 8–10 animals were sacrificed when batch preparation of high-speed supernatant (see below) was planned. All subsequent operations were carried out in the cold room. The pieces of tissue were blotted on absorbent paper, and, in a tissue press, were forced through a stainless steel plate with 1-mm perforations. The pulp was weighed, slurried with 2 ml sucrose solution/g (0 25 M for preparation of high-speed supernatant or 1.0 M for cell fractionation), and was homogenized (8–10 passes) with a Teflon-pestle, motor-driven tissue grinder (Arthur H Thomas Co., Philadelphia, Pa, size C, pestle rotating at 1000–2000 rpm) Slightly more or less vigorous homogenization than this did not affect the fractionation significantly.

For preparation of high-speed supernatant to be used as a source of ribonuclease inhibitor (45), the 0.25 M sucrose homogenate was centrifuged 15–20 min-25K-A211 (~65,000) The supernatant was recentrifuged 2 h-44K-A211 (200,000) A syringe and steel cannula were used to remove the (second) clear supernatant, excluding the milky scum at the top of the tubes This supernatant (designated IS for inhibitory supernatant) was stored in 15–20-ml samples at −20°C for up to 2 mo before use in cell fractionation

The fractionation scheme devised is described below with reference to the numbered steps in flow diagrams I and II (Figs 1 and 2) The scheme essentially involves removing nuclei from a liver homogenate, the density of which is adjusted so that most other membranous organelles either float, are isopycnic, or sediment very slowly during the appropriate centrifugation. No ionic components (KCl, Tris, $MgCl_2$, etc.) are introduced into the homogenate The postnuclear supernatant is then diluted to a density low enough to allow sedimentation of mitochondria which are washed with relatively ion-free sucrose solutions. The combined PMS is then fractionated on a discontinuous sucrose density gradient (which contains no ions) into smooth and rough microsome and free ribosome fractions

### *Flow Diagram I (Fig. 1)*

(*a*) The liver pulp was homogenized in 2 vol of 1.0 M sucrose (2 ml/g). Use of 1 0 M sucrose was somewhat arbitrary a higher sucrose concentration made homogenization more difficult, while a lower concentration necessitated greater dilution of the homogenate in the subsequent density adjustment step. Filtering the homogenate through a single layer of Nytex cloth (No 130, Tobler, Ernst and Traber, Inc , New York) removed connective tissue debris and improved the subsequent separation of nuclei. Addition to the filtered homogenate of an equal volume of 2.5 M sucrose followed by thorough mixing (repeated inversion in a stoppered measuring cylinder) produced a mixture of suitable density. Normally, 50 ml of filtered homogenate (representing ~17 g of liver pulp derived from four or five rats) was processed.

(*b*) The 100 ml of density-adjusted homogenate was transferred to four SB110 tubes and each was overlaid with 1 ml of 1.0 M sucrose to assure that material which floated to the top during the ensuing centrifugation was not exposed to an air-water interface. After centrifugation (45 min-24K-SB110 [~100,000]), each tube contained a well-packed, mottled, pinkish-gray pellet, a tan supernatant, and a thick, reddish-tan pellicle. Shorter centrifugation (15–30 min) often failed to give well-packed pellets; longer centrifugation did not increase the yield of nuclei. Recentrifugation of the rehomogenized supernatant (see below) gave a small pellet with only marginal increase in the overall yield of nuclei. Separation of nuclei under these conditions of minimal ion content did lead to some swelling and gelation. However, since the elimination of ions lessened the aggregation of membranous organelles, this disadvantage was acceptable. Attempts to remove nuclei by step gradient centrifugation of the entire homogenate (27) were unsuccessful, in that with the large volumes used, considerable DNA was trapped at the lower interface and did not sediment further, even after prolonged centrifugation This trapping occurred whether or not the underlay contained ions (e.g , TKM).

(*c*) The pellicle was dislodged from the walls of the centrifuge tube with a metal spatula, and, together with the viscous supernatant, was carefully transferred into the tissue grinder and homogenized (two or three passes) to disperse all clumps. To the ~100 ml of this dispersed postnuclear supernatant 50 ml of water was added, with thorough mixing, to achieve a dilution sufficient to allow subsequent sedimentation of the mitochondria. The mixture was divided into six portions and centrifuged 15 min-15K-A211 (~22,000).

(*d*) The pink, turbid supernatant was decanted and stored in a beaker (to which were subsequently added

