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Studies on Ribosome-membrane Interaction

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STUDIES ON RIBOSOME-MEMBRANE INTERACTION

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PREFACE

This work was conducted in the laboratory of cell biology. I am grateful to all members of the laboratory for their encouragement and for providing me with a pleasant environment in which to work. I would especially like to thank my research advisor, David Sabatini, for his interest in this work and for being constantly available for helpful discussions and guidance. I would also like to thank Belinda Ulrich for general helpfulness, and Barbara Frames for typing most of this thesis.

ABSTRACT

Several aspects of the relationship between free and membrane-bound ribosomes and of the ribosome-membrane interaction were investigated in a rat liver cell-free system.

a) When nascent polypeptide chains were terminated by incubating rough microsomes in a medium optimal for amino acid incorporation, a subsequent incubation in a solution containing 0.5 M KCl and 0.0025 M $MgCl_2$ resulted in a partial detachment of ribosomes, as 40 S and 60 S particles.

b) Solutions containing high concentrations of monovalent ions (1 M KCl) and no magnesium ions detached essentially all membrane-bound ribosomes as unfolded subunits, while the peptidyl tRNA molecules remained associated with the microsomal vesicles.

c) An exchange of small subunits between free and membrane-bound ribosomes was found to occur in vitro upon release of polypeptide chains under conditions thought to approximate the physiological one. The exchange resulted in the transfer of tritium-labeled subunits to rough microsomes and, vice versa, the detachment of labeled subunits from rough microsomes upon addition of competing, unlabeled subunits. Up to 60% of the membrane-associated small subunits exchanged with excess added small subunits obtained from free polysomes, when polypeptide chains of membrane-bound ribosomes were released by puromycin. The exchange required a macromolecular fraction of the cell sap, was stimulated by ATP or GTP, and occurred at low concentrations of magnesium ions. Addition of large subunits to the system caused a transfer of small subunits of membrane-bound ribosomes into a newly formed pool of free monomers. However, in the time period studied, membrane-bound large subunits did not exchange efficiently with added large subunits obtained from either free or bound ribosomes. These results were confirmed by analyzing the material bound to membranes after incubation for exchange.

d) The binding of ribosomes to rough microsomes stripped of their ribosomes was studied at a low ionic strength. The binding reached a maximal value after incubation for five minutes at 37° C. The RNA to protein and RNA to phospholipid ratios in reconstituted rough microsomes were 65% and 55% of untreated rough microsomes respectively. The binding of ribosomes to smooth microsomes treated for stripping was half that observed for stripped rough microsomes, and binding to similarly treated erythrocyte ghosts was virtually nil. The capacity of heat treated stripped rough microsomes to bind ribosomes was reduced approximately sevenfold with respect to untreated controls.

e) Comparison of the buoyant densities of ribosomal subunits by CsCl density gradient centrifugation revealed no differences between subunits obtained from free and bound ribosomes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis also showed close similarities in the protein patterns of subunits from free and bound ribosomes, with the exception of one protein band which was more intense in free large subunits.

These findings are discussed in relation to possible mechanisms of the assembly of membrane-bound ribosomes.

TABLE OF CONTENTS

ABBREVIATIONS USED

RM = rough microsomes

SM = smooth microsomes

AAs = amino acids

PEP = phosphoenolpyruvate

PK = pyruvate kinase

SDS = sodium dodecyl sulfate

DOC = deoxycholate

EDTA = ethylenediaminetetraacetic acid

tRNA = transfer RNA

mRNA = messenger RNA

rRNA = ribosomal RNA

DTT = dithiothreitol

TEMED = N,N,N',N'-Tetramethylenediamine

I. INTRODUCTION

1.1. Free and Membrane-Bound Ribosomes

In mammalian cells, cytoplasmic ribosomes exist in two states: bound to membranes of the rough endoplasmic reticulum or free in the cytoplasm. Early morphological evidence indicated that membrane-bound ribosomes are abundant in cell types specialized for secretion (Palade, 1955, 1956; Porter, 1961). For example, in the pancreatic exocrine cell, which is specialized in the secretion of digestive enzymes, the bulk of the ribosomes are membrane-bound, whereas in the reticulocyte, a cell producing haemoglobin, which is not exported, most ribosomes are free in the cytoplasm. An intermediate case is that of the hepatocyte, a cell which carries out many important metabolic processes, but is also involved in the secretion of several serum proteins; correspondingly a large population of membrane-bound ribosomes coexists, in the hepatocyte, with a sizeable population of free ribosomes. Figs. 1 and 2 show electron micrographs of sections of a hepatocyte, demonstrating the typical disposition in parallel arrays of the cisternae of the rough endoplasmic reticulum, in between which free ribosomes are apparent.

The early morphological observations which led to the recognition of free and bound ribosomes in the liver cell were soon confirmed by biochemical analysis on cell fractions. Fragments of the endoplasmic reticulum, isolated from cell homogenates as a fraction of membrane-bound vesicles, the microsomes, provided the material from which bound ribosomes can be recovered after treatment with detergents, which solubilize the membranes (Palade and Siekevitz, 1956a and b). Biochemical analysis on rat liver homogenates and cell fractions showed that in the rat hepatocyte $\sim$75% of the ribosomes are membrane-bound while $\sim$25% are free (Blobel and Potter, 1967a). Chemical studies showed that the ribosomal RNAs of free and bound ribosomes are similar in their sedimentation coefficients, base composition and metabolic half-lives (Loeb, Howell and Tomkins, 1967; Talal and Kaltreider, 1968;

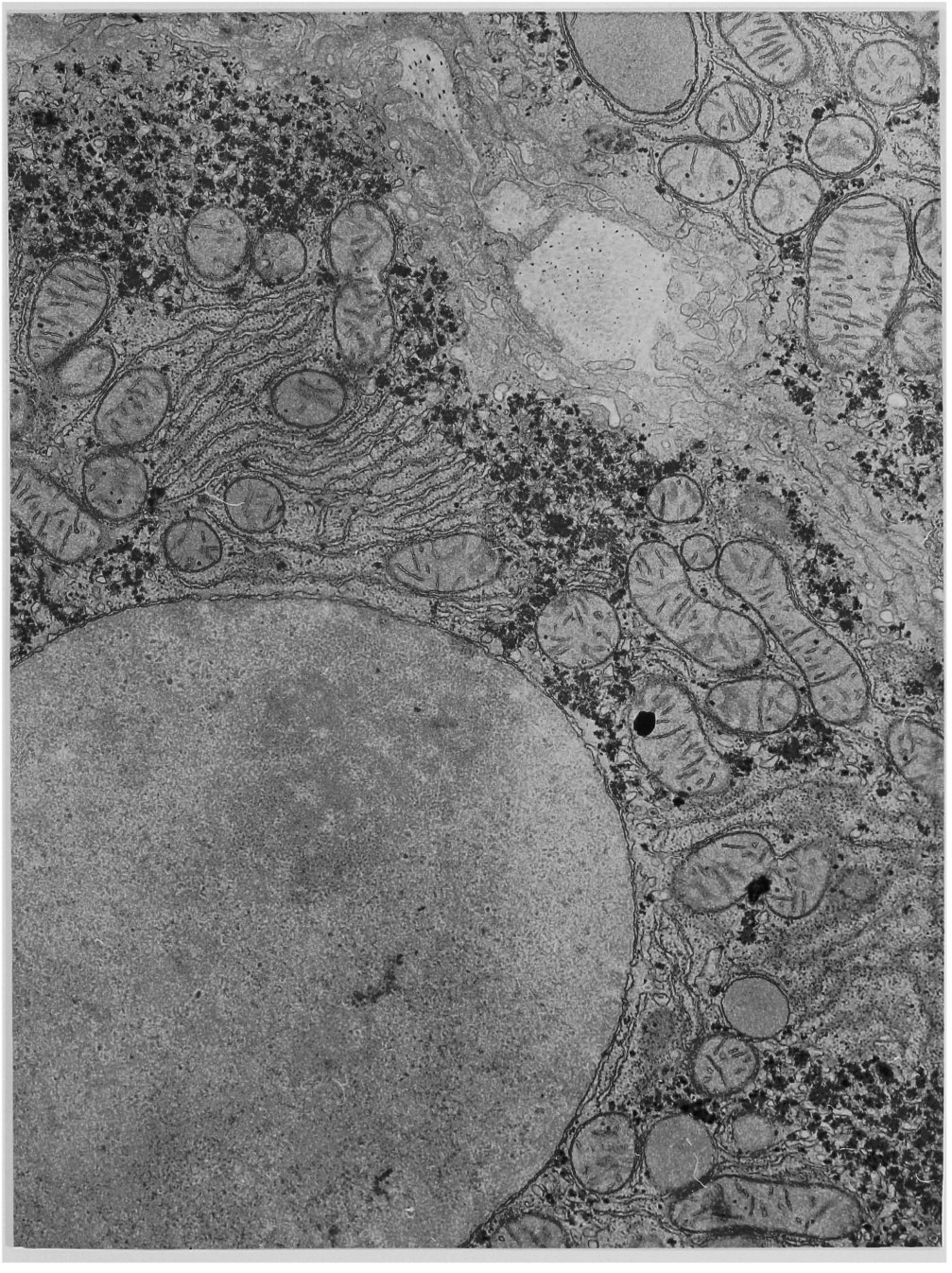

Figure 1. Electronmicrograph of a section through parts of two rat hepatocytes. Arrows point to an area of rough endoplasmic reticulum cut transversally. In the lower right hand corner of the electronmicrograph ER cisternae are cut tangentially and patterns of membrane-bound polysomes are seen.

Tissue fixed in 2% gluteraldehyde, 0.1 M phosphate buffer, pH 7.4 and postfixed in 1% OsO_4, 0.1 M phosphate buffer; block stained with 0.5% uranyl acetate in veronal acetate buffer; sections stained with uranyl acetate and lead citrate.

N = nucleus; Nu = nucleolus; G = glycogen area.

x 19,000.

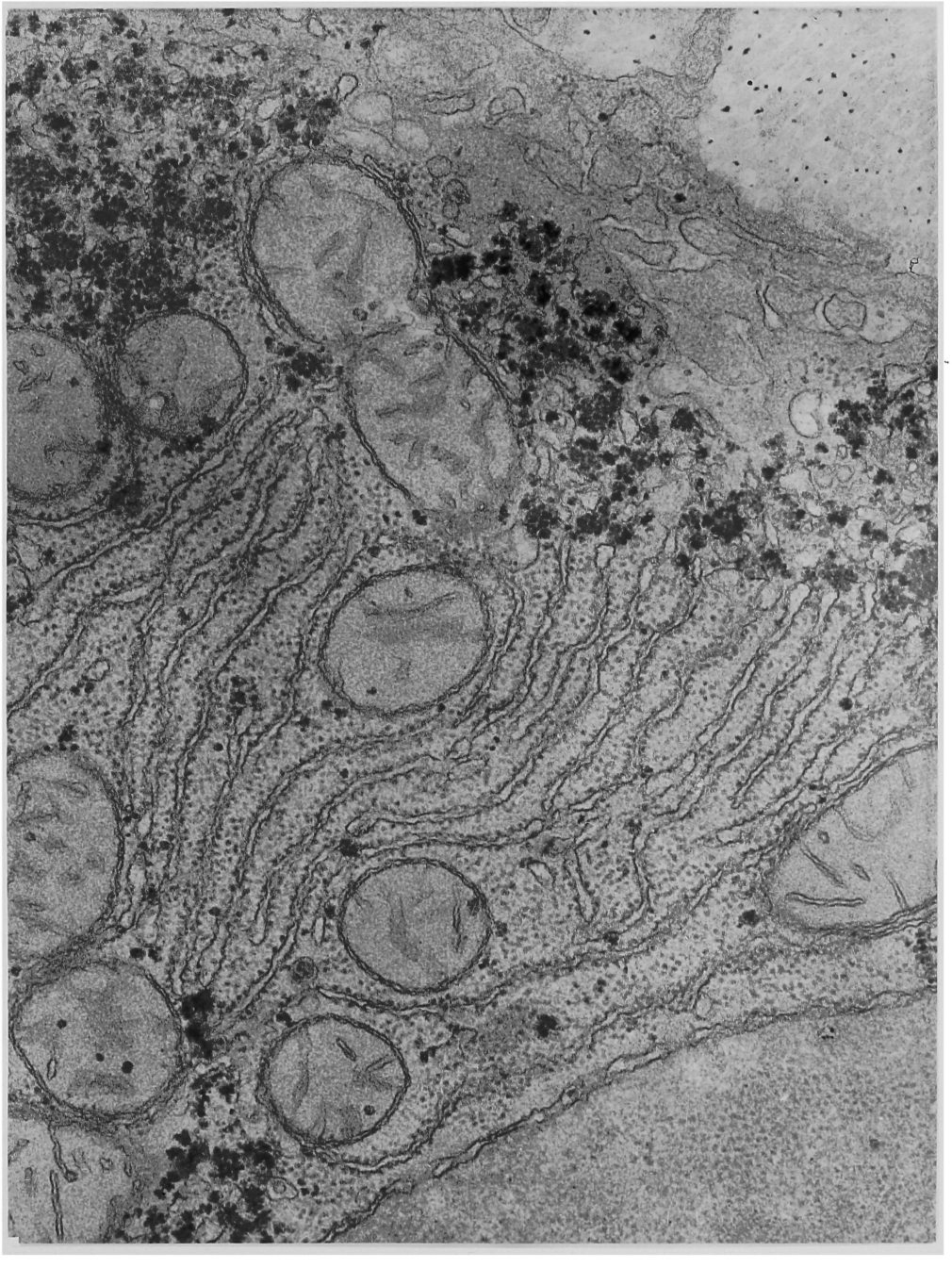

Figure 2. Higher magnification view of the area of rough endoplasmic reticulum marked by arrows in Figure 1. Ribosomes are bound to the membranes of parallel cisternae or free in the intervening cytoplasm.
x 48,500.

Tanaka, Takagi and Ogata, 1970; but for metabolic half-life of rRNAs see also: Bouvet and Moulé, 1964; Murty and Hallinan, 1968).

During the past twelve years evidence has accumulated that membrane-bound ribosomes are responsible for the translation of specific messengers directing the synthesis of secretory proteins (Siekevitz and Palade, 1960; Campbell, Greengard and Kernot, 1960; Peters, 1962a and b; Ganoza, Williams and Lipmann, 1965; Williams, Ganoza and Lipmann, 1965; Redman, Siekevitz and Palade, 1966; Sargent and Campbell, 1965; Takagi and Ogata, 1968; Redman, 1968; Scherr and Uhr, 1971; Permutt and Kipniss, 1972). Direct comparison, by immunochemical techniques, of the products of synthesis of free and membrane-bound ribosomes has indicated that, in the liver, free ribosomes may be active in the synthesis of proteins retained in the cell, while bound ribosomes are engaged in the synthesis of secretory proteins (Hicks, Drysdale, and Munro, 1969; Takagi, Tanaka and Ogata, 1969; Redman, 1969a and b; Ganoza and Williams, 1969; Takagi, Tanaka and Ogata, 1970; but see also: Pitot et al., 1969; Ragnotti, Lawford and Campbell, 1969).

It has also been suggested that membrane-bound ribosomes are involved in the synthesis of organelle proteins, such as membrane proteins of the endoplasmic reticulum (Dallner, Siekevitz and Palade, 1966a and b; Omura and Kuriyama, 1970), peroxisomal catalase (Higashi and Peters, 1963a and b) and mitochondrial malate dehydrogenase (Bingham and Campbell, 1972).

Although some knowledge has accumulated regarding the synthetic products of free and bound ribosomes, the mechanism of assembly of the polysome-membrane complex is as yet unclear. An understanding of the mechanism by which polysomes, operating in the translation of specific messengers, recognize the membrane, would be of interest to elucidate the process by which specific proteins are distributed intracellularly.

1.2. Studies on the Mode of Interaction of Ribosomes with Microsomal Membranes

A knowledge of the factors involved in maintaining the ribosome-membrane complex would be a first step toward an understanding of the process of assembly of the rough endoplasmic reticulum. Some knowledge of these factors has been obtained by studying conditions which lead to the disassembly *in vitro* of rough microsomes.

The implication of divalent cations in the attachment of ribosomes to the endoplasmic reticulum came from the early finding that the chelating agent EDTA detached 50% of the microsomal RNA (Siekevitz and Palade, 1956). It was shown in 1966 (Sabatini, Tashiro and Palade, 1966) that in guinea-pig liver, ribosomes are attached to microsomal membranes via their large subunits. The evidence supporting this conclusion was both morphological and biochemical, the biochemical experiments involving the selective detachment of small ribosomal subunits from microsomes by the chelating agent EDTA. Subsequent morphological observations on neoplastic mouse plasma cell microsomes (Shelton and Kuff, 1966) and on intact mouse liver cells (Florendo, 1969) were in agreement with the finding of Sabatini *et al*. The biochemical data were also corroborated by other workers with microsomes from other types of mammalian cells (Bennett and Hallinan, 1966; Attardi, Cravioto and Attardi, 1969; Rosbash and Penman, 1971a; but see also: Azcurra and Sellinger, 1967; Lee, Krsmanovic and Brawerman, 1971).

Sabatini *et al*. (Sabatini *et al*., 1966) also found that the nascent polypeptide chain is contained in the large ribosomal subunit of membrane-bound ribosomes. The polypeptide chain can be discharged into the internal space of microsomal vesicles upon termination in an amino acid incorporation medium (Redman, Siekevitz and Palade, 1966) or upon puromycin induced release from the ribosome (Redman and Sabatini, 1966; Redman, 1967; Andrews and Tata, 1968; Bevan, 1971). The close association of the nascent chain with the microsomal vesicles protects

it from the action of proteolytic enzymes (Sabatini and Blobel, 1970). The role of the nascent chain in maintaining the ribosome-membrane complex has recently been thoroughly investigated in our laboratory (Adelman, Blobel and Sabatini, 1970; Sabatini, Blobel, Nonomura and Adelman, 1971; Adelman, Sabatini and Blobel, in preparation). It was found that release of nascent chains by puromycin in vitro causes the release of ribosomes from rough microsomes, if the concentration of monovalent ions is sufficiently high. There are thus two factors involved in maintaining the ribosome-membrane association: 1) ionic bonds, disruptable by high concentrations of monovalent ions, and 2) the nascent polypeptide chain, which anchors the ribosome to the membrane. At low ionic strengths (25 to 150 mM KCl) the nascent chain is not necessary to maintain the ribosome-membrane complex, since ribosomes remain bound to membranes even after release of their nascent chains by puromycin.

A second experimental approach to the problem of the mode of interaction of ribosomes with microsomal membranes is the attempt to associate ribosomes with membrane fractions in vitro. This kind of approach could answer questions concerning the character of membrane binding sites, the requirements for ribosome binding, the existence of a class of ribosomes that bind to membranes preferentially over others. This approach has been undertaken by Pitot and coworkers (Süss, Blobel and Pitot, 1966; Ragland, Shires and Pitot, 1971; Shires, Narurkar and Pitot, 1971a and b) using "conditioned" rough microsomes (i.e. microsomes stripped of their ribosomes by treatment with a chelating agent and, more recently, ribonuclease) and free polysomes from rat liver, and by a group in Rabin's laboratory (James, Rabin and Williams, 1969; Sunshine, Williams and Rabin, 1971; Blyth, Freedman and Rabin, 1971; Roobol and Rabin, 1971), using rat liver smooth microsomes or degranulated rough microsomes and polysomes. The results obtained from this approach have supplied as yet limited information, which will be discussed in Chapter IV.

A third experimental approach to the problem has been to devise experimental conditions that alter the distribution of free and bound ribosomes in vivo. Agents such as hormones (Tata, 1967a and b, 1968 and 1970; Cox and Mathias, 1969; Rancourt and Litwack, 1968), carcinogens (Porter and Bruni, 1959; Lafontaine and Allard, 1964; Benedetti and Emmelot, 1966; Ketterer, Holt and Ross-Mansell, 1967; Butler, 1966) and inhibitors of protein synthesis (Blobel and Potter, 1967b; Rosbash and Penman, 1971a; Bleiberg, Zauderer and Baglioni, personal communication), and experimental conditions such as starvation (Lee, Krsmanovic and Brawerman, 1971; Sidrawsky, Verney and Shinozuka, 1969) have been used, and possible resulting alterations in the distribution of free and bound ribosomes have been studied electronmicroscopically or biochemically. In addition, the effects of inhibition of protein synthesis on the transport in vivo of newly synthesized mRNA (Rosbash, personal communication) or ribosomal particles (Baglioni, Bleiberg and Zauderer, 1971) to the rough endoplasmic reticulum have been studied. Although this type of approach may be promising, the results obtained to the present date have generally suffered from inadequate cell fractionation procedures, such as the failure to isolate reasonably pure microsomal fractions from tissue culture cells, and a lack of serious attempts to provide quantitative data.

1.3. Outline of the Project

Free ribosomes, both in prokaryotic and eukaryotic cells, are known to undergo cyclic dissociations into subunits and subsequent reassociation in between termination and reinitiation of synthesis of polypeptide chains. This dissociation-association reaction results in ribosomal subunit exchange, which can be followed using ribosomes labeled with different isotopes (Kaempfer, Meselson and Raskas, 1968; Kaempfer, 1968 and 1969; Ceccarini, Campo and Andronico, 1970; Jacobs-Lorena and Baglioni, 1970; Howard, Adamson and Herbert, 1970; Falvey and Staehlin, 1970). Our main effort has been directed to the behavior of membrane-bound ribosomes upon polypeptide chain release

under conditions which are presumed to approximate the physiological one. Under these conditions it has been shown that ribosomes are not efficiently detached from rough microsomes, even after release of their nascent chains by puromycin (Adelman et al., 1970). Our question has been: upon polypeptide chains release in vitro, induced by natural termination in a medium optimal for amino acid incorporation or by the action of puromycin, 1) do the subunits of membrane-associated ribosomes undergo a dissociation-association reaction similar to the one occurring in free ribosomes, and 2) does the large subunit-membrane complex undergo a similar reaction? Reaction (1) should result in exchange of membrane-associated small subunits with added small subunits (small subunit exchange); reaction (2) should result in the exchange of membrane-associated large subunits with added large subunits (large subunit exchange). Experiments conducted to detect these reactions are reported in section 3.2. We have also carried out some experiments on the disassembly of rough microsomes in vitro at high ionic strength (section 3.1) and on the reassembly of rough microsomes from its constituent parts (section 3.3). Finally we have investigated possible structural differences between free and membrane-bound ribosomes (section 3.4).

All experiments have been carried out with microsomes and free polysomes from rat liver. Among the obvious advantages offered by the rat liver system are the suitable relative proportions of free and membrane-bound ribosomes, which are known to synthesize different products, the existence of well developed cell fractionation procedures, and the extensive studies already carried out in this system on ribosome-membrane interaction.

