

TWO FRACTIONS OF ROUGH ENDOPLASMIC RETICULUM FROM RAT LIVER

II. Cytoplasmic Messenger RNA's Which Code for Albumin and Mitochondrial Proteins are Distributed Differently between the Two Fractions

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ABSTRACT

Subcellular fractions were obtained from rat liver homogenates under conditions which prevented degradation of polysomes (pH 8.5 and high ionic strength). Rough endoplasmic reticulum (RER) was recovered in high yields from a low-speed nuclear pellet (rapidly sedimenting endoplasmic reticulum, RSER) and from a postmitochondrial supernate (rough microsomes). The polysomal RNA content of these two fractions was very similar. When polyA⁺-RNA's were translated in the mRNA-dependent wheat embryo cell-free system, both fractions yielded polypeptide products which had similar electrophoretic patterns on sodium dodecyl sulfate (SDS)-polyacrylamide gels. Activities of messenger RNA's which code for albumin and for polypeptides destined for transport to the inner membrane and matrix of mitochondria (i.e. 'mitoplasts') were assayed by translating in the more active rabbit reticulocyte cell-free system followed by immunoprecipitation of radioactive products and coelectrophoresis with immunoprecipitated marker proteins on SDS-polyacrylamide gels. These tests indicated that albumin mRNA is about equally distributed between the two fractions of RER, or slightly enriched in the RSER fraction when activity is expressed as a percentage of total polypeptide synthesis. Activities of cytoplasmic mRNA's which code for at least some mitoplast proteins could be detected in both fractions, but all were enriched in the rough microsome fraction, not the RSER (two- to threefold when corrected for differences in total polypeptide synthesis in the lysate). Comparisons of mRNA's from free vs. membrane-bound polysomes indicated that most of the albumin mRNA activity (86-91%) and mitoplast protein mRNA activities (75%) were present in the bound fraction.

Assuming that RSER and rough microsomes do not derive exclusively from different cell types, the evidence suggests that, compared to albumin and most other membrane-bound mRNA's, cytoplasmic mRNA's coding for mitoplast proteins may be preferentially segregated or compartmentalized within the cell on the microsomal class of RER.

A massive literature has accumulated over the past 10–20 years concerning the rough endoplasmic reticulum (RER) and, to a large extent, the function of this membrane in cells which are specialized for protein secretion has been well characterized (43). Wherever it has been tested, proteins which are destined for export out of the cell are found to be made primarily on polysomes attached to this membrane. It has been emphasized, however, that the converse—all membrane-bound polysomes are involved in the synthesis of secretory proteins—may not be true (2–4, 49, 56, 58). For example, in cells which do not actively secrete proteins (e.g. brain), bound polysomes have been shown to be very active in protein synthesis (2–4). In bacteria, membrane-bound ribosomes may synthesize intracellular proteins (27). Histone messenger RNA is found in both free and bound fractions in cultured plasmacytoma cells (65). Even in liver tissue, there are well-documented examples of both soluble and membrane proteins which are synthesized on the rough endoplasmic reticulum, e.g., serine dehydratase (31, 44, 45), microsomal proteins (25, 47), catalase isozyme (50, 55), mitochondrial proteins (23–25, 34), plasmalemmal 5'-nucleotidase (6). It seems likely, therefore, that additional functions for the RER may very well exist in both secretory and nonsecretory cells (43, 56, 58). In this respect, however, it may be misleading to distinguish between extracellular secretion and the intracellular transport of proteins to other organelles which are not involved in secretion (e.g., mitochondria, plasma membrane, lysosomes in nonsecretory cells). The same mechanism could account for the two processes (43), although, as yet, there is no direct evidence that this is so.

Thus, it has been proposed (56, 58) that the RER may serve to topographically segregate or compartmentalize the synthesis of certain intracellular proteins in particular regions of the cell. This could be achieved in a number of ways. For example, RER itself may be compartmentalized. The close topographical association *in vivo* between RER and certain organelles—most notably, mitochondria and nuclei—is well known to occur (17). In plant cells, RER is found concentrated near the cell plate only during cell division (46). At a more complex level, the ER could also provide a network for the nonrandom segregation of specific mRNA's by creating 'microenvironments' for different populations of polysomes that synthesize different classes of proteins. If true, it might be

expected that heterogeneous fractions of rough endoplasmic reticulum would exhibit heterogeneity in their mRNA content. The present study is concerned primarily with investigating this possibility.

In the previous paper, characteristics of a rapidly sedimenting fraction of rough endoplasmic reticulum (RSER) from rat liver were described. Here, we compare the mRNA content of RSER with the messenger content of the fraction of RER which can be recovered from postmitochondrial supernates (rough microsomes). Subcellular fractions were prepared using media of high pH (8.5) and relatively high ionic strength in order to prevent polysome degradation (15, 36). Translation of mRNA's in a heterologous cell-free system followed by specific immunoprecipitation of labeled polypeptides indicates that, whereas mRNA for a secreted protein (albumin) is distributed about equally between the two fractions of RER, mRNA's coding for proteins which are destined for the mitochondrion are relatively enriched in the rough microsome fraction.

MATERIALS AND METHODS

General

Young male rats (~110–120 g, Sprague-Dawley strain) which had been maintained on standard food pellets and water-fed *ad lib.* were used throughout. For experiments described in this paper, all glassware was rinsed exhaustively in double-distilled H₂O and oven-heated at 160°C for 4–12 h before use. Stock solutions were filtered (Millipore Corp., Bedford, Mass., 0.45 μ m pore size) and those with organic constituents were stored at –20°C. Chemical assays were performed as described in the preceding paper (54), but in certain instances RNA determinations were performed directly by spectrophotometry of solutions containing purified samples (1.0 A₂₈₀, u = 50 μ g RNA).

Isolation of 'Mitoplasts' from

Rat Liver

A mitochondrial fraction containing 350 mg of protein was obtained from 30 g of liver tissue (51). Treatment with digitonin yielded mitoplasts, i.e. mitochondria from which the outer membrane and soluble intermembrane proteins have been removed. The procedure followed was exactly as described in reference 26.

Preparation of Antiserum to Mitoplasts

Mitoplasts were suspended in 1.0 ml of 0.15 M NaCl (1.0 mg of protein), sonicated briefly, and mixed with

1.0 ml of Freund's complete adjuvant. Aliquots were injected into rabbits (Sandy Lops crosses) on days 0 and 7 (intramuscular) and again after 6 wk (subcutaneous). After 3 mo, rabbits were boosted with a series of intravenous injections containing a total of 10 mg of mitoplast protein which had been precipitated with potassium alum. Blood was collected 5 and 7 days later. Serum was prepared and stored containing 3 mM NaN_3 at -20°C .

Antiserum to Purified Rat Serum Albumin

Antiserum to rat serum albumin was raised in goats by Dr. H. Gordon of this institute. When antiserum was reacted against total serum protein, albumin accounted for greater than 97% of the precipitated antigenic product, as judged by Laurell cross-immunoelectrophoresis tests.

Ouchterlony Double Diffusion

Gels were formed in petri dishes and contained phosphate-buffered saline (PBS) (0.15 M NaCl, 4 mM KCl, 2 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 1 mM CaCl_2 , 1 mM MgCl_2), 1.5% (wt/vol) agar (Oxoid Ltd., London), 0.1% deoxycholate, 1.0% Triton X-100 (Koch-Light Laboratories, England) and 3 mM NaN_3 . Samples from subcellular fractions were suspended in PBS containing 1% Triton X-100 (10–16 mg protein/ml) and tested for their reaction against antisera over a 12-h period at 4°C .

Subcellular Fractionation of Liver Homogenates

The scheme used routinely is depicted in Fig. 1. Briefly, the procedure used to prepare RSER and postmitochondrial (6,000 g) supernate was similar to the procedure described in the previous paper (54) except that tissue was washed and homogenized in 0.35S T_{200} K_{50} M_{10} (0.35 M sucrose, 200 mM Tris acetate, pH 8.5 at 2°C , 50 mM KCl, 10 mM Mg acetate) containing 6 mM 2-mercaptoethanol added just before use. A 'total cytoplasmic' (postnuclear) fraction was obtained by removing 2.0 ml from a filtered homogenate, diluting with 0.35S T_{200} K_{50} M_{10} , pH 8.5, 6 mM 2-mercaptoethanol to 20 ml, adding Triton X-100, (1.5% final conc.) and centrifuging at 2,000 g (10 min) to remove nuclear material. The supernate contained all the ribosomes present in the original homogenate aliquot.

The 6,000-g (postmitochondrial) supernate (30 ml from 5 g of tissue) was made 1.6 M sucrose by adding 48 ml of 2.3S T_{200} K_{50} M_{10} , pH 8.5, 6 mM 2-mercaptoethanol. Aliquots (9.0 ml) were layered over 3.0 ml of 2.0S T_{200} K_{50} M_{10} , pH 8.5, 6 mM 2-mercaptoethanol, and the remainder of the tube was filled with 0.35S T_{200} K_{50} M_{10} , pH 8.5. Centrifugation was carried out for 4 h (2°C) at 40,000 rpm in a Beckman SW40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (284,000 g max.). Material which collected at the 1.6/2.0 M sucrose

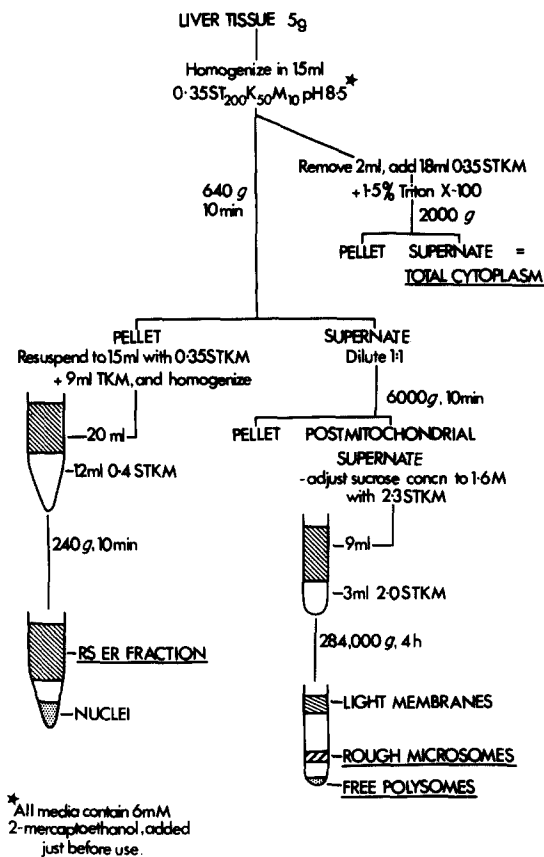


FIGURE 1 Schematic representation of the procedure for obtaining total cytoplasm, RSER, rough microsomes, and free polysomes from rat liver homogenates (see Materials and Methods for details).

interface (rough microsomes) or which passed through the 2.0 M sucrose cushion (free polysomes) was removed.

Analysis of Polysomes

Samples from subcellular fractions were diluted with T_{200} K_{50} M_{10} , pH 8.5, and Triton X-100 was added to a final concentration of 1.0–1.5%. Aliquots (equivalent to 0.5–1.0 g of tissue) were layered over 2.0 ml of 1.5S T_{200} K_{50} M_{10} , pH 8.5, and centrifuged for 90 min (2°C) at 40,000 rpm in a Beckman SW40 rotor. Pellets were resuspended in 0.5 ml of T_{200} K_{50} M_{10} , pH 8.5, and sedimented in 15–50% sucrose (wt/vol) gradients containing the same buffer. After centrifuging for 75 min at 40,000 rpm (Beckman SW40 rotor), optical density profiles were recorded at 260 nm using a Gilford 2480 density gradient scanner (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Isolation of RNA

Tissue homogenates were fractionated as shown in

Fig. 1. Subcellular fractions were diluted to a volume of 40–50 ml with 0.35S T₂₀₀ K₅₀ M₁₀, pH 8.5, i.e., equivalent to a 5–10% tissue homogenate. Triton X-100 was added to 1.0%, SDS to 1%, and EDTA to 2.0 mM. After bringing to room temperature, the mixture was extracted by shaking (10 min) with 2 vol of chloroform:phenol (1:1, vol/vol), and centrifuged for 10 min at 10,000 g. The phenol phase (including any interface material) was collected and shaken (10 min) with 1 vol of 0.1 M Tris acetate, pH 9.0, 0.1 M Na acetate, and 2 mM EDTA, and centrifuged. The combined aqueous phases were reextracted with 1 vol of chloroform:phenol. After centrifuging, RNA was precipitated from the aqueous phase (made 0.2 M K acetate, pH 5.5) with 2.5 vol of redistilled ethanol at –20°C. Precipitates were washed two to three times with 3 M Na acetate, pH 6.0, followed by 70% ethanol containing 0.1 M Na acetate, dried under N₂, and finally dissolved in 10 mM Tris acetate, pH 7.6, and stored at –70°C.

Isolation of PolyA-Containing RNA

2–4 mg RNA was dissolved in 2.0 ml of 0.4 M NaCl, 10 mM Tris acetate, pH 7.4, 0.5% SDS, and applied to an oligo(dT)-cellulose column (21) which had been equilibrated and well washed with the same medium (0.3 ml of packed volume, retention capacity > 1 mg of polyA/ml) (5). An additional 2.0 ml of the same medium without SDS was passed through the column, and then polyA⁺-RNA was eluted with 2.0 ml of H₂O, precipitated, and dissolved in 10 mM Tris acetate, pH 7.6, and stored at –70°C.

[³H]polyU Hybridization Assays

The presence of polyA in high molecular weight RNA was tested by hybridization with excess [³H]polyU (22). Reaction mixtures contained in a final volume of 0.5 ml: 20–40 μg of unfractionated RNA or 0.2–0.6 μg of polyA⁺-RNA, 10 mM Tris acetate, pH 7.4, 0.2 M NaCl, 5 mM Mg acetate, and 0.02 μCi [³H]polyU (5.42 μCi/mol P). After incubating for 15 min at 25°, pancreatic ribonuclease (0.4 μg) was added and, after a further 30 min, 5% TCA containing carrier RNA (from wheat germ) was added. Reaction mixtures were left on ice for 20 min. Precipitates were collected on Whatman GF/C filters, washed, dried, and radioactivity was measured in scintillation fluid (1,500–4,000 cpm/reaction).

Translation of Rat Liver Messenger RNA

RABBIT RETICULOCYTE CELL-FREE SYSTEM: Reticulocyte lysates were prepared from 2 to 3-kg New Zealand white rabbits as described elsewhere (11). Reaction mixtures (30) contained in a total volume of 100 μl: 1.0 mM ATP, 0.2 mM GTP, 75 mM KCl, 2.0 mM Mg acetate, 10 mM Tris acetate, pH 7.6, 20 μM

hemin, 9 mM creatine phosphate, 10–20 μg of creatine phosphokinase, 16 μg of tRNA (from wheat germ, reference 39), 18 amino acids (minus methionine and cysteine), 5–20 μCi [³⁵S]methionine (150–260 Ci/mM), 10–40 μg of RNA (or its equivalent polyA⁺-fraction) and 50 μl of reticulocyte lysate. Mixtures were incubated in plastic Eppendorf microtubes (Netheler and Hinz Co., Hamburg, W. Germany) (1.5 ml capacity) for 60 min at 26°C, then placed on ice, and diluted with 0.4 ml of ice-cold PBS containing 1.0% Nonidet P-40 (Shell Chemical Co., New York), 20 mM unlabeled methionine, and 3 mM NaN₃. Under the assay conditions employed, RNA was present in rate-limiting amounts, and radioactive incorporation into albumin and mitoplast polypeptide products proceeded linearly for over 60 min.

To determine total incorporation of radioactive precursor, aliquots (10 μl) were spotted on Whatman No. 1 filter paper and washed consecutively with cold (2°C) 5% TCA (containing 2 mM unlabeled methionine) for 20 min, hot (85°C) 5% TCA for 15 min, 5% TCA at room temperature, twice, and finally ethanol. Filters were dried and radioactivity was measured in scintillation fluid using a Beckman LS-250 Liquid Scintillation System (Beckman Instruments, Fullerton, Calif.) (90% efficiency).

Immunoprecipitation of specific polypeptide products was carried out using the remainder of each reaction. Excess antiserum was added (50 μl for antimitoplast and 25 μl for antialbumin), and mixed thoroughly before adding 48 μg of mitoplast protein or 3 μg of albumin. These conditions yielded maximum precipitation of antigen carrier and radioactive polypeptide products. After incubating for 18 h at 4°C, precipitates were collected by centrifugation and pellets were washed two to three times with 500 μl of the PBS-Nonidet-methionine mixture. For analysis by gel electrophoresis, the final pellet was dissolved directly 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) (see below). For quantitating radioactivity in immunoprecipitated products, pellets were first dissolved in 0.1 N NaOH and then reprecipitated with hot 5% TCA containing unlabeled methionine. In these experiments, performing 'clearing' reactions, i.e. immunoprecipitating with one antiserum before analyzing the lysate with the other, did not alter the results obtained. All reactions were performed in duplicate to quadruplicate and the results averaged.

WHEAT EMBRYO CELL-FREE SYSTEM: RNA was translated in a standard system derived from wheat embryos (40). Reactions contained in a volume of 280 μl: 80 mM KCl, 1.5 mM ATP, 36 μM GTP, 12 mM creatine phosphate, 16 μg of creatine phosphokinase, 16 μg of tRNA (39), 3.6 mM Mg acetate, [³⁵S]methionine (5–15 μCi, ~ 260 Ci/mM), 120 μl of wheat embryo S₂₀ (40), and polyA⁺-RNA obtained from 135–145 μg of total RNA. After 60 min at 30°C, ribosomes and nascent peptides were removed by centrifugation at

42,000 rpm (105,000 g_{avr}) for 1 h in a Beckman type 65 rotor, and radioactive products in the supernate were precipitated with hot (85°C) 5% TCA.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

Electrophoresis was performed with thin slab gels essentially as described in the previous paper (54), but with certain modifications: acrylamide and bis-acrylamide were recrystallized from chloroform and acetone, respectively, and electrophoresis was performed either overnight at 10 V or for shorter periods at 80–120 V. Gels were then stained with Coomassie Brilliant Blue, dried in contact with Whatman No. 1 filter paper, and pressed onto Kodak Xomat AP54 film for periods of up to 10 days, at which time film was developed with Ilford PQ Universal developer (Ilford Ltd. Ilford, Essex, England). In certain instances, gels were impregnated with 2,5-diphenyloxazole (PPO, Koch-Light Laboratories) just before drying (see legend, Fig. 10).

Chemicals

[³⁵S]Methionine (150–350 Ci/mol) and [³H]polyU (54.2 μ Ci/mol P) were purchased from the Radiochemical Centre, Amersham, England and Miles Laboratories, Kankakee, Ill., respectively. Chemicals for electron microscopy were purchased from TAAB Laboratories, Reading, England. Chemicals for routine use were of analytical grade and obtained from BDH Chemicals Ltd., Poole, England or Sigma London Chemical Co., Surrey, England.

RESULTS

Conditions for Subcellular Fractionation of Rat Liver without Degradation of mRNA

The scheme depicted in Fig. 1 for separation of free from membrane-bound polysomes represents a compromise between conditions which yield efficient separation and conditions which allow recovery of intact mRNA's. Polysome profiles obtained from the different subcellular fractions were monitored to detect degradation of polysomal mRNA.

When RSER was prepared according to methods described in the previous paper, i.e. using 0.35S T₅₀ K₂₅ M₁₀, pH 7.6, for homogenization, membrane-bound polysomes were found to be almost totally degraded (Fig. 2*a*). This occurred even in the presence of rat liver ribonuclease inhibitor (Searle Diagnostic, Des Plaines, Ill.). Intact polysomes were recovered from RSER only when tissue homogenization and polysome isolation were performed under conditions of high pH (8.5) and relatively high ionic strength (0.35S T₂₀₀ K₅₀ M₁₀ medium, Fig. 2*b* and *c*). Moreover, the size of polysomes associated with this membrane varied according to the food intake of animals. After starvation (16 h), polysomes were recovered from RSER (Fig. 2*b*), but these contained relatively few ribosome monomer units per polysome, especially when compared to polysomes from a

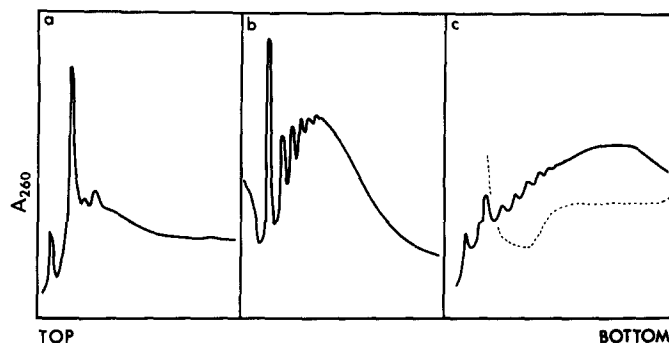


FIGURE 2 Effects of starvation and different homogenization media on the integrity of polysomes recovered from RSER. RSER fractions were prepared as follows: (a) by the pH 7.6 procedure outlined in the previous paper (54) using animals starved for 16 h before death; (b) by the pH 8.5 procedure outlined in Fig. 1 and Materials and Methods using animals starved for 16 h, and (c) by the pH 8.5 procedure outlined in Fig. 1 and Materials and Methods but using nonstarved animals. Polysomes were isolated from each of the three preparations (see Materials and Methods) and resolved on 15–50% (wt/vol) sucrose gradients (panels a, b, and c, respectively). Absorbance at 260 nm was recorded, and it yielded maximum values of 0.5, 0.6, and 0.9 A_{260} U for a, b, and c, respectively. —, 20 mM EDTA added to polysomes; ---, no EDTA added.

postmitochondrial supernate prepared from starved animals (Fig. 3a). Large polysomes (heavily contaminated with glycogen) were obtained from RSER (Fig. 2c) only when nonstarved animals were used.

In this study, RSER was recovered without significant contamination by free polysomes. For example, when a dilute sample of RSER was sedimented at 48,000 g for 75 min in a linear 25–60% (wt/vol) sucrose gradient, most of the ribosomes (>95%) sedimented with the membrane and not to a region of the gradient expected for unbound ribosomes (data not shown).

Free polysomes were recovered from postmitochondrial supernates, but their efficient separation from rough microsomes while leaving polysomes intact proved exceedingly difficult. Procedures involving long centrifugation times (24 h) resulted in isolation of free ribosomes which accounted for approximately 16% of total cytoplasmic RNA (Table I), a value which is in reasonable agreement with other reports (1, 8, 9). Nevertheless, even under conditions of high pH, polysomes recovered from rough microsomes were somewhat degraded (data not shown). The procedure shown in Fig. 1 which involved centrifuging postmitochondrial supernates for only 4 h, however, gave

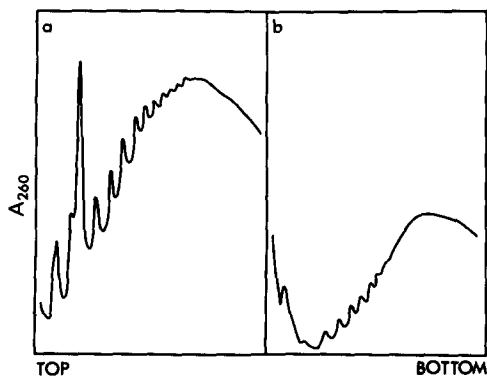


FIGURE 3 Polysome profiles from postmitochondrial supernates (starved animals) and from rough microsomes (nonstarved animals). Polysomes were isolated from (a) postmitochondrial supernates prepared from animals starved for 16 h before death or (b) rough microsomes prepared from nonstarved animals. Medium containing $T_{200} K_{50} M_{10}$, pH 8.5, and 6 mM 2-mercaptoethanol was used throughout (see Materials and Methods and Fig. 1). Polysome profiles were displayed on 15–50% (wt/vol) sucrose gradients as described in Materials and Methods and gave maximum A_{260} readings of 0.26 and 0.14 for (a) and (b), respectively.