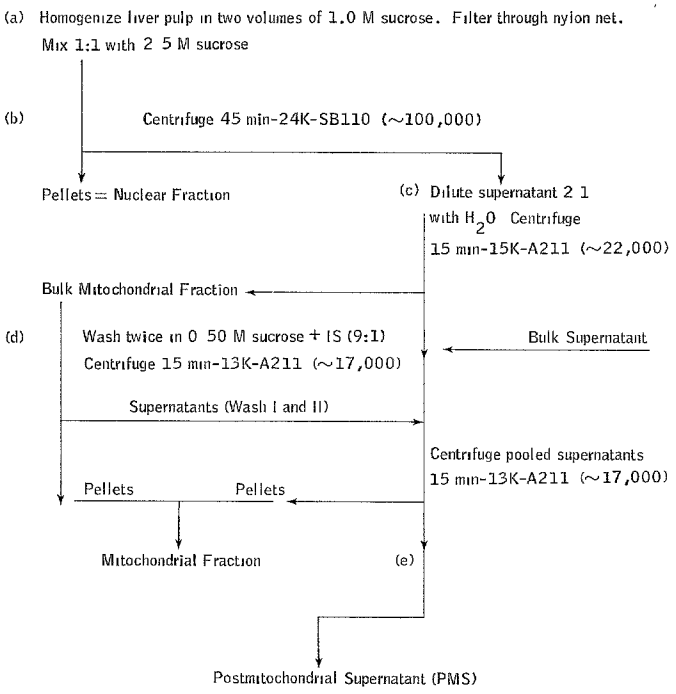


**FIGURE 1 Flow diagram I.**

the mitochondrial washes) The pellets, tan with a small red bottom layer (presumably erythrocytes), were suspended and homogenized in 25 ml of a mixture of 9 parts 0.50 M sucrose and 1 part IS. The inclusion of IS at this point was a precaution to minimize nuclease attack on bound polysomes The mitochondrial suspension was placed in two centrifuge tubes and sedimented 15 min-13K-A211 (~17,000) The supernatant was decanted and saved and the entire mitochondrial washing was repeated. The pellets obtained after each wash showed a dark tan, tightly packed lower layer and a lighter tan, less tightly packed upper layer. In addition, there was an appreciable amount of pinkish, fluffy material which was only lightly packed and which was decanted with the supernatants. Further washes of the mitochondrial fraction were ineffective in removing residual RNA (see Results and Discussion). If the two washes were carried out with 0 25 M STKM, the contamination of the mitochondrial fraction with RNA was even greater

(*e*) The combined mitochondrial supernatants were put in eight tubes and centrifuged 15 min-13K-A211 (~17,000). Each tube contained a tiny two-layer pellet (as in step *d* above) and a large layer of loosely packed, pinkish, fluffy material The supernatants were decanted with gentle swirling to assure transfer of the pinkish fluff, and were then gently homogenized to disperse any clumps. The pooled supernatants constituted the final PMS All of the two-layer pellets (steps *d* and *e*) were combined and suspended for analysis as the "mitochondrial" fraction.

## *Flow Diagram II (Fig. 2)*

The PMS (~180–190 ml, derived from ~17 g of liver) was separated into free ribosomes, rough and smooth microsomes, and a supernatant fraction by centrifugation on a discontinuous sucrose density gradient

(*a*) The total PMS was distributed evenly to eight centrifuge tubes, and, with a syringe and large steel cannula, was underlaid with (*i*) 4 ml of a mixture of 3 parts 2 0 M sucrose plus 1 part IS, and (*ii*) 1 ml of 2.0 M STKM. Layer *i* serves to separate rough from relatively smooth microsomes (see Results and Discussion) and is approximately equivalent in density to 1 5 M sucrose Numerous experiments (data not included) involving zone sedimentation of PMS on linear sucrose density gradients were conducted with the aim of separating discrete smooth and rough microsomal populations. These yielded membranous bands distributed over the range 1.0–2 0 M sucrose The exact position and number of bands varied, depending on sample load, rotor speed, tube size, ionic composition of the gradient, etc , but, in general, the distribution of membranous material was bimodal, with maxima near 1 1–1 3 M and 1 6–1 8 M sucrose When RNA was measured it was found to be primarily associated with the more dense membrane bands Experiments in which the PMS was made more dense than 2 0 M sucrose and layered below the continuous gradient (so that membranes floated up into the gradients during centrifugation) served to rule out the possible contribution of free ribosomes to these RNA analyses. Based on

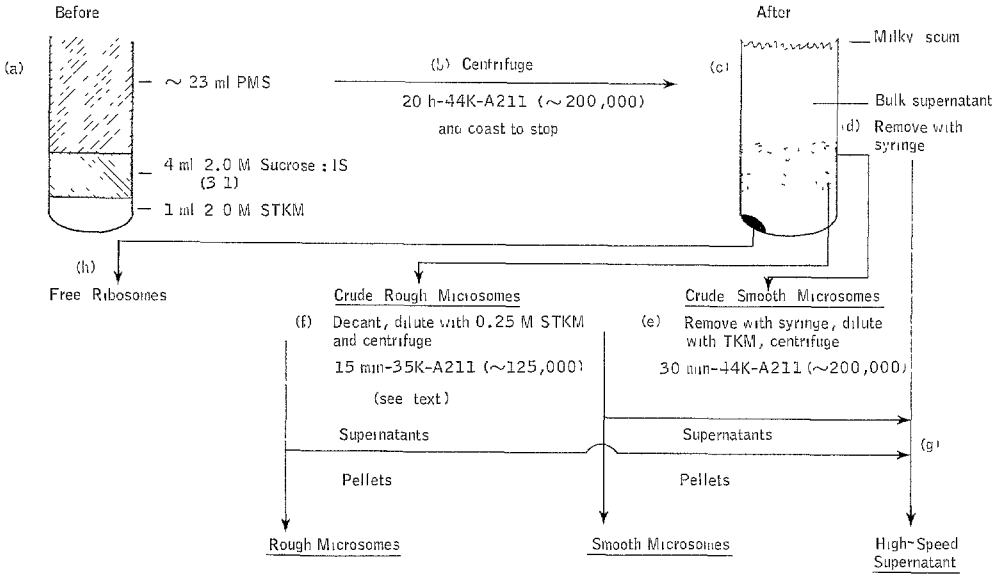


FIGURE 2 Flow diagram II.

these observations, a step gradient separation of smooth and rough microsomes was adopted with the density cutoff of 1.5 M sucrose for the separating underlay (layer *i*). While the choice of this density cutoff resulted in an appreciable loss of membrane-bound RNA to the SM fraction, it served to minimize the extent to which mitochondrial fragments contaminated the RM. The addition of ions (e.g., TKM) to the underlay was avoided, since this led to poorer separation of RM from SM, as shown by the RNA distribution. When the IS was not present in the 1 5 M sucrose underlay, free polysomes were more extensively degraded, sedimented more slowly, and heavily contaminated the RM fraction.