II. MATERIALS AND METHODS

2.1. Fractionation and Labeling of Liver Cells

2.1.1. Rough Microsomes, Smooth Microsomes and Free Polysomes. Rough microsomes (RM), smooth microsomes (SM) and free polysomes were prepared by either of the following two procedures:

Procedure A (Adelman, Blobel and Sabatini, manuscript in preparation). The fractionation scheme is summarized in the flow diagram of Fig. 3. The method, recently developed in this laboratory, consists of the following operations. 100-150 g rats were starved for 15-20 hours before decapitation with a guillotine (Harvard Apparatus Co., Dover, Mass.). The livers were quickly removed, immersed in ice-cold 0.25 M sucrose and transferred to a 4°C cold room. The tissue was passed through a stainless steel press and homogenized in a Potter-Elvehjem homogenizer with 2 volumes of 1 M sucrose using 4 strokes of a motor driven Teflon pestle. The homogenate was filtered through a nylon net (Nitex, nylon monofilament, 130 μ, Tobler Ernst and Traber, New York, N.Y.) and diluted twofold with 2.5 M sucrose. All centrifugations were carried out in an International B60 preparative ultracentrifuge (International Equipment Co., Needham Heights, Mass.) at 3° C. 25 ml portions of the homogenate were poured into centrifuge tubes of the SB110 rotor. The content of each tube was overlayed with 1 ml 1 M sucrose, so that material which floated to the top during centrifugation would not be exposed to a liquid-air interface. A nuclear fraction was sedimented after centrifugation for 45 min at 25,000 rpm. The postnuclear supernatant derived from 100 ml of homogenate was diluted by the addition of 50 ml deionized water and centrifuged for 15 minutes at 15,000 rpm in the A211 rotor to sediment mitochondria and large particles. The postmitochondrial supernatant (PMS) was decanted and saved. The mitochondrial pellets were washed twice (15 min at 13,000 rpm) with a 9:1 mixture of 0.5 M sucrose and rat liver high speed supernatant (for preparation, see below). The first postmitochondrial supernatant and the two washes were combined and

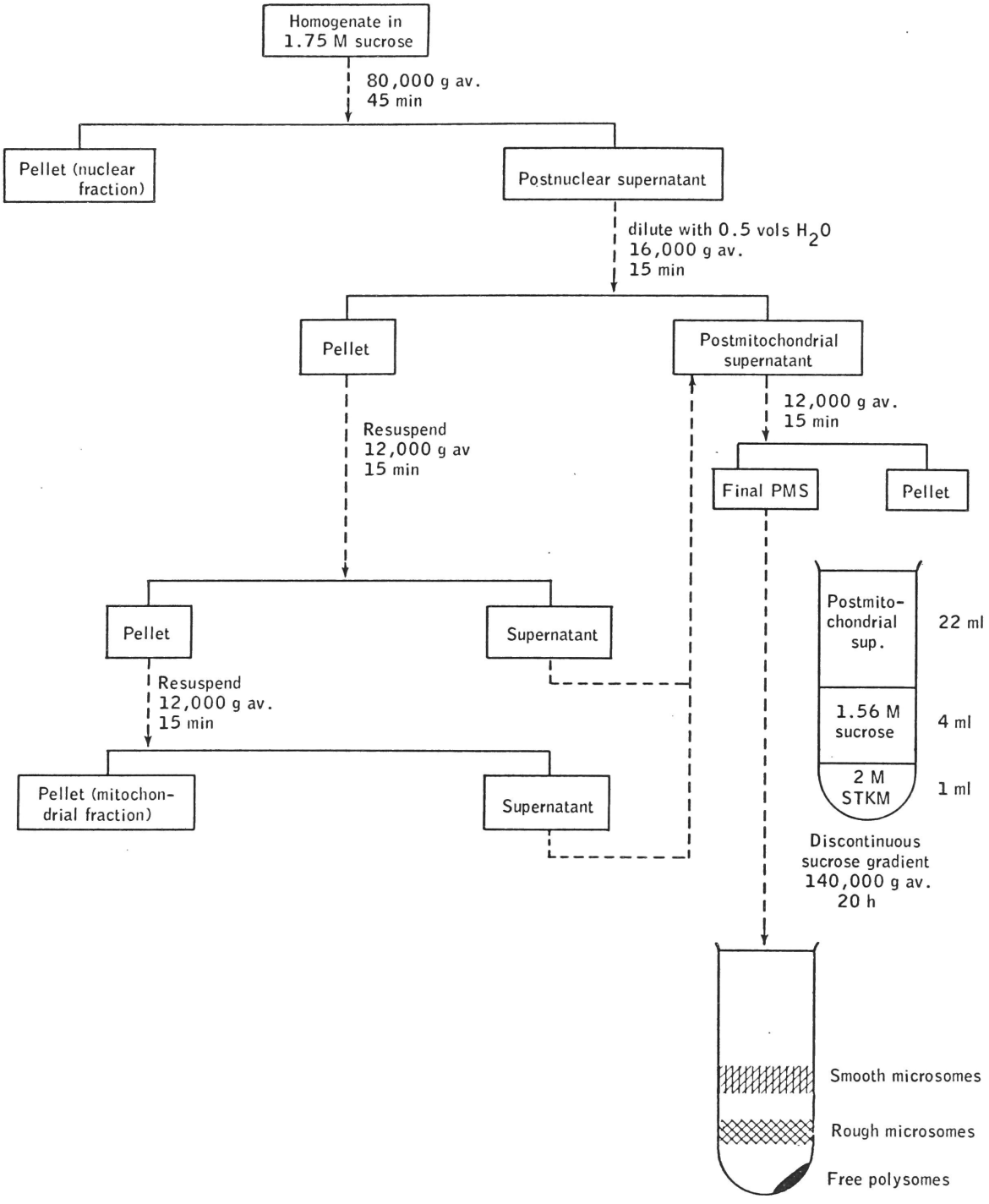

Figure 3. Flow diagram summarizing fractionation of liver cells by procedure A. For details, see text.

centrifuged for 15 min at 13,000 rpm in the A211 rotor, to obtain a final postmitochondrial supernatant.

Discontinuous sucrose density gradients were used to separate rough microsomes, smooth microsomes and free polysomes from the final postmitochondrial supernatant. The gradients, prepared in tubes of the A211 rotor, consisted of 1 ml 2 M sucrose-TKM (TKM = 50 mM Tris HCl, pH 7.5 at 20° C, 25 mM KCl, 5 mM $MgCl_2$), overlayed with 4 ml of a 3:1 combination of 2 M sucrose and high speed supernatant (final sucrose concentration: 1.56 M). Approximately 22 ml of the postmitochondrial supernatant were loaded onto each gradient. After centrifugation for 20 hr at 44,000 rpm, the rotor was stopped without braking. Free polysomes were recovered in the pellets and smooth and rough microsomes formed two bands at the liquid interfaces on each side of the 1.56 M sucrose layer. The top 18 ml of the gradients were removed and discarded and the two microsome bands were collected separately. The ribosomal pellet was rinsed with distilled water, drained and stored at -20° C. Each pellet contained ∿2.5 mg RNA

The pooled smooth microsome fraction was diluted 6:1 with 0.25 M sucrose-TKM and the microsomal vesicles were sedimented into pellets by centrifugation for 30 min at 44,000 rpm in the A211 rotor. The pellets were resuspended gently by hand in 0.25 M sucrose-TKM, using a Potter-Elvehjem homogenizer. One ml aliquots of the suspension, containing approximately 12 mg protein, were diluted 2:1 with glycerol and stored at -20° C for up to two months.

The pooled rough microsome fraction was diluted 6:1 either with 0.25 M sucrose-TKM (non-washed RM) or with a solution of high ionic strength containing 0.25 M sucrose, 0.5 M KCl, 0.05 M Tris-HCl and 0.010 M $MgCl_2$ (washed RM). This latter treatment was used to remove inactive ribosomes (i.e. ribosomes lacking nascent polypeptide chains) from the microsomal vesicles (Adelman et al., 1970), which in both cases were recovered by centrifugation at 35,000 rpm for 15 min in the A211 rotor, and resuspended by hand in 0.25 M sucrose-TKM. One ml aliquots of the suspension, containing approximately 12 mg protein,

were diluted 2:1 with glycerol and stored at -20° C for up to two months. The RNA to protein ratios of non-washed and washed RM were equal to ∿0.2 and 0.175 respectively.

Before use, rough or smooth microsomes were recovered from the microsomal suspensions, which were diluted 2:1 with TKM and centrifuged at 40,000 rpm for 15 min in the A321 rotor.

An electron micrograph of non-washed rough microsomes obtained by this procedure is shown in Fig. 4.

Procedure B (Blobel and Potter, 1967c; Blobel and Sabatini, 1970). The fractionation scheme is summarized in the flow diagram of Fig. 5. The preparation of the homogenate was as described for procedure A, except that the excised livers were chilled in 0.25 M sucrose-TKM and the liver pulp was homogenized in two volumes of 0.25 M sucrose-TKM, without subsequent dilution. A postmitochondrial supernatant was prepared by centrifuging the homogenate for 15 min at 16,000 rpm in the number 40 rotor of the Spinco L centrifuge (Beckman Instruments, Inc., Palo Alto, California). Four ml portions of the postmitochondrial supernatant were layered over three-layer discontinuous sucrose gradients, containing 2.0 ml 2.0 M sucrose, 1.5 ml 1.6 M sucrose and 1.5 ml 1.35 M sucrose, all in TKM. After the gradients were centrifuged for 14 to 20 hours in a Spinco No. 40 rotor at 40,000 rpm, the top 4 ml of the gradients were removed and discarded. The 1.35 M and 1.6 M sucrose layers, corresponding to the smooth and rough microsome fractions respectively, were collected separately and diluted 1:1 with TKM. The pellets, corresponding to the free polysome fractions, were rinsed with distilled water and stored at -20° C. Each pellet contained ∿3.5 mg RNA.

The smooth microsome suspension was centrifuged for 4 hr at 40,000 rpm in the Spinco No. 40 rotor. Eight ml portions of the rough microsome suspension were layered over 1.0 ml 1.35 M sucrose cushions, containing either TKM (non-washed RM) or 0.5 M KCl, 0.05 M Tris-HCl, 0.010 M $MgCl_2$ (washed RM) and centrifuged for 4 hr at 40,000 rpm in the Spinco No. 40 rotor. The resulting smooth and rough microsomal

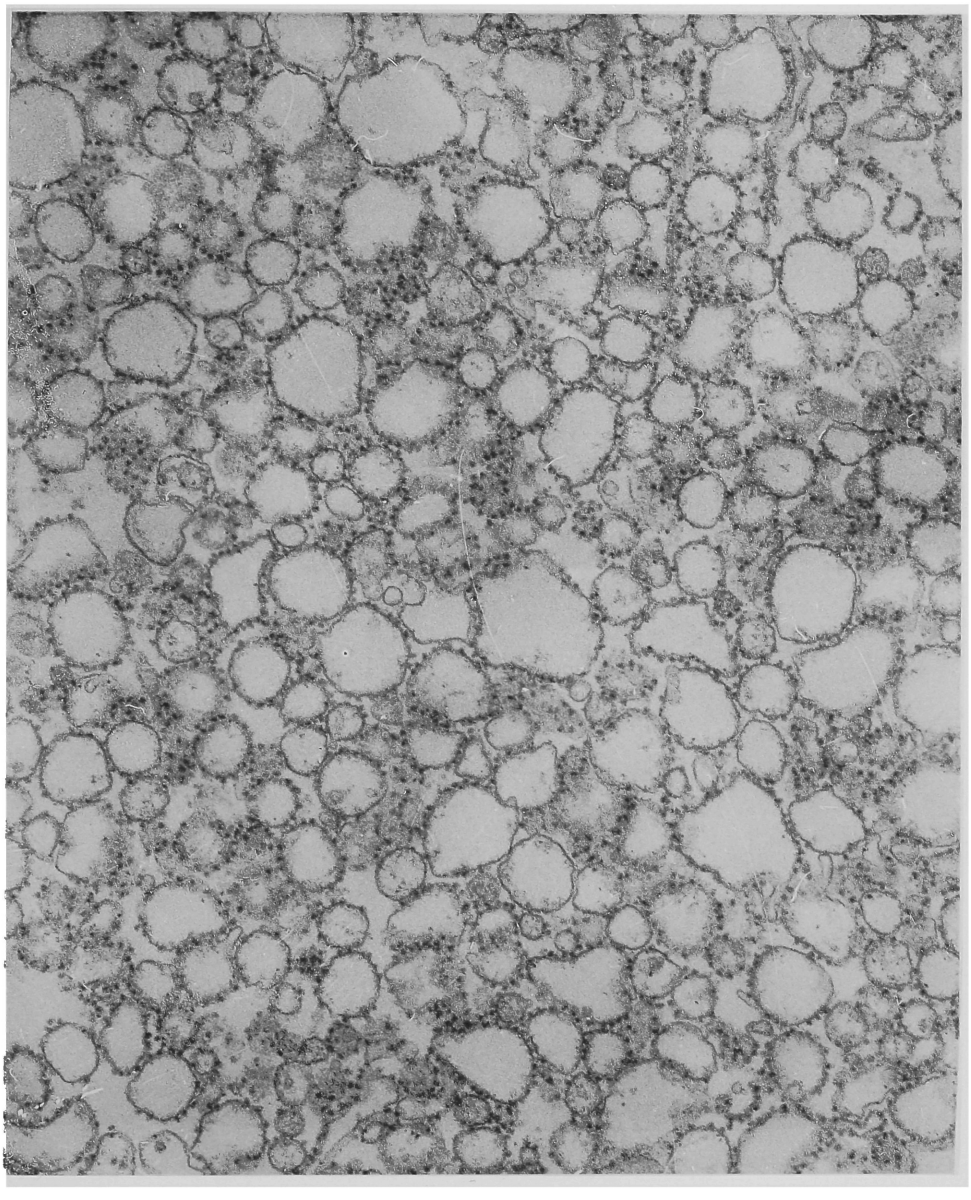

Figure 4. Electronmicrograph of a section through a pellet of the rough microsome fraction prepared by procedure A. Pellet fixed in 2% glutaraldehyde, 0.1 M Na^{+} cacodylate buffer and postfixed in 2% OsO_4, 0.1 M Na^{+} cacodylate; block stained with 0.5% Mg^{++} uranyl acetate in saline; sections stained with uranyl acetate and lead citrate.

x 44,000

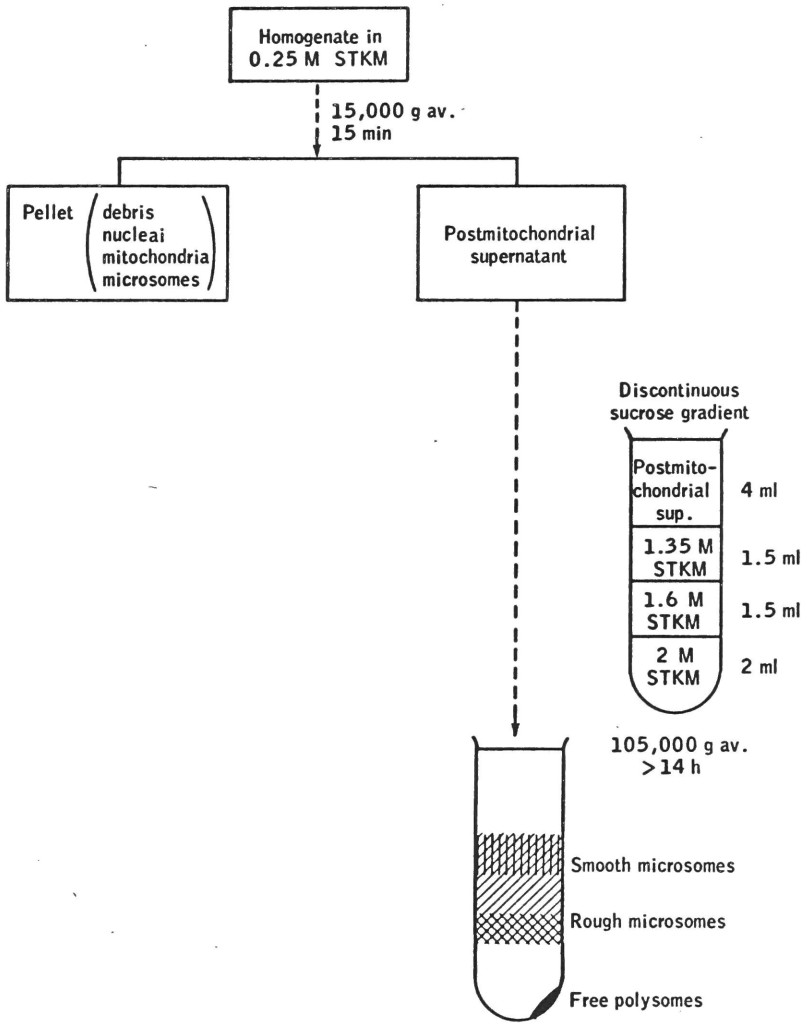

Figure 5. Flow diagram summarizing fractionation of liver cells by procedure B. For details, see text.

pellets were resuspended by hand in 0.25 M sucrose TKM, and, after addition of glycerol, were stored in aliquots and recovered from the suspensions before use, as described for procedure A.

Procedure A has the advantage of permitting a much higher recovery of rough microsomes (∿50% of the total RM) than procedure B (∿10% of the total RM), but the disadvantage of involving more manipulations and of producing polysomes partially degraded into ribosomal monomers. Unless otherwise specified in the text, procedure A was employed for the preparation of cell fractions used in the experiments described in this thesis.

2.1.2. High speed supernatant. The high speed supernatant, which was used as a source of RNase inhibitor (Blobel and Potter, 1966) for cell fractionation, was obtained from homogenates prepared using 2 ml 0.25 M sucrose per g of liver. The homogenate was centrifuged at first for 20 min at 25,000 rpm to remove large particles, and then for 2 hrs at 40,000 rpm in the A211 rotor of the International centrifuge, to remove microsomes and ribosomes. The final supernatant (∿20 ml per tube) was collected, avoiding the fatty layer at the meniscus and stored at -20° C.

The G-50 supernatant fraction used for amino acid incorporation in vitro was obtained from homogenates prepared in 0.25 M sucrose-TKM. The high speed supernatant was passed through a G-50 Sephadex column (2 cm x 50 cm) equilibrated with TKM containing 1 mM DTT. The material excluded from the column (G-50 supernatant fraction) was collected and stored in 1 ml aliquots at -80° C for up to two weeks. Each aliquot contained ∿15 mg protein.

2.1.3. Radioactive labeling in vivo. a) Free polysomes and rough microsomes were labeled with tritium in their RNA by injecting 200-250 μC orotic-5-(^{3}H) acid intraperitoneally to each rat 36 to 40 hrs before sacrifice. The specific radioactivity in ribosomes was 6×10^5 to 1.2×10^6 dpm/mg RNA. We will refer to ribosomes and rough microsomes labeled in this way as ^{3}H-labeled rough microsomes or ^{3}H-labeled ribosomes.

b) ^{3}H-labeled nascent polypeptide chains. 200 μC (^{3}H)-leucine were injected into the portal vein of anesthetized rats. Two minutes after beginning the injection the portal vein was cut and the liver excised. Specific radioactivity in the ribosomes was 1.5 to 2.5 x 10^{5} dpm/mg RNA. In a typical preparation (procedure A) 10% of the total acid insoluble radioactivity of the homogenate was recovered with free polysomes and 27% with rough microsomes, of which 60% was ribosome associated and 40% represented by finished chains. Eighteen percent of the applied isotope was recovered as acid insoluble material in the homogenate.

2.2. Preparation of Ribosomal Subunits

Ribosomal subunits from free polysomes (Blobel and Sabatini, 1970) or rough microsomes (Adelman et al., 1970) were obtained as previously described. Microsome or polysome suspensions (1 to 2 mg RNA/ml) were incubated for 10 min at 37° C with puromycin (10^{-3} M) in a high salt buffer (0.5 M KCl, 0.05 M Tris-HCl pH 7.6, 0.0025 M $MgCl_2$). Aliquots (1.65 ml) were layered onto 33 ml linear sucrose (5 to 20%)[1] gradients containing HSB (HSB = 0.5 M KCl, 0.05 M Tris-HCl, 0.005 M $MgCl_2$), which were centrifuged for 5 hrs at 20° C and 25,000 rpm in the SB110 rotor of the International centrifuge. The gradients were withdrawn from the top and the optical density profiles were recorded (for details, see below). Fig. 6 shows the optical density profile obtained for a typical preparation. The effluents corresponding to the 40 S and 60 S subunit peaks (shaded areas in Fig. 6) were collected separately, and diluted 1:1 with TKM. The subunits were sedimented by an overnight centrifugation at 3° C and 40,000 rpm in a Spinco No. 40 rotor. The pellets were rinsed with distilled water and stored for up to one month at -80° C. Fig. 7 shows the analysis of purified 40 S and 60 S particles after incubation in minimal exchange medium (for composition, see Results section).

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[1] % sucrose will be used for % sucrose weight/volume.

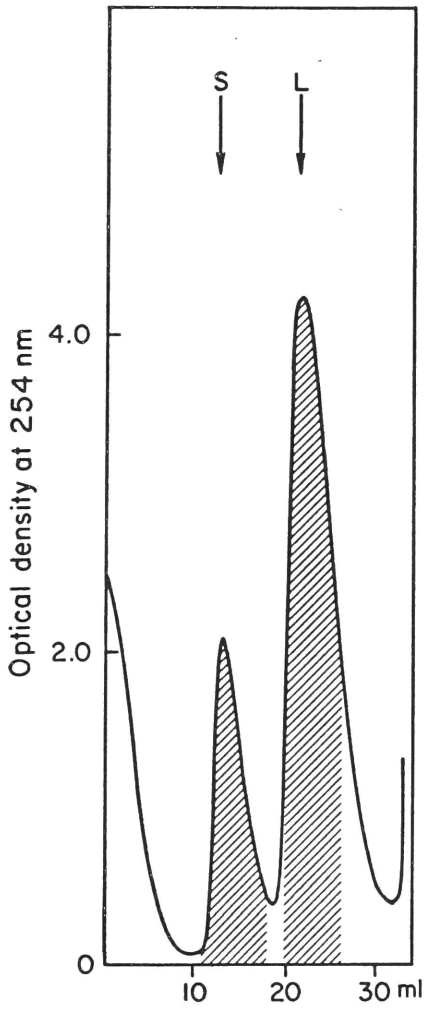


Figure 6. Separation of ribosomal subunits on a high salt 5 to 20% sucrose gradient.

Sedimentation properties of isolated ribosomal subunits after incubation in minimal exchange medium

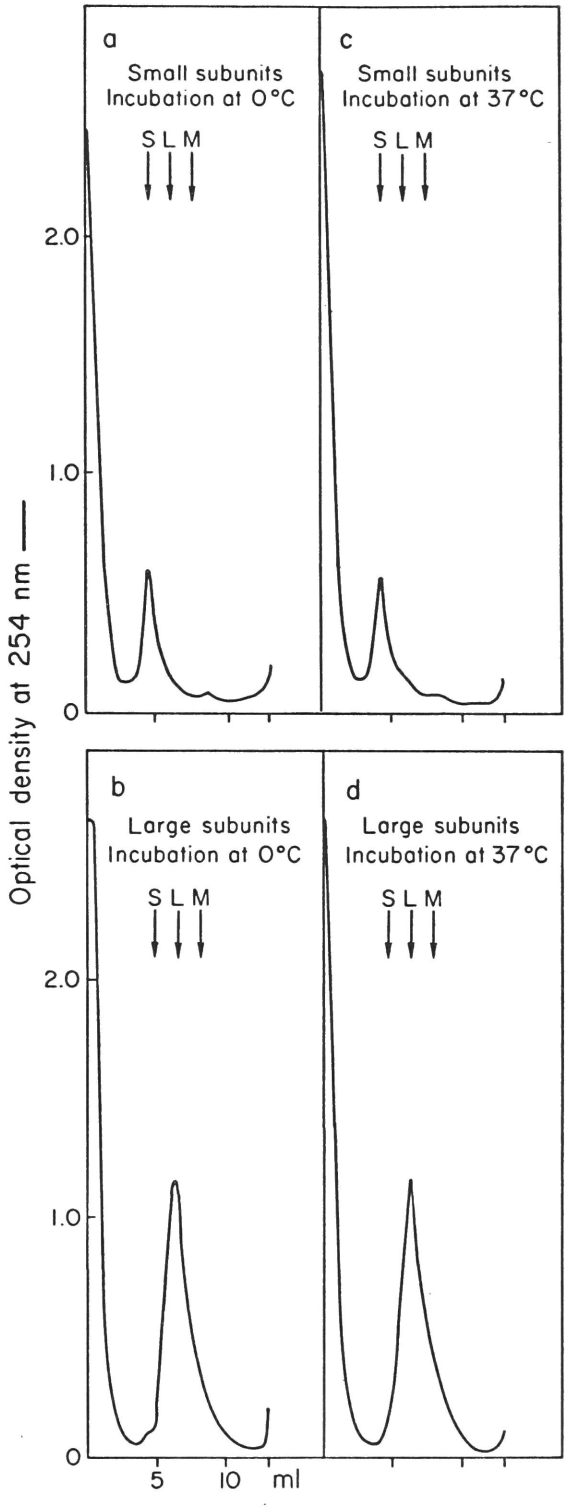


Figure 7. Sucrose density gradient analysis of purified ribosomal subunits after incubation in minimal exchange medium (for composition, see Results section). The subunits were prepared from free polysomes by the KCl-puromycin procedure. a and b, 2.8 OD_{260} units of small subunits; c and d, 7.0 OD_{260} units of large subunits; a and c, incubation at 0° C; b and d, incubation at 37° C.