TABLE I
Separation of Free from Membrane-Bound
Ribosomes*

Fraction	Recovery of RNA	
	%	
Total cytoplasm	100	
RSER	27	
PMS	55	
Fractionation of PMS	Procedure described in Fig. 1	Procedure modified from Blobel and Potter (8, 9)
Rough microsomes	24	28
Free polysomes	8	16

* Total cytoplasm, RSER, and a postmitochondrial supernate (PMS) were prepared from rat liver as described in Materials and Methods and Fig. 1. A portion of the PMS was used to prepare rough microsomes and free polysomes by the procedure described in Fig. 1, i.e., by adjusting the sucrose concentration of the PMS to 1.6 M, layering over a 2.0 M sucrose cushion and centrifuging for 4 h ($284,000 g_{max}$). Alternatively, free polysomes and rough microsomes were prepared from the PMS according to a procedure modified from Blobel and Potter (8, 9) in which $T_{200} K_{50} M_{10}$, pH 8.5, 6 mM 2-mercaptoethanol medium was used throughout. PMS was layered over a discontinuous gradient consisting of 9 ml of 2.0 M sucrose, 6 ml of 1.35 M sucrose, and 6 ml of 0.5 M sucrose, and centrifuged for 24 h ($2^\circ C$) at 27,000 rpm in a Beckman SW27 rotor ($131,000 g_{max}$). RNA in the various fractions was estimated by the Fleck and Munro method (see previous paper) and yielded a value of 6.5 mg of RNA/g liver for the total cytoplasmic fraction. In this experiment, RNA in the PMS and RSER fractions together accounted for 82% of total cytoplasmic RNA. Approximately 12% was lost when nuclei were removed from the 640 g pellet during preparation of RSER (Fig. 1), and the remainder (6%) was present in the 640–6,000-g pellet.

relatively good recoveries of rough microsomes (Table I) with polysomes intact (Fig. 3b). Free polysomes, on the other hand, were recovered in relatively low yield (8% of total cytoplasmic RNA, Table I). Under the conditions of this procedure, the small size class of free polysomes would not be expected to sediment as a pellet in 4 h and was probably retained in the 1.6 M sucrose overlay. The experiment described in Fig. 4 indicated that free polysomes did not contaminate the rough microsome fraction to a significant extent (<15%).

Specificity of Antiserum to Mitoplast Proteins

Fig. 5 shows an electron micrograph of a typical thin section from a mitoplast preparation. Mitochondria appear devoid of outer membrane and exhibit morphologies similar to those demonstrated for purified mitoplasts in other studies (26). Nevertheless, smooth membrane vesicles are clearly present in this fraction, but these may arise from the inner mitochondrial membrane itself because marker enzyme analysis indicated a com-

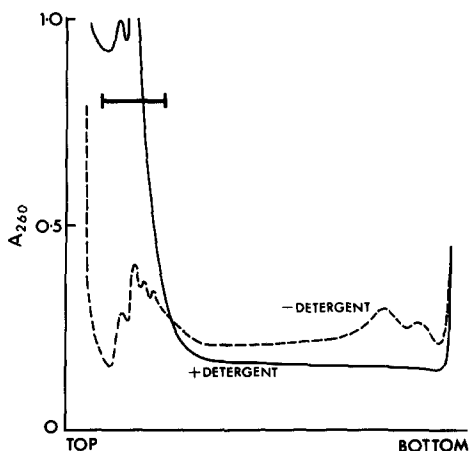


FIGURE 4 Determination of the extent of contamination of the rough microsomal fraction by free ribosomes. Rough microsomes were prepared using discontinuous sucrose gradients as described in Fig. 1. Material banding at the 1.6/2.0 M sucrose interface was removed, diluted with $T_{200} K_{50} M_{10}$, pH 8.5, until sucrose concentrations reached 0.2–0.4 M, and particulate material was collected by centrifuging at 45,000 rpm for 60 min in a Beckman type 65 rotor ($130,000 g_{av}$). Pellets were resuspended using a tight-fitting Teflon pestle in 0.5 ml of $0.35S T_{200} K_{50} M_{10}$, pH 8.5, and Triton X-100 was then added (1% final concn.) to half the samples. The resuspensions were layered on 12 ml of 15–60% (wt/vol) sucrose gradients and centrifuged for 90 min at a relatively low speed ($75,000 g_{av}$) in a Beckman SW41 rotor. Gradients were then scanned at 260 nm. The increase in absorbancy which occurred near the top of the gradient as a result of detergent treatment (denoted by a bar in the figure) was due to release of membrane-bound ribosomes. In the absence of detergent, a portion of RER sedimented through the gradient and was recovered as a pellet.

plete absence of the two most likely sources of contamination, the ER (glucose-6-phosphatase) and the outer mitochondrial membrane (monoamine oxidase).

Detection of mitochondrial polypeptide products by immunological procedures after translation of cytoplasmic messenger RNA requires that the antibodies used be specific for mitochondrial proteins. Whole mitochondria were not used for preparing antiserum because of the probability that mitochondrial outer membrane and ER contain common antigenic determinants. Moreover, the use of digitonin in preparing mitoplasts ensures against contamination by nonmitochondrial organelles. Antiserum which was raised in rabbits against constituents of this and other subcellular

fractions (Fig. 6 A). At the protein concentrations used for Ouchterlony double diffusion tests (10–16 mg/ml), antiserum was found to react against solubilized mitoplasts, but not against smooth membrane (i.e., ER, Golgi apparatus, lysosomes, etc), rough microsomes, or cell sap. When control serum was tested against mitoplasts, no reactivity was detected. Also, reaction between mitoplast antiserum and the rabbit reticulocyte lysates used for *in vitro* protein synthesis experiments could not be detected, even after lysates had been concentrated 20- to 25-fold (Fig. 6 B).

Translation of PolyA⁺-RNA in the Wheat Embryo Cell-Free System

PolyA⁺-RNA was prepared from total RNA obtained from subcellular fractions by oligo(dT)-cellulose chromatography, and recovery was monitored by hybridization tests using excess

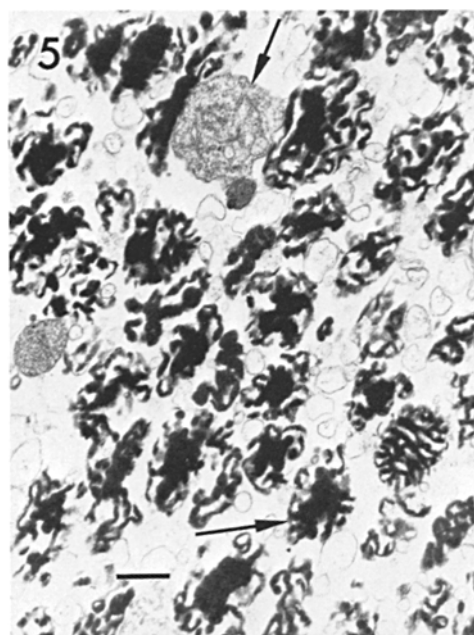


FIGURE 5 Electron micrograph of the mitoplast preparation used for raising antiserum to mitochondrial proteins. Mitoplasts were pelleted and fixed overnight at 2°C in 2% glutaraldehyde, 0.2 M sucrose, and 0.1 M Na phosphate buffer, pH 7.2. Material was washed, post-fixed (2 h) in 2% OsO₄, 7% glucose, and 0.1 M Na phosphate buffer, pH 7.2. Thin sections were stained with uranyl acetate and lead citrate. In this figure, mitoplasts are seen to exhibit different morphological configurations (arrows) depending upon their respiratory state (26). $\times 14,200$. Bar, 0.5 μm .

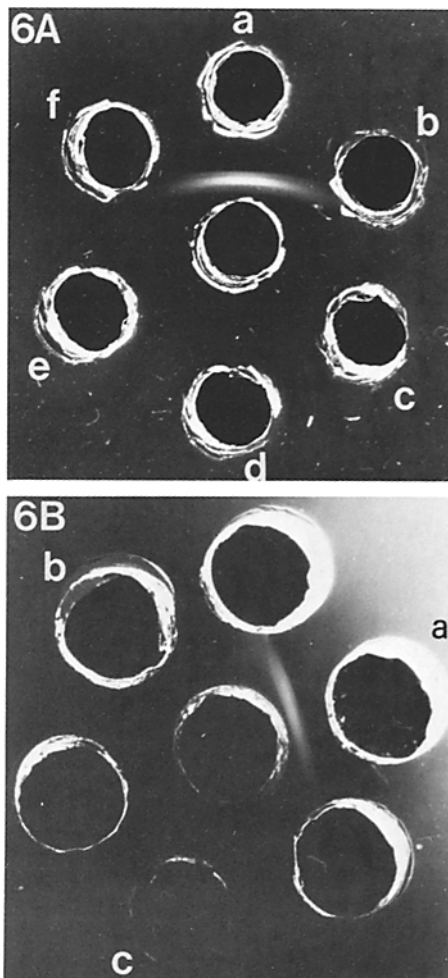


FIGURE 6 Ouchterlony double-diffusion analysis of the reactivity of different subcellular fractions to antimitoplast antiserum. As described in Materials and Methods, antiserum was prepared against a purified mitoplast fraction and tested (Fig. 6 A, center well) against the following fractions (10–16 mg protein/ml): mitoplasts (a), rough microsomes (c), smooth membranes (d), cell sap (e). Serum from control rabbits was placed in wells (b) and (f). All fractions recovered from relatively low-speed sediments of rat liver homogenates contained mitochondria and consequently were not tested for cross-reactivity with antimitoplasts. In Fig. 6 B, antiserum (center well) was reacted against mitoplasts (a), reticulocyte lysate concentrated to 2 times (b), and to 20–25 times (c) the concentration used for *in vitro* protein synthesis.

[³H]polyU (Table II). Unfractionated RNA from total cytoplasm, RSER and rough microsomes hybridized [³H]polyU to a similar extent, but RNA from free polysomes exhibited lower (~55%)

reactivity, thus indicating a lower content of polyA. When equivalent amounts of polyA⁺-RNA (as judged by [³H]polyU hybridization) from RSER and rough microsomes were translated in a wheat embryo cell-free system, protein synthesis was stimulated to a greater extent by the former (354,000 vs. 264,000 cpm incorporated, legend Fig. 7). The distribution of polypeptide products on SDS-polyacrylamide gels, however, was qualitatively very similar for the two RNA populations (Fig. 7). This is interpreted to mean that, insofar as electrophoretic patterns of polypeptide products accurately reflect mRNA content, the majority of membrane-bound mRNA's in rat liver are present in both RSER and rough microsomes. Nevertheless, large quantitative differences could still exist for certain messenger species and not be detected in experiments which measure total products of translation. When synthesis of specific polypeptides was tested using antibodies to albumin and mitoplast proteins, however, the wheat embryo system described here proved unsatisfactory. The main problem was premature termination of polypeptide elongation for the larger molecular weight proteins which made it impossible to accurately identify all the products by gel electrophoresis and which resulted in low incorporation of radioactivity into full-size chains. These analyses, therefore, were performed using the more active rabbit reticulocyte system.

Translation of mRNA's Coding for Albumin and Mitoplast Proteins in the Rabbit Reticulocyte Cell-Free System

Translation of polyA⁺-RNA obtained from the various subcellular fractions using the rabbit reticulocyte cell-free system followed by immunoprecipitation of radioactive products with antialbumin yielded a polypeptide product which coelectrophoresed on polyacrylamide gels with rat serum albumin (Fig. 8). Densitometer tracings of autoradiographs exposed to gels for short periods of time (4 h) indicated that this single polypeptide accounted for greater than 80% of the radioactivity present in the resolving region of these gels (data not shown). Two minor bands were detected, but these accounted for only 10% of total radioactivity.

Although the autoradiograph shown in Fig. 8 was obtained using polyA⁺-RNA isolated from the rough microsome fraction, an identical distri-

TABLE II
*Recovery of PolyA⁺-RNA from Oligo(dT)-Cellulose Columns**

RNA isolated from	³ H]PolyU hybridized			Recovery of polyA ⁺ -RNA %
	Before fractionation on oligo(dT)-cellulose	PolyA ⁻ fraction	PolyA ⁺ fraction	
		<i>(cpm/initial 20 μg RNA)</i>		
Total cytoplasm	4,615	32	2,720	59
RSER	4,185	17	2,955	71
Rough microsomes	4,635	1	3,085	67
Free polysomes	2,536	0	1,360	54

* Samples of RNA from the different subcellular fractions were fractionated on oligo(dT)-cellulose columns and hybridized against excess [³H]polyU as described in Materials and Methods. Assays contained either 20 μg of unfractionated RNA, or an amount of polyA⁺-RNA or polyA⁻-RNA which was recovered from 20 μg of RNA (see Materials and Methods). A comparison of the extent of hybridization before and after fractionation of RNA on oligo(dT)-cellulose is a measure of the recovery of the polyA⁺-fraction from the column.

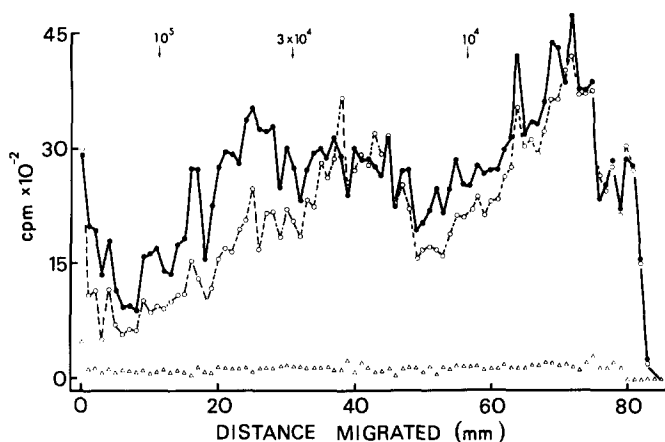


FIGURE 7 Translation of polyA⁺-RNA from RSER and rough microsomes using the wheat embryo cell-free system. PolyA⁺-RNA (equivalent to 95 μg of unfractionated RNA, see Table II) was translated in the wheat embryo system containing [³⁵S]methionine as described in Materials and Methods. Released polypeptide chains were analyzed on 12.5% SDS-polyacrylamide gels. Gels were dried, cut into 1-mm sections, dissolved in 30% H₂O₂ (60°C overnight), and radioactivity was measured. (Δ) no RNA added; (●) polyA⁺-RNA from RSER (354,000 cpm incorporated); (○) polyA⁺-RNA from rough microsomes (264,000 cpm incorporated). Approximate molecular weight calibrations are indicated by the arrows.

bution of radioactive products was obtained using either total or polyA⁺-RNA from total cytoplasm, RSER, and free polysome fractions. PolyA⁻-RNA did not contain any apparent sequences which coded for either completed or noncompleted albumin polypeptides (Fig. 8).

After immunoprecipitation of translation products with antimitooplast antiserum, a number of prominent radioactive bands were resolved on SDS-polyacrylamide (10%) gels, and at least some of these apparently coelectrophoresed with unlabeled mitoplast markers (Fig. 9). This experiment was performed using unfractionated RNA, but identical results were obtained using the

polyA⁺ fraction. PolyA⁻-RNA did not support the synthesis of any of these polypeptides. When mitoplast proteins which had been prelabeled *in vivo* with [³⁵S]methionine and then precipitated with antimitooplast antiserum were displayed on gels (12%), at least two mitoplast proteins exactly comigrated with polypeptide products of translation (arrows, Fig. 10). One reason why more immunoprecipitable polypeptides synthesized *in vivo* and *in vitro* did not comigrate may be the fact that, *in vivo* mitochondrial proteins and membrane proteins in general, can be subject to extensive posttranslational modifications (e.g. glycosylation, proteolytic processing, etc) before being

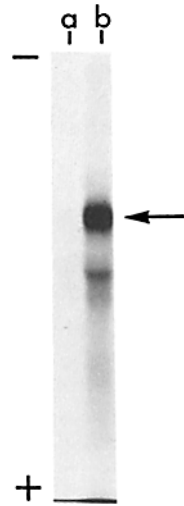


FIGURE 8 Translation of albumin mRNA in the rabbit reticulocyte cell-free system. RNA was isolated from subcellular fractions and fractionated by oligo(dT)-cellulose chromatography. PolyA⁻-RNA or polyA⁺-RNA (equivalent to 40 μ g of unfractionated RNA, see Table II) was incubated with the reticulocyte lysate system for 60 min. Radioactive products which were precipitated by antiserum to rat albumin were electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed (see Materials and Methods for details). (a) PolyA⁻-RNA, (b) polyA⁺-RNA. The position of authentic rat serum albumin on the gel is indicated by the arrow (apparent mol wt, 65,000–70,000 daltons) and was determined by coelectrophoresis with the purified protein. Results are shown for RNA isolated from rough microsomes, but an identical electrophoresis pattern was obtained using polyA⁺-RNA from total cytoplasm, RSER, or free polyosome fractions. Also, translation of unfractionated RNA yielded products with the same electrophoretic pattern as for the polyA⁺-fractions.

transported to their final destination. Such modifications of the newly formed polypeptide will, of course, lead to altered electrophoretic properties.

Figs. 9 and 10 show (bars) that some immunoprecipitable radioactive products appeared on gels even when cell-free protein synthesis occurred in the absence of added rat liver mRNA. It is unlikely that antiserum against rat mitoplasts recognizes any mitochondrial polypeptides which may be coded for by endogenous reticulocyte messengers, since reactivity of the antiserum used in these tests with even highly concentrated reticulocyte lysate could not be detected (Fig. 6 B). The possibility must be considered, therefore, that these bands arise due to nonspecific trapping of nonmi-

toplast radioactive polypeptides. Extensive efforts to eliminate this high background (e.g. centrifugation through sucrose cushions, precipitating with goat antiserum to antimitoplast, etc) have failed. Nevertheless, there were long stretches in the resolving region of these minus-mRNA control gels which did not contain any trace of this background (Figs. 9 and 10), yet more than eight polypeptides coded for by rat messenger RNA and precipitated by specific antimitoplast migrated here.

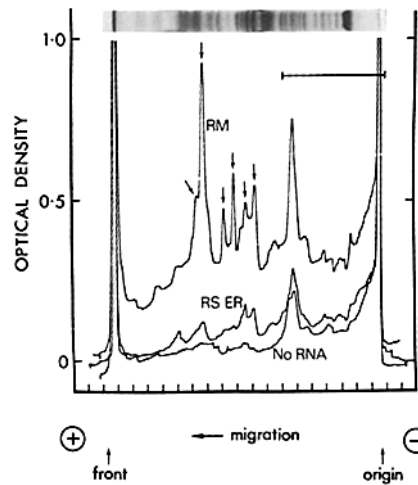


FIGURE 9 Translation of mRNA's coding for mitoplast proteins in the rabbit reticulocyte cell-free system. Samples of RNA (40 μ g) from RSER and rough microsomes (RM) were incubated with the reticulocyte lysate for 60 min, and radioactive products which precipitated upon addition of antimitoplast antiserum were electrophoresed on an SDS-polyacrylamide (10%) slab gel (see Materials and Methods). Controls were performed in which the lysate was incubated without added mRNA (No RNA). The gel was dried and exposed to autoradiograph film for a short period of time (10 h). The film was developed and gave bands whose density was proportional to radioactivity (cf. 90–100 h exposure which was routinely used for visual examination of autoradiographs). Densitometer tracings were obtained using a Joyce-Loebl 3CS microdensitometer (Joyce, Loeb & Co., Inc., Burlington, Mass.), where the zero OD reading represents unexposed regions of the film. Arrows indicate products whose synthesis is approximately five-fold greater using RM-RNA compared to RSER-RNA. Coelectrophoresis with mitoplasts dissolved in SDS sample buffer gave the profile shown above upon staining with Coomassie Brilliant Blue. The bar denotes a region of the gel in which background radioactive bands appeared in the 'No RNA' control.

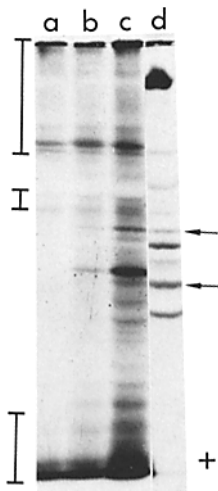


FIGURE 10 Coelectrophoresis of immunoprecipitated mitoplast proteins labeled *in vivo* and *in vitro*. Translation of mitoplast mRNA's was performed as described in Materials and Methods and Fig. 9. *In vivo* labeled mitoplasts were purified from liver (26) 12 h after giving rats a single intraperitoneal injection of 1.5 mCi [³⁵S]methionine in 0.15 M NaCl. Samples (150 μg of protein, 5,000 cpm) were precipitated with 75 μl of antimitoplast antiserum (3,250 cpm recovered), dissolved in SDS sample buffer and coelectrophoresed with *in vitro* translation products (recovered as described in Materials and Methods) on an SDS-polyacrylamide (12%) slab gel. The gel was prepared for autoradiography in the usual way except that it was first impregnated with PPO as follows (Bonner, W. M., and R. A. Laskey, 1974. *Eur. J. Biochem.* 46:83-88): the gel was soaked in dimethylsulfoxide (DMSO) for two 30-min periods, soaked in DMSO containing PPO (20%, wt/wt) for 3 h, and rinsed three times (1 h each) in H₂O. Autoradiography was performed at -70°C for 36 h (*in vitro* translation products) or 10 days (*in vivo* labeled mitoplast markers). (a) no RNA added to lysates, (b) 43 μg of RSER RNA added, (c) 43 μg of RNA from a postmitochondrial supernate added (see Table I, Fig. 1), (d) *in vivo* labeled mitoplast markers. The bars denote regions of the gel in which background radioactive bands appeared in the minus-mRNA control (a). Arrows indicate comigration of *in vivo* and *in vitro* labeled polypeptides. RNA from rough microsomes and free polysomes gave the same pattern of mitoplast polypeptide products as shown above for PMS-RNA.

Membrane-Bound mRNA's Which Code for Albumin vs. Mitoplast Proteins Exhibit Different Subcellular Distributions

Table III describes an experiment in which total and polyA⁺-RNA were extracted from the four

subcellular fractions (Fig. 1) and tested for their ability to direct synthesis of albumin *in vitro* using the rabbit reticulocyte system. In the absence of added RNA, high levels of amino acid precursor were incorporated into protein (primarily as globin). Addition of the various samples of RNA (40 μg each) either stimulated (rough microsomes) or depressed (RSER) this incorporation. Immunoprecipitation by antiserum to albumin showed that synthesis of this polypeptide was similar when RNA from RSER or rough microsomes was used. When expressed as a percentage of total radioactive incorporation by the lysate, however, incorporation into albumin was slightly enriched (50%) in the RSER fraction compared to rough microsomes. RNA from free polysomes supported albumin synthesis to a relatively low extent compared to other fractions. These results have been obtained consistently in numerous tests.

In contrast to the situation for albumin polypeptide synthesis, incorporation of radioactivity into polypeptides precipitated by antimitoplast was very significantly stimulated by rough microsomal RNA compared to RSER-RNA (Fig. 9), by as much as fivefold for many of those polypeptides which migrated to a region of polyacrylamide gels where there was little background radioactivity (arrows, Fig. 9; see also Fig. 10). Quantitation was performed by densitometer tracings of autoradiographs which had been exposed to gels for relatively short periods so that radioactivity and optical density of individual bands on the developed film are proportional (legend, Fig. 9). In the experiments described in Table III, the radioactivity in total precipitates using antimitoplast antiserum was also measured (legend, Table III). On average, this was three to four times higher for rough microsomal RNA compared to RSER-RNA, and even though these precipitates may contain variable levels of trapped nonspecific radioactive products, the overall conclusion derived from Fig. 9, i.e. that at least certain cytoplasmic mitoplast mRNA's are enriched in the rough microsome fraction, is not altered.

Synthesis of Mitoplast Proteins

In Vitro is due to mRNA's of Cytoplasmic Origin, not of Mitochondrial Origin

Total RNA from the fraction richest in mitochondria (RSER) demonstrated the lowest specific activity for synthesis of mitoplast proteins *in vitro* (Fig. 9; legend, Table III). Rough microsomes were recovered free of mitochondria and

TABLE III
Subcellular Distribution of Albumin mRNA Assayed in the Reticulocyte Cell-Free System*

RNA isolated from	[³⁵ S]Methionine incorporated (cpm)/60 min into:			
	Total protein		Albumin	
	Total RNA	PolyA ⁺ -RNA	Total RNA	PolyA ⁺ -RNA
	($\times 10^{-3}$)		(stimulation over minus-mRNA control)	
Total cytoplasm	47,100	36,315	39,000 (0.83)‡	59,945 (1.65)
RSER	38,125	37,080	59,850 (1.57)	64,960 (1.75)
Rough microsomes	74,070	56,685	70,575 (0.95)	65,740 (1.15)
Free polysomes	62,280	44,915	40,140 (0.64)	22,875 (0.51)
No addition (control)	51,690	45,355	7,505	7,120

* RNA was isolated from subcellular fractions as described in Materials and Methods. Samples (40 μ g of RNA, or polyA⁺-RNA which hybridized [³H]polyU to an extent equal to 40 μ g of total RNA, Table II) were incubated for 60 min in the rabbit reticulocyte lysate mixture containing 15 μ Ci [³⁵S]methionine (~ 260 Ci/mM) per reaction (see Materials and Methods). Radioactivity was measured in products which were precipitated with TCA (total protein) or which precipitated with antiserum to albumin. Background radioactivity has been subtracted from the values presented (stimulation over minus-mRNA control) and was measured by adding antiserum and carrier albumin to lysates which had been incubated without added rat liver RNA (no addition). The experiments with total and polyA⁺-RNA were performed on separate occasions, which accounts for differences in lysate activity under control conditions (no addition). All values are an average of three determinations.

In these same experiments, immunoprecipitation was also performed using antiserum to mitoplast proteins and yielded values (stimulation over minus-mRNA control) for (a) total RNA which were (counts per minute): 6,675 (total cytoplasm); 4,640 (RSER); 20,115 (rough microsomes); 14,455 (free polysomes), and for (b) polyA⁺-RNA: 10,735 (total cytoplasm); 8,015 (RSER); 22,690 (rough microsomes); 15,520 (free polysomes). Background incorporation (minus-mRNA control) was 6,385 and 9,440 cpm for (a) and (b), respectively.

‡ Percentage of total TCA-insoluble radioactivity which was recovered in albumin precipitates.

contained RNA which coded for all the mitoplast polypeptides which were also coded for by total cytoplasmic RNA (data not shown). Finally, when polysomes obtained from the various fractions were incubated in the mRNA-dependent wheat embryo cell-free system \pm chloramphenicol, the inhibitor had little effect on total radioactive incorporation (data not shown). Thus, the level of mitochondrial polyribosomes in any given subcellular fraction was too insignificant to interfere with the present tests.

Ratios of Free to Membrane-Bound mRNA's Coding for Albumin and Mitoplast Proteins

The following assumptions are made: total RNA in rat liver consists of 5% nuclear RNA, 15% nonsedimentable RNA, 18% free polysomal RNA (8), and the remainder (~62%) is associated with membrane-bound polysomes which are equally distributed between RSER and rough microsomes (Table I). On the basis of the values presented in Table III for mRNA activities in the reticulocyte cell-free system, it can be calculated that albumin mRNA is enriched (86–91%) in

membrane-bound polysomes, compared to free polysomes (see also 20, 28, 48, 55, but cf. 52, 53), and that mRNA's coding for mitoplast polypeptides are likewise enriched in bound polysomes but to a lower extent (~75%, see legend, Table III). These calculations did not take into consideration the variations in incorporation of radioactivity into total protein by the reticulocyte system.

DISCUSSION

In all procedures used to date for isolating RER from rat liver homogenates, 50% or more of total membrane-bound ribosomes are found to sediment with nuclei (1, 7, 8, 14, 29, 37, 54). This rapidly sedimenting fraction of RER, however, is almost impossible to recover free of contaminating organelles (54). Analyses are generally performed, therefore, with only the microsomal fraction of RER which can relatively easily be obtained free of other organelles from postmitochondrial supernates. But even the most careful studies (e.g. reference 1) have resulted in recovery of only 50% of total membrane-bound ribosomes in the rough microsome fraction, and the exhaustive manipulations that were required may result in considerable degradation of associated polysomes (1,

but see reference 9). It was not the intention of the present study to analyze purified fractions of RER per se, but rather to recover the membrane in acceptable yields while still retaining polysome integrity (Figs. 1-3, Table I).

Modifications were introduced in the usual procedures for fractionating RER and for separating free from membrane-bound polysomes to enable their recovery without significant degradation of mRNA. It was essential that tissue homogenization and polysome isolation be performed using media of relatively high ionic strength and high pH (8.5) to inhibit endogenous ribonucleases (Figs. 2 and 3). The methods were first developed for plant systems (15) where they have been successfully used for analyzing specific mRNA's recovered from either total or membrane-bound polysomes (59, 60). Such media were particularly necessary to prepare RS.ER, where even rat liver ribonuclease inhibitor could not prevent degradation of polysomes when this fraction was isolated using standard media at pH 7.6 (Fig. 2). Non-starved animals must be used, however, in order to retain large polysomes on this membrane (Fig. 2, see also 57, 62-64). The use of high pH and high ionic strength, instead of the usual media (pH 7.6, see previous paper), did not significantly alter the distribution of protein, RNA, DNA, glucose-6-phosphatase, or cytochrome *c* oxidase between subcellular fractions.

Another important modification was to introduce dense sucrose (1.6 M) to the postmitochondrial supernate (42), and then separate free from bound polysomes by layering over 2.0 M sucrose and centrifuging for only 4 h (Fig. 1). Smooth membranes (including lysosomes) floated to the surface whereas rough microsomes sedimented to the 1.6/2.0 M sucrose interface and formed a loosely packed membrane layer which presumably presented little resistance to sedimenting free polysomes. Rough microsomes were thus recovered without polysome degradation (Fig. 3 *b*), and free polysomes were recovered intact (Fig. 3 *a*) but in rather low yields (Table I). A small portion of the ribosomes in the rough microsomal fraction, however, derived from contamination by free polysomes (~ 15%, Fig. 4).

The levels of mRNA's which code for mitoplast and albumin polypeptides were estimated by their activities in the rabbit reticulocyte cell-free system. The wheat embryo system, which has a very low level of endogenous polypeptide synthesis (Fig. 7), proved unsatisfactory for these estima-

tions due to premature termination during synthesis of large molecular weight polypeptides, e.g. greater than 25% of radioactivity present in released chains was recovered in polypeptides with a molecular weight less than 10,000 (Fig. 7). This value (25%) is much higher than that for polypeptides labeled in vivo (unpublished observations). In the experiments described here, mRNA's from different subcellular fractions were assayed for activity in a system which synthesizes primarily completed chains but which also has a high level of endogenous polypeptide synthesis. Meaningful comparisons, therefore, are subject to certain assumptions. (*a*) The need for recovering undegraded mRNA in high yield has already been emphasized. The present study has largely been successful in meeting this criterion. Not only were polysomes shown to remain intact during tissue fractionation (Figs. 2 and 3), but translation of mRNA's coding for albumin and mitoplast polypeptides yielded products consisting primarily of completed polypeptides (Figs. 8-10), not a series of smaller polypeptides which would have accumulated if the mRNA's had been degraded. Also, when expressed per gram of tissue, the activities of these mRNA's in free polysomes, rough microsomes, and RSER fractions together added up to the activity calculated for total cytoplasm (from Table III). (*b*) The detection of specific translation products by antibody must be reliable. The antiserum used against albumin was monospecific and, following translation of RNA, it reacted with a polypeptide which coelectrophoresed with rat serum albumin and which contained >80% of precipitated radioactivity (Fig. 8). Antiserum to mitoplasts did not react with other subcellular fractions or with the reticulocyte lysate itself (Fig. 6) and detected in vitro translation products, some of which coelectrophoresed with mitoplast markers (Figs. 9 and 10). Antiserum against whole mitochondria was not used in these tests because mitochondrial outer membrane may be continuous with ER in vivo (18, 41), and thus the two may share common antigenic determinants. Nevertheless, antibody precipitated a number of in vitro polypeptide products which did not coelectrophorese with immunoprecipitated mitoplast proteins prelabeled in vivo; and vice versa, there were examples of the latter which did not coelectrophorese with any of the in vitro labeled 'mitoplast' polypeptides (Fig. 10). There was exact comigration, however, between two in vivo and in vitro labeled products (Fig. 10). As described in

the previous paper (54), synthesis of all *in vivo* labeled mitochondrial products which could be resolved on these gels was inhibited by cycloheximide so that mitochondrial ribosomes were not responsible for synthesizing any of the labeled markers used here. Thus, in view of the specificity of our antiserum, the simplest interpretation for these discrepancies is that many of the putative mitoplast polypeptides synthesized *in vitro* (Figs. 9 and 10) are not subject to the kinds of posttranslational modifications which occur in many mitochondrial proteins, and membrane proteins in general, in the intact cell, and which affect their electrophoretic mobilities in SDS-gels (e.g., glycosylation, proteolytic cleavage, lipidation, covalent attachment of heme to cytochrome *c*, etc)¹. (c) A serious problem with measuring mRNA levels by their ability to support polypeptide synthesis *in vitro* is the possibility that crude RNA preparations from different subcellular fractions may contain different levels of activating or inhibiting components which influence the cell-free system. This is usually avoided in the reticulocyte system by expressing the radioactivity incorporated into specific polypeptides as a percentage of total incorporation (see Table III). In the case of mitoplast polypeptides, however, their synthesis could not accurately be assayed by measuring radioactivity incorporated into immunoprecipitates due to possible contamination by nonspecific trapping (Figs. 9 and 10). But the differences between mitoplast polypeptide synthesis using RSER and rough microsomal RNA, as recorded by densitometer tracings of gels (up to fivefold, Fig. 9), were large compared to differences for isotope incorporation into total protein by the lysate (at most 1.5- to 1.9-fold). The assumption remains, however, that different mRNA's in the same fraction are translated with equal efficiency.

With the above considerations in mind, the results of the *in vitro* translation experiments described in this study indicate that albumin mRNA is about equally distributed between RSER and rough microsomes, or slightly enriched (50%) on RSER when albumin synthesis is expressed as a percentage of total lysate activity (Table III). Mitoplast mRNA's, however, were clearly enriched in the rough microsome fraction, not in RSER (Fig. 9). The same conclusion was obtained when

¹ Shore, G. C., and J. R. Tata. Functions for polyribosome:membrane interactions in protein synthesis. Manuscript in preparation.

RSER-RNA was compared to total RNA derived from postmitochondrial supernates (Fig. 10), even though mitoplast mRNA activity in the latter was diluted by the presence of free polysomal RNA (see legend, Table III). This enrichment was obtained for the two *in vitro* products which comigrated with immunoprecipitated mitoplast and also for all of the putative mitoplast polypeptide products which reacted with antiserum but did not comigrate with a marker (Figs. 9 and 10). Because RSER and rough microsomes contain about the same amount of polysomal RNA per gram liver (Table I, Figs. 2c and 3b; or per unit glucose-6-phosphatase (ER marker) activity, reference 37), and because total messenger content of these two fractions is similar (as judged by polyU hybridization, Table II; or activity in the wheat embryo system, Fig. 7), the difference obtained *in vitro* for the distribution of albumin and mitoplast mRNA's between these two fractions of RER may reflect a similar difference in the functional distribution of these messengers *in vivo*. Certainly, the fact that two fractions of ER were obtained which contained different ratios for the activity of mRNA's coding for albumin and mitoplast proteins must mean that the two fractions did not arise merely as the result of unequal fragmentation of the same membrane during homogenization. Thus, assuming that RSER and rough microsomes do not exist exclusively in different liver cell types (16, 61), this study demonstrates that at least one class of cytoplasmic mRNA's, those coding for polypeptides destined for transport to the mitochondrion, may be segregated or compartmentalized preferentially in one fraction of RER within the cell, whereas mRNA coding for a secreted protein (albumin) is not so compartmentalized. Indeed, experiments which tested products of the total polyA⁺-RNA population in the two fractions (Fig. 7) revealed that the majority of membrane-bound mRNA's are distributed about equally between the two ER fractions, or are slightly enriched in the RSER fraction. It may be very significant, therefore, that enrichment on rough microsomes involved mRNA's which code for polypeptides destined for intracellular transport, and not export out of the cell. But, again, it must be emphasized that whereas heterogeneity in the composition of hepatic RER has been demonstrated in this and other studies (12, 13, 38), it is not at all certain whether such heterogeneity exists within the same cell. This question must be resolved before considering a role for RER in the segregation within the

same cell of mRNA's which code for mitochondrial proteins, relative to other membrane-bound mRNA's. Toward this end, recent advances (16) in separating rat hepatocytes according to differences in cell density, a property which may reflect in part differences in ER morphology (16, 61), should be very useful.

The previous paper (54) provided evidence that RER is closely associated with mitochondria in the RSER fraction, but that these mitochondria are not particularly active in incorporating radioactive proteins newly made in the cytoplasm, and the present study demonstrates that this fraction of ER has relatively low levels of mRNA associated with it which code for at least some mitochondrial proteins. Taken together, these results are somewhat surprising, especially since evidence from yeast (10, 35) suggests that cytoplasmic synthesis of mitochondrial proteins may be compartmentalized on 80S ribosomes attached to the outer surface of mitochondria, and morphological evidence in higher organisms shows that ER cisternae may be continuous with mitochondrial outer membrane *in vivo* (18, 41). On the other hand, a number of studies on liver tissue provide evidence that mitochondrial proteins are formed on the rough microsome fraction recovered from postmitochondrial supernates (23, 25, 32-34, but see 19). Such products which are present in microsomal membrane may even be induced to transfer to mitochondria *in vitro* (32). It is true that a major fraction of total mitochondria are found *in vivo* in association with large stacks of RER cisternae, especially in the perinuclear region of the cell (54), and that these may be recovered in the RSER fraction (37, 54), but mitochondria are also frequently seen in association with single cisternae of ER distributed throughout the cytoplasm (17, 54, see especially micrographs in references 16 and 61). It could be that these single cisternae are preferentially involved in the synthesis of mitochondrial proteins and are recovered primarily in the rough microsome fraction following tissue homogenization (54).

Compartmentalization of cytoplasmic synthesis of mitochondrial proteins on ER cisternae closely apposed to mitochondria could be an important aspect of the mechanism whereby the endocellular membrane system discriminates between polypeptides which are destined for transport to different cellular loci. Deposition of newly formed mitochondrial proteins near their final destination might be important for the polypeptide to interact

with those forces, whatever they may be, which result in transfer of the polypeptide to the proper 'sink', in this case of mitochondrion. Recent studies (G.C. Shore, unpublished observations) show that nascent chains on undegraded mitoplast polyosomes interact strongly with the membrane of rough microsomes. Attachment of these polyosomes is just as resistant to high concentrations of KCl as attachment of albumin polyosomes. If it turns out that, in analogy with albumin, such interaction between nascent chains and membrane reflects vectorial discharge across the ER, then the process for transporting proteins to the mitochondrion may be similar to the initial process of extracellular protein secretion (43), with transport occurring within the confines of ER cisternae which in turn have access to the mitochondrion because they can be continuous with the mitochondrial outer membrane (18, 41).

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