Layer *ii* separated RM from free ribosomes, which sedimented through the 2 0 M STKM into a pellet. Addition of IS to the 2 0 M STKM layer did not improve the yield or preservation of free polysomes, so long as IS was present in layer *i*, above. Use of 2.0 M sucrose (without ions) gave low yields of free ribosomes.

(*b*) The step gradients were centrifuged 20 h-44K-A211 (~200,000), and the rotor was allowed to coast to a stop. Shorter centrifugation times greatly decreased the yield of free ribosomes, and, if short enough (<4–8 h), resulted in poor separation of RM from SM. Even after 20 h centrifugation, sedimentation of free ribosomes was only ⅔–¾ complete (see Results and Discussion). These incompletely sedimented free ribosomes (mostly monomers) were easily removed from the RM during the subsequent differential centrifugation (step *f*, below).

(*c*) As indicated in flow diagram II (Fig. 2), after centrifugation, each tube contained a clear, pink-to-red supernatant above which floated a thin, milky scum. Membranous material was accumulated in the lower part of the tube, and, upon close examination, it was seen that there were two reddish-brown membranous bands, one at each interface with a small, relatively clear zone between them. Under these conditions of separation, the upper band (crude smooth microsomes) was uniform, with no sign of clumping or adherence to the tube walls. If TKM was present in the ~1.5 M sucrose underlay (or if the microsomes in the PMS had been centrifuged and resuspended to allow application of a more concentrated sample to the discontinuous gradient), the upper membrane band was not uniform Clumping of this band was always associated with higher contamination of SM with RM and lower yields of purified RM. The lower membrane band (crude rough microsomes), having been in contact with the 2.0 M STKM, was somewhat clumped Free ribosomes had sedimented through the 2.0 M STKM and formed a small, pale-orange pellet.

(*d*) A syringe with a large steel cannula was used to remove the bulk clear supernatant (including the floating scum) from each tube and to transfer this to a beaker.

(*e*) Using the same syringe and cannula, the upper membrane layer was removed and transferred to a graduated cylinder, care being taken not to disturb the lower membrane layer. Including the residual supernatant fluid removed with this layer, the total crude SM suspension thus obtained was 50–60 ml. This was diluted with TKM to ~150 ml, distributed in six tubes, and centrifuged 30 min-44K-A211 (~200,000). The clear supernatants were added to the bulk supernatant (step *d*) while the pellets constituted the SM fraction

(*f*) The residual fluid contents of the step gradient tubes were decanted into a graduated cylinder. Each tube was then gently rinsed with ~5 ml of 0.25 M STKM, and the rinses added to the same cylinder, care being taken to maximize transfer of the turbid fluid while minimizing disturbance of the ribosome pellets. This crude RM suspension was brought to a volume of ~100 ml with 0.25 M STKM, gently homogenized, and centrifuged (in six tubes) 15 min-35K-A211 (~125,000). The supernatant was added to that stored from steps *d* and *e*. The pellets were homogenized in 100 ml of 0 25 M STKM and recentrifuged (six tubes) 15 min-30K-A211 (~ 95,000). The supernatants were saved as above, while the pellets constituted the RM fraction.

(*g*) The combined supernatants (steps *d–f*) constituted the high-speed supernatant.

(*h*) The small, pale-orange, slightly opalescent pellets left in the step gradient tubes constituted the free ribosome fraction. In general, one pellet each of SM, RM, and free ribosomes was used for analysis. The remaining pellets were immediately frozen (−20°C) for future experiments. If the fractionation procedure was begun at approximately 9:00 a.m. on day 1, it was possible to complete it by approximately 2:00 p.m. on day 2.

## RESULTS AND DISCUSSION

Analytical data on the various cell fractions are presented in Tables I and II. Tables I A and I B display the distribution of RNA, DNA, protein, and cytochrome oxidase, while Table II presents analyses on various ancillary parameters. The nuclear fraction which accounted for ~80% of the total DNA was only slightly contaminated with mitochondria, as judged by the low cytochrome oxidase activity. The small amount of RNA present, being not much higher than that found in nuclei purified by other procedures (36, 37), suggested minimal trapping of ribosomal and/or rough microsomal elements. The mitochondrial fraction, which accounted for ~85% of the re-

TABLE I A

*Distribution of RNA, DNA, Protein, and Cytochrome Oxidase Activity in Cell Fractions Derived from 50 ml of Filtered Homogenate (=17 g liver)**

| Fraction | RNA | DNA | Protein | Cytochrome oxidase |
|---|---|---|---|---|
| | *mg* | *mg* | *mg* | *U* |
| Homogenate | 137 0 ± 5 | 33 50 ± 2 8 | 2526.0 ± 148 | 9.920 ± 1.40 |
| Nuclei | 9 8 ± 1.1 | 27.70 ± 2.7 | 244.0 ± 12 | 0.559 ± 0.264 |
| Mitochondria | 25 7 ± 4.0 | 5 58 ± 0.38 | 828.0 ± 66 | 12.000 ± 1 4 |
| PMS | 97.6 ± 9 2 | 0.96 ± 0.25 | 1565.0 ± 222 | 1 820 ± 0 38 |
| SM | 7 0 ± 2.4 | 0.21 ± 0 03 | 191.0 ± 42 | 0.671 ± 0.199 |
| RM | 35.6 ± 3.6 | 0.45 ± 0.10 | 179.0 ± 10 | 0.623 ± 0.081 |
| Ribosome | 22.4 ± 3.6 | 0.09 ± 0 03 | 33.1 ± 9.0 | 0.008 ± 0 002 |
| Supernatant | 25.0 ± 2 8 | 0.38 ± 0.17 | 990.0 ± 113 | 0.113 ± 0 022 |

* The procedure followed was exactly that described in Materials and Methods During the development of this procedure, numerous other assays were carried out, all of which gave results similar to these insofar as could be expected, considering the procedural differences involved Values given are mean ± $\sigma$ for $N$ determinations, $N$ = 7, 4, 6, and 3 for RNA, DNA, protein, and cytochrome oxidase respectively.

TABLE I B

*Percent Distribution of RNA, DNA, Protein, and Cytochrome Oxidase in Cell Fractions**

| Fraction | % RNA | % DNA | % Protein | % Cytochrome oxidase |
|---|---|---|---|---|
| Nuclei | 7.7 | 80 5 | 9.2 | 4.0 |
| Mitochondria | 20.4 | 16.2 | 33.9 | 85.9 |
| SM | 5.6 | 0.6 | 7.8 | 4.8 |
| RM | 28 3 | 1.3 | 7.3 | 4 5 |
| Ribosome | 17.8 | 0.3 | 1.4 | 0 05 |
| Supernatant | 19.8 | 1.1 | 40.5 | 0.81 |

* Calculated from means of Table I A The amount in each fraction is expressed as a percent of the sum of the amounts recovered in all fractions. Expressed as a percent of the contents of the homogenate, recoveries of RNA, DNA, protein, and cytochrome oxidase were 92, 103, 97, and 141% respectively The apparent overrecovery of cytochrome oxidase presumably reflects the difficulty of accurately assaying the activity in the homogenate.

covered cytochrome oxidase, contained the bulk of the residual DNA In addition, this fraction contained ~20% of the total RNA. The assumption that this RNA reflected the presence of RM was supported by the observation that treatment of the mitochondrial fraction with puromycin, under appropriate ionic conditions, led to release of ribosomal subunits and "stripped" membranes, in accordance with our results on purified RM (28, 29). The significance of and the reasons for the persistent contamination of the mitochondrial fraction with RM remain unclear; numerous modifications of the fractionation scheme have failed to minimize the contamination. Because this RNA represents an appreciable loss of RM, we continue to test possible alternative purification schemes.

Taken together, the SM and RM fractions contained ~35% of the total RNA; roughly 4/5 of this, or 28% of the RNA in rat liver, was recovered in the RM fraction. Data not shown here (but see reference 28) indicated that virtually all of the RNA in these RM was ribosomal, and that all ribosomes were bound to the membranes (i e , contamination with free ribosomes was negligible). Assuming that 60% of all liver RNA is ribosomal RNA of membrane-bound ribosomes (19), the RM fraction contained nearly 50% of all the mem-

TABLE II

*Ancillary Analytical Data on Cell Fractions*

| Fraction | [$^3$H]RNA* | [$^4$H]Leucine‡ | mg PLP / mg Protein | Catalase % units recovered | Acid phosphatase % units recovered |
|---|---|---|---|---|---|
| | *cpm/mg RNA* | *cpm* × $10^{-4}$*/mg protein* | | | |
| Homogenate | 22,600 | 0.321 | 0.248 | § | ‖ |
| Nuclei | 19,700 | 0.186 | 0.170 | 3.3 | 4 3 |
| Mitochondria | 22,300 | 0.204 | 0.219 | 7.6 | 32 9 |
| SM | 22,200 | 0.372 | 0.660 | 3.4 | 18.4 |
| RM | 23,100 | 1.38 | 0 679 | 5.4 | 10.8 |
| Ribosome | 22,000 | 2.49 | 0.078 | 0.9 | 0.3 |
| Supernatant | 22,400 | 0.169 | 0.071 | 80.5 | 33.4 |

* Animals were injected 36 h before sacrifice with [$^3$H]orotic acid (Materials and Methods). The slightly low value for the specific activity of RNA in the nuclear fraction is probably not significant since in another fractionation involving similar labeling conditions, all fractions had ~25,000 cpm/mg, except the supernatant which had ~20,600 cpm/mg.

‡ Animals received an injection of [$^3$H]leucine 2 min before removal of liver (Materials and Methods).

§ Homogenate contained 810 U. Total recovered = 1033.

‖ Homogenate contained 167 U. Total recovered = 196.

brane-bound ribosomes If the density of the intermediate underlay (layer *i*) in the step gradient was lowered to 1.3 or 1.4 M sucrose, the RNA contamination of the SM fraction was decreased Although the resulting RM contained more RNA, the contamination with mitochondrial fragments (cytochrome oxidase) was disproportionately increased. As can be seen from the data in Table I B, the RM contained ~5% of the cytochrome oxidase and a small amount of DNA, the significance of which remained obscure (46).

The free ribosome fraction contained somewhat less than the expected 20% of the total RNA (19) As mentioned above, some of the free ribosomes failed to sediment through the 2.0 M STKM layer of the step gradient and were left in the crude RM layer. During the washing of the RM these free ribosomes, along with some small RM elements, were transferred to the combined high-speed supernatant fraction. Prolonged centrifugation of the supernatant led to sedimentation of ⅓–½ of the RNA in this fraction, primarily as a mixture of free and bound ribosomes.

Table II includes the results of some additional analyses of the various fractions. The distribution of $^3$H label incorporated from orotic acid into RNA verified the chemically assayed RNA distribution. The similar specific activities of the RNA in the various fractions suggested that there were no gross differences in turnover characteristics between (for example) RNA in the RM and mitochondrial fractions. The results of a 2-min in vivo pulse of [$^3$H]leucine indicated, as expected, that the RM and free ribosome fractions were the primary amino acid incorporation sites. The apparent relatively high specific activity (in terms of [$^3$H]leucine counts per minute per milligram RNA) of the SM fraction must be interpreted with some caution since even with this brief a pulse, significant chain termination and release of labeled completed polypeptides occurs. Experiments involving detergent solubilization suggest that ~50% of the acid-insoluble [$^3$H]leucine counts per minute in the RM is in completed chains which have been released from the ribosomes and sequestered within the microsome. Such nonribosomal label presumably accounts for the bulk of the counts in the SM.

Phospholipid phosphorus analysis indicated the expected distribution of lipids (6, 7). Both SM and RM fractions contained 0.6–0.7 mg PLP/mg protein; although most workers have reported ratios of 0.3–0.4 mg/mg, higher values have been published, and, in view of the somewhat unconventional definition of standards used here (see Materials and Methods), we attach no special significance to the particular PLP to protein ratio obtained here. Both free ribosome and supernatant fractions contained ~0.07 mg PLP/mg protein; the contamination of the former with some membranous elements was not surprising (47), and the addition to the bulk supernatant of the

SM and RM washes would be expected to lead to some transfer of small microsomes to the high-speed supernatant. Analysis of catalase, as a peroxisomal marker (32), revealed ~80% of the activity in the supernatant fraction, which suggested (as might be expected in view of the repeated homogenization involved in this procedure) extensive rupture of peroxisomes. However, since a rather large fraction of rat liver catalase may exist in nonparticulate form (48) the exact extent of peroxisome rupture is difficult to assess. The RM fraction contained ~5% of the recovered catalase activity. Damage to lysosomes was apparently less extensive, since only ~33% of the acid phosphatase activity was released to the supernatant, while an equal amount was found in the mitochondrial fraction (which is equivalent to the M + L fraction of de Duve et al [22]) The RM accounted for ~10% of the recovered acid phosphatase activity. It should be pointed out that while the RM had more catalase activity than the SM, they contained less acid phosphatase.

Electron microscopic examination of the SM and RM fractions corroborated the biochemical analyses As might be predicted from consideration of the fractionation scheme, the SM (Fig. 3) consisted of a fairly heterogeneous population of membranous vesicles Most were smooth-surfaced, but occasional ribosome-studded vesicles were found. In addition, mitochondrial fragments, presumptive lysosomes, and large, flattened sheets (presumably plasma membrane) were present. The RM fraction (Figs. 4 and 5) was considerably more homogeneous, consisting primarily of ribosome-studded vesicles, which, when sectioned tangentially (Fig. 5), showed a fairly high surface density of ribosomes. Very few free ribosomes or smooth-surfaced vesicles were found, but occasional mitochondrial fragments, damaged lysosomes, and peroxisomal cores were detected.

It should be noted that the micrograph in Fig. 4 is representative of the bulk of the material in a fixed and sectioned RM pellet. Sections cut from top or bottom regions of the pellet did not show a significantly higher degree of heterogeneity. Figs 3 and 5 were obtained from material prepared, using a modification of the Millipore filter technique (44), for random sampling of the fractions and therefore provide a direct, reliable estimate of the quality and homogeneity of the SM and RM fractions. The functionality of the ribosomes in the RM and free ribosome fractions was shown by their activity in an in vitro amino acid incorporation mixture (Fig. 4), both fractions were able to utilize endogenous messenger but were significantly stimulated by the addition of polyuridylic acid. The RM were ~½ as active as their counterpart fractions, prepared by more conventional procedures (17, 18) and assayed under identical conditions (data not shown). Examination of the free polyribosomes and of the polyribosomes released from RM by detergent treatment indicated fairly extensive degradation of messenger RNA, since the sucrose density gradient profiles (not included here) showed maximal absorption in the trimer-pentamer region (cf. profiles in reference 45). The degradation was more severe if high-speed supernatant (IS) was not used in the fractionation. Inclusion of IS in the media used to resuspend RM and ribosome pellets before detergent treatment and/or gradient analysis improved the polysome profiles, suggesting that the degradation was, at least in part, due to ribonuclease present in the final fractions

The data included here indicate the possibility of isolating rough microsomal fractions which contain a large proportion of all membrane-bound ribosomes in rat liver and are suitable for many biochemical studies. Our procedure is in no way a radical departure from previously published ones. Procedures involving sedimentation through concentrated sucrose media to purify nuclei (26, 27), washes with simple sucrose media to reduce the microsomal contamination of mitochondria (22, 23), and sedimentation through discontinuous sucrose density gradients to separate SM, RM, and free ribosome fractions (6, 7, 17, 18) are in common use. Similarly, it is fairly common knowledge that in most standard procedures (19–21), much of the microsomal population cosediments with the nuclei and mitochondria, and that the presence of mono- or divalent cations in the sucrose media used for fractionation induces clumping and aggregation (3, 24) of membranous organelles.

Making use of the available information, we have simply attempted to design a fractionation scheme which would allow the preparation of a rough microsome fraction more representative of the total rough endoplasmic reticulum and therefore better suited to our studies of ribosome-membrane interaction (28, 29) than the more routinely used fractions The RM fraction obtained by the procedure described here represents

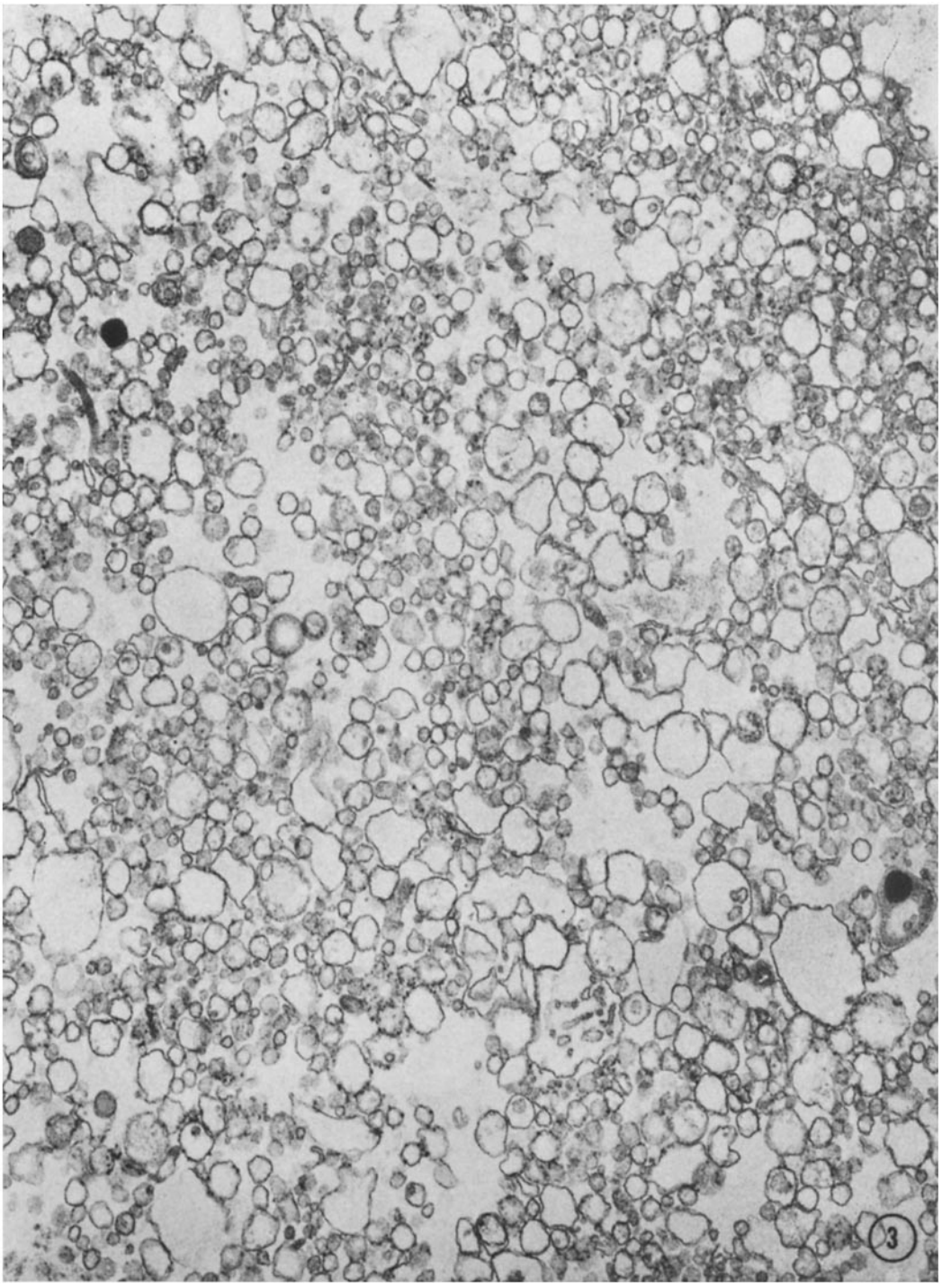

FIGURE 3 Random sample of a smooth microsome fraction prepared by the technique of Baudhuin et al. (44). Most vesicles are smooth, but a few ribosome-studded vesicles, mitochondrial fragments, and presumptive lysosomes are also seen. × 30,000.

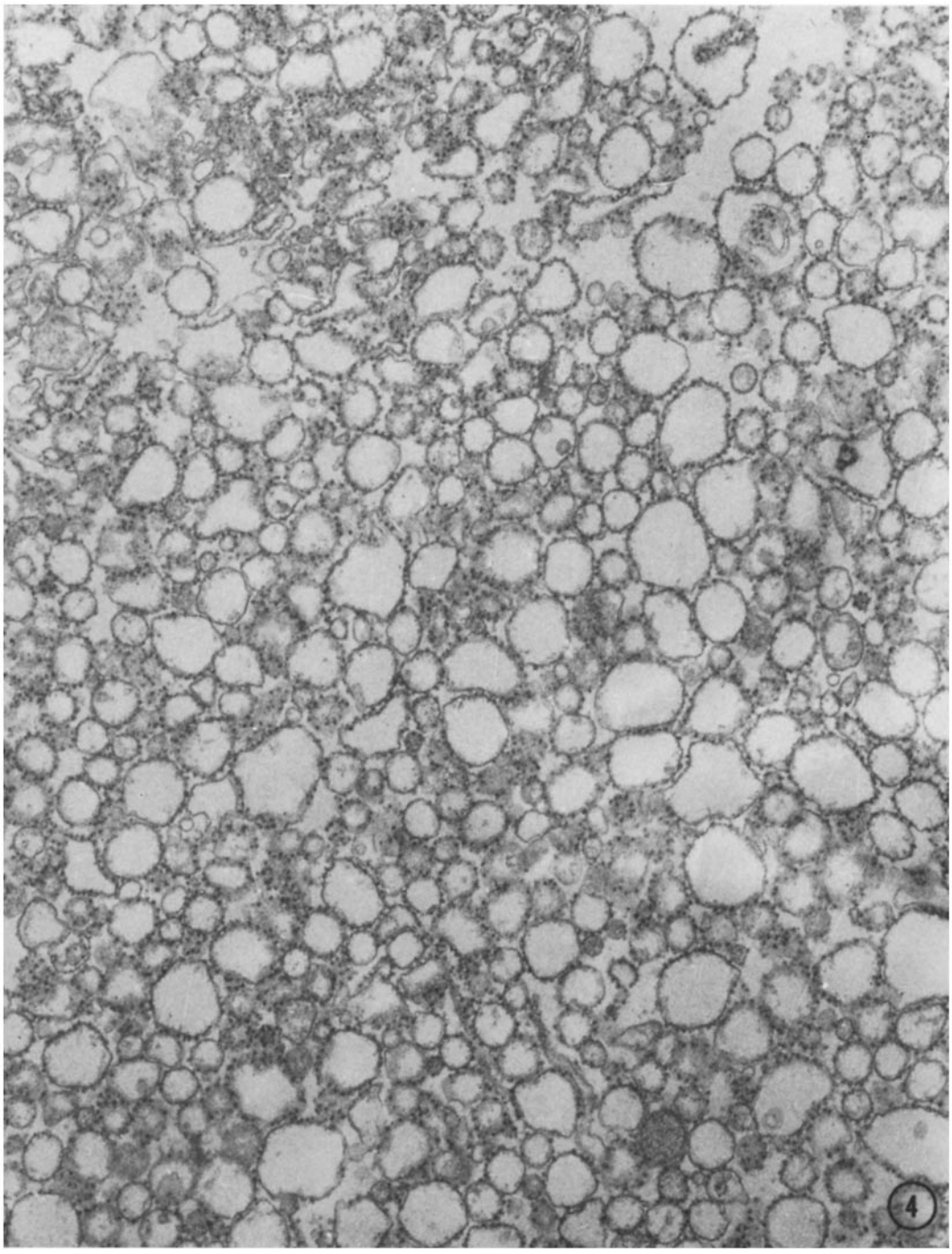

FIGURE 4 Representative view of section through a pellet of rough microsomes. Most vesicles are studded with ribosomes. A few contaminating dense bodies are seen in the field. × 30,000.

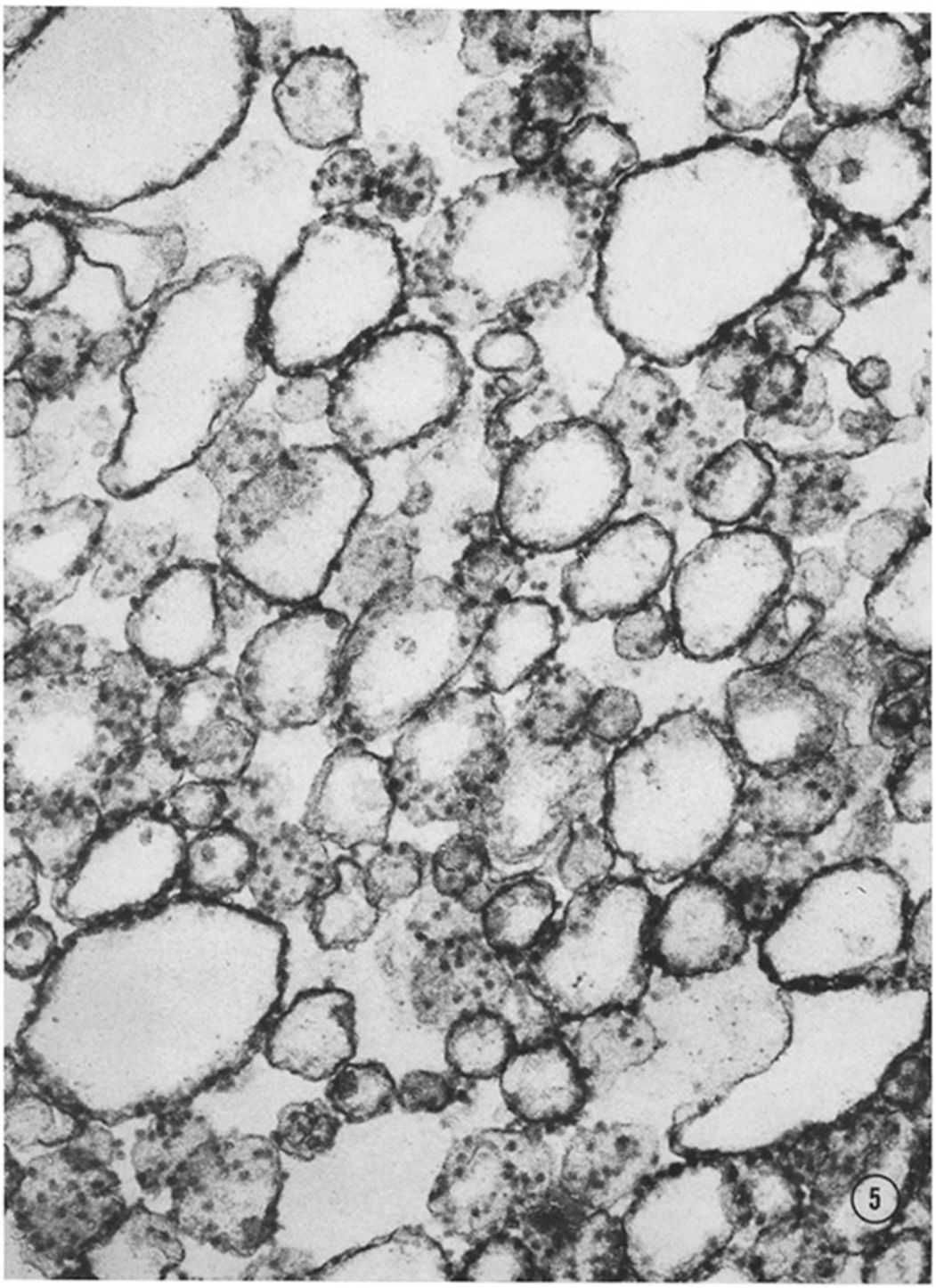


FIGURE 5 Random sample of a rough microsome fraction prepared as the smooth microsomes shown in Fig. 3. Surface view of the vesicles show numerous membrane-bound ribosomes. × 80,000.

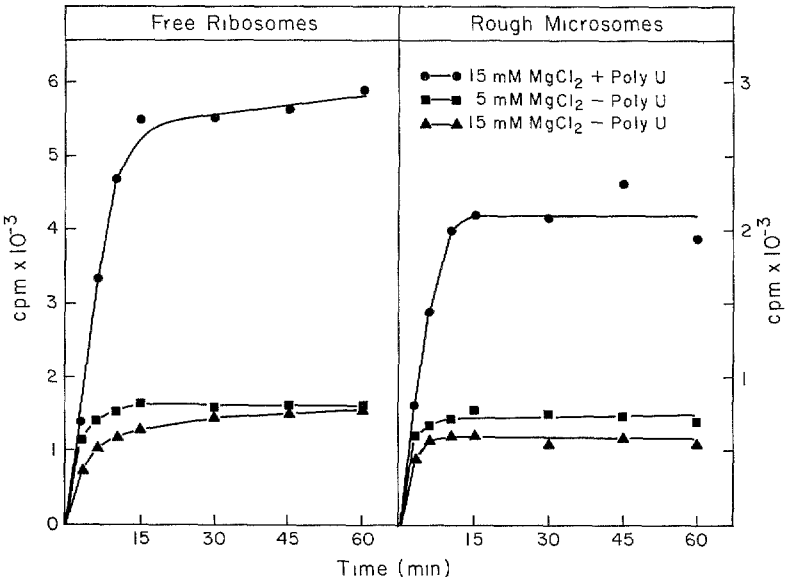


FIGURE 6 In vitro amino acid incorporation by free ribosomes and membrane-bound ribosomes (rough microsomes). At the indicated time points, 100-μl samples containing 84 μg RNA (free ribosomes) or 76 μg RNA (rough microsomes) were assayed for incorporation of [$^{14}$C]phenylalanine into hot acid-insoluble material. ■—■, 5 mM Mg, no polyuridylic acid; ▲—▲, 15 mM Mg, no polyuridylic acid; ●—●, 15 mM Mg plus polyuridylic acid.

almost 50% of the total membrane-bound ribosomes of rat liver. The RNA/protein ratio of 0 20–0.25 mg/mg is in good agreement with published values (6, 7). Other analytical and enzymatic data also suggest a fair degree of purity for the fraction, a conclusion which is supported by the electron microscopic observations. The RM are reasonably active in in vitro amino acid incorporation (Fig. 6), and, as shown elsewhere (28, 29), can be stripped of their bound ribosomes in a simple, nondestructive, and functionally significant manner. These rough microsomes seem, therefore, well-suited for studies of the site or sites at which bound ribosomes interact with the membranes of the endoplasmic reticulum

The disadvantages of this fractionation procedure are that it is somewhat time-consuming, and that the repeated homogenizations lead to fragmentation of mitochondria, lysosomes, and peroxisomes, and to significant cross-contamination of the RM fraction Significant degradation of polysomal messenger RNA also occurs We have not succeeded in eliminating the trapping of endoplasmic reticulum elements by the mitochondrial fraction, nor have we elucidated the nature of this interaction. These difficulties are the subject of continuing investigation

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