2.3. Preparation of Stripped Membrane Fractions

Rough and smooth microsomes were stripped of their ribosomes essentially as described by Adelman et al. (Adelman et al., 1970; Adelman, Sabatini and Blobel, in preparation). Freshly prepared non-washed rough microsomes or smooth microsomes were resuspended in 0.25 M sucrose and a compensating buffer was added so that the final composition of the suspensions was 0.25 M sucrose, 0.5 M KCl, 0.05 M Tris-HCl, 0.0025 M $MgCl_2$, 10^{-3} M puromycin. The final membrane protein concentration was ∿5 mg/ml. The suspensions were incubated for 1 hr at 3° C, followed by 10 min at 25° C, after which they were diluted sixfold with 0.25 M sucrose-HSB and centrifuged for 15 min at 3° C and 35,000 rpm in the A211 rotor of the International centrifuge. The pellets were resuspended in 0.25 M sucrose-HSB (∿0.8 mg protein/ml) and the suspensions were again centriguged for 15 min at 35,000 rpm in the A211 rotor. The resulting microsomal pellets were resuspended in 0.25 M sucrose-TKM (∿8 mg protein/ml), diluted 1:1 with glycerol and stored at -20° C. Before use, microsomes were recovered from the suspensions as described for rough and smooth microsomes (section 2.1). Table I presents the results of chemical analysis of two preparations of non-washed RM and SM and of stripped RM and SM. If phospholipid is taken as a measure of membrane content, it can be seen that the stripping procedure resulted in the removal from rough microsomes of 85 to 90% of their RNA. Fig. 8 shows an electron micrograph of a typical preparation of stripped RM.

2.4. Preparation of Erythrocyte Ghosts

Erythrocyte ghosts, prepared as described by Dodge et al. (Dodge, Mitchell and Hanahan, 1963) were treated with high salt and puromycin in the same way as described for stripped RM and SM.

Erythrocytes were obtained from whole human blood by centrifugation for 20 min at 1,000 g_{av} in an IEC model SBV centrifuge in the cold room. The packed erythrocytes were washed three times and finally resuspended in an equal volume of isotonic NaH_2PO_4-Na_2HPO_4 buffer (pH

Table I

Chemical analysis of rough and smooth microsomes before and after removal of ribosomes by the KCl-puromycin procedure*

Sample	RNA/protein	RNA/PLP	PLP/protein
Rough Microsomes	0.205	0.565	0.315
	0.197	0.526	0.374
Stripped RM	0.037	0.084	0.440
	0.032	0.064	0.490
Smooth Microsomes	0.046	0.098	0.416
		0.110	
Stripped SM	0.005	0.011	0.430
	0.007	0.016	0.422

*Rough microsomes and smooth microsomes, prepared by the method of Adelman et al. (in preparation), were stripped of ribosomes as described in the text. Appropriate aliquots were taken in duplicate for RNA, protein and PLP determinations. Each number represents the average of values obtained from duplicate assays. These generally agreed to within 5% for RNA and protein and to within 10% for PLP assays run from separate extracts. The table gives values obtained from two experiments.

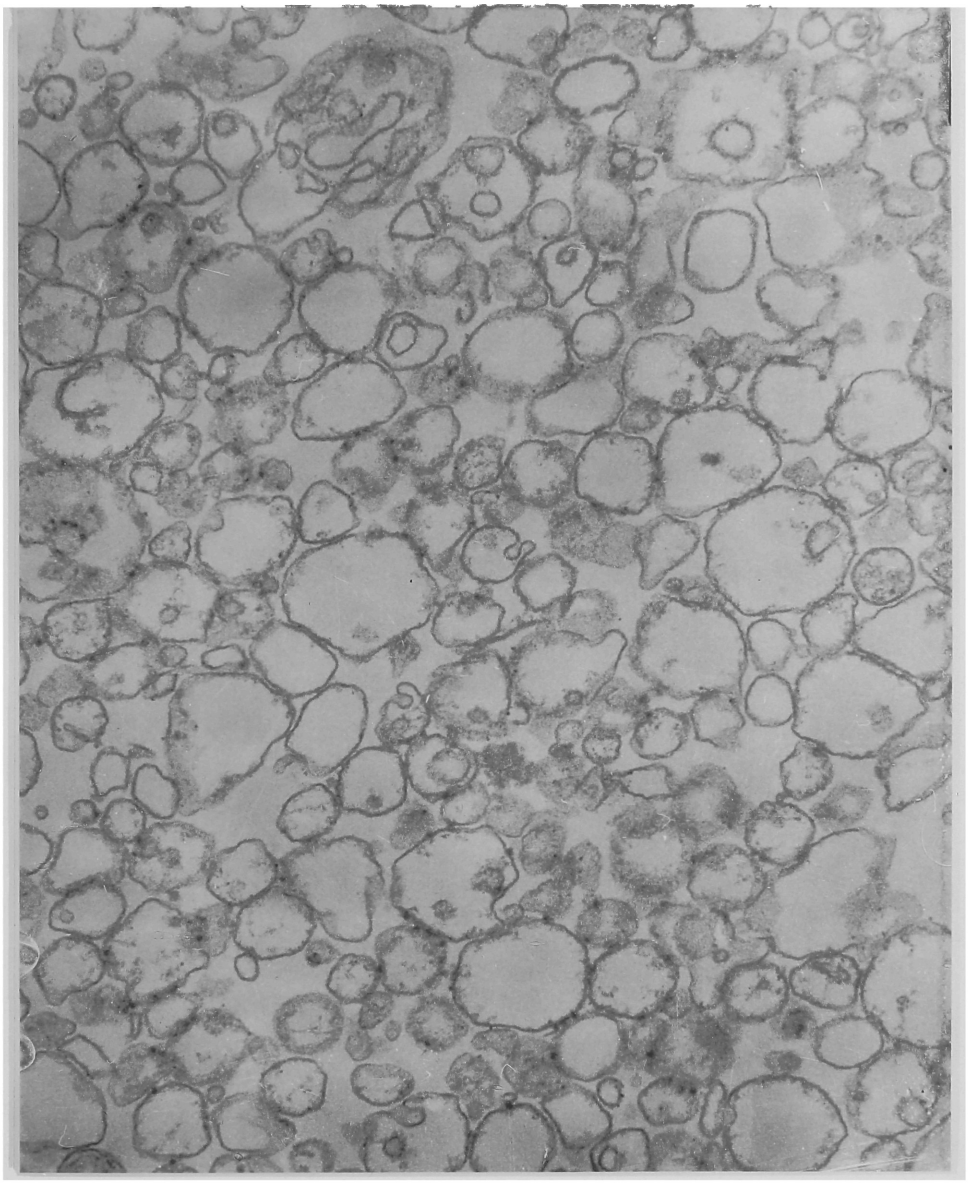


Figure 8. Electronmicrograph of a section through a pellet of rough microsomes, prepared by procedure A and stripped by the KCl-puromycin procedure. Pellet fixed and stained as described in the legend to Fig. 4.
x 44,000

7.6, 310 ideal milliosmolar). Hemolysis was performed by pipetting 2 ml of the erythrocyte suspension into 27.0 ml hypotonic NaH_2PO_4-Na_2HPO_4 buffer (pH 7.6, 15.5 ideal milliosmoles) in centrifuge tubes of the Spinco No. 30 rotor. The erythrocyte ghosts were sedimented into a loose pellet by centrifugation for 15 min at 3° C and 30,000 rpm. The pellets were washed twice with an equal volume of hypotonic $Na_2H_2PO_4$-Na_2HPO_4 buffer (15 min, 30,000 rpm) and finally resuspended in 0.25 M sucrose (∿2 mg protein/ml). A compensating buffer was added, so that the final composition of the suspension was 0.25 M sucrose, 0.5 M KCl, 0.05 M Tris-HCl, 0.0025 M $MgCl_2$, 10^{-3} M puromycin. The membrane suspension was incubated, and the membranes recovered and washed in HSB, as described for the preparation of stripped microsome fractions. The final membrane pellets were resuspended in 0.25 M sucrose-TKM (∿10 mg protein/ml), diluted 1:1 with glycerol and stored at -20° C.

2.5. Amino Acid Incorporation in vitro

The incorporation medium contained in one ml: 1 µMole ATP, 0.5 µMoles GTP, 10 µMoles PEP, 5 µl pyruvate kinase, 25 µl amino acid supplement (12.5 µl of a solution of essential amino acids at molarities 10 times those described by Eagle (1959), except for leucine, which was present at 0.14 mM, plus 12.5 µl of a solution of nonessential amino acids each at 1.0 mM), 50 µC 4,5-(^{3}H)-leucine or 2.5 µC (^{14}C)-leucine, 150 µl G-50 high speed supernatant fraction, 150 µMoles NH_4Cl, 20 µMoles Tris-HCl pH 7.6, 5 µMoles $MgCl_2$, 0.15 µMoles DTT, 250 µMoles sucrose and rough microsomes corresponding to 0.5 to 1.0 mg RNA. The microsomes were added last and the incubation was carried out in a 37° C water bath with shaking. To study incorporation kinetics, 100 µl aliquots were withdrawn at the indicated time intervals and pipetted onto Whatman 3 MM filter paper discs, which were transferred after 10 sec into a beaker containing ice cold 10% TCA. After hot acid hydrolysis and phospholipid extraction (Mans and Novelli, 1961), the residual radioactivity due to protein and polypeptides was determined by liquid scintillation counting in 5 ml of toluene-Liquifluor (40 ml of

Liquifluor and 960 ml of toluene) in a Mark I Nuclear Chicago Counter (Nuclear Chicago Corp., Des Plaines, Ill.).

2.6. Sucrose Density Gradient Centrifugation

Linear sucrose gradients were prepared in centrifuge tubes of the SB110, SB283 or SB405 rotors of the International centrifuge. Details of gradient analysis (composition of the gradients, time, temperature and speed of centrifugation) are given in the figure legends or in the text. An Auto Densi-Flow probe (Buchler Instruments, Fort Lee, N.J.), connected to a Perpex Peristaltic Pump (LKB-Producter, AB, Bromma-1, Sweden), via an LKB Uvicord II, type 8303A, equipped with a log converter, was used to collect the gradients from the top and to obtain the optical density profiles which were recorded on a Hewlett Packard linear recorder (7101 BM Strip Chart Recorder). A pump speed of 2.4 or 1.35 ml/min and a chart speed of 1.25 cm/min were generally used.

In order to determine the distribution of radioactivity throughout the gradients, timed fractions from the effluent of the recording system were collected in glass conical tubes. Material in the pellets was resuspended in water, quantitatively transferred to glass conical tubes and processed in the same way as the fractions. Each fraction received one mg of bovine serum albumin as carrier and three volumes of ice cold 10% TCA. Fractions were centrifuged at 4° C for 10 min at 1,000 x g in a model SBV International centrifuge. Supernatants were discarded and the acid insoluble material was dissolved in 0.5 ml of NCS solubilizer. Samples were transferred to glass scintillation vials and counted with 8 ml of Toluene-Liquifluor as scintillator in the Mark I Nuclear Chicago Counter. Efficiency for tritium was 30 to 35%. For double label experiments (^{3}H and ^{14}C), the counter was set for 0.01% overlap of tritium into the ^{14}C channel. Efficiency for ^{14}C was 50 to 55%. Since in these experiments the ratio of ^{3}H to ^{14}C was well over 10, no corrections for ^{14}C overlap into the ^{3}H channel were necessary. All values were corrected for background. Recovery of radioactivity on the gradients was 85 to 95%. In all figures of gradient

analyses, the direction of sedimentation is from left to right, and the large point at the right of the optical density tracing represents radioactivity recovered in the pellet. For quantitation of the distribution of ultraviolet absorbing material, areas under the optical density tracing were cut out and the paper was weighed.

2.7. Comparison of Buoyant Densities of Ribosomal Subunits obtained from Free and Bound Ribosomes

Rough microsomes and free polysomes were resuspended (∿3.5 mg RNA/ml) in TEAKM buffer (TEAKM = 50 mM Triethanolamine-HCl, pH 7.6 at 20° C, 25 mM KCl, 5 mM $MgCl_2$) and made 0.5% in Deoxycholate (DOC). One ml aliquots of the suspensions were layered over 12 ml 10 to 40% sucrose gradients containing TEAKM and prepared in centrifuge tubes of the SB283 rotor. After centrifugation for one hr at 3° C and 40,000 rpm, the effluents corresponding to the monomer and polysome regions of the gradients were collected. In this way, the ribosome preparations were freed of heavy aggregates and soluble proteins. A compensating buffer was added to the ribosome solutions, so that the final composition was 0.5 M KCl, 0.05 M TEA, 0.0025 M $MgCl_2$, 10^{-3} M puromycin. The samples, which contained 0.5 mg RNA/ml, were incubated for 10 min at 37° C and then passed through a Sephadex G-25 column (10 x 1 cm) equilibrated in HSB containing TEA instead of Tris, to free the preparations from puromycin and sucrose. The excluded material was collected, made 4% in formaldehyde, and fixed for 17 hrs at 4° C. 1.6 ml aliquots of the samples were then added to 10 ml of a CsCl stock solution (ρ = 1.8) and 0.05 M TEA-HCl was added to adjust the density of the solutions to 1.62. The refractive index of the solutions was monitored on a Bausch-Lomb refractometer (Bausch and Lomb Inc., Rochester, N.Y.) and the densities calculated from the equation $\rho^{25.0} = 10.8901n^{25.0}_D - 13.4974$ (Ifft, Voet and Vinograd, 1961). The presence of formaldehyde (0.04%) in the CsCl solutions caused a slight shift to higher values in the refractive index, so that the calculated densities were higher than the true values by 0.008. Values given in the Results section have been corrected for this error.

Aliquots (6 ml) of the final CsCl solution containing the fixed ribosomes were pipetted into oak ridge screw cap tubes of the A321 rotor of the International centrifuge. The gradients were centrifuged at 52,000 rpm and 3° C for 30 hrs. The use of an angle head rather than a swinging bucket rotor permits a better resolution on CsCl gradients (Flamm, Bond and Burr, 1966).

After the rotor was allowed to stop without braking, the gradients were collected from the top by means of the Buchler probe, as described for sucrose gradients (section 2.6). The optical density profiles were recorded and 0.3 ml fractions were collected on ice for immediate analysis on the refractometer.

2.8. Polyacrylamide Gel Electrophoresis

Gel electrophoresis of ribosomal proteins was carried out in sodium dodecyl sulfate (SDS) discontinuous polyacrylamide gels (spacer gel 4% acrylamide; resolving gel 12.5% acrylamide), according to Maizel (Maizel, 1971). A vertical electrophoresis cell (E-C Apparatus Corp., Philadelphia, Pa.) was used with slabs 3 mm thick and provided with eight slots. 150 ml of the resolving gel contained: 62.5 ml of an acrylamide solution (30 g acrylamide, 0.8 g N,N'-methylenebisacrylamide, to 100 ml with water), 30 ml 2.0 M Tris-HCl pH 8.9, 75 µl TEMED and 47 ml H_2O. 0.5 ml of 0.025% riboflavin and 0.2 ml 10% ammonium persulfate were added to 29 ml of the mixture, which was used for polymerizing a plug at the bottom of the electrophoresis apparatus and for sealing the sides. To the remaining 120 ml were added 0.6 ml 10% ammonium persulfate and 1.2 ml 10% SDS. The mixture was degassed, poured into the sealed electrophoresis apparatus standing in the vertical position, and carefully overlaid with isobutanol. 2.5 cm from the top of the gel to the upper rim were left for the spacer gel. After polymerization (∿20 min), the isobutanol was removed and the electrophoresis apparatus was placed in the horizontal position. After positioning the slot-former, the space left over the resolving gel was filled with spacer gel. The spacer gel contained in 50 ml: 10 ml of an acrylamide solution

(19 g acrylamide and 1 g N,N'-methylenebisacrylamide to 100 ml with water), 6 ml 0.5 M Tris-HCl pH 6.8, 33 ml H_2O, 25 µl TEMED, 0.2 ml 10% ammonium persulfate and 0.5 ml 10% SDS. The mixture was degassed before use. After allowing one hour for polymerization, the excess spacer gel was removed with a spatula and the slotformer was carefully withdrawn.

The samples for analysis were resuspended in 15% sucrose (w/v) at a concentration of ∿200 OD_{260} units/ml. 5 µl of an SDS solution (containing in 5 ml: 4.0 ml 25% SDS, 0.2 ml 0.5 M Tris-HCl pH 6.8, 0.3 ml Bromophenolblue pH 7, 0.1 ml mercaptoethanol, 0.4 ml H_2O) were added to 20 µl of the ribosome suspensions, which were then heated at 100° C for two minutes.

After filling the electrophoresis apparatus with 2 liters of electrode buffer, the samples were underlaid into the slots. The electrode buffer contained in 1 liter: 6 g Tris, 28.8 g glycine and 1% SDS. The gels were run at a constant current of 17.5 mA for 14 hrs or until the bromophenolblue front had run 13 to 15 cm from the top.

After electrophoresis, the slab was removed from the apparatus and stained and fixed with five volumes of a Coomassie Brilliant Blue R250 solution (0.2 g Coomassie dissolved in 50 ml methanol and 50 ml H_2O to which 7 ml glacial acetic acid were added before use). After staining for ∿8 hrs, the gels were destained with 7% acetic acid 20% methanol.

2.9. Ribosomal Subunit Exchange

The exchange was followed either (1) by adding ^3H-labeled large or small subunits to washed rough microsomes, or (2) by adding non-labeled subunits to washed ^3H-labeled rough microsomes. Since in case (1) a transfer of radioactivity from free subunits to rough microsomes is followed we will refer to the exchange detected in this way as exchange "in." Vice-versa we will refer to the exchange detected in case (2) as exchange "out." Incubation was carried out either in the amino acid incorporation medium described above or in the minimal

exchange medium described in the Results section. Mixtures were incubated for 10 min in a 37° C water bath with shaking. Aliquots (0.5 ml) were taken in which the subunit distribution was determined by sucrose density gradient analysis in the SB283 rotor of the International B60 centrifuge, which was run at 20° C to avoid the formation of subunit aggregates in the cold. 20 to 25 fractions were collected and processed for scintillation counting.

2.10. Binding of Ribosomes to Stripped Membrane Fractions

^{3}H-labeled ribosomes obtained from rough microsomes by high salt puromycin treatment were incubated with stripped rough microsomes, stripped smooth microsomes, or erythrocyte ghosts. The ribosomes were prepared as follows. ^{3}H-labeled rough microsomes were incubated in 0.5 M KCl, 0.05 M Tris-HCl, 0.0025 M $MgCl_2$, 10^{-3} M puromycin for 10 min at 37° C (∿3 mg RNA/ml). After incubation the microsomes were sedimented into a pellet by a 12 min centrifugation at 3° C and 40,000 rpm in the A321 rotor of the International centrifuge. The supernatant, containing the released ribosomes, was decanted, diluted fivefold with TKM, and layered over 1 ml 1.3 M sucrose-TKM cushions in centrifuge tubes of the A321 rotor. The ribosomes were sedimented into pellets by an overnight centrifugation at 59,000 rpm and 3° C. The pellets were rinsed with water and stored at -20° C.

The incubation mixtures for ribosome binding were in TKM and contained 0.2 to 0.4 mg membrane protein in a volume of 0.12 ml. At the end of the incubation, 2.08 ml of cold 2.2 M sucrose-TKM were added to the samples. After thorough mixing, 0.8 ml of the solution were underlayed, by means of a syringe, below a 2.6 ml continuous sucrose gradient, prepared in centrifuge tubes of the SB405 rotor. The gradients were in TKM, and the sucrose concentration range varied, according to the membrane fraction used, so that the membranes would float to the upper part of the gradient (1.2 to 1.9 M sucrose for stripped RM, 1.0 to 1.9 M sucrose for stripped SM, 0.9 to 1.9 M sucrose for erythrocyte ghosts). All gradients were overlayed with 0.050 ml

0.25 M sucrose-TKM, to avoid exposure of any material to a liquid-air interface. The gradients were centrifuged for 30 min at 3° C and 60,000 rpm and the rotor allowed to stop without braking. The gradients were withdrawn from the top, as described above, and three fractions were collected and processed for scintillation counting: a top fraction (1.85 ml), an intermediate fraction (0.45 ml), and a bottom fraction (1.1 ml). Material in the pellet was resuspended in water and combined with the bottom fraction. The acid insoluble radioactivity recovered in the top fraction was considered to be membrane-associated. When ribosomes were analyzed in the absence of added membranes approximately 25 cpm were recovered in the top fraction. All values presented in the results section have been corrected for this background. A typical optical density profile showing the separation obtained between stripped RM and free ribosomal material is presented in Fig. 9. The stripped RM form a sharp band at the top of the gradient and the free ribosomal material is found at the bottom. Centrifugation for 6 hrs did not lead to any change of the results obtained with a 30 min centrifugation, for the three membrane fractions used.

2.11. Analytical Procedures

Optical densities were determined in a Zeiss PMQ II spectrophotometer.

Protein was assayed by the method of Lowry et al. (Lowry, Rosenbrough, Farr and Randall, 1951), with bovine serum albumin as standard.

RNA was determined by a modified Schmidt-Tannhauser procedure (Schmidt and Tannhauser, 1945; Fleck and Munro, 1962), using $E^{1\%}_{1\ cm} = 313$ (Munro and Fleck, 1966). Alternatively, ribosome concentrations were determined directly, using $E^{1\%}_{1\ cm} = 135$ at 260 nm (Tashiro and Siekevitz, 1965) and correcting for ferritin (Jackson, Munro and Korner, 1964). To estimate the ribosomal content of microsomal suspensions, aliquots were made 0.5% in DOC and read at 260 nm against a 0.5% DOC blank. This procedure resulted in a 10 to 15% overestimation of the RNA content of rough microsomes, when compared to values obtained by RNA

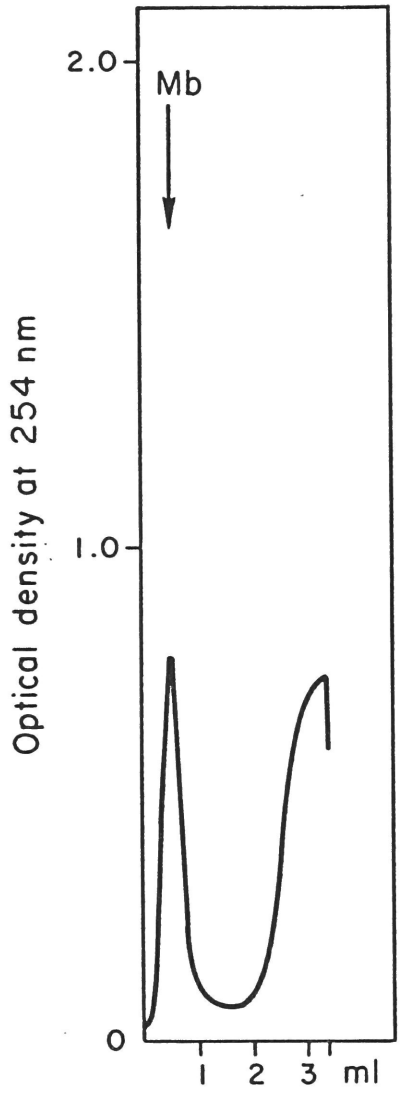


Figure 9. Separation of stripped RM from unattached ribosomes. Stripped rough microsomes (0.120 mg of membrane protein) and 1.0 OD_{260} units of ribosomes were mixed in the cold, immediately diluted with heavy sucrose, and layered under a 1.9 to 1.1 M sucrose gradient, containing TKM. The optical density at the bottom of the gradient is due to unattached ribosomes. For details, see text.

determination.

For phospholipid phosphorus determinations, lipids were extracted from resuspended cell fractions with 20 volumes of chloroform-methanol 2:1 (v/v) and purified according to Folch et al. (Folch, Lees and Sloane Stanley, 1957). Appropriate aliquots of the extract were brought to dryness in a dessicator, and assayed for phosphorus as described by Ames (Ames, 1966). Values for phospholipid phosphorus were converted to phospholipid using a factor of 25.

2.12. Materials

Male albino rats of the Sprague-Dawley strain, maintained on a Purina Chow diet, were used.

Whole human blood was obtained from the New York Blood Center (courtesy of Dr. C. Redman).

Chemicals were obtained from the following sources: Dithiothreitol (DTT), puromycin dihydrochloride and cycloheximide (actidione) from Nutritional Biochemicals Corp., Cleveland, Ohio; 5-(^{3}H)-orotic acid (1 mC/0.156 mg) and Liquifluor from New England Nuclear, Boston, Mass.; equine muscle ATP (Na^{+} salt), GTP (type IIS, Na^{+} salt) and Sodium Dodecyl Sulfate (SDS) from Sigma Chemical Co., St. Louis, Mo.; phosphoenolpyruvate (PEP, Na^{+} salt) and pyruvate kinse (PK) (2230 International Units/ml) from Calbiochem, San Diego, Calif.; amino acids (AAs), L-4,5-(^{3}H)-leucine (40 to 50 C/mM) and L-(^{14}C)-leucine (316 mC/mM) from Schwarz Bioresearch Inc., Orangeburg, N.Y.; sodium deoxycholate (DOC) from Matheson, Coleman and Bell, Cincinnati, Ohio; Sephadex G-50 medium from Pharmacia, Piscataway, N.J.; NCS solubilizer from Amersham Searle, Arlington Heights, Ill.; Coomassie Brilliant Blue R250 from Schwarz/Mann, Orangeburg, N.Y.; acrylamide, N,N,N'N'-Tetramethylenediamine and N,N'-methylenebisacrylamide from Eastman Kodak Co., Rochester, N.Y.; Cesium Chloride, optical grade power, from Harnshaw Chemical Co., Solon, Ohio.

III. RESULTS

3.1. Disassembly of the Polysome-Membrane Complex

Polypeptide chain release *in vitro* can be induced artifically by puromycin or allowed to proceed naturally by amino acid incorporation. Recently it has been shown that 85 to 90% of the membrane-bound ribosomes can be detached as ribosomal subunits by moderately high concentrations of monovalent ions (0.5 M KCl) in the presence of Mg^{++} (0.0025 M), if their nascent polypeptide chains are released by puromycin (Adelman *et al.*, 1970). We attempted to determined whether natural termination of polypeptide chains in an amino acid incorporation medium would also lead to the subsequent release of ribosomes from microsomal membranes when transferred to a solution containing 0.5 M KCl and 0.0025 M $MgCl_2$. Freshly prepared washed rough microsomes, obtained by procedure B, were used for this experiment. In order to obtain maximal preservation of polysomes, high speed supernatant was present in all layers of the discontinuous sucrose gradient used for fractionation of the postmitochondrial supernatant (1 part high speed supernatant + 3 parts of the appropriate sucrose solution). After a 15 min incubation in the amino acid incorporation medium with or without cycloheximide, the incubation mixtures were diluted 1:1 with a compensating buffer, so that the final composition of the suspensions was 0.5 M KCl, 0.05 M Tris-HCl, 0.0025 M $MgCl_2$, and incubated again for 10 min at 37° C. Aliquots were layered onto 5 to 20% sucrose gradients containing HSB, which were centrifuged so that the microsomes were sedimented into a pellet and the ribosomal subunits displayed on the gradients. Fig. 10 shows that preincubation in the amino acid incorporation medium at 37° C (Fig. 10b) resulted in a larger release of subunits from microsomal vesicles than that observed when preincubation was at 0° C (Fig. 10a) or at 37° C with cycloheximide (Fig. 10c). Thus, like puromycin-induced polypeptide chain release, natural chain termination also results in a higher salt sensitivity of the ribosome-membrane complex. The ribosome rlease observed after natural termination was about 55% of that obtained with puromycin, presumably because of a low efficiency

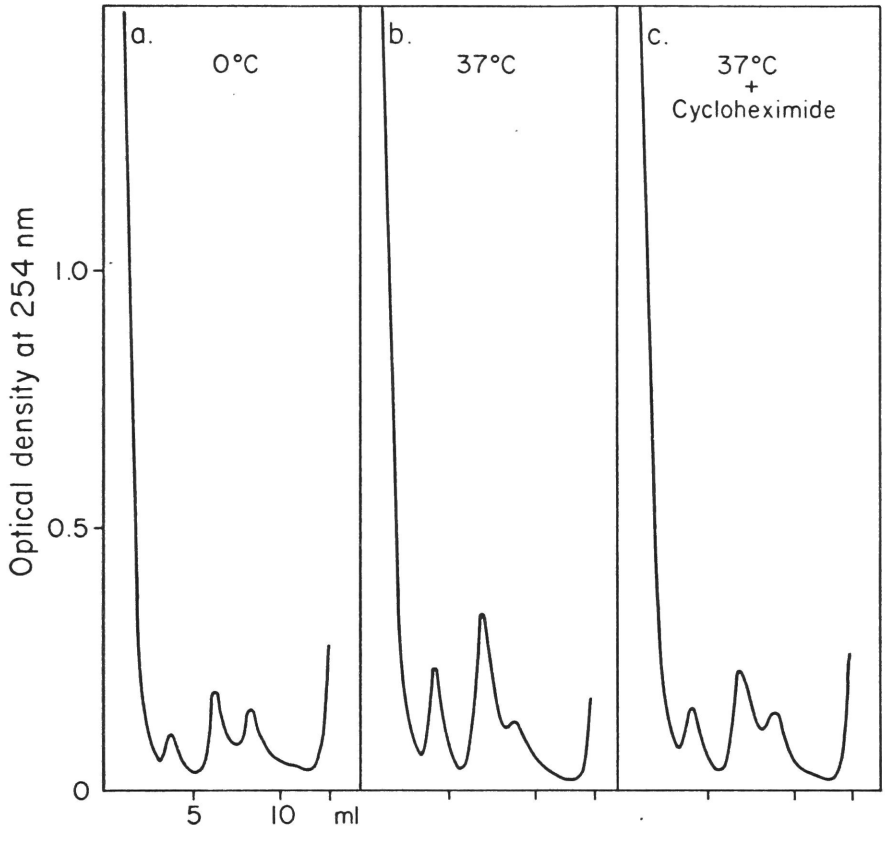


Figure 10. Detachment of ribosomes from rough microsomes in a buffer of high ionic strength after preincubation for amino acid incorporation. Rough microsomes (10.4 OD_{260} units/ml) were incubated in the amino acid incorporation medium described in the Methods section, but containing 0.1 M KCl instead of 0.150 M NH_4Cl. After incubation for 15 min, 0.4 ml aliquots were withdrawn and chilled. 0.2 ml of a compensating buffer were then added so that the final composition of the solutions was 0.5 M KCl, 0.05 M Tris-HCl, 0.0025 M $MgCl_2$. After incubation for 10 min at 37° C, 0.5 ml aliquots were layered onto 5 to 20% sucrose gradients containing HSB, which were centrifuged for 2 hrs and 30 min at 20° C in the SB 283 rotor.

a, incubation in the AA incorporation medium at 0° C; b, incubation in the AA incorporation medium at 37° C; c, incubation in the AA incorporation medium at 37° C with 10^{-2} M cycloheximide.

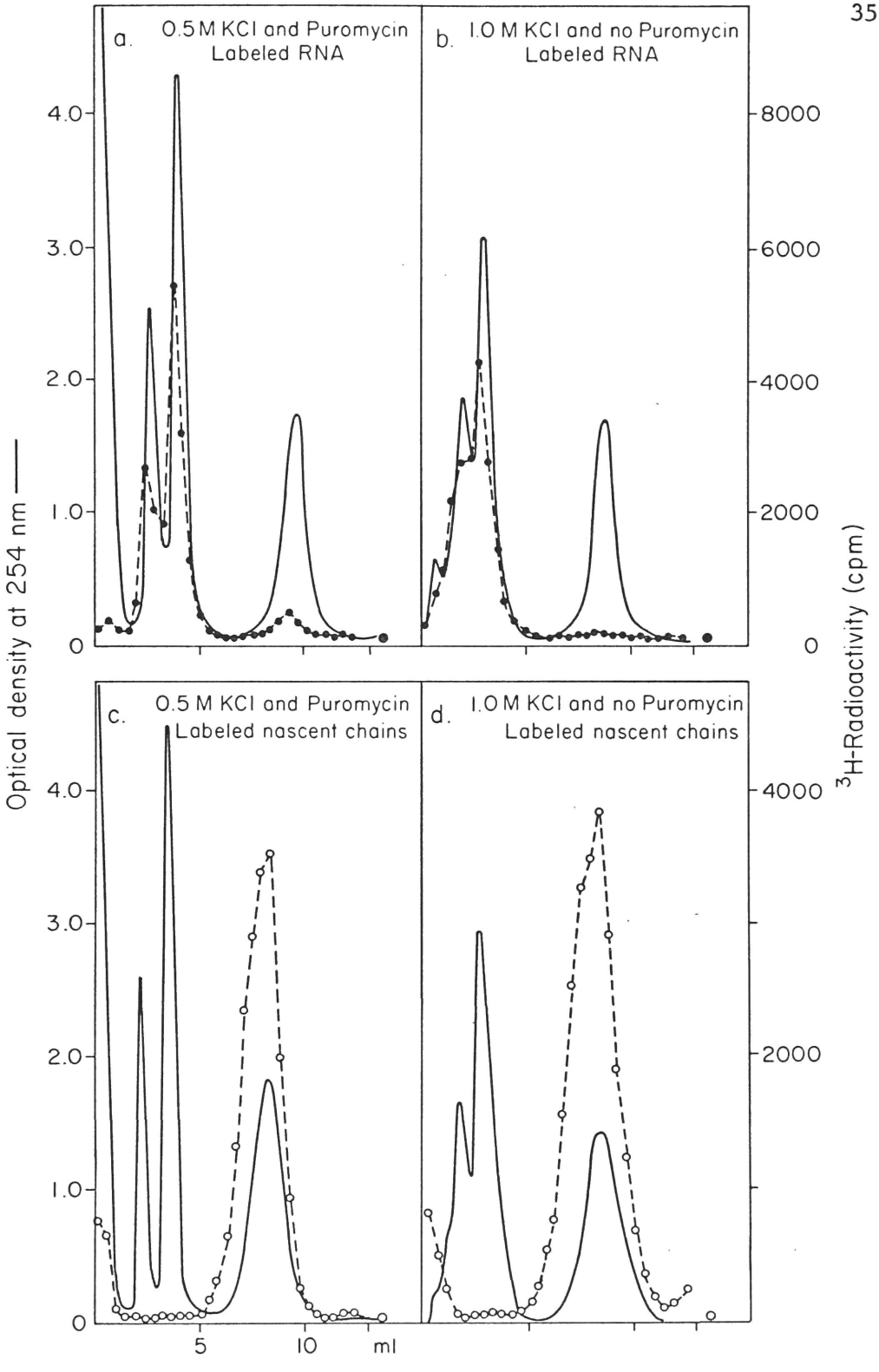
of the microsomal system in polypeptide chain termination. Incubating for longer times in the amino acid incorporation medium did not lead to increased KCl-induced release of ribosomes (see also section 3.2.1, for kinetics of amino acid incorporation by rough microsomes).

The ribosomal subunits, detached in 0.5 M KCl in the presence of Mg^{++} after release of polypeptide chains by natural termination or by the action of puromycin, have sedimentation coefficients of 60 S and 40 S and retain their native conformation, since in the case of puromycin induced release they have been shown to be active in the poly U directed synthesis of polyphenylalanine. The experiment in Fig. 11 demonstrates that an even more efficient release of ribosomes, which does not require puromycin or amino acid incorporation, can be achieved by incubating rough microsomes in 1 M KCl in the absence of Mg^{++}. This is apparent from comparison of Figs. 11a and b, which show sucrose gradient analyses of the distribution of ^{3}H-labeled subunits, after incubation for detachment from ^{3}H-labeled rough microsomes. Whereas after incubation with 0.5 M KCl and 10^{-3} M puromycin in the presence of 0.0025 M $MgCl_2$ (Fig. 11a), 10% of the total ^{3}H-labeled RNA remained associated with the membranes (which band isopycnically in the lower part of the gradient), only 5% remained with the membranes after incubation with 1.0 M KCl in the absence of Mg^{++} (Fig. 11b). Release of polypeptide chains from tRNA previous to ribosome detachment was unnecessary under the latter condition, presumably because the ribonucleoprotein particles in the absence of Mg^{++} were unfolded into derivatives of the subunits, which sediment more slowly than the particles released in the presence of Mg^{++} and puromycin (Fig. 11a). Thus, the more efficient procedure for the disassembly of the polysome-membrane complex is also destructive of the integrity of the subunits.

The fate of the nascent polypeptide chains after the 1.0 M KCl-no Mg^{++} treatment was also investigated, using rough microsomes labeled with (^{3}H)-leucine for two minutes *in vivo*. Adelman *et al.* demonstrated that after puromycin induced release of ribosomes, the nascent polypeptide chains remain in association with the microsomal membranes

Figure 11. Comparison between the KCl-puromycin and the 1.0 M KCl-no Mg^{++} procedure for detaching ribosomes from rough microsomes. Non-washed rough microsomes were resuspended in 0.5 M KCl, 0.05 M Tris-HCl, 0.0025 M $MgCl_2$ with 10^{-3} M puromycin (a and c) or 1.0 M KCl, 0.05 M Tris-HCl (b and d) at a concentration of ∿26 OD_{260} units/ml. After incubation for 10 min at 37° C, 0.5 ml aliquots were layered onto sucrose gradients of the following composition: a and c, 10 to 50% sucrose containing HSB; b and d, 10 to 40% sucrose containing 1.0 M KCl, 0.05 M Tris-HCl, 0.05 M $MgCl_2$. The gradients were centrifuged for 1 hr and 24 min (a and b) or 1 hr and 48 min (c and d) at 20° C and 40,000 rpm in the SB 283 rotor.

a and b, rough microsomes labeled with tritium in their RNA (●----●); c and d, rough microsomes labeled with tritium in the nascent polypeptide chains (o----o).


a. 0.5 M KCl and Puromycin Labeled RNA
b. 1.0 M KCl and no Puromycin Labeled RNA
c. 0.5 M KCl and Puromycin Labeled nascent chains
d. 1.0 M KCl and no Puromycin Labeled nascent chains
Optical density at 254 nm
^{3}H-Radioactivity (cpm)
4.0
3.0
2.0
1.0
0
8000
6000
4000
2000
0
5
10
ml

(Adelman, Sabatini and Blobel, in preparation). This can also be seen in Fig. 11c. Fig. 11d shows that also after the 1.0 M KCl-no Mg^{++}-no puromycin treatment virtually all the labeled nascent chains were dislodged from the ribosomes and remained associated with the microsomal vesicles. Since these polypeptide chains must still be attached to the tRNA molecules, it remains to be investigated whether they were, or not, vectorially transferred across the microsomal membranes.

3.2. In vitro Exchange of Ribosomal Subunits with Membrane-bound Ribosomes

3.2.1. Exchange of free and membrane-bound small subunits. We first investigated whether small subunits of membrane-bound ribosomes would exchange in an *in vitro* amino acid incorporation system with added ^{3}H-labeled small subunits (Exchange "in"), derived from free polysomes (free small subunits). The experiments were carried out in incorporation media containing unlabeled leucine. The kinetics of amino acid incorporation by the rough microsomes was followed in parallel in separate samples containing ^{3}H-leucine and no labeled small subunits. Fig. 12 shows that the rate of incorporation leveled off rapidly after the first 5 min of incubation and that cycloheximide added at zero time had a strong inhibitory effect on incorporation.

In order to artificially increase termination of the polypeptide chains, and thereby possibly promote subunit exchange (Kaempfer and Meselson, 1969), puromycin was added to the incubation medium. At the relatively low ionic strength used for *in vitro* amino acid incorporation it is known that puromycin reacts extensively with the nascent peptide chains, but does not lead to the release of ribosomes from membranes (Adelman *et al.*, 1970).

Fig. 13 and Table II show the results of one experiment, in which the ratio of added labeled subunits to microsomal subunits was ~1:1. The conditions of sucrose gradient analysis (see legend) were chosen so that microsomes sedimented to an isopycnic position in the lower third

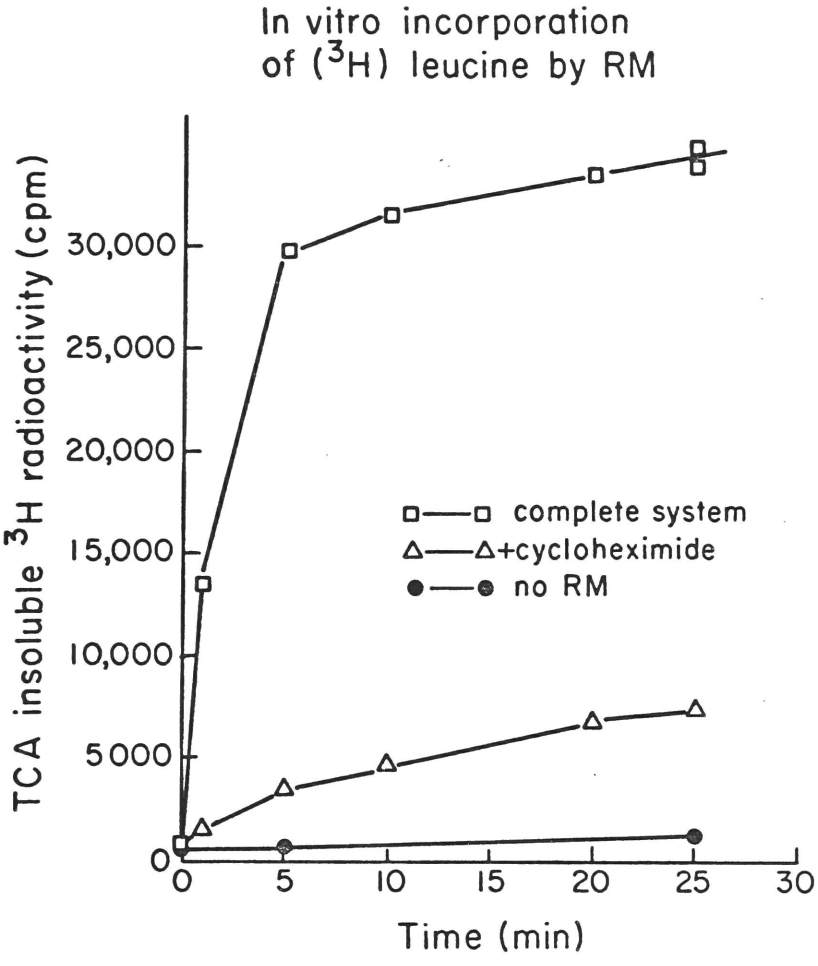


Figure 12. In vitro incorporation of (^{3}H)-leucine by RM. Incubation mixtures contained in 1 ml 12 OD_{260} units of RM and 50 µC (^{3}H)-leucine. The ordinate represents hot acid insoluble radioactivity recovered in 100 µl of the incubation mixtures. —— , complete system; Δ-Δ-Δ, complete system with 10^{-2} M cycloheximide; ●-●-●, no RM.

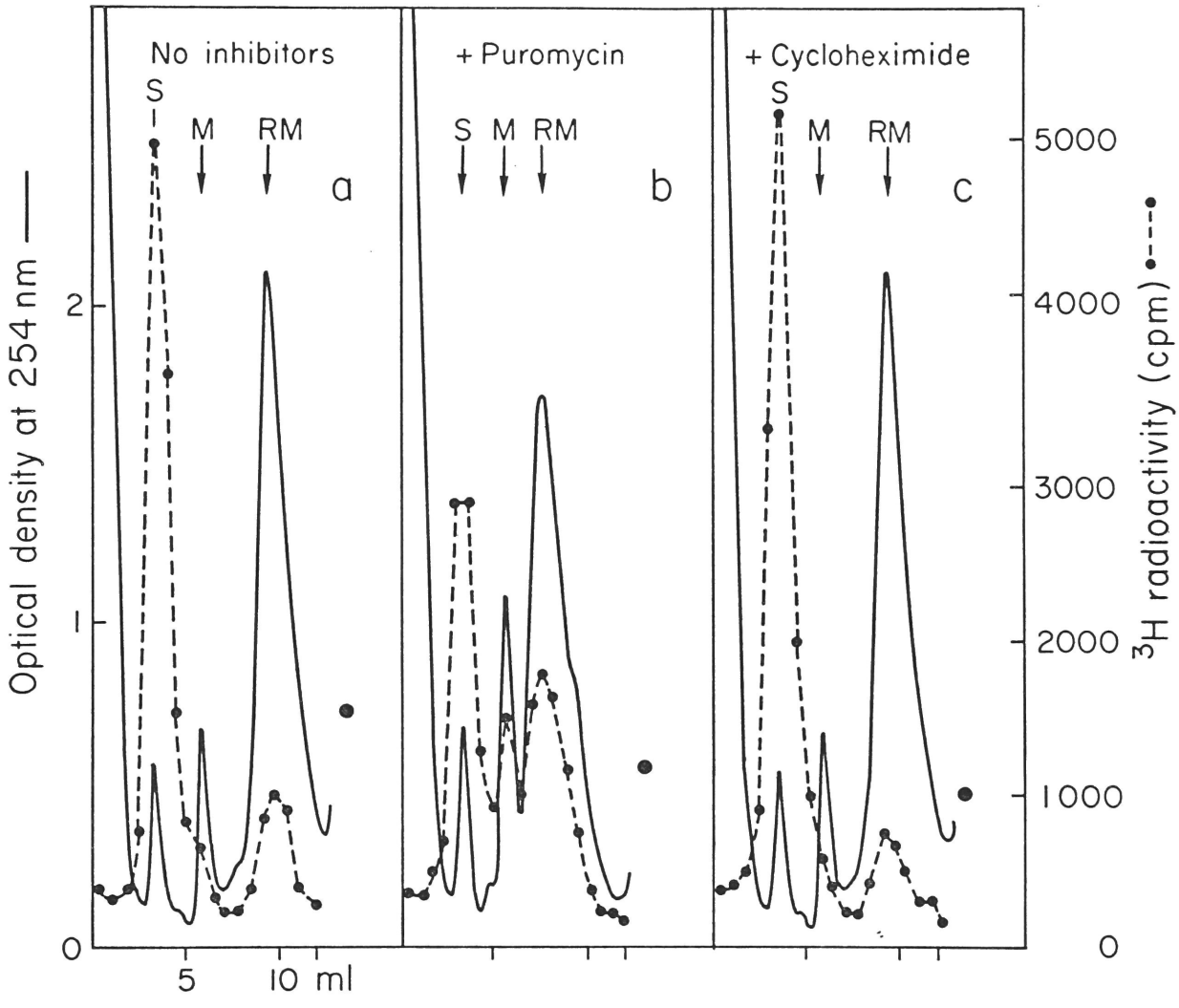


Figure 13. Exchange of ^{3}H-labeled small subunits with membrane-bound ribosomes in amino acid incorporation medium. Incubation mixtures contained in 1 ml 11.2 OD_{260} units of RM and 2.2 OD_{260} units of ^{3}H-labeled small subunits (specific activity, 22,400 cpm/OD_{260} unit). After incubation, 0.5 ml aliquots were layered onto 15 to 60% sucrose gradients, containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$. Centrifugation was for 2 hrs 36 min in the SB 283 rotor at 40,000 rpm.

a, no inhibitors; b, 10^{-3} M puromycin; c, 10^{-2} M cycloheximide. ----, optical density; ●---●---●, ^{3}H radioactivity.

Table II

Distribution of radioactivity (cpm) after incubation of ^{3}H-labeled small subunits with RM in amino acid Incorporation Medium*

Region of Gradient	Experiment I						Experiment II					
	Incubation at 0°C		Incubation at 37°C		Incubation at 37°C with puromycin†		Incubation at 37°C		Incubation at 37°C with puromycin†		Incubation at 37°C with cycloheximide§	
Top fractions	691	(4%)	1778	(7%)	1466	(6%)	1028	(5%)	1209	(6%)	1279	(7%)
40 S	19058	(83%)	17253	(70%)	11065	(47%)	12421	(61%)	8677	(41%)	12549	(65%)
Monomers	1513	(7%)	2705	(11%)	4346	(18%)	856	(4%)	2470	(12%)	961	(5%)
Microsomes	1180	(5%)	2499	(10%)	6219	(26%)	3966	(20%)	7420	(35%)	3108	(16%)
Pellet	426	(2%)	386	(2%)	438	(2%)	2053	(10%)	1502	(7%)	1428	(7%)
Total cpm	22869	(100%)	24623	(100%)	23531	(100%)	20315	(100%)	21278	(100%)	19225	(100%)

*Time of incubation and conditions of sucrose gradient centrifugation were as in Fig. 13. Specific activity of small subunits was: Exp. I: 26150 cpm/OD_{260} unit; Exp. II: 22400 cpm/OD_{260} unit. Input of rough microsomes was: Exp. I: 5.3 OD_{260} units; Exp. II: 5.6 OD_{260} units.

†10^{-3} M

§10^{-2} M

of the gradient, while ribosomal subunits and monomers are displayed in the top third. Fig. 13a shows that some membrane-bound ribosomes (∿20% of total) are detached in the incorporation medium, and that after incubation for 10 min at 37° C ∿15% of the added labeled small subunits are bound to the microsomes. Table II shows that the membrane-bound radioactivity after 10 min of incubation at 37° C is approximately twice that found in control microsomes incubated with labeled subunits for 10 min at 0° C in the incorporation medium.

Puromycin (10^{-3}M) added to the incubation medium (Fig. 13b, Table II) caused a large shift in the radioactivity distribution from the small subunit to the monomer and microsome regions of the gradient. However, the area of the peak in the optical density profile corresponding to small subunits was not reduced and in fact appears slightly larger after puromycin addition. Thus, one must conclude that the labeled small subunits disappearing from the 40 S region were all replaced by non-radioactive small subunits released from the microsomes. As can be seen from a comparison of Figs. 13a and b, puromycin also caused a release of monomers from the microsomal membranes somewhat larger than that which normally occurs in the incorporation medium (Fig. 13a). As a result, the isopycnic position of the band of microsomes is displaced to a lighter region of the gradient.

Fig. 13c shows that, in contrast to the effect of puromycin, cycloheximide (10^{-2} M) inhibited the exchange of small subunits. In several experiments the inhibition by cycloheximide referred to the value in the microsomes incubated at 37° C in the absence of the inhibitor was reproducibly 20 to 25% (Table II). If, however, the value of the membrane associated radioactivity found in controls incubated at 0° C (Table II) is considered as background or unspecific binding, and is subtracted from the experiments, then the inhibition by cycloheximide during the first 10 min of incubation is ∿50%. Nevertheless, this effect is still weaker than may be expected on the basis of the effectiveness of cycloheximide as an inhibitor of amino acid incorporation (Fig. 12). This could be explained by a weaker effect of cycloheximide

on termination than on overall amino acid incorporation in the in vitro system.

The significance of the background binding (membrane bound radioactivity found in samples incubated at 0° C in the amino acid incorporation medium) is unclear. At least part of the background binding may be due to exchange of subunits which follows natural termination of chains, occurring when the system is brought to room temperature before centrifugation, which is at 20° C. As will be discussed below (sections 3.2.3 and 3.2.5), at least part of the background binding is also due to nonspecific adsorption of altered small subunit material to microsomal membranes.

In order to confirm the results of the experiments just described, we tested for small subunit exchange "out" (Fig. 14 and Table III), i.e. using ^{3}H-labeled rough microsomes and non-labeled small subunits (also in a ∿1:1 subunit molar ratio). In these experiments (^{14}C)-leucine was added directly to the exchange reaction mixture, so that amino acid incorporation was monitored in the same mixture as the subunit exchange. As expected (Fig. 14 and Table III), addition of small subunits to the amino acid incorporation system caused a release of labeled subunits from the microsomes into the 40 S region of the gradient (Figs. 14a and b). When puromycin was present in the medium (Figs. 14c and d), incorporation was effectively inhibited (∿90%), but the non-labeled small subunits were more than twice as effective in removing labeled small subunits from the monomer-microsome region of the gradient than in the absence of puromycin (Table III). The extent to which the subunit exchange was inhibited by cycloheximide (Figs. 14e and f) ranged from 50 to 60%, compared to the exchange in samples without added drugs incubated for 10 min at 37° C (60% in Table III).

3.2.2. Requirements for the puromycin induced exchange. The previous results indicate that in an amino acid incorporation medium puromycin promotes small subunit exchange between bound ribosomes and a population of added small subunits.

Figure 14. Exchange of small subunits with ^{3}H-labeled membrane-bound ribosomes in (^{14}C)-leucine amino acid incorporation medium. All incubation mixtures contained in 1 ml 8.0 OD_{260} units of ^{3}H-labeled RM (specific activity, 22,000 cpm/OD_{260} unit) and 2.5 μC (^{14}C)-leucine. Samples b, d and f contained 3.3 OD_{260} units of small subunits. After incubation, 0.5 ml aliquots were layered onto sucrose gradients. Composition of the sucrose gradients and conditions of centrifugation were the same as described in Fig. 13. Fractions collected from the gradients were processed for scintillation counting as described in the methods section, except that they were washed three times with 10% TCA, to eliminate all (^{14}C)-leucine soluble in cold acid.

a and b, no inhibitors; c and d, 10^{-3} M puromycin; e and f, 10^{-2} M cycloheximide. ——, optical density; ●---●---●, ^{3}H radioactivity; x···x···x, (^{14}C) radioactivity.

Exchange of small subunits with ^{3}H-labeled membrane-bound ribosomes in (^{14}C)-leu AA incorporation medium

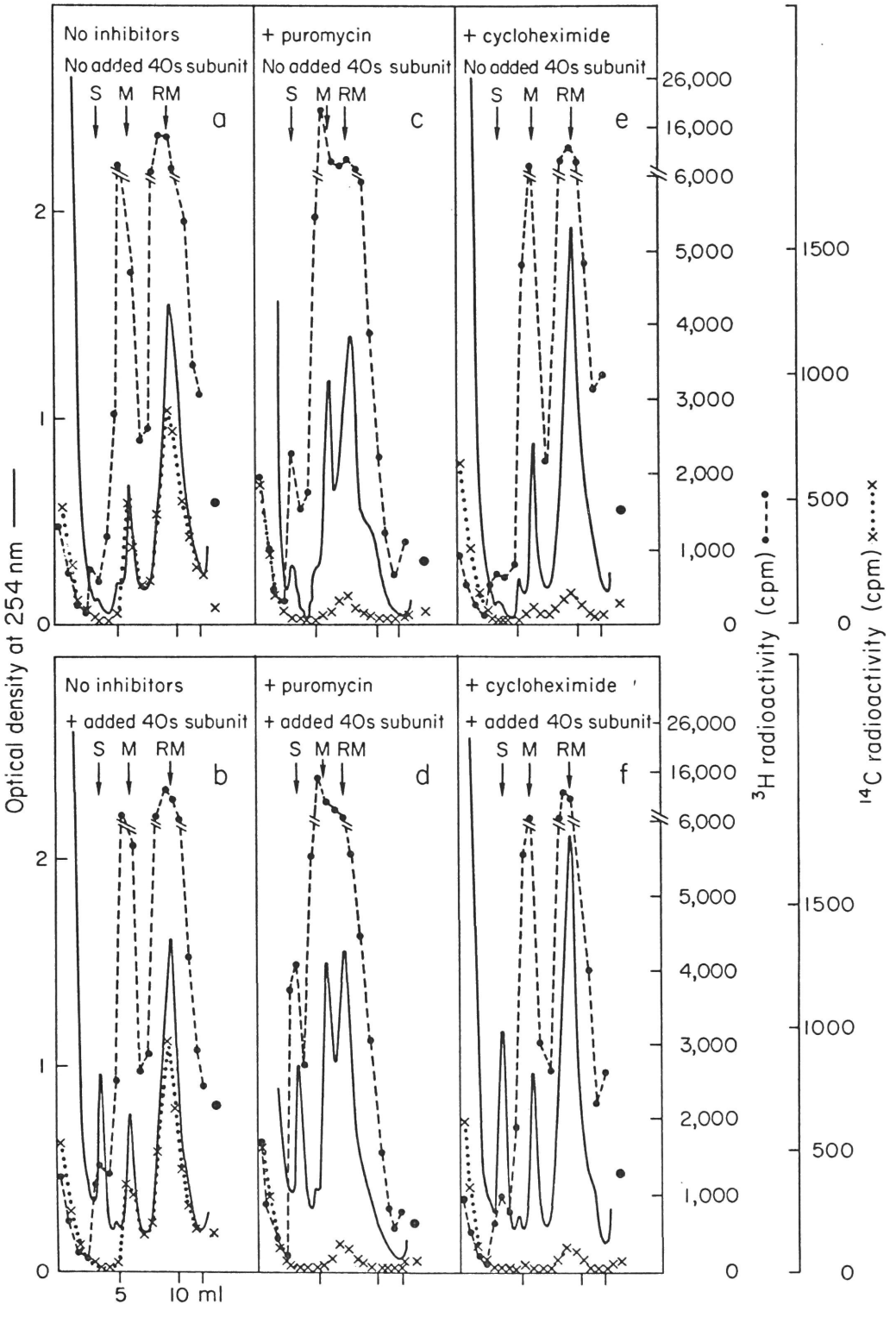


Table III

Effect of added unlabeled small subunits on the release of ^{3}H-labeled subunits from RM in amino acid incorporation medium*

Condition of Incubation	cpm in 40s region		Δcpm
	- cold 40s subunit	+ cold 40s subunit	
No Drug	1298	2826	1528
+ Puromycin†	3786	7946	4160
+ Cycloheximide§	1124	1750	626

*Medium of Incubation, inputs of rough microsomes and added small subunits, and conditions of sucrose gradient centrifugation are described in the legend to Fig. 14. The table shows the total radioactivity in fractions corresponding to the 40S region. Total radioactivity recovered in each gradient was ∿75.500 cpm.

†10^{-3} M

§10^{-2} M

To elucidate the mechanism of the exchange, we attempted to establish its minimum requirements and systematically tested for exchange after omitting components of the amino acid incorporation medium. The results are given in Table IV, for exchange followed with ^{3}H-labeled small subunits and non-labeled RM. The difference (Δcpm) in membrane plus monomer associated radioactivity in the presence and absence of puromycin is considered as a measure of exchange.

Table IV (Exp. 1) shows that the addition of amino acids is not required for the puromycin induced exchange. It further demonstrates (Exp. 2) that if PEP and pyruvate kinase are also omitted, no exchange is induced at 5 mM $MgCl_2$. PEP, however, is a chelating agent, and therefore its function in promoting subunit exchange at 5 mM Mg^{++} may be due to a decrease in the effective Mg^{++} concentration which promotes ribosome dissociation, rather than to the supply of chemical energy. The chelating effect of PEP could also explain the higher value of the background binding at 5 mM Mg^{++} (Exp. 2b) when PEP and PK were absent. For this reason we examined whether exchange might be restored in the absence of PEP and PK by lowering the Mg^{++} concentration. We tested a series of Mg^{++} concentrations and found that between 1.0 and 2.0 mM $MgCl_2$ the exchange did indeed occur in the absence of PEP and PK. The results for 1.5 mM $MgCl_2$ are shown in Table IV (Exp. 2c). It can be seen that in the absence of PEP at this lower Mg^{++} concentration puromycin is as effective in promoting exchange as it is at 5 mM Mg^{++} in the complete system with no amino acids (Exp. 2a). However, in the absence of puromycin, more labeled subunits material is bound to the microsomes in the incomplete (Exp. 2c) than in the complete system (Exp. 2a). This effect was unexpected since in the absence of PEP and PK amino acid incorporation and chain termination are totally suppressed. Upon examination of the microsomes, however, we found (see section 3.2.5) that the radioactivity bound to the membranes in the absence of puromycin at 1.5 mM $MgCl_2$ (Exp. 2c) was due to degraded material and therefore represented a higher background of unspecific adsorption to the membranes.

Table IV

Requirements of puromycin induced exchange of ^{3}H-labeled small subunits with rough microsomes*

Experiment #†		Condition	cpm in monomer-microsome region		
			- puromycin	+ puromycin	Δcpm
1	a	Complete system	2313	4910	2597
	b	- AAs	2822	5081	2259
2	a	- AAs	1556	3261	1705
	b	- AAs - PEP - PK	2539	2836	297
	c	- AAs - PEP - PK 1.5 mM Mg_2Cl	2513	4175	1662
3	a	as 2c	3047	5754	2707
	b	as 2c but no G-50 fraction	3652	4167	515
4	a	- AAs	3081	5144	2063
	b	- AAs + heat treated G-50 fraction§	2115	2527	412
5	a	as 2c	4568	7598	3012
	b	as 2c but no ATP	3862	7826	3964
	c	as 2c but no GTP	3364	6671	3309
	d	as 2c but no ATP, no GTP	3323	5550	2227
6	a	as 5b	4472	9513	5041
	b	as 5b but no GTP	4338	7644	3306
	c	as 5b but no GTP and 1.0 mM $MgCl_2$	4636	7755	3119

*Exchange was analyzed on sucrose gradients as described in the legend to Fig. 13. Radioactivity (cpm) was measured in the pooled fractions from the monomer-microsome region of the gradients. For each row, Δcpm indicates the difference between radioactivities in the presence and absence of puromycin

†Specific activity of small subunits was: Exp. 1, 17,700 cpm/OD_{260} unit; Exp. 2, 11,045 cpm/OD_{260} unit; Exp. 3, 23,500 cpm/OD_{260} unit; Exp. 4, 19,800 cpm/OD_{260} unit; Exps. 5 and 6, 22,500 cpm/OD_{260} unit. Total radioactivity recovered on gradients was: Exp. 1, ∿10,800 cpm; Exp. 2, ∿8,400 cpm; Exp. 3a, ∿10,800 cpm; Exp. 3b, ∿8,750 cpm; Exp. 4a, ∿11,000 cpm; Exp. 4b, ∿8,100 cpm; Exp. 5, ∿14,800 cpm; Exp. 6, ∿16,100 cpm. Inputs of RM were: Exps. 1 and 2, 4.0 OD_{260} units; Exp. 3, 4.5 OD_{260} units; Exps. 4 and 5, 3.0 OD_{260} units; Exp. 6, 5.5 OD_{260} units.

§55° C, 15 min.

The effect of raising the monovalent ion concentration was tested in a medium containing 200 mM NH_4Cl (not shown). The background binding (radioactive material present in the microsomes in the absence of puromycin) was reduced but the total extent of the exchange reaction was not altered. As expected (Adelman et al., 1970), at this higher ionic strength puromycin produced a larger detachment of monomers from the membranes and thus led to a proportional increase of small subunit exchange into the pool of free monomers.

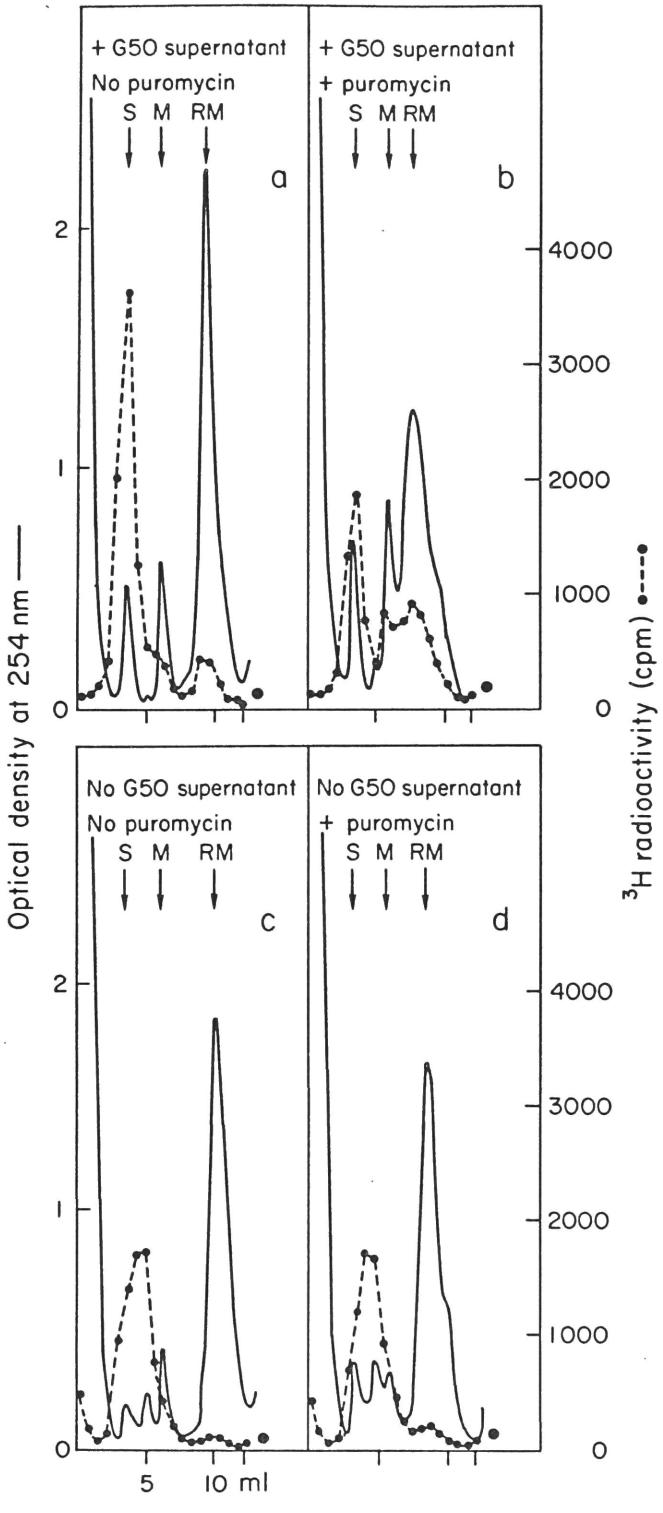
The effect of the G-50 fraction of the high speed supernatant on the puromycin induced exchange is shown in Fig. 15 and in Table IV (Exp. 3). A comparison of Figs. 15 b and d shows that the G-50 fraction, which contains macromolecular components larger than 30,000 in molecular weight, has a large effect in promoting the exchange. Furthermore, Table IV (Exp. 4) shows that the cell sap macromolecules responsible for this action are thermolabile. However, the mode of action of the G-50 supernatant fraction remained obscure. Fig. 15 shows that in the absence of the G-50 fraction the sedimentation properties of small subunits are altered. About half of the subunits sediment at a faster rate, as indicated by a second peak in the sedimentation profile, which may correspond to dimers of the 40 S particles. Moreover, the supernatant fraction served to protect small subunits from degradation during incubation. There was more radioactivity at the top of the gradient, and the recovery of radioactivity was consistently lower ($\sim$20%) when the G-50 high speed supernatant fraction was omitted. For these reasons it could not be decided if the role of the G-50 supernatant fraction in promoting exchange is due entirely to its effect in protecting the integrity of the small subunits and preventing their aggregation, or if an additional exchange-promoting macromolecular component exists in this fraction.

The requirements for ATP and GTP were also examined (Table IV, Exps. 5 and 6). Addition of either of these compounds had a stimulatory effect in promoting exchange, but their effects were not additive. It is therefore unlikely that the nucleoside triphosphates act simply by chelating Mg^{++}. Moreover, lowering the Mg^{++} concentration to 1.0 mM in

Figure 15. Effect of G-50 supernatant fraction on puromycin induced small subunit exchange. Incubation mixtures contained in 1 ml 9.0 OD_{260} units of RM and 1.1 OD_{260} units of ^{3}H-labeled small subunits (specific activity, 23,400 cpm/OD_{260} unit). After incubation, 0.5 ml aliquots were layered onto sucrose gradients. Composition of the gradients and conditions of centrifugation were as described in the legend to Fig. 13.

a and c, no puromycin; b and d, 10^{-3} M puromycin; a and b, with G-50 supernatant fraction; c and d, no G-50 supernatant fraction. ——, optical density; ●----●, ^{3}H radioactivity.

Effect of G50 supernatant on puromycin induced small subunit exchange



the absence of ATP and GTP did not restore the exchange to the control value. Because of these observations, 0.5 mM GTP was kept as a component of the minimal exchange medium. A similar stimulatory effect of GTP and ATP on the activity of Bacterial Dissociation Factor was reported by Subramanian and coworkers (Subramanian, Davis and Beller, 1969), who later (Subramanian and Davis, 1970) attributed the effect to chelation.

From the results described in this section a minimal exchange medium for the puromycin induced exchange was designed, the composition of which is as follows: G-50 supernatant fraction 0.15 ml/ml, 0.5 mM GTP, 150 mM NH_4Cl, 20 mM Tris-HCl pH 7.6, 1.5 mM $MgCl_2$, 0.25 M sucrose.

3.2.3. Quantitation of the exchange. In order to determine how many membrane-bound subunits are exchangeable after artificial termination, we incubated ^{3}H-labeled rough microsomes in minimal exchange medium with puromycin and increasing amounts of unlabeled free small subunits. Exchange "out" was then followed from the release of radioactivity into the 40 S region of 15 to 30% sucrose density gradients, which were centrifuged to sediment the microsomes to the bottom of the tube, while small subunits and monomers were well separated within the gradient. Theratios of added subunits to microsomal small subunits and the percentages of small subunits released from microsomes were calculated, taking a value of 2.6 for the molecular weight ratio of 28 S to 18 S RNA (Loening, 1968). The results from these experiments are plotted in Fig. 16, where it can be seen that in the presence of puromycin the release of bound small subunits increased with the addition of unlabeled free subunits and approached a limit value of ∿60% at the highest ratio of added to microsomal subunits (∿10:1). In the absence of puromycin, on the other hand, addition of unlabeled small subunits to the system caused no release of radioactivity into the 40 S region of the gradient. From these observations we concluded that the radioactivity which appeared in the membrane band when the reaction was followed for exchange "in" (Table IV) was due to unspecific adsorption of ribonucleoprotein to membranes and not to exchange (i.e. it represented background binding). It is not clear, however, why in the presence of puromycin the exchange did not involve more than 60%

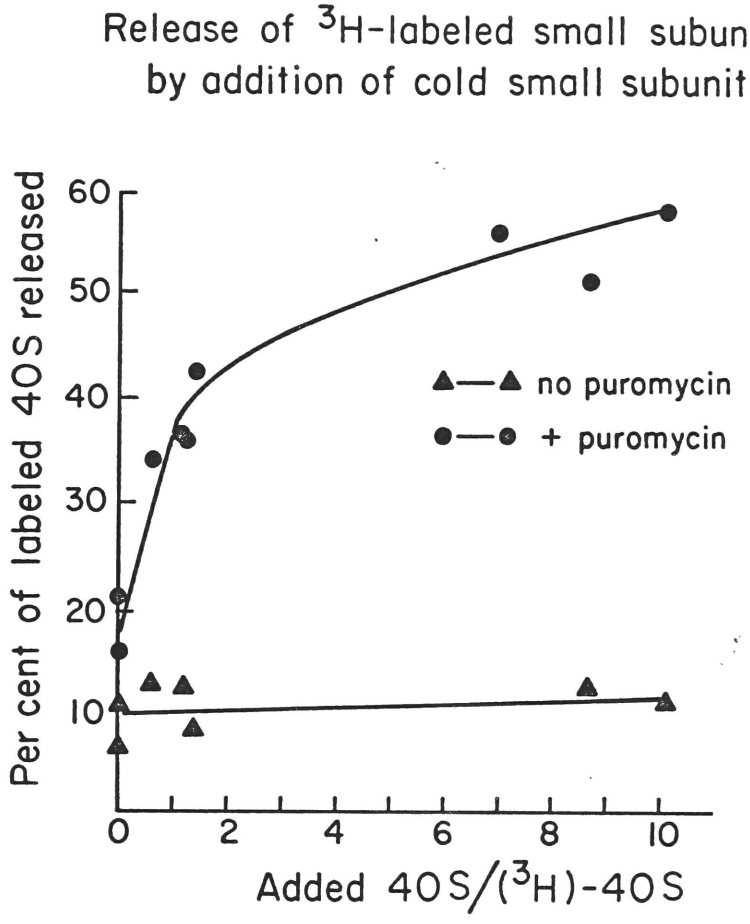


Figure 16. Effect of cold subunits and puromycin on the release of ^{3}H-labeled small subunits from RM. Incubation mixtures contained in 1 ml 7.5 OD_{260} units of ^{3}H-labeled RM (specific activity 11,000 cpm/OD_{260} unit). Small subunits were added in the amounts indicated. The ratio of added subunits to microsomal small subunits, as well as the extent of microsomal subunit release, were calculated by assuming a molecular weight ratio of 2.6 of 28 S to 18 S RNA. After incubation, 0.5 ml aliquots were layered onto 15 to 30% sucrose gradients, containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$. Centrifugation was for 2 hrs and 45 min at 40,000 rpm in the SB 283 rotor. The ordinate represents radioactivity recovered in pooled fraction corresponding to the small subunit optical density peak, as percentage of total radioactivity recovered on the gradient multiplied by 3.6.

●——●, with 10^{-3} M puromycin; ▲----▲, no puromycin.

of the bound small subunits. Prolonging the incubation time did not increase the extent of the exchange, which was found to proceed only during the first 5 minutes of incubation. Increasing the amount of high speed supernatant had no effect. A probable explanation for the incomplete exchange may be that the system is rapidly inactivated during incubation at 37° C, because of ribosome degradation. Incubation for 30 minutes at 0° C produced only 15% of the effect at 37° C. At least part of the exchange in samples incubated at 0° C may have occurred when the temperature was raised to 20° C before gradient centrifugation.

3.2.4. Experiments with the large subunit. Bound ribosomes attach to the microsomal membranes through large subunits which contain the nascent polypeptide chain. The latter is a factor which stabilizes the ribosome-membrane interaction (Adelman et al., 1970). Therefore it was of special interest to investigate the behavior of large subunits in the in vitro system described in the preceding sections. ^{3}H-labeled large subunits, obtained from free or bound polysomes, were added to non-labeled rough microsomes in minimal exchange medium. The ratio of labeled to non-labeled large subunits was approximately 1:1. The results are shown in Fig. 17 and Table V. In both instances, results with added labeled small subunits are included for comparison. To this effect, the radioactivity scale in Fig. 17 is twofold expanded for those panels showing the 40 S subunit exchange (Figs. 17 a and b). It can be seen that addition of puromycin to the system containing added free or bound large subunits has the following effects:

1) A large decrease in the amount of added 60 S subunits which remained single and free is indicated by a parallel diminution in height of the 60 S peaks in the optical density and radioactivity profiles (marked L in Figs. 17 c and e, versus d and f). This effect is in marked contrast to the effect when small subunits were added, in which case, the height of the optical density peak in the 40 S region actually increased upon addition of puromycin (Figs. 17 a and b). The 60 S subunits which remained free and uncombined after incubation with puromycin had approximately the same specific activity as the initially added subunits.

Figure 17. Puromycin induced exchange of ^{3}H-labeled small and large subunits with membrane-bound ribosomes in minimal exchange medium. Incubation mixtures contained in 1 ml 10.2 OD_{260} units of RM and: a and b, 1.9 OD_{260} units of ^{3}H-labeled small subunits; c and d, 3.76 OD_{260} units of ^{3}H-labeled large subunits obtained from free polysomes; e and f, 3.46 OD_{260} units of ^{3}H-labeled large subunits obtained from bound ribosomes. Specific activity of the subunits was 23,700 cpm/OD_{260} unit. After incubation, 0.5 ml aliquots were layered onto sucrose gradients containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$. The sucrose concentration ranges were: a, c, and e, 15 to 60%; b, 15 to 55%; d and f, 15 to 50%. Centrifugation was for 2 hrs 36 min at 40,000 rpm in the SB 283 rotor.

a, c, and e, no puromycin; b, d, and f, 10^{-3} M puromycin.
——, optical density; o---o---o, ^{3}H radioactivity.

Puromycin induced exchange of ^{3}H-labeled small and large subunits with membrane-bound ribosomes in minimal exchange medium

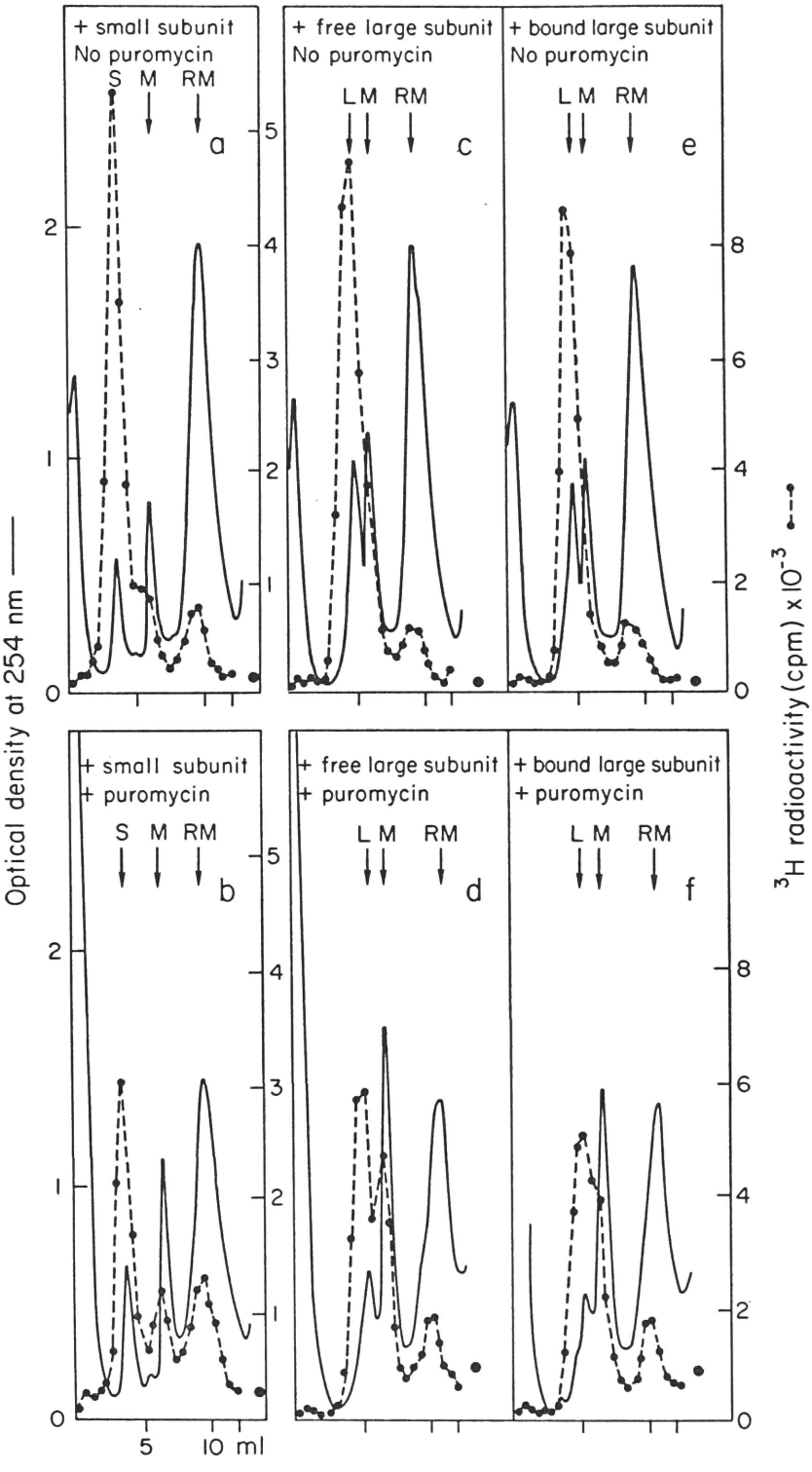


Table V

Distribution of radioactivity (cpm) after incubation of ^{3}H-labeled small and large subunits with RM in Minimal Exchange Medium*

Region in gradient	+ ^{3}H-labeled small subunits					+ ^{3}H-labeled free large subunits					+ ^{3}H-labeled bound large subunits				
	- puromycin		+ puromycin		Δcpm	- puromycin		+ puromycin		$\frac{\Delta cpm}{2.6}$	- puromycin		+ puromycin		$\frac{\Delta cpm}{2.6}$
Top	683	(3%)	801	(4%)		1354	(3%)	1148	(3%)		1127	(3%)	1042	(2%)	
Subunits	13868	(67%)	8604	(41%)	-5264	27708	(62%)	15961	(38%)	-4525	26068	(67%)	15172	(40%)	-4200
Monomers	2742	(13%)	4055	(20%)	+1313	8219	(18%)	14565	(34%)	+2440	6262	(16%)	12248	(32%)	+2300
Microsomes	3604	(17%)	7264	(35%)	+3660	5339	(17%)	10695	(25%)	+2040	5605	(14%)	9964	(26%)	+1680
Total cpm	20897	(100%)	20724	(100%)		42680	(100%)	42680	(100%)		39062	(100%)	38426	(100%)	

*Medium of incubation, inputs of rough microsomes and added subunits, and conditions of sucrose gradient centrifugation are described in the legend to Fig. 17.

Therefore, the incubation resulted in little replacment by bound large subunits of the added subunits which were shifted to other regions of the gradient.

2) A large increase in the amount of free monomers, as indicated by the optical density profile in the 80 S region. This increase was considerably larger (at least twice) than the one observed in the presence of puromycin and small subunits (Figs. 17 d and f versus Fig. 17b). As indicated by the radioactivity peaks in the 80 S region (peaks marked M in Figs. 17 d and f) the free monomer contained labeled added large subunits, which recombined with unlabeled small subunits released from the rough microsomes. The transfer of bound small subunits to a monomer pool should be expected from their exchangeability, demonstrated in section 3.2.1, and from the operation of a ribosome cycle in ribosomes which are attached to the membranes via large subunits only (Sabatini et al., 1966).

3) An increase in binding of labeled large subunit material to the membranes (peak labeled RM in Figs. 6 d and f versus c and e). This effect is approximately half of the one observed for small subunits, if the data are compared taking into account the factor of 2.6 for the molar ratio of 28 S and 18 S RNAs (Table V). The nature of the membrane bound material is further investigated in section 3.2.5.

Finally, it should be noted that in experiments performed in the complete incorporation medium (Table VI), the behavior of the large subunits is similar to their behavior in minimal exchange medium.

Complimentary experiments, designed to test for exchange of large subunits "out," were also performed. Since in these experiments it was critical to achieve good resolution between large subunits and monomers, 5 to 20% sucrose gradients were used, in which microsomes were sedimented into the pellet. To avoid dissociation during centrifugation of inactive monomers (i.e. monomers lacking nascent chains) into subunits--which we observed at high centrifugal fields as described by Infante (Infante and Baierlein, 1971; Infante and Krauss, 1971; Infante and Graves, 1971)--

Table VI

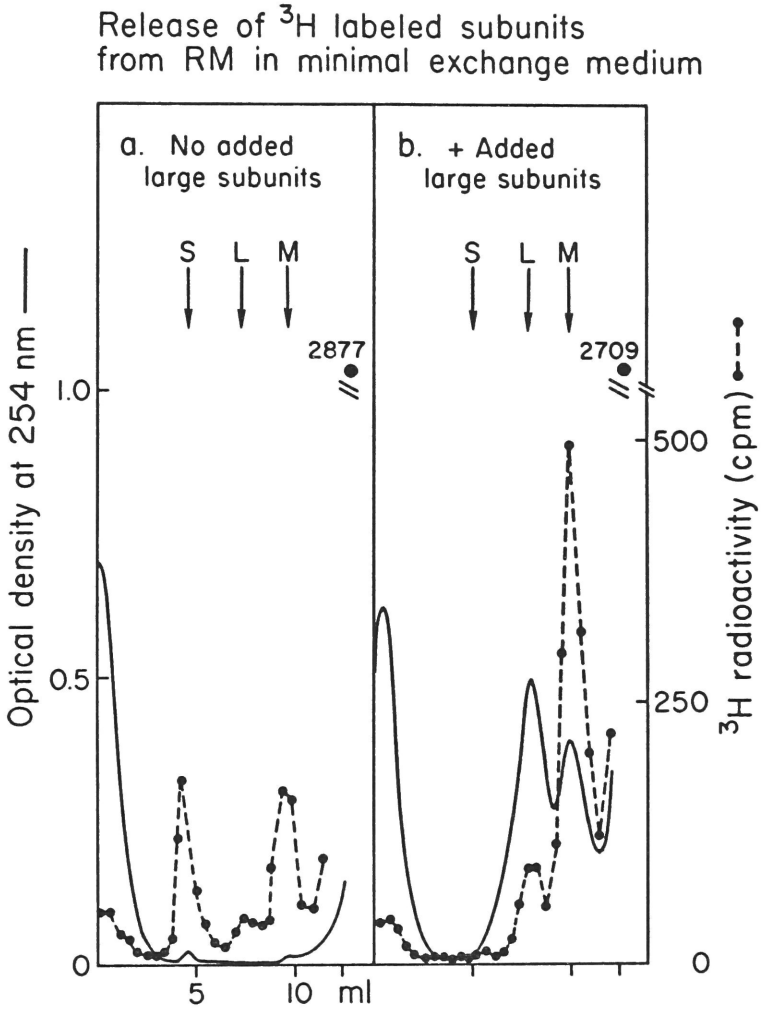
Distribution of radioactivity (cpm) after incubation of ^{3}H-labeled large subunits with RM in AA incorporation medium*

Region in gradient	- puromycin	+ puromycin	Δcpm
Top	889 (2%)	794 (2%)	
Large subunit	32475 (63%)	20134 (40%)	- 12341
Monomer	14220 (28%)	18880 (38%)	+ 4660
Microsomes	3411 (7%)	9684 (20%)	+ 6273
Total cpm	50995 (100%)	49492 (100%)	

*4.9 OD_{260} units of rough microsomes were incubated with 2.3 OD_{260} of ^{3}H-labeled large subunits (specific activity 26.000 cpm/OD_{260} uni obtained from free polysomes in the complete amino acid incorporation medium with or without the addition of 10^{-3} M puromycin. Volumes of the incubation mixtures were 0.5 ml and incubation was for 10 min at 37° C. The samples were layered onto 15 to 55% sucrose gradients containing 150 mM NH_4Cl, 20 mM Tris, 5 mM $MgCl_2$. Centrifugation was for 3 hr at 40,000 rpm. Radioactivities from pooled fractions from different regions in the gradient are given in the table.

gradients were centrifuged at 18,000 rpm for 10 hrs. ^{3}H-labeled rough microsomes were incubated in minimal exchange medium in the presence of puromycin with or without the addition of unlabeled large subunits. The ratio of added large subunits to microsomal large subunits was of approximately 6:1. This high ratio was chosen so that if exchange of large subunits occurred, nearly all released subunits would be recovered in the 60 S peak. In this experiment the concentration of rough microsomes was decreased four times with respect to previous experiments. This higher dilution allowed us to test for spontaneous release of subunits from puromycin treated microsomes in the absence of added competing subunits. As can be seen in Fig. 18, under these conditions microsomes released ∿39% of the small subunits as free 40 S particles, but only ∿7% of the large subunits as free 60 S particles. Approximately 13% of the membrane-bound ribosomes were recovered as free monomers. The preferential release of small subunits from diluted microsomes treated with puromycin provides an independent confirmation of the results on small subunit exchange presented in preceding sections (section 3.2.1, 3.2.2 and 3.2.3). The much smaller amount of 60 S particles released (more than fourfold smaller) indicates that the binding constant of large subunits to the membrane is considerably higher than that of the small subunits to the bound large subunits. As in the previous experiments (Fig. 17), addition of large subunits (Fig. 18b) to the microsome-puromycin system led to the formation of free monomers (peak marked M in Fig. 18b). These monomers were hybrids of added unlabeled 60 S particles and labeled bound 40 S particles released from the membranes. This is apparent from the specific activity (cpm/OD) of the 80 S particles in Fig. 18b, which is lower than the specific activity of the pure labeled ribosomal material in Fig. 18a by approximately the dilution factor 1:3.6 expected from a 1:1 molar ratio of unlabeled large and labeled small subunits. The small amount of labeled 60 S particles (∿6.5% of the radioactivity, i.e. ∿9.0% of the labeled large subunits) which remain as free large subunits in Fig. 18b should be contrasted to the 50 to 60% of microsomal small subunits which can be released by

Figure 18. Effect of added unlabeled large subunits on the release of ^{3}H-labeled subunits from RM in minimal exchange medium. Incubation mixtures contained in 1 ml 4.75 OD_{260} units of ^{3}H-labeled RM (specific activity, 6200 cpm/OD_{260} unit). Sample b contained in 1 ml 20.8 OD_{260} units of free large subunits. After incubation, the samples were diluted by addition of an equal volume of 150 mM NH_4Cl, 20 mM Tris-HCl, 1.5 mM $MgCl_2$, and 0.4 ml aliquots were layered onto 5 to 20% sucrose gradients containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$. Centrifugation was for 10 hrs at 18,000 rpm in the SB 283 rotor. ——, optical density; ●---●---●, ^{3}H radioactivity.


Release of ^{3}H labeled subunits
from RM in minimal exchange medium
a. No added
large subunits
b. + Added
large subunits
S
L
M
2877
2709
Optical density at 254 nm
1.0
0.5
0
5
10
ml
500
250
0
^{3}H radioactivity (cpm)

adding excess free small subunits. Moreover, the release of large subunits observed could be accounted for by the exchange of the added subunits with the labeled monomers shown in Fig. 18a, which were spontaneously released after puromycin.

The results described in this section indicate that in the in vitro system, during the time interval studied, puromycin does not induce an exchange of free and bound large subunits similar to the exchange of small subunits.

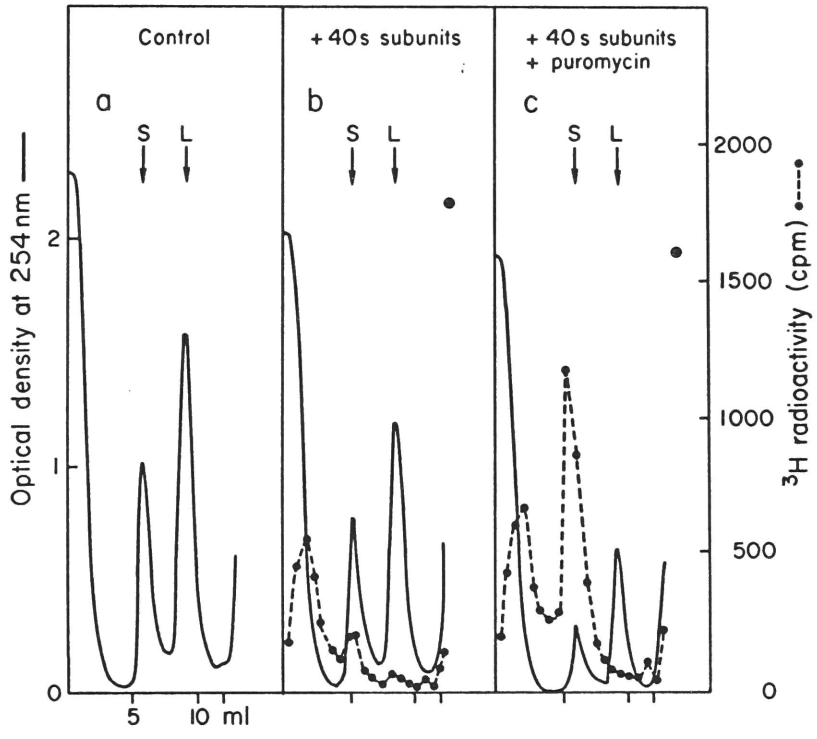
3.2.5. Analysis of membrane-bound ribosomes in microsomes recovered after subunit exchange. The occurrence of exchange should be corroborated by the analysis of membrane-bound ribosomes recovered in microsomes preincubated for exchange with added subunits. To this purpose, the microsomes were sedimented, incubated in HSB to detach all bound ribosomes (Adelman et al., 1970), and analyzed on sucrose density gradients. Puromycin was added during the reincubation in HSB for the sake of uniformity, but its addition was only necessary in the case of controls which contained no puromycin during the first incubation. Experiments were carried out with both subunits in each case for exchange "in" and exchange "out." Chemical determinations, however, showed that only ∿50-60% of the RNA in the preincubated microsomes was released from the membranes by the high salt-puromycin treatment, as opposed to ∿85% which is released from non-incubated controls (Adelman et al., 1970). It is likely that the lower release of RNA in recovered microsomes is due to deleterious effects of the preincubation.

Fig. 19 shows the results of a sucrose gradient analysis of the material released from microsomes incubated for exchange with ^{3}H-labeled small subunits, in a 1:1 ratio to microsomal small subunits. It can be seen (Figs. 19 c and b) that addition of puromycin has a striking effect in increasing the relative amount of labeled membrane-bound small subunits. Labeled material bound without puromycin was degraded, aggregated or could not be released, since it was mainly found in the upper fractions of the gradients or in the pellet. It is therefore this altered material

Figure 19. KCl-puromycin analysis of RM recovered after incubation in minimal exchange medium with ^{3}H-labeled subunits. Incubation mixtures contained in 1 ml 9.5 OD_{260} units of RM. Samples b and c contained in 1 ml 2.0 OD_{260} units of ^{3}H-labeled small subunits (specific activity, 23,500 cpm/OD_{260} units). Sample c contained 10^{-3} M puromycin. Volumes of the incubation mixtures were 5 ml. After incubation, each sample was layered onto two 10.5 ml sucrose gradients containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$. The sucrose concentration ranges were: a and b, 15 to 60%; c, 15 to 55%. After centrifugation for 1 hr at 40,000 rpm in the SB 283 rotor, the membrane bands from the three samples were collected from the lower third of the gradient, diluted 1:1 with TKM and sedimented into pellets by a 15 min centrifugation at 59,000 rpm and 3° C in the A 321 rotor of the International centrifuge. The microsome pellets were resuspended in 0.7 ml of water and diluted 1:1 with a compensating buffer, so that the final composition of the microsome suspensions was HSB and 10^{-3} M puromycin. The samples were then incubated for 10 min at 37° C, after which 0.4 ml aliquots were layered onto the final analytical 10 to 30% sucrose gradients in HSB. Centrifugation was for 3 hrs and 30 min at 40,000 rpm and 20° C in the SB 283 rotor.

——, optical density; ⊙----⊙, ^{3}H radioactivity.

KCl-puromycin analysis of RM recovered after incubation in minimal exchange medium with ^{3}H-labeled small subunits.



which constitutes most of the background binding described in sections 3.2.1 and 3.2.2. It can be computed from Fig. 19c and the value of the specific activity of the added subunits that in this experiment the labeled added subunits represent after exchange 20% of all the membrane associated small subunits. However, it should be noted that the ratio of large to small bound subunits is unchanged with respect to a control incubated in the absence of puromycin and added subunits (Fig. 19a). From this experiment it can be concluded that the main effect of puromycin is to promote replacement of released small subunits by undegraded added labeled subunits (small subunit exchange).

An analysis of the distribution of radioactive material released from microsomes after incubation with ^{3}H-labeled large subunits (in a 1:1 ratio to microsomal large subunits) with and without the addition of puromycin can be seen in Fig. 20. It is apparent that only 16% of the radioactive material released from microsomes incubated with large subunits and without puromycin remained as undegraded 60 S particles. Puromycin caused an increase in the amount of membrane bound radioactivity from a total of 7,800 cpm to 11,300 cpm, but the increase occurred in all regions of the gradient and not selectively in the 60 S region. This unselective increase of bound labeled ribonucleoprotein after addition of ^{3}H-labeled 60 S particles and puromycin should be contrasted with the selective increase in membrane associated labeled 40 S particles, which was observed when ^{3}H-labeled small subunits were added.

A summary of the results obtained by analyzing the membrane-bound material released by treatment with puromycin-HSB from ^{3}H-labeled microsomes, which were previously incubated for exchange "out" with unlabeled small and large subunits is shown in Fig. 21. The control sample (Fig. 21 ●—●—●) consisted of labeled rough microsomes preincubated in minimal exchange medium with puromycin and no added subunits. From parallel direct analysis of aliquots of the incubation mixtures it was learned that in this experiment, after incubation with small or large subunits, the amount of radioactivity which remained membrane associated

Figure 20. KCl-puromycin analysis of RM recovered after incubation in minimal exchange medium with ^{3}H-labeled large subunits. Incubation mixtures contained in 1 ml 9.6 OD_{260} units of RM and 6.0 OD_{260} units of ^{3}H-labeled free large subunits (specific activity, 16,000 cpm/OD_{260} unit). The incubation for exchange, the procedure for recovering the microsomes, the high salt-puromycin incubation of the recovered RM, and final sucrose gradient centrifugation conditions were as described in the legend to Fig. 19. ●——●, no puromycin in minimal exchange medium; ▲----▲, 10^{-3} M puromycin in minimal exchange medium.

KCl-puromycin analysis of RM recovered after incubation in minimal exchange medium with ^{3}H-labeled large subunits

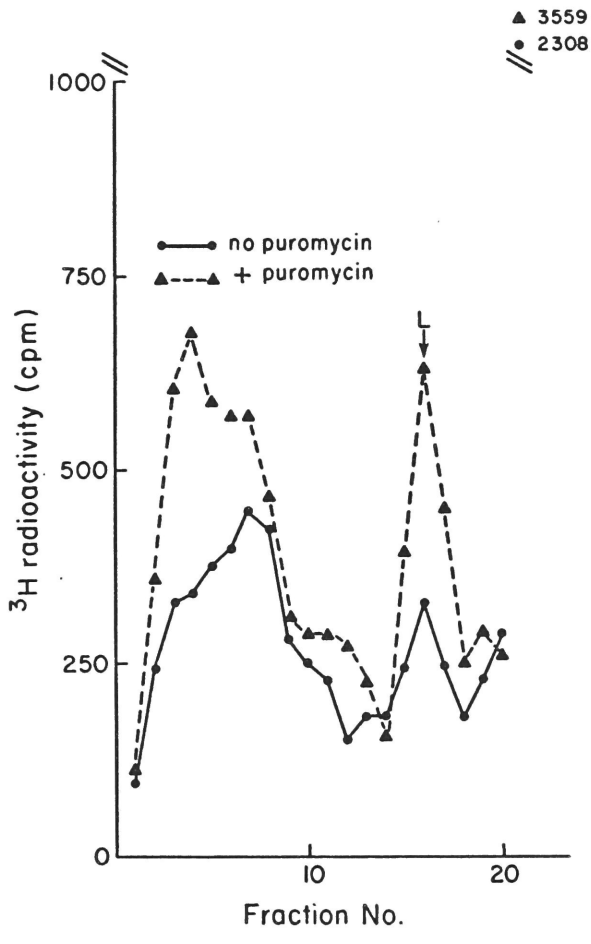
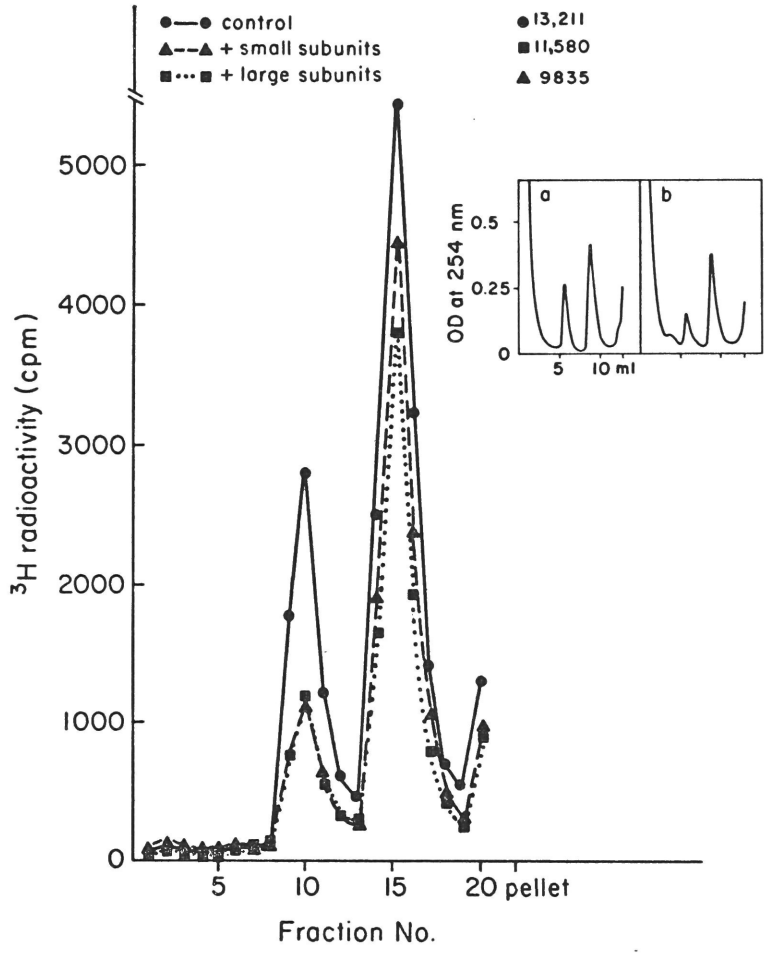


Figure 21. KCl-puromycin analysis of ^{3}H-labeled RM recovered after incubation in minimal exchange medium. Incubation mixtures contained in 1 ml 6.7 OD_{260} units of ^{3}H-labeled RM (specific activity 12,800 cpm/OD_{260} unit) and 10^{-3} M puromycin, with or without added unlabeled subunits. The total volumes of the incubation mixtures were 4.0 ml. After incubation, 0.25 ml aliquots of each sample were layered onto 15 to 30% sucrose gradients containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$, for direct analysis of the incubation mixtures. The remaining 3.75 ml were layered onto 11.5 ml 15 to 55% sucrose gradients containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$, to recover the microsomes, as described in the legend to Figure 19. The microsomal pellets were resuspended in 0.6 ml H_2O and incubated in HSB-puromycin for detachment of ribosomes, as described in the legend to Figure 19. 0.5 ml aliquots of the high salt incubation mixtures were layered onto 10 to 30% sucrose gradients. Composition of the final analytical gradients and conditions of centrifugation were as described in the legend to Fig. 19. ●——●, no added subunits in minimal exchange medium; ▲----▲, 11.5 OD_{260} units of small subunits/ml in minimal exchange medium; ■·····■, 26.5 OD_{260} units large subunits/ml in minimal exchange medium. The inset shows optical density profiles corresponding to ▲----▲ (a), and ■·····■ (b).

KCl-puromycin analysis of ^{3}H-labeled RM recovered after incubation in minimal exchange medium



was 72% of the radioactivity which was membrane-bound in the control. Therefore the radioactivity value of each experimental point in Fig. 21 within the gradient and in the pellet (Fig. 21 ▲---▲, ■····■) has been multiplied by a normalizing factor which makes the total radioactivity in each case correspond to 72% of the control (Fig. 21 ●——●——●). In this manner small differences in the recovery of microsomes after the incubation for exchange have been compensated and the data become directly comparable on the basis of equal input of microsomal membranes on the final analytical gradient. The percentage of radioactivity released into the gradient after puromycin-HSB was: control (Fig. 21 ●—●), 60%; sample incubated with small subunits (Fig. 21 ▲--▲), 58%; sample incubated with large subunits (Fig. 21 ■····■ , 51%. The distribution of radioactivity in the subunits demonstrates that added small and large subunits are equally effective in removing bound small subunits from microsomes (∿50% in Fig. 21 with respect to the control without added subunits). The effect of small and large added subunits on removal of large subunits (22 and 35% of control respectively in Fig. 21) was considerably weaker than their effect on small subunits. Most likely the decrease of large subunits after incubation with either subunit was unspecific and the difference between the effect of each subunit was not significant, since, as mentioned before, the puromycin-KCl procedure was not equally effective in each case.

Optical density profiles corresponding to radioactivity profiles have been plotted as an inset in Fig. 21 for the samples incubated with added subunits. Table VII compares the radioactivity and optical density ratios of large to small subunits from data in this figure. It is clear that only in the case of preincubation with small subunits do these ratios differ significantly from each other. The difference indicates that in this case true subunit exchange occurs, since competing unlabeled subunits replace released subunits and the optical density ratio remains unaltered with respect to the control. In the case of preincubation with large subunits, on the other hand, both ratios are changed with respect to the control samples and do not differ significantly from each

Table VII

Radioactivity and optical density ratios of large to small subunits released from RM recovered after incubation for exchange*

Sample	Large/Small	
	Optical Density	Radioactivity
Control ^{3}H-RM	2.5	2.4
^{3}H-RM + small subunits	2.3	3.4
^{3}H-RM + large subunits	3.2	3.0

*The radioactivity ratios are calculated from the data plotted in Fig. 9. The optical density ratios were estimated from the corresponding areas in the optical density profiles.

other. Therefore, in this case the released material has not been replaced by competing unlabeled subunits.

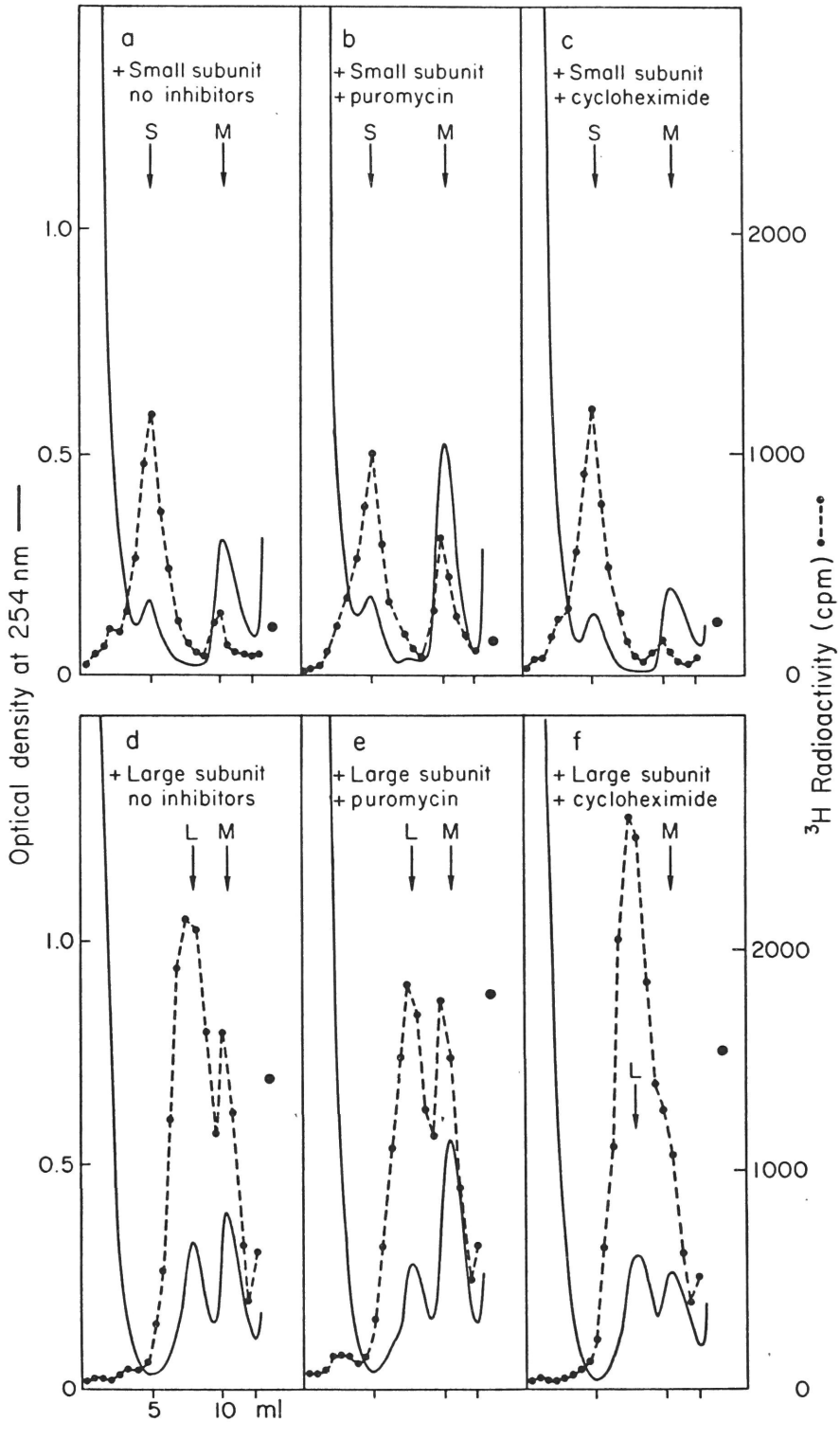
3.2.6. Exchange of small and large subunits with free polysomes. The previous results demonstrate that upon natural or artificial termination of peptide chains, membrane-bound ribosomes can undergo extensive exchange of small subunits with added subunits derived from free polysomes. However, a comparable exchange was not observed for large subunits. To establish if this difference is specifically due to the fact that in bound ribosomes large subunits are attached to the membranes, or rather whether it is due to a general incompetence of our preparations of large subunits, we investigated whether small and large subunits are equally efficient in exchanging with a system of free polysomes. The sucrose gradient analysis in Fig. 22 shows the natural and puromycin induced exchange of both subunits in an amino acid incorporation system with free polysomes (exchange "in"). Particles with sedimentation coefficients greater than 80 S were sedimented to the bottom of the tube, while ribosomal subunits and monomers were still displayed within the gradients, which were centrifuged at low speed to avoid pressure induced dissociation of inactive monomers. The ratio of added subunits to subunits in the polysomes was ∿2:1. Measurements of the areas under the monomer peaks of the optical density profiles (M in Fig. 22) and determinations of RNA in the samples, showed that incubation during 10 min for amino acid incorporation resulted in a partial conversion of polysomes into monomers (∿60%). Cycloheximide inhibited the conversion of polysomes into monomers (Figs. 22 c and f). In the presence of puromycin, on the other hand, the conversion was essentially complete (Figs. 22 b and e). Examination of the radioactivity patterns in Fig. 22 shows an increase in the amount of labeled added subunits in the monomer peak, which parallels the increase in monomers generated during incubation. It can be computed from the optical density and radioactivity profiles that, when puromycin was added, the specific activity of subunits within monomers was 87% of that expected if total equilibration had occurred. The degree of equilibration reached with the puromycin induced monomers

Figure 22. Exchange of small and large subunits with free polysomes in amino acid incorporation medium. Free polysomes were repurified by centrifuging the polysome suspension containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$ for 5 min at 10,000 rpm and 3° C in the A 321 rotor of the International centrifuge, to eliminate heavy aggregates. The resulting supernatant was layered over a 4 ml cushion of 2 M sucrose containing 150 mM NH_4Cl, 20 mM Tris-HCl, 5 mM $MgCl_2$, and centrifuged for 2 hrs at 59,000 rpm in the A 321 rotor at 3° C. The polysomes in the resulting pellet were used for the exchange experiment.

Each incubation mixture contained in 1 ml 2.6 OD_{260} units of purified polysomes. Samples a, b, and c contained in 1 ml 1.32 OD_{260} units of ^3H-labeled small subunits; samples d, e, and f contained in 1 ml 2.95 OD_{260} units of ^3H-labeled large subunits. Specific activity of the subunits was 15,000 cpm/OD_{260} unit. After incubation, 0.4 ml aliquots were layered onto 5 to 20% sucrose gradients, containing 150 mM NH_4Cl, 20 mM Tris, 5 mM $MgCl_2$. Centrifugation was for 10 hrs at 18,000 rpm in the SB 283 rotor.

a and d, no inhibitors; b and c, 10^{-2} M cycloheximide; c and f, 10^{-3} M puromycin. ——, optical density; ●----●, ^3H radioactivity.

Exchange of small and large subunits with free polysomes in Amino Acid incorporation medium



was similar for both subunits, as indicated by the ratio of the radioactivities in monomers, obtained after large and small subunit exchange, which was ∿2.7 (expected theoretical value: 2.6). In no case was the optical density peak of added 40 S and 60 S subunits significantly affected by the addition of puromycin, indicating for both subunits, equal replacement of labeled by unlabeled subunits and, therefore, true exchange with free ribosomes.

3.3. Ribosome Attachment to Stripped Membrane Fractions in vitro

The different behavior of small and large subunits of membrane-bound ribosomes in the exchange reaction indicates that there are ribosome binding sites on microsomal membranes, which strongly interact with large ribosomal subunits. Quantitation and characterizations of these sites would greatly contribute to an understanding of the role of membranes in the process of assembly of the polysome-membrane complex.

Preliminary work in our laboratory (M. Adelman, unpublished) indicated that ribosomes, detached from rough microsomes by the KCl-puromycin procedure, could rebind to the stripped rough microsomes at low ionic strengths (TKM). It was found that at ionic strengths (0.1 M KCl) closer to physiological conditions the rebinding was weaker and that it was not affected by the addition of non-sedimentable proteins released from the microsomes by the high salt treatment.

As a first attempt to characterize the in vitro binding occurring at a low ionic strength (TKM), we have determined the time course of binding and amount of RNA bound to the membranes on a phospholipid basis for three different membrane fractions (RM, SM and erythrocyte ghosts, all three treated for stripping). Fig. 23 shows the time course of ^{3}H-labeled ribosome binding to the three membrane fractions at 37° C (Fig. 23a) and at 0° C (Fig. 23b). Binding of the ^{3}H-labeled ribosomes to RM and SM treated for stripping reached a maximal value after incubation for 5 to 10 minutes at 37° C. A longer incubation in the cold (30 min to 1 hr) was required to approach the amount of binding obtained

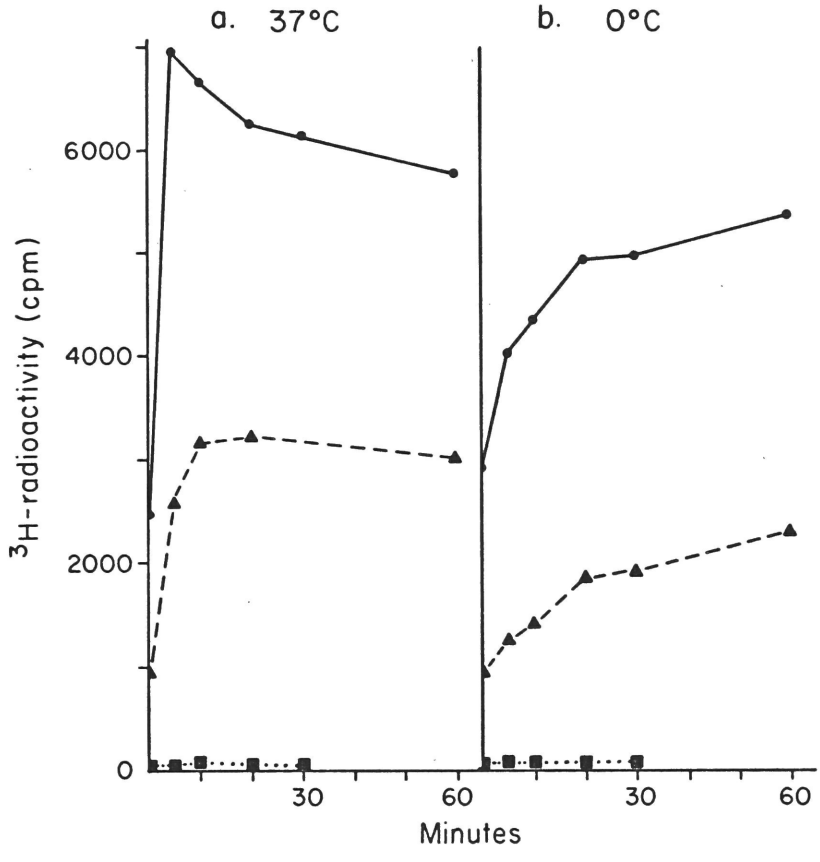


Figure 23. Time course of binding of ribosomes to membrane fractions. ●——●, stripped RM (0.186 mg protein) and 0.72 OD_{260} units of ^{3}H-labeled ribosomes (specific activity, 12,000 cpm/OD_{260} unit); ▲---▲, stripped SM (0.188 mg protein) and 0.73 OD_{260} units of ^{3}H-labeled ribosomes (specific activity, 12,000 cpm/OD_{260} unit); ■···■, stripped erythrocyte ghosts (0.213 mg protein) and 0.56 OD_{260} units of ^{3}H-labeled ribosomes (19,000 cpm/OD_{260} unit). a, 37° C; b, 0° C.

at 37° C. Because binding did occur in the cold, it was not possible to obtain a true zero time point, since ∿20 minutes elapsed from the time of addition of heavy sucrose to the incubation mixtures to the time at which centrifugation was started. A 37° C incubation did not increase the binding of ^{3}H-labeled ribosomes to erythrocyte ghosts, which was virtually nil at both temperatures examined (see also Table VIII).

In order to quantitate the amount of RNA that can bind to stripped RM, it was necessary to determine whether all available sites on the membranes were saturated. Fig. 24 shows the results obtained when increasing amounts of ^{3}H-labeled ribosomes were added to a fixed amount of stripped RM (0.184 mg protein) and the membrane-associated radioactivity was determined after incubation for 10 minutes at 37° C. When the input of ribosomes was increased from 0.095 to 0.130 mg, the amount of membrane-associated radioactivity remained approximately constant (5040 and 5267 cpm respectively), indicating that the available binding sites must be nearly saturated. On the other hand, at lower inputs of ribosomes, less radioactivity was bound to the membranes (for example, 4136 out of 5167 cpm for an input of 0.045 mg of ribosomes), indicating that at lower ratios of input ribosomes to stripped RM, saturation of binding sites was not attained. However, it can be seen that at the lowest ratio of input ribosomes to membranes virtually all the ribosomes were bound.

Table VIII summarizes the results obtained for the quantitation of ribosome binding to RM, SM and erythrocyte ghosts, all three treated for stripping. In all experiments, excess ribosomes were added, so that only 20 to 50% of the total radioactivity was bound to the membranes and, in the case of stripped RM, saturation of binding sites was attained. It can be seen that approximately twice as much RNA binds to stripped RM as to similarly treated SM under the same conditions of incubation (10 min at 37° C) and with similar ratios of input ribosomes to membrane phospholipid. On the other hand, ribosome binding to erythrocyte ghosts was virtually nil, and binding to heat treated stripped RM (55° C 15 min) was much reduced. The RNA to phospholipid ratio of

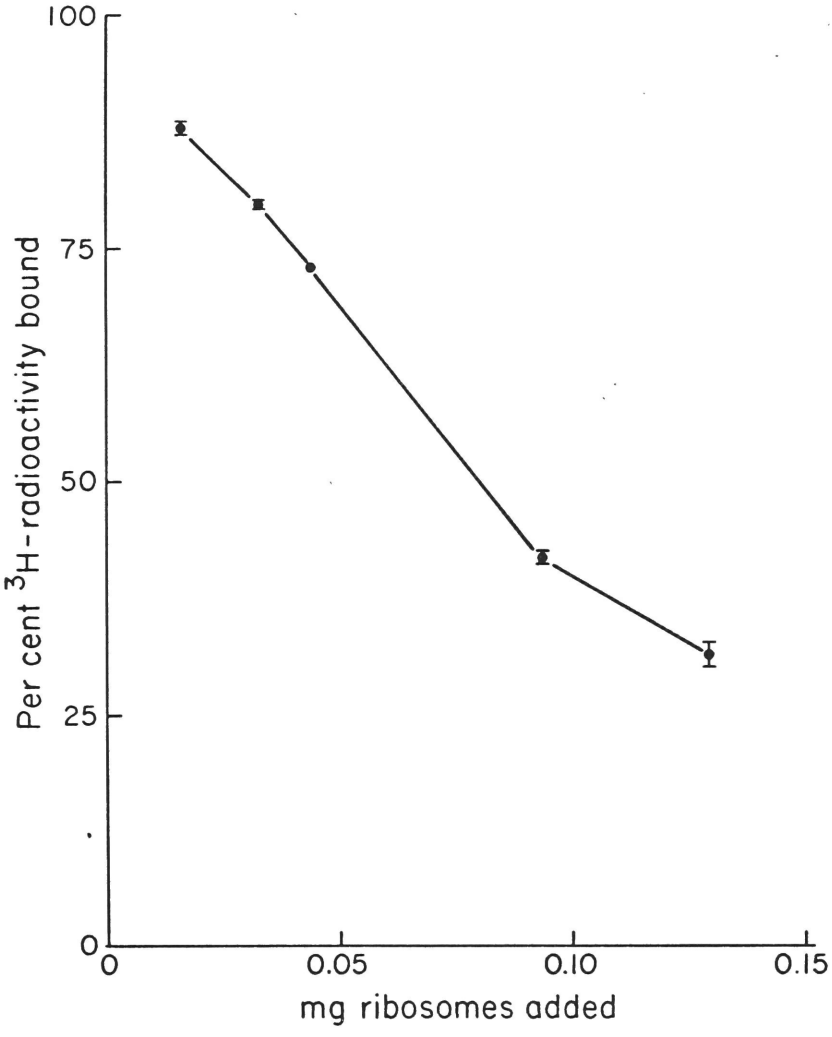


Figure 24. Percentage of ribosomes bound to stripped RM as a function of the amount of ^{3}H-labeled ribosomes added to a fixed amount of membranes (0.184 mg protein). Specific activity of the ribosomes was 10,000 cpm/OD_{260} unit. Each point represent the average of values obtained from two separate binding assays. The length of the bars represents the range of the values obtained.

Table VIII

Binding of ribosomes to different membrane fractions*

Membrane Fraction**		mg RNA bound / mg protein	mg RNA bound / mg PLP††	ΣRNA / PLP§
Stripped RM	(5)	0.1 ± 0.012†	0.229	0.303
Heat treated*** stripped RM	(1)	0.016	0.034	
Stripped SM	(3)	0.044 ± 0.007†	0.103	0.116
Erythrocyte Ghosts	(2)	---	0.0025 ± 0.0005†	---

*Binding was assayed as described in the methods section. All values are for binding obtained after 5 or 10 min incubations at 37° C.

**In this column numbers in parenthesis represent number of experiments.

***55° C, 15 min.

†Mean deviations

††Except for the case of erythrocyte ghosts, the values in this column are computed from the data of Table I.

§ΣRNA is the sum of RNA bound during the incubation and residual RNA of the stripped membranes before incubation for rebinding (see Table I).

the "reconstituted RM" (ΣRNA/PLP in Table VIII) was ∿55% of the ratio in natural non-washed RM (see Table I).

The implications of these as yet preliminary findings will be discussed in Chapter IV.

3.4. Structural Studies on Free and Bound Ribosomes

3.4.1. CsCl density gradient centrifugation. We first examined the buoyant densities of subunits obtained from free and bound ribosomes on CsCl density gradients. The results are shown in Fig. 25. Two optical density peaks, banding at densities (1.545 and 1.610) close to those expected for ribosomal subunits are apparent both in the case of free and bound ribosomes. However, the ratio of large to small subunits is in excess of what would be expected (2.6) on the basis of the molar ratios of 28 S to 18 S RNA. This preferential loss of small subunits at some step during the procedure remains unexplained. However, within the limits of resolution of this technique, there is no observable difference in the buoyant densities of the ribosomal subunits obtained from free or bound ribosomes.

3.4.2. Electrophoresis of ribosomal proteins. To further investigate possible differences between free and bound ribosomes, their protein composition was compared by SDS polyacrylamide gel electrophoresis (in collaboration with G. Blobel). The subunits were obtained from washed RM and washed free polysomes (free polysomes resuspended in 0.5 M KCl, 0.050 M Tris-HCl, 0.010 M $MgCl_2$ in the cold, and recovered from the suspension by centrifugation for 2 hrs at 60,000 rpm in the A321 rotor of the International centrifuge). Figs. 26 a and b show the electrophoretic patterns for bound and free small subunits respectively. Although as many as 17 protein bands are resolved on these gels, no difference between the two types of subunits is apparent. However, a complete resolution of all proteins would be required to conclude that free and bound small subunits have identical protein complements. The electrophoretic patterns obtained from bound and free large subunits respectively are shown in Figs. 26 c and d. In this case, also, there

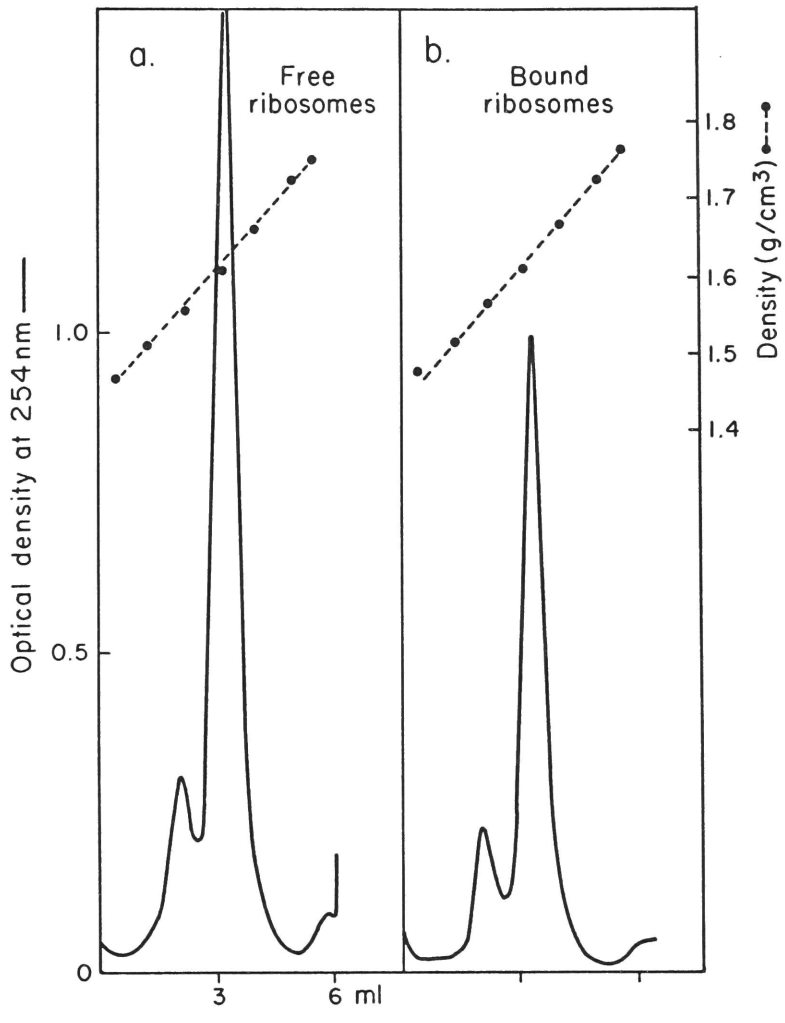


Figure 25. CsCl density gradient analysis of subunits obtained from free and bound ribosomes, prepared by procedure B. a, ∿1.4 OD_{260} units of free dissociated ribosomes; b, ∿1.15 OD_{260} units of bound dissociated ribosomes.

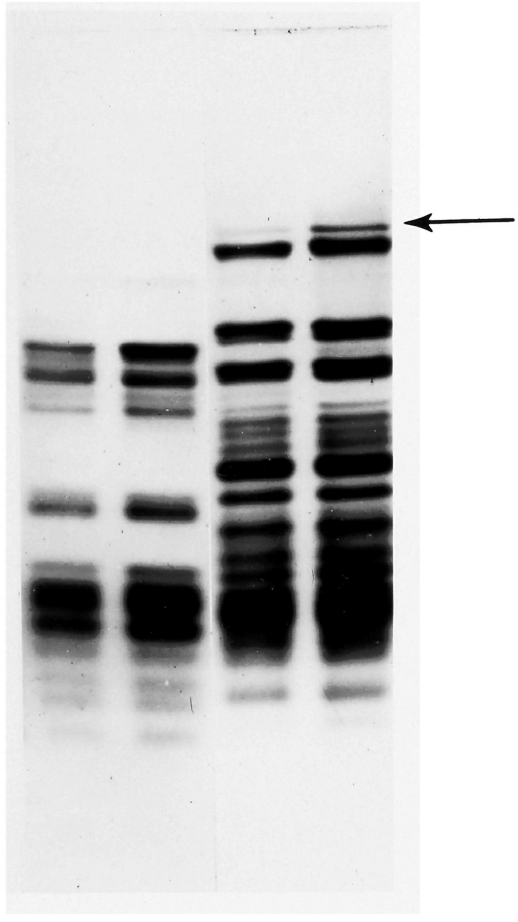


Figure 26. Electrophoresis of subunits, obtained from free and bound ribosomes, on 12.5% polyacrylamide SDS gels. Free polysomes and rough microsomes were prepared by procedure B and washed in HSB prior to high salt-puromycin treatment to obtain subunits. Slot a, ∿0.4 OD_{260} units of small subunits obtained from bound polysomes; slot b, ∿0.5 OD_{260} units of small subunits obtained from free polysomes; slot c, ∿1.25 OD_{260} units of large subunits obtained from bound polysomes; slot d, ∿1.5 OD_{260} units of large subunits obtained from free polysomes.

is a close correspondence between both sets of protein bands. However, a more intense band in the upper part of the gel is apparent in the case of the free large subunit (arrow, Fig. 26). A band at the corresponding position in the bound large subunit was in most cases absent or weak. Control experiments demonstrated that the more intense band in free ribosomes was not due to contamination of the preparations with ferritin. A similar extra protein band was observed in undissociated chick embryo free ribosomes (Fridlender and Wettstein, 1970).

IV. DISCUSSION

4.1. Disassembly of the Polysome-Membrane Complex

Fig. 27 schematically represents the two main factors involved in maintaining the binding of ribosomes to membranes of the endoplasmic reticulum, as has been deduced from the results of Adelman et al. (Adelman et al., 1970). The ionic bonds (crosses) between ribosomes and membranes are disruptable by high concentrations of monovalent ions (0.5 M KCl). The disruption of these bonds, however, is not sufficient to release the ribosomes from microsomal membranes, if the integrity of ribosomal structure is preserved by magnesium ions, since the nascent polypeptide chain remains as an anchor, which holds the ribosomes on the membrane. The release of nascent chains can be achieved by chain termination in a medium optimal for amino acid incorporation, or, much more efficiently, by the action of puromycin. Chain release, however, does not result in the detachment of ribosomes, unless the microsomes are incubated in a medium which disrupts the ionic bonds. The scheme of Fig. 27 also indicates the finding that the peptidyl-puromycin molecules are vectorially discharged into the interior of the microsomal vesicles (Redman and Sabatini, 1966), as well as the possibility that after reaction with puromycin, some nascent polypeptides may remain associated with the membranes rather than being discharged into the cisternal cavities (Kreibich and Sabatini, manuscript in preparation).

We have found that in solutions containing no Mg^{++} ions and very high concentrations of monovalent ions, which unfold the ribosomes, essentially all bound ribosomes are detached from microsomal membranes, without addition of puromycin. In this case also, the nascent chains, presumably still bound to tRNA molecules, remain associated with microsomal membranes. (The recovery of tRNA with microsomal vesicles, completely stripped of their ribosomes by means of 2 M LiCl, has been reported (Scott-Burden and Hawtrey, 1969).) The detachment of ribosomes under these conditions results from a dual action of monovalent ions in 1) unfolding the ribosome in such a way that the anchoring nascent chain

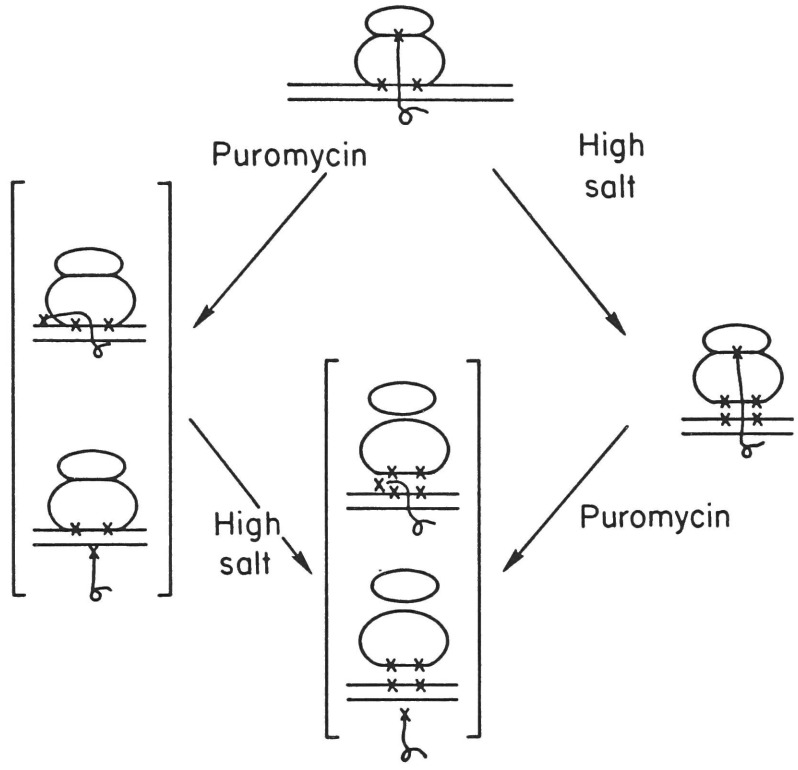


Figure 27. Scheme explaining the disassembly of the ribosome-membrane association by treatment with high salt and puromycin. Ribosomes with no nascent chains are released from the membranes and are dissociated into subunits by high salt alone. Ribosomes containing nascent chains must be treated with puromycin as well as with high salt.

is dislodged from the ribosomes and 2) disrupting the ionic bonds between ribosomes and membranes.

The unfolding of the ribosomes at 1 M KCl is most probably paralleled by a loss of RNA and/or protein. Removal of divalent ions is known to affect ribosome structure. For example, the loss of 5 S RNA from large subunits caused by chelation of Mg^{++} was shown for fungal (Comb and Sarkar, 1967) and rat liver (Peterman and Pavlovec, 1969) ribosomes. Recently it was also demonstrated that the loss of 5 S RNA is accompanied by the loss of a protein (Blobel, 1971; Lebleu *et al.*, 1971). Furthermore, removal of "split" proteins from bacterial ribosomes by centrifugation in CsCl is a well established phenomenon (Traub and Nomura, 1968).

The chelating agent, EDTA, has been commonly used to disrupt the polysome-membrane complex. EDTA is known to unfold ribosomes and to disrupt ionic bonds mediated by magnesium ions. However, EDTA is incapable of detaching all large subunits from the membranes (Sabatini *et al.*, 1966; Bennett and Hallinan, 1968; Attardi *et al.*, 1969; Rosbash and Penman, 1971a), probably because the unfolding of the ribosome caused by chelation of Mg^{++} is insufficient to release nascent chains which are still linked to a bulky tRNA molecule. It is also possible that some ionic bonds between ribosomes and membranes, not involving Mg^{++} ions, remain unaffected.

Proteolytic enzymes can also detach ribosomes from membranes (Lust and Drochmans, 1963; Chefurka and Hayashi, 1966; Sabatini and Blobel, 1970). In this process, however, the anchoring nascent chains are split (Sabatini and Blobel, 1970) and ribosomal or membrane proteins involved in formation of the ionic bonds are probably degraded.

Low concentrations of ribonuclease, under conditions which degrade polysomes but are mild enough so that ribosomes remain intact, judging by their sedimentation coefficients, are ineffective in detaching ribosomes from liver microsomes (Blobel and Potter, 1967b; Morimoto and Sabatini, unpublished results). However, a partial disassembly of rough

microsomes by low concentrations of ribonuclease has been reported for HeLa (Rosbash and Penman, 1971a) and mouse sarcoma (Lee et al., 1971) cells. This observation has been taken to suggest that in these cells a class of bound ribosomes is attached to membranes via the messenger RNA only (Rosbash and Penman, 1971a; Lee et al., 1971).

Carcinogens have also been reported to partially detach ribosomes from liver rough microsomes (Williams and Rabin, 1969 and 1971). However, it has not been established whether bound ribosomes containing nascent polypeptide chains are affected.

Although it would be of great interest to follow the fate of the messenger RNA when the polysome-membrane complex is disassembled, this question remains open, because of the insufficient characterization and the difficulty of avoiding degradation of messenger RNAs in most mammalian systems. Rosbash and Penman (Rosbash and Penman, 1971a), on the basis of the criterion that rapidly labeled, Actinomycin D and ethidium bromide resistant RNA represents cytoplasmic mRNA, have reported that about 70% of the messenger can be released from HeLa cell rough microsomes by the combined action of puromycin in vivo and EDTA in vitro.

4.2. In vitro Exchange of Ribosomal Subunits between Free and Membrane-Bound Ribosomes

Our results show that, upon termination of polypeptide chains in vitro, small subunits of membrane-bound ribosomes are capable of undergoing extensive exchange with added small subunits derived from free ribosomes. The small subunit exchange was inferred from the transfer of added labeled small subunits to microsomal membranes, or, vice versa, from the release of labeled microsomal small subunits upon addition of cold small subunits. This exchange is depicted in Fig. 28, which also presents possible effects resulting from the addition of large subunits. Considering that small subunits become exchangeable upon termination of polypeptide chains, addition of large subunits could have resulted in one of two alternative situations, depending on whether large subunit

Schematic representation of exchange of small and large subunits with membrane-bound ribosomes

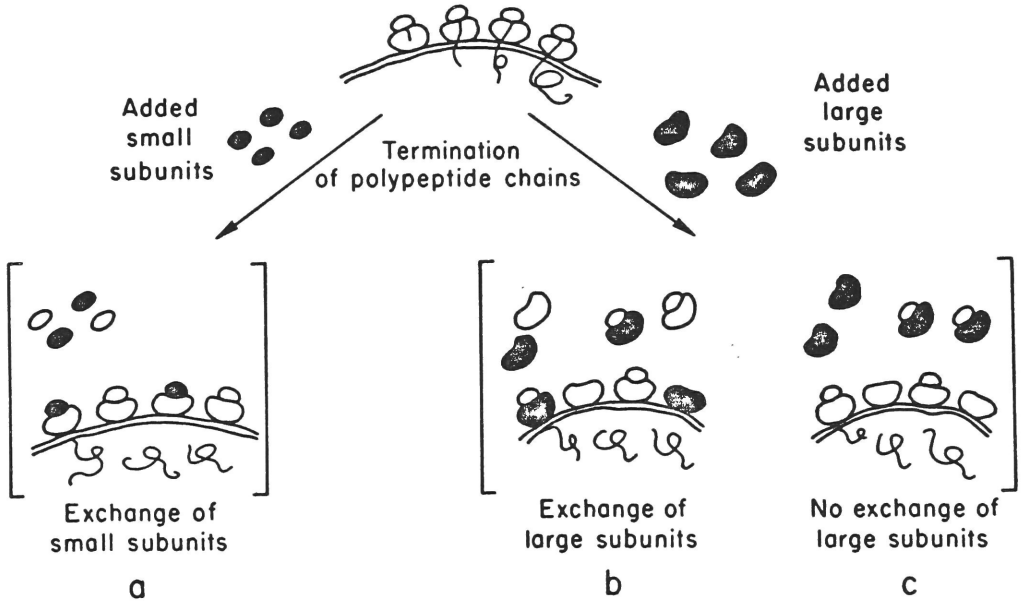


Figure 28. Schematic representation of exchange of small and large subunits with membrane-bound ribosomes.

exchange occurred or not (Figs. 28 b and c). Large subunit exchange "in" would have resulted in a dilution of the specific activity of the added subunits, whereas large subunit exchange "out" would have resulted in the release of radioactivity from the microsomes (Fig. 28b). Since we were unable to demonstrate either of these effects, which were clear in the case of small subunits, our results are better interpreted by the scheme of Fig. 28c, showing no *in vitro* exchange of large subunits. As indicated in Fig. 28c, the main effect of adding large subunits and puromycin to rough microsomes was a large net removal of small subunits from bound ribosomes, due to the trapping of small subunits into a newly formed monomer pool. This effect is consistent with the observation that small subunits become exchangeable once polypeptide chains have been released and with previous reports (Sabatini *et al.*, 1966), that ribosomes are attached to membranes via the large subunits only.

Details of the process of the ribosomal subunit exchange which we have observed remain to be elucidated, but possible mechanisms will be discussed after considering current concepts on the ribosome cycle. The conclusion that ribosomal subunit exchange constitutes an essential part of the mechanism of protein synthesis and that ribosomes probably undergo subunit exchange after each round of translation resulted from studies with bacterial cells (Kaempfer, Meselson and Raskas, 1968) and cell free systems (Kaempfer, 1968), which first demonstrated the formation of ribosome hybrids from differently labeled ribosome populations, dependent on protein synthesis. The existence of a subunit cycle, however, was implicit in the earlier finding, that the small ribosomal subunit participates in the formation of an initiation complex (Nomura and Lowry, 1967; Ghosh and Khorana, 1967), which only subsequently joins to the large subunit (Nomura, Lowry and Guthrie, 1967) to form an active 70 S monomer, and with the earlier report (Mangiarotti and Schlessinger, 1966) that ribosomal subunits and not 70 S monomers represent the true state of inactive ribosomes within the cell. The latter conclusion was derived from sucrose gradient analysis of bacterial lysates, obtained from a fragile form of *E. coli*, which revealed

the existence of polysomes and ribosomal subunits, but not of 70 S monomers. Based on the above observations, Kaempfer (Kaempfer, 1968) proposed a model according to which ribosomes, upon termination of polypeptide chains, dissociate to join a pool of free subunits, which reassociate only after formation of an initiation complex. According to this model, the ribosome in its active form is part of a polysome, and in its inactive form is dissociated into subunits; inactive monomers, that is, monomers lacking nascent polypeptide chains, do not exist in the cell.

Evidence opposing the view that ribosomal monomers originate only from the breakdown of polysomes during lysis, was provided by Kohler et al. (Kohler, Ron and Davis, 1968) and supported by work in other laboratories (Flessel et al., 1967; MacDonald and Yeater, 1968; Algranati, Gonzales and Bade, 1969). The conclusion that 70 S monomers do not exist in the cell (Mangiarotti and Schlessinger, 1966) was attributed to the dissociation of inactive ribosomes induced by sodium ions present in the lysing medium (Beller and Davis, 1971; Davis, 1971). It was found instead that in bacterial cell lysates prepared in solutions containing potassium, there existed very small amounts of free subunits, which remained constant, regardless of culture conditions. On the other hand, the relative proportions of polysomes and monomers could be changed by altering culture conditions in short term experiments (Kohler et al., 1968). Subsequently, it was reported (Subramanian, Ron and Davis, 1968; Bade, Gonzales and Algranati, 1969) that a factor, extracted from bacterial ribosomes, could dissociate inactive monomers by binding to small subunits. This dissociation factor was later found to be identical to an initiation factