

TWO FRACTIONS OF ROUGH ENDOPLASMIC RETICULUM FROM RAT LIVER

I. Recovery of Rapidly Sedimenting Endoplasmic Reticulum in Association with Mitochondria

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ABSTRACT

Low-speed centrifugation (640 *g*) of rat liver homogenates, prepared with a standard ionic medium, yielded a pellet from which a rapidly sedimenting fraction of rough endoplasmic reticulum (RSER) was recovered free of nuclei. This fraction contained 20–25% of cellular RNA and approximately 30% of total glucose-6-phosphatase (ER marker) activity. A major portion of total cytochrome *c* oxidase (mitochondrial marker) activity was also recovered in this fraction, with the remainder sedimenting between 640 and 6,000 *g*. Evidence is provided which indicates that RSER may be intimately associated with mitochondria. Complete dissociation of ER from mitochondria in the RSER fraction required very harsh conditions. Sucrose density gradient centrifugation analysis revealed that 95% dissociation could be achieved when the RSER fraction was first resuspended in buffer containing 500 mM KCl and 20 mM EDTA, and subjected to shearing. Excluding KCl, EDTA, or shearing from the procedure resulted in incomplete separation.

Both electron microscopy and marker enzyme analysis of mitochondria purified by this procedure indicated that some structural damage and leakage of proteins from matrix and intermembrane compartments had occurred. Nevertheless, when mitochondria from RSER and postnuclear 6,000-*g* pellet fractions were purified in this way from animals injected with [³⁵S]methionine ± cycloheximide, mitochondria from the postnuclear 6,000-*g* pellet were found to incorporate approximately two times more cytoplasmically synthesized radioactive protein per milligram mitochondrial protein (or per unit cytochrome *c* oxidase activity) than did mitochondria from the RSER fraction. Mitochondria-RSER associations, therefore, do not appear to facilitate enhanced incorporation of mitochondrial proteins which are newly synthesized in the cytoplasm.

During homogenization of rat liver tissue, rough endoplasmic reticulum (ER) is fragmented to form a heterogeneous population of vesicles and cisternae which exhibit a highly heterodisperse sedimentation pattern upon centrifugation, pellet-

ing anywhere between 100 and 100,000 *g*. This has been demonstrated in several studies (for example, see references 1–3, 9, 15, 18), but the question still remains whether such variability in sedimentation *in vitro* reflects heterogeneity in

either structure or function of this organelle in vivo. Problems arise particularly in studies concerned with isolating rough ER free of other organelles, but under conditions which do not disrupt ribosome-membrane attachments. Consequently, biochemical tests are often restricted to the component of total rough ER which can be easily purified (1, 3, 27) from postmitochondrial supernates even though this fraction may represent a relatively small portion (10–50%) of total.

Earlier studies in this laboratory (18) compared the distribution and morphology of rough ER between low- and high-speed sediments from rat liver homogenates which had been prepared with a standard ionic medium (designated rapidly sedimenting rough endoplasmic reticulum, RSER, and microsomal rough ER, respectively). Even after vigorous homogenization of liver tissue, the RSER fraction contained rather large stacks of cisternae which retained the familiar bilamellar configuration normally seen in vivo, whereas microsomal rough ER was recovered, as expected, consisting only of small vesicles. Moreover, when RSER was separated from nuclei by differential centrifugation, electron microscopy revealed that the fraction also contained a large number of mitochondria in close association with the rough ER lamellae. The present paper is concerned primarily with extending this observation, whereas the following paper (27) compares the mRNA content of RSER and microsomal ER.

Mitochondria in rat hepatocytes exhibit heterogeneity in both size and morphology, conditions which are considered to reflect differences in respiratory state and biosynthetic activity (for recent reviews, see references 4, 21). It is possible that such differences may correspond to different topographical positions of mitochondria within the cell, particularly as this pertains to associations of mitochondria with different organelles. In the present study, for example, evidence is also provided which indicates that mitochondria which are closely associated with RSER exhibit relatively low incorporation of cytoplasmically synthesized proteins compared to mitochondria recovered from a postnuclear 6,000-g pellet.

MATERIALS AND METHODS

Animals

110–130-g male rats (Sprague-Dawley strain) were maintained in wire-floored cages and provided with standard food pellets and water ad lib. Starvation procedures were specifically avoided in this study. Animals were

sacrificed by cervical dislocation and the livers were quickly removed for analysis.

Subcellular Fractionation

The procedure described here is similar to one previously reported by Lewis and Tata (18). Freshly excised liver (5.0–5.5 g) was placed in 50 ml of cold (2°C) homogenization medium containing 0.35 M sucrose, 50 mM Tris acetate, pH 7.6, 25 mM KCl, and 10 mM Mg acetate (designated 0.35S T₅₀ K₂₅ M₁₀). After determining fresh weight, tissue was minced with scissors and washed twice more. Homogenization (2°C) was carried out in 3 vol of medium in a glass-Teflon Potter-Elvehjem homogenizer (Jencors Scientific Ltd., Hemel Hempstead, Herts., England) (radial clearance 0.15–0.20 mm when cold) rotating at ~1,100 rpm (10 up-and-down strokes). After passing through nylon cloth, the homogenate was centrifuged at 640 g (10 min) to yield a low-speed pellet from which a membrane fraction designated rapidly sedimenting endoplasmic reticulum (RSER [18]) was recovered as follows: the pellet was suspended to 15 ml in 0.4S T₅₀ M₅ (0.4 M sucrose, 50 mM Tris acetate, pH 7.6, 5 mM Mg acetate), diluted with 9 ml of H₂O, given five strokes with a glass-Teflon homogenizer, and the suspension layered on 12 ml 0.4S T₅₀ M₅ in a conical tube and centrifuged at 240 g for 10 min. Nuclei, cell debris, and erythrocytes were collected as a large pellet while the supernate retained the RSER fraction. For sucrose gradient analyses, particulate material in this fraction was first collected by centrifuging at 15,000 g for 15 min.

A second membrane fraction was obtained from the first 640-g supernate. After diluting with 1 vol of homogenization medium, material sedimenting at 6,000 g (10 min) was collected, resuspended, and recentrifuged. This fraction was denoted “640–6,000 g mitochondrial pellet”.

Sucrose Density Gradient Analysis of the RSER and 640–6,000-g Mitochondrial Pellet Fractions

Routinely, RSER and 640–6,000-g pellet fractions were resuspended in 0.35S T₅₀ M₅ (2.5–4.0 mg protein/ml) in a glass-Teflon homogenizer (four strokes). 1.0-ml Aliquots were layered on 12.0-ml linear gradients of 25–60% (wt/vol) sucrose containing T₅₀ M₅ buffer. Centrifugation was carried out for 2 h (2°C) at 20,000 rpm in a Beckman SW-40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 0.5-ml Fractions were collected by pumping dense sucrose (1 ml/min) through the bottom of gradient tubes.

In experiments which were specifically designed to separate the ER and mitochondria components present in the two membrane fractions, the latter were resuspended in 0.35S T₅₀ K₅₀₀ E₂₀ (0.35 M sucrose, 50 mM Tris acetate, pH 7.6, 500 mM KCl, and 20 mM EDTA), left on ice for 15 min, and 6.0-ml aliquots were then

subjected to shearing at 2°C for three 2-s periods using an Ultra-Turrax Disintegrator (Janke and Kunkel, Staufen, W. Germany) set at 130 V. 1.0-ml Samples were analyzed as described above except that sucrose gradients contained T₅₀ K₅₀₀ E₂₀ medium.

Marker Enzyme Assays

In all cases, rate-limiting levels of enzyme protein were present in the assays performed. With the exception of glucose-6-phosphatase determinations, measurements were made after adding the detergent Brij 35 (Atlas Chemical Industries, Inc., Wilmington, Del.) (0.5–1.0% final concentration) to the various membrane fractions. Enzyme determinations were made only on freshly prepared membrane fractions.

Glucose-6-phosphatase is present in ER and the nuclear envelope in rat hepatocytes (29) and was assayed (10) using particulate material collected by centrifugation from subcellular fractions or diluted gradient fractions. After suspending in 50 mM cacodylate buffer, pH 6.0, aliquots (100–400 µg protein) were incubated in 1.0 ml of 50 mM cacodylate buffer, pH 6.0, 20 mM glucose-6-phosphate for 20 min at 30°C. TCA was added (5% final concentration) and soluble P_i content was determined (7). Activity is expressed as micromoles P_i liberated per 20 min.

Cytochrome *c* oxidase is located in mitochondria at the inner membrane (25). Assays (8) were performed by incubating enzyme samples (10–60 µg protein) in 2.5 ml of 50 mM phosphate buffer, pH 7.4, 20 µM cytochrome *c* (Sigma type 111, reduced with sodium dithionite). Activity was measured by the loss in absorbance of cytochrome *c* at 550 nm (room temperature), using a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) coupled to a digital absorbance meter. The rate of cytochrome *c* oxidation was calculated using an extinction coefficient of 18.5/mM per cm (14).

Monoamine oxidase (located in the outer membrane of mitochondria, [25]) was measured (30) in a reaction mixture containing 25 µl from gradient fractions (up to 50 µg protein), 250 µl of 0.2 M phosphate buffer, pH 7.4, and 25 µl of [³H]tryptamine (0.25 mM, 0.58 µCi). Incubation was carried out for 20 min at 37°C. Activity was estimated as described in reference 30 by measuring the conversion of [³H]tryptamine to [³H]indoleacetic acid. HCl (0.4 ml, 1.0 N) and toluene (6.0 ml) were added to reaction tubes and the contents were vigorously mixed. The mixtures were centrifuged at 1,000 *g* for 10 min, and the radioactivity in the toluene phase was determined (up to 40,000 cpm incorporated per reaction).

Adenylate kinase activity present in mitochondria is located in the space between the inner and outer membranes (25). It was assayed at room temperature by a coupled reaction as described in reference 25. Reactions contained in 1.5 ml: up to 150 µg of protein from gradient fractions, 0.75 mM NADP⁺, 15 mM glucose, 3

mM ADP, 0.45 mM KCN, 70 mM Tris acetate, pH 9.0, 5 mM Mg acetate, 15 U of hexokinase, and 0.6 U of glucose-6-phosphate dehydrogenase. The increase in absorbance (340 nm) which occurred between 1.5 and 2.5 min after the start of the reaction was recorded (up to 0.14 OD U per reaction).

Malate dehydrogenase in mitochondria is located in the matrix (5). Assays (22) contained 10–100 µg of protein from gradient fractions in 3.0 ml of a medium containing 25 mM Tris acetate, pH 7.4, 50 µM NADH, and 250 µM oxaloacetate. The loss in absorbance of NADH was followed at 340 nm (up to 0.2 OD U/min per reaction).

In Vivo Radioisotope Incorporation into Mitochondria

Nonstarved rats were given a single intraperitoneal injection of [³⁵S]methionine (100–200 µCi, 260 Ci/mM) contained in 0.6 ml of 0.15 N NaCl 2 h before death. In experiments with cycloheximide, the inhibitor (5 mg in 0.5 ml of saline) was injected 15 min before and again 1 h after the injection containing label. RSER and 640–6,000-g pellet fractions were resuspended in 0.35S T₅₀ K₅₀₀ E₂₀ medium, subjected to Ultra-Turrax treatment, and sedimented in sucrose gradients as described above, except that 5.0-ml aliquots of resuspension (12–18 mg protein) were layered on 33 ml, 25–60% sucrose gradients and centrifuged for 2 h at 20,000 rpm in a Beckman SW27 rotor. Regions of the gradient corresponding to mitochondria, as located by cytochrome *c* oxidase activity, were removed and diluted with H₂O containing 50 mM unlabeled methionine. An equal volume of 10% TCA was added and mixtures were left on ice for 30 min and then placed in a hot (90°C) water bath for 15 min. TCA-insoluble material was collected by centrifugation (10,000 *g*, 10 min) and further extracted with hot 5% TCA (4 mM methionine) for 15 min, cooled, collected on glass-fiber filters (Whatman GF/C), washed twice with 5% TCA, dried, and the radioactivity was measured in scintillation fluid using a Beckman LS-250 Liquid Scintillation System (Beckman Instruments, Inc., Fullerton, Calif.) (90% efficiency).

Electrophoresis of Mitochondrial Proteins

Solubilized mitochondrial proteins were analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis using a discontinuous buffer system by a procedure modified from Laemmli (17). Mitochondria (free of ER) were isolated by sucrose gradient centrifugation as described above, extracted with TCA, and dissolved at 80°C in SDS sample buffer (5% SDS [wt/vol], 0.7 M 2-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl, pH 6.7, 0.01% bromophenol blue). Slab gels were prepared by standard procedures to give a stacking region (3% acrylamide, 0.08% bis-acrylamide, 60 mM Tris-HCl, pH 6.7, 0.1% SDS) and a resolving region

(12.5% acrylamide, 0.33% bis-acrylamide, 0.375 M Tris-HCl, pH 8.9, 0.1% SDS). Electrophoresis was carried out for 12–15 h (room temperature) at 30 V in a buffer containing 50 mM Tris-HCl, pH 8.3, 380 mM glycine and 0.1% SDS. Gels were stained with 0.2% Coomassie Brilliant Blue. For determination of radioactivity in gels, 2-mm slices were collected, dissolved in 0.5 ml of hydrogen peroxide (30% wt/vol) at 60°C, and ³⁵S measured in scintillation fluid (70% toluene, 30% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) and 5 g/liter, 2,5-diphenyloxazole).

Chemical Assays

Protein estimations were made on TCA-insoluble material by the method of Lowry et al. (19). RNA was measured by the Fleck and Munro procedure (11). DNA was measured with the diphenylamine reagent (6), and inorganic phosphate was assayed with the ammonium molybdate reagent (7).

Electron Microscopy

Freshly excised liver tissue was quickly diced at 2°C in 2.5% glutaraldehyde, 0.1 M cacodylate, pH 7.2, and incubated for 5 min with three changes of solution. After washing briefly (2–3 min) with buffer at 2°C, the tissue was postfixed (1 h, room temperature) with 2% osmium tetroxide buffered with 0.1 M cacodylate, pH 7.2.

Membrane preparations were pelleted and fixed overnight at 2°C in 2% glutaraldehyde, 0.2 M sucrose, and 0.1 M cacodylate, pH 7.2. Pellets were diced, washed, and postfixed (2 h, room temperature) in a 2% osmium tetroxide solution containing 7% glucose and 0.1 M cacodylate, pH 7.2. In certain instances (Figs. 3 and 4), fixations were performed with 0.1 M phosphate buffer, pH 7.2.

After dehydrating in a graded series of alcohol concentrations (30–100% over 3–4 h), material was embedded in an Araldite mixture (13), sectioned, stained with 1% uranyl acetate and alkaline lead citrate (23), and examined in a Philips EM model 300 or 201.

Chemicals

[³⁵S]Methionine was purchased from the Radiochemical Centre, Amersham, England. Chemicals for electron microscopy were purchased from TAAB Laboratories, Reading, England. All other chemicals used in this study were of analytical grade and were purchased from either BDH Chemicals Ltd., Poole, England or Sigma London Chemical Co., Surrey, England.

RESULTS

Fig. 1 shows an electron micrograph of a typical thin section through a rat hepatocyte. It is included here to emphasize the distinctive morphology of hepatic rough ER. This membrane is not distributed uniformly throughout the cytoplasm,

but rather it exists primarily as clusters of cisternae which are in turn closely associated with mitochondria.

Distribution of Mitochondria and ER between Subcellular Fractions

Table I records the distribution of glucose-6-phosphatase (ER marker), cytochrome *c* oxidase (mitochondrial marker), and RNA after fractionation of liver homogenates by differential centrifugation. No attempt was made to include ingredients in the homogenization medium which are widely used to “free” mitochondria from adhering ER (e.g., EDTA [24]; or low ionic-strength media [26]).

Consequently, the majority of mitochondria (>75%, as judged by marker enzyme levels) were found to cosediment with a substantial portion of total ER (>45%), and were recovered in a low-speed (640 g) pellet. Moreover, upon further fractionation of this low-speed pellet, these mitochondria were found primarily (70–80%) in the RSER fraction. This fraction also contains 20–25% of total cellular RNA and approximately 30% of total glucose-6-phosphatase activity.

A striking feature of the RSER fraction (Fig. 2 and reference 18) is that rough ER largely retains the bilamellar configuration in which this membrane is normally encountered in intact cells (Fig. 1). Fig. 2 also shows the close association between mitochondria and ER in pelleted RSER fractions, which again is reminiscent of the situation *in vivo*.

In contrast to RSER, microsomal rough ER recovered from 6,000-g (postmitochondrial) supernates is well known to exist as small vesicles under standard ionic conditions (e.g., references 1, 3, 9, 28). In Table I, 40–45% of total glucose-6-phosphatase activity is present in the postmitochondrial supernate. The small amounts of ER which are found in postnuclear mitochondrial pellets (640–6,000 g pellet, see Table I) exhibit a heterogeneous morphology (Fig. 4) consisting of vesicles and small cisternae.

Separation of ER and Mitochondria Components of the RSER and 640–6,000-g Pellet Fractions

To investigate whether mitochondria in the RSER fraction are physically attached to ER membrane, attempts were made to separate the two components by means of linear sucrose gradients. When total RSER membrane was sedi-

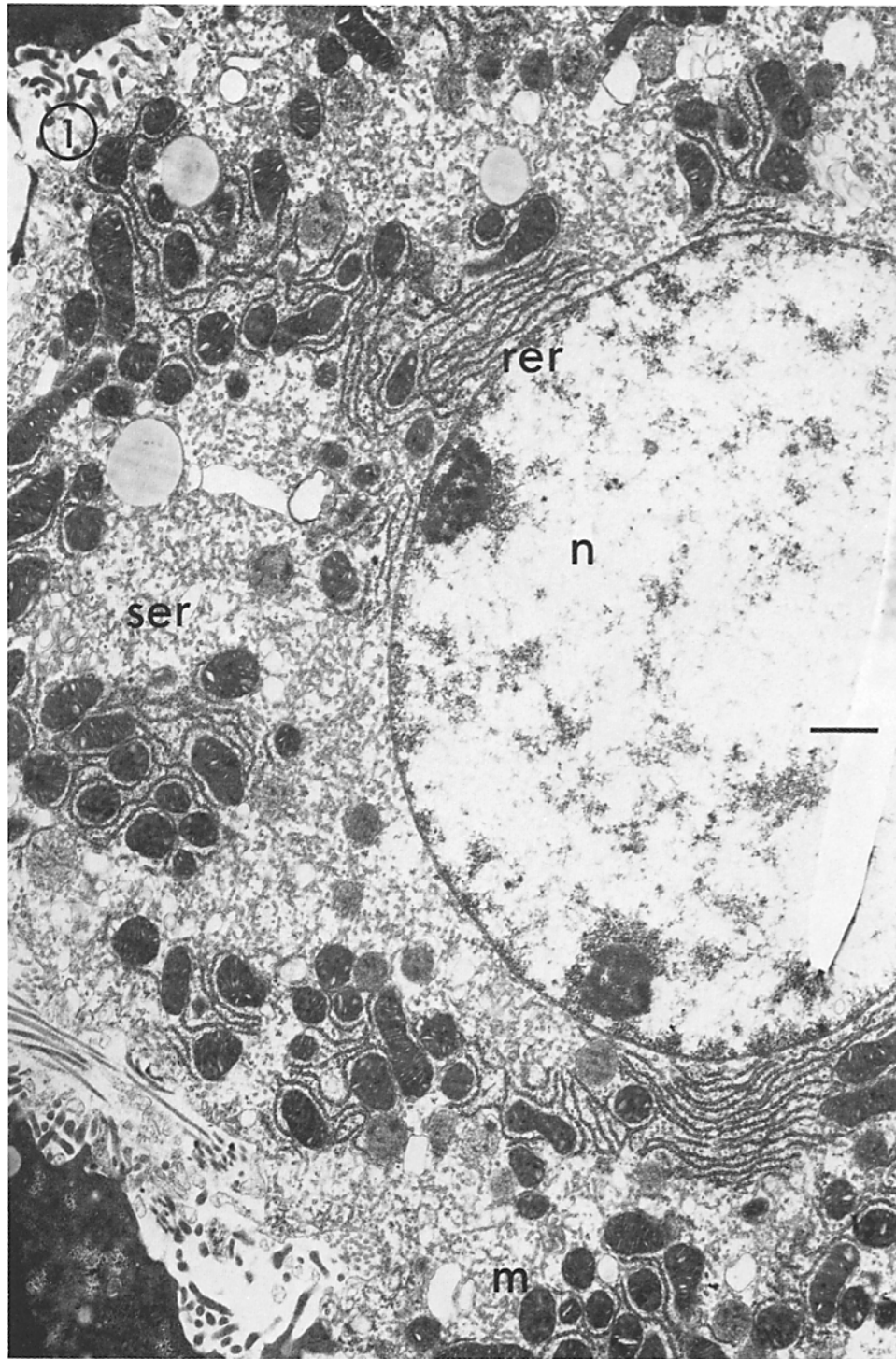


FIGURE 1 The morphology of hepatic rough endoplasmic reticulum in vivo. Thin sections of liver tissue from nonstarved rats were prepared as described in Materials and Methods. A typical hepatocyte is shown. nucleus (*n*); rough endoplasmic reticulum (*rer*); smooth endoplasmic reticulum (*ser*) mitochondrion (*m*). $\times 9,500$. Bar, $1.0 \mu\text{m}$.

TABLE I
Distribution of RNA and Marker Enzyme Activities for ER and Mitochondria between Various Subcellular Fractions Obtained from Rat Liver*

Fraction	RNA	Glucose-6-phosphatase	Cytochrome <i>c</i> oxidase
	mg/g liver	U/g liver	U/g liver
Homogenate	8.6	190	22.7
640 g pellet	3.6	96	16.0
Nuclear fraction	1.8	45	3.0
RSER fraction	1.8	56	11.2
640-6,000 g pellet	0.67	24	4.0
6,000 g supernate	3.9	80	0.2
% Recovery	95	105	89

* Liver tissue (5 g) was homogenized in 3 vol of 0.35S T_{50} K_{25} M_{10} medium, pH 7.6, and fractionated according to the scheme described in Materials and Methods. Glucose-6-phosphatase and cytochrome *c* oxidase activities are expressed as micromoles P_i liberated per 20 min and micromoles cytochrome *c* oxidized per minute, respectively. In this experiment, DNA was found only (>95%) in association with nuclei.

mented in 25-60% sucrose gradients containing T_{50} M_5 buffer, the majority of glucose-6-phosphatase activity in this fraction was found to cosediment with mitochondria at an approximate density of 1.2 g/cm³ (fractions 20-24, Fig. 5 a). If the RSER fraction was first resuspended in 0.35S T_{50} K_{500} E_{20} buffer (i.e. conditions which resulted in almost complete release of 18 and 28S rRNA from ER, data not shown), then a major portion of ER marker (62%) still cosedimented with mitochondria, although the complex was recovered at a lower density (approximately 1.16 g/cm³) in the gradient (fractions 10-13, Fig. 5 b). The shift to the lighter density was presumably a consequence of stripping of ribosomes from the ER and shrinkage of the mitochondria matrix due to hypertonicity of the medium. Complete separation (>95%) of RSER membrane from mitochondria was achieved (Fig. 5 c) by subjecting the RSER fraction to shearing in the presence of KCl and EDTA using an Ultra-Turrax disintegrator. In this instance, mitochondria were again recovered at density 1.16 g/cm³ (fractions 10-14, Fig. 5 c) whereas less than 5% of total glucose-6-phosphatase activity present in RSER was recovered in these same fractions. Rather, glucose-6-phosphatase now banded at approximately 1.11 g/cm³ (fractions 2-5), typical of vesicularized ER devoid of attached ribosomes. Separation of these components was not achieved if either KCl or EDTA was absent.

The sequence of procedures described in Fig. 5 a-c for dissociating ER and mitochondria was repeated for the 640-6,000-g pellet fraction (Fig. 6 a-c). Under all of these conditions, the sedimentation patterns of cytochrome *c* oxidase were similar

to those obtained for the RSER fraction. And even though a relatively small fraction of total ER is recovered in 640-6,000-g pellets (Table I), glucose-6-phosphatase activity in this fraction still approximately cosedimented with mitochondria under nondissociating conditions (no KCl and EDTA, Fig. 6 a), but complete (95%) separation was achieved by shearing in the presence of 500 mM KCl and 20 mM EDTA (Fig. 6 c), i.e., similar to the situation for RSER shown in Fig. 5 a and c. Differences were obtained, however, in the sedimentation characteristics of glucose-6-phosphatase activity in 640-6,000-g pellets compared to RSER under conditions of only partial dissociation (i.e., KCl, EDTA, but no Ultra-Turrax treatment). Whereas 62% of glucose-6-phosphatase activity in RSER cosedimented with mitochondria under these conditions (Fig. 5 b), the corresponding value in Fig. 6 b is at most 20%. A major fraction (40%) of ER marker enzyme was recovered from near the top of the gradient, with the remaining 60% sedimenting broadly throughout the gradient. Thus, if physical associations exist between ER and mitochondria in the 640-6,000-g mitochondrial pellet fraction, these are evidently more labile than interaction between ER and mitochondria in the RSER fraction.

Electron micrographs of mitochondria isolated from RSER fractions using the Ultra-Turrax technique show (Fig. 3) that they were recovered free of ER, but also reveal that a certain amount of shrinkage of inner matrix and at least partial disruption of outer membrane occurred. To further test the extent of this loss of structural integrity, experiments were performed similar to those described for Fig. 5 a and c, except that the distribution in these gradients of a variety of mitochondrial enzymes, each of which occupies a different compartment within the mitochondrion, was determined (Fig. 7). Leakage of enzymic protein (20-30%) evidently occurred from the intermembrane compartment (as distinguished by adenylate kinase activity) and from the inner matrix (malate dehydrogenase), whereas less than 8% of monoamine oxidase activity (outer membrane) and no cytochrome *c* oxidase activity (inner membrane) were released. Similar results were obtained for the 640-6,000-g pellet fraction (data not included).

Cytoplasmic Synthesis of Mitochondrial Proteins In Vivo

Table II compares the incorporation of radioactive protein into mitochondria recovered from the

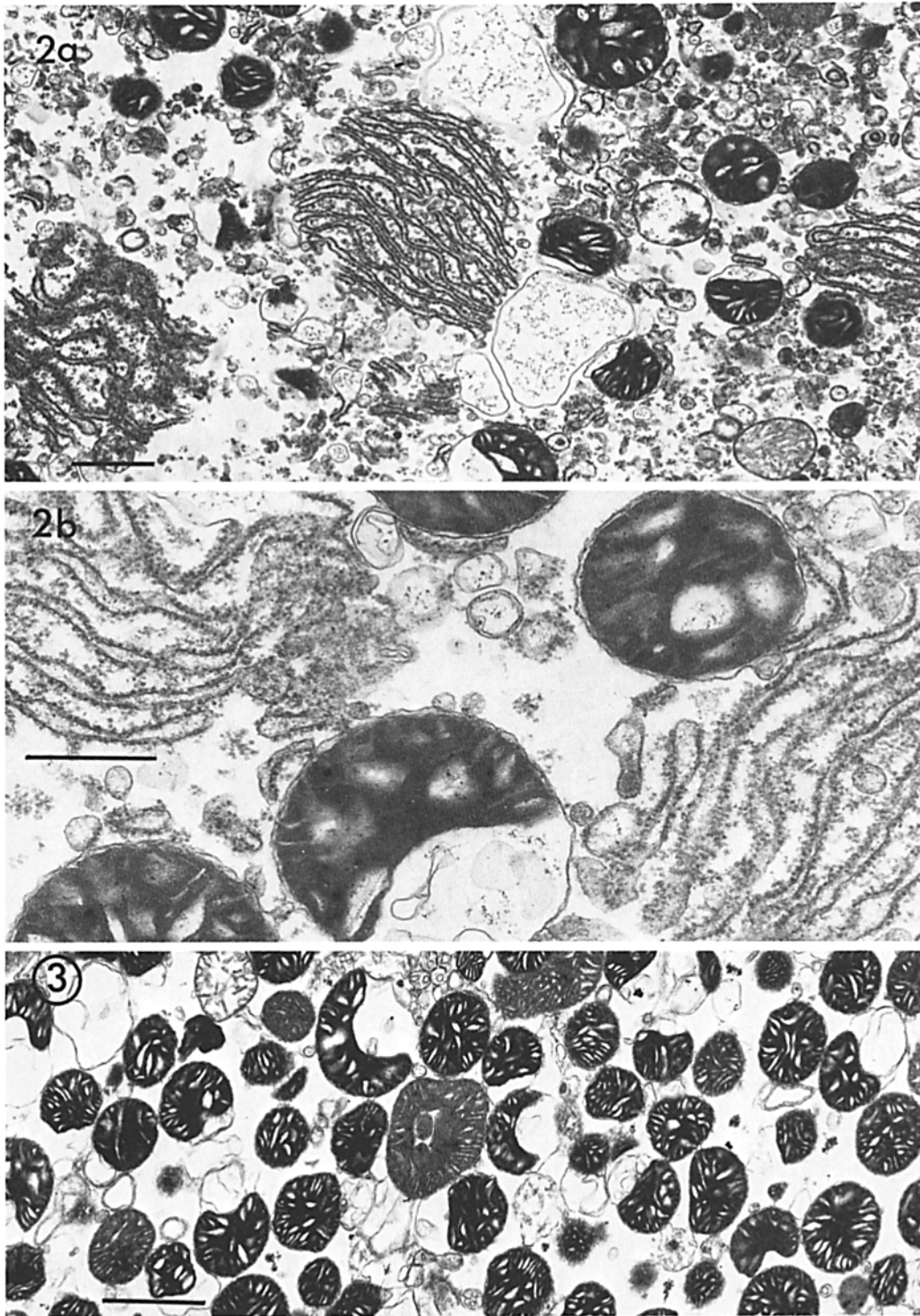


FIGURE 2 Electron micrographs of the RSER fraction. (a) $\times 12,300$. Bar, $1.0 \mu\text{m}$. (b) $\times 38,400$. Bar, $0.5 \mu\text{m}$.

FIGURE 3 Electron micrograph showing mitochondria purified from the RSER fraction by sucrose density gradient sedimentation after treatment with 500 mM KCl , 20 mM EDTA , and shearing with the Ultra-Turrax. In many instances, the inner membrane and matrix have contracted and consequently are seen well separated from the outer membrane. $\times 15,300$. Bar, $1.0 \mu\text{m}$.

RSER fraction and from the 640–6,000-g pellet fraction after injection of animals with [³⁵S]methionine. The radioactivity in mitochondria isolated from the RSER fraction was 50% greater than in mitochondria present in the 640–6,000-g pellet fraction from an equivalent amount of tissue. However, the former has a mitochon-

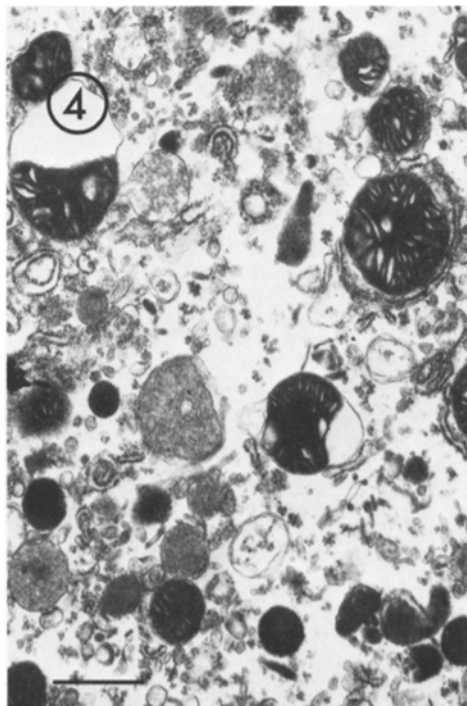


FIGURE 4 Electron micrograph of the 640–6,000-g pellet fraction. $\times 22,000$. Bar, 0.5 μm .

drial content which is three times greater than the latter. This is based on protein measurements (Table II), but a comparison of cytochrome *c* oxidase activity in the two fractions (Table I) yields a similar value. Thus, per milligram protein, mitochondria purified from the 640–6,000-g pellet fraction incorporated radioactivity to an extent 2.2 times greater than did mitochondria from the RSER fraction. When the data are expressed per unit cytochrome *c* oxidase activity, the magnitude of this ratio is again very similar (i.e. 1.9 times greater); no cytochrome *c* oxidase activity is lost during the purification of mitochondria by the Ultra-Turrax technique (Figs. 5–7). The two fractions of mitochondria, therefore, may not derive from a common population of mitochondria within the liver. Moreover, assuming that a common or similar sized precursor pool of amino acid contributes to the synthesis of mitochondria proteins in the two fractions, mitochondria present in the 640–6,000-g pellet fraction exhibit greater biosynthetic activity than mitochondria associated with RSER. In both instances, however, the radioactive proteins were of cytoplasmic origin. Cycloheximide prevented incorporation of radioactive precursor by 90%, and the remaining 10% did not account for any radioactive peaks when mitochondrial proteins were resolved on an SDS-polyacrylamide gel (see Materials and Methods, data not shown).

DISCUSSION

The portion of total hepatic ER which can be recovered as RSER from low-speed sediments of

TABLE II
*Differences in the In Vivo Incorporation of [³⁵S]Methionine into Proteins of Mitochondria Isolated from the RSER Fraction Compared to Mitochondria from the 640 to 6,000-g Pellet Fraction**

Fraction	Protein		[³⁵ S]Methionine (cpm) incorporated/2 h	
	mg/g liver	per g liver	per mg protein	per U cytochrome <i>c</i> oxidase [†]
RSER	15.3	91,417	5,975	8,160
640–6,000 g pellet	9.8	106,310	10,848	26,575
Mitochondria purified from:				
RSER	3.5	12,803	3,658	1,145
640–6,000 g pellet	1.1	8,846	8,042	2,210

* The table describes a typical experiment in which animals were injected with 100 μCi [³⁵S]methionine and, after 2 h, the RSER and 640–6,000-g pellet fractions were prepared. Mitochondria from the two fractions were purified by sucrose gradient centrifugation in $T_{50} K_{500} E_{20}$ buffer after Ultra-Turrax treatment (Materials and Methods, Figs. 5 and 6) and extracted with TCA. Radioactivity in the insoluble material was measured as described in Materials and Methods. This experiment was repeated twice and yielded results very similar to those presented.

† Enzyme assays were not performed in this experiment but rather were obtained from Table I (11.2 U/g liver for RSER and 4.0 U/g liver for the 640–6,000-g pellet). Table I lists a distribution for cytochrome *c* oxidase activity which has been obtained repeatedly in numerous tests. Under the conditions of mitochondria purification, loss of this enzyme could not be detected (Figs. 5–7).

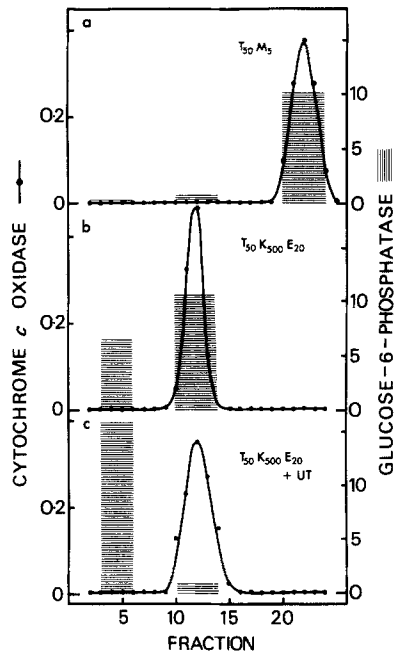


FIGURE 5 Separation of the ER and mitochondria components present in RSER. An RSER fraction was obtained as described in Materials and Methods, pelleted at 15,000 g (15 min), and resuspended in either 0.35S $T_{50} M_5$ (panel a) or 0.35S $T_{50} K_{500} E_{20}$ (panel b) to yield a protein concentration of 3.5 mg/ml. After 15 min on ice, an aliquot of the latter was subjected to shearing with the Ultra-Turrax (UT, panel c). Two 1.0-ml aliquots from each of the three fractions (a, b, and c) were analyzed by centrifuging in a pair of tubes containing matched density gradients (12.0 ml, 25–60% (wt/vol) sucrose and containing the appropriate buffer, $T_{50} M_5$ or $T_{50} K_{500} E_{20}$). Centrifugation was carried out for 2 h (2°C) at 20,000 rpm in a Beckman SW40 rotor after which 0.5-ml fractions were collected from each pair of tubes. One set was analyzed directly for cytochrome *c* oxidase activity (micromoles cytochrome *c* oxidized per minute per fraction). Fractions from the second gradient of each pair which corresponded to regions of the gradient containing a membrane band were pooled, diluted, and particulate material was pelleted by centrifuging for 45 min at 45,000 rpm (Beckman type 65 rotor). Pellets were resuspended in 20 mM cacodylate buffer, pH 7.0, and assayed immediately for glucose-6-phosphatase activity (micromoles P_i liberated/20 min per pooled fractions). The only visible membrane-containing bands in the gradients were those associated with glucose-6-phosphatase or cytochrome *c* oxidase activity. Recovery of total glucose-6-phosphatase activity in the pooled fractions assayed was 65, 98, and 89% for panels a, b, and c, respectively. Ribosomal RNA (18 and 28S) banded with glucose-6-phosphatase in panel a, but in panels b and c it was entirely recovered in the overlay above the gradient.

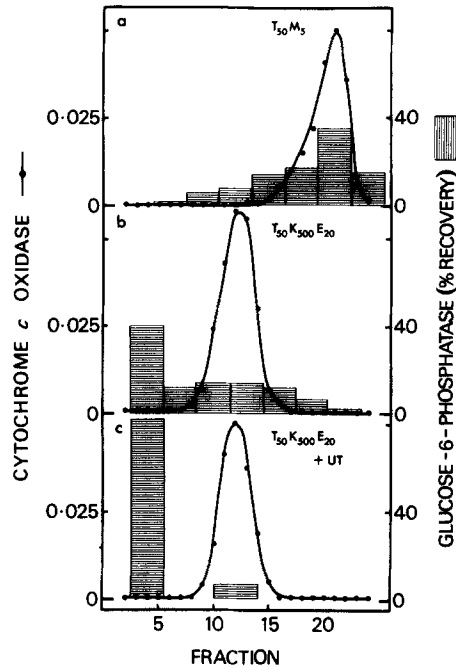


FIGURE 6 Separation of ER and mitochondria components present in the 640–6,000-g pellet fraction. Conditions were exactly as described for Fig. 5. Glucose-6-phosphatase determinations were performed on pooled fractions as indicated in the figure and expressed as percentage of the total activity (~2.2 U) recovered from the gradient.

liver homogenates yields a fraction which is particularly enriched in mitochondria as well (Table I, Fig. 2, and reference 18). In view of the evidence for direct attachment between ER cisternae and mitochondria in the intact hepatocyte (12, 20), an obvious question is whether such attachment exists between RSER and mitochondria *in vitro* and, if so, whether association with RSER is of some physiological significance. In particular, we were especially concerned with the possibility that such an intimate association might serve to facilitate the *in vivo* transfer to mitochondria of proteins newly synthesized on membrane-bound cytoplasmic ribosomes.

As a first step toward investigating possible functions for the association between mitochondria and ER membranes in the RSER fraction, it was necessary to accomplish a quantitative separation of the two organelles. It was found that harsh treatments were required to separate completely RSER from mitochondria. A comparison of data in Table I and Fig. 5 b indicates that even in the presence of 500 mM KCl and 20 mM EDTA,

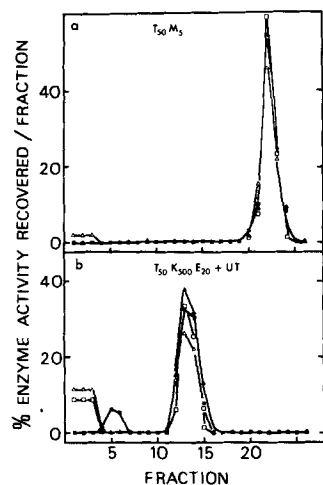


FIGURE 7 Effects of 0.5 M KCl, 20 mM EDTA, and Ultra-Turrax treatment on the structural integrity of mitochondria during their isolation from RSER. Samples from an RSER fraction were resuspended in either 0.35S $T_{50} M_5$ (a), or 0.35S $T_{50} K_{500} E_{20}$ followed by Ultra-Turrax treatment (b), and analyzed by centrifugation through 25–60% (wt/vol) sucrose gradients containing either $T_{50} M_5$ or $T_{50} K_{500} E_{20}$ buffers (conditions the same as for Fig. 5 a and c). Gradient fractions (0.5 ml) were assayed for the following mitochondrial enzymes (see Materials and Methods): monoamine oxidase (■, located in the outer membrane); adenylate kinase (Δ, located between the inner and outer membrane); cytochrome *c* oxidase (▲, located in the inner membrane); and malate dehydrogenase (□, located in the matrix). Enzyme activity per fraction is expressed as the percent of total activity which was recovered from the gradient. The distribution of glucose-6-phosphatase activity in a and b above was the same as in Fig. 5 a and c, respectively.

62% of RSER (equivalent to approximately 18% of total liver ER as judged by glucose-6-phosphatase measurements) still cosediments with mitochondria in sucrose gradients, even though these conditions remove entirely the ribosomes attached to the ER (legend, Fig. 5). A complete separation could be achieved only if the RSER fraction was subjected to a shearing procedure in the presence of KCl and EDTA (Fig. 5 c). A certain amount of damage to the structural integrity of these mitochondria resulted from this treatment (Figs. 3 and 7) but, nevertheless, the method represents the minimal requirements. Complete separation is not achieved if KCl, EDTA, or Ultra-Turrax grinding is excluded, or if RSER fractions are merely washed repeatedly with KCl and EDTA, and recovered by differential centrifugation. That such

harsh conditions are required for separation is interpreted to mean that RSER cisternae and mitochondria membrane are continuous.

In yeast, Kellems et al. (16) have suggested that a large proportion of mitochondrial proteins are synthesized by cytoplasmic ribosomes attached to the surface of mitochondria. A main conclusion from the present study, however, is that ribosomes associated with RSER probably do not fulfill a similar function in rat liver since mitochondria associated with RSER do not preferentially incorporate newly made proteins to an extent predicted by such a scheme. In fact, these mitochondria exhibit cycloheximide-sensitive incorporation of [35 S]methionine *in vivo* at only half the apparent rate exhibited by mitochondria purified from a 640–6,000-g pellet (Table II). Moreover, this conclusion is enhanced by results presented in the following paper (27) which indicate that cytoplasmic mRNA's that code for mitochondrial proteins are present in RSER in relatively low levels compared to the microsomal class of rough ER which is normally obtained *in vitro* from postmitochondrial (6,000 g) supernates. It may be, however, that close associations also exist *in vivo* between this microsomal rough ER and the 'active' mitochondria recovered in the 640–6,000-g pellet fraction, and that these function in the synthesis and transport of proteins to the mitochondrion (see reference 27 for discussion). If true, these associations are presumably more labile during tissue homogenization than RSER mitochondria associations, a conclusion which is supported by the evidence provided in Fig. 6. Finally, it should be emphasized that further work is required to establish whether the different populations of mitochondria and rough ER identified in this study (see also reference 27) occupy different regions within the same cell or derive from different cell populations in the liver lobule.

Even if RSER is not particularly active in the formation of mitochondrial protein (biogenesis), it is difficult to imagine that its close association with mitochondria is without functional significance. An alternative explanation, therefore, is that such an association provides a mechanism for compartmentalizing protein synthesis near the biosynthetic source of ATP, or that the close association facilitates oxidative detoxification processes. Whatever the case, however, the fact that RSER and microsomal rough ER appear to have different levels of mRNA's associated with them which code for mitochondrial proteins (27) strongly implies that

these two fractions in vitro do not arise merely as the result of random and incomplete fragmentation of the ER during homogenization of liver tissue.

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REFERENCES

1. ADELMAN, M. R., G. BLOBEL, and D. D. SABATINI. 1973. An improved cell fractionation procedure for the preparation of rat liver membrane-bound ribosomes. *J. Cell Biol.* **56**:191-205.
2. BERGSTRAND, A., and G. DALLNER. 1969. Isolation of rough and smooth microsomes from rat liver by means of a commercially available centrifuge. *Anal. Biochem.* **29**:351-356.
3. BLOBEL, G., and V. R. POTTER. 1967. Studies on free and membrane-bound ribosomes. I. Distribution as related to total cellular RNA. *J. Mol. Biol.* **26**:279-292.
4. BOELL, E. J., and P. C. GREENFIELD. 1975. Mitochondrial differentiation during animal development. In *The Biochemistry of Animal Development*. R. Weber, editor. Academic Press Inc., New York. **3**:337-386.
5. BRDICZKA, D., D. PETTE, G. BRUNNER, and F. MILLER. 1968. Kompartimentierte verteilung von enzymen in rattenlebermitochondrien. *Eur. J. Biochem.* **5**:294-304.
6. BURTON, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315-323.
7. CHEN, P. S., T. Y. RURIBARA, and H. WARNER. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28**:1756-1758.
8. COOPERSTEIN, S. J., and A. LAZAROW. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* **189**:665-670.
9. DALLNER, G., and L. ERNSTER. 1968. Subfractionation and the composition of microsomal membranes: A review. *J. Histochem. Cytochem.* **16**:611-632.
10. DE DUVE, C., J. BERTHET, H. G. HERS, and L. DUPRET. 1949. Le systeme hexose-phosphatasique. I. Existence d'une glucose-6-phosphatase specifique dans le foie. *Bull. Soc. Chim. Biol.* **31**:1242-1253.
11. FLECK, A., and H. N. MUNRO. 1962. The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. *Biochim. Biophys. Acta.* **55**:571-583.
12. FRANKE, W. W., and J. KARTENBECK. 1971. Outer mitochondrial membrane continuous with endoplasmic reticulum. *Protoplasma.* **73**:35-41.
13. GLAUERT, A. M., and R. H. GLAUERT. 1958. Araldite as an embedding medium for electron microscopy. *J. Biophys. Biochem. Cytol.* **4**:191-194.
14. HATEFI, Y., and J. S. RIESKE. 1967. The preparation and properties of DPNH-cytochrome c reductase (complex 1-111 of the respiratory chain). *Methods Enzymol.* **10**:225-231.
15. HOWELL, R. R., J. N. LOEB, and G. M. TOMKINS. 1964. Characterization of ribosomal aggregates isolated from liver. *Proc. Natl. Acad. Sci. U. S. A.* **52**:1241-1248.
16. KELLEMS, R. E., V. F. ALLISON, and R. A. BUTOW. 1975. Cytoplasmic type 80S ribosomes associated with Yeast mitochondria. IV. Attachment of ribosomes to the outer membrane of isolated mitochondria. *J. Cell Biol.* **65**:1-14.
17. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (Lond.)*. **227**:680-685.
18. LEWIS, J. A., and J. R. TATA. 1973. A rapidly-sedimenting fraction of rat liver endoplasmic reticulum. *J. Cell Sci.* **13**:447-459.
19. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
20. MORRÉ, D. J., W. D. MERRIT, and C. LEMBI. 1971. Connections between mitochondria and endoplasmic reticulum in rat liver and onion stem. *Protoplasma.* **73**:43-49.
21. MUNN, E. A. *The Structure of Mitochondria*. Academic Press, Inc., New York, 465 pp.
22. OCHOA, S. 1955. Malic dehydrogenase from pig heart. *Methods Enzymol.* **1**:735-739.
23. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208-212.
24. ROODYN, D. B., P. J. REIS, and T. S. WORK. 1961. Protein synthesis in mitochondria. *Biochem. J.* **80**:9-12.
25. SCHNAITMAN, C., and J. W. GREENWALT. 1968. Enzymatic properties of the inner and outer membrane of rat liver mitochondria. *J. Cell Biol.* **38**:158-175.
26. SCHNEIDER, W. C. 1959. Mitochondrial metabolism. *Adv. Enzymol. Relat. Areas Mol. Biol.* **21**:1-72.
27. SHORE, G. C., and J. R. TATA. 1976. Two fractions of rough endoplasmic reticulum from rat liver. II. Cytoplasmic mRNA's which code for albumin and mitochondrial proteins are distributed differently between the two fractions. *J. Cell Biol.* **72**:726-743.

28. TATA, J. R. 1972. Preparation and properties of microsomal and submicrosomal fractions from animal cells. *In* Subcellular Components; Preparation and Fractionation. G. D. Birnie, editor. Butterworth & Co. (Publishers) Ltd., London. 185-213.
29. TICE, L. W., and R. L. BARNETT. 1962. The fine structural localization of glucose-6-phosphatase in rat liver. *J. Histochem. Cytochem.* **10**:754-762.
30. WURTMAN, R. J., and J. AXELROD. 1963. A sensitive and specific assay for the estimation of monoamine oxidase. *Biochem. Pharmacol.* **12**:1439-1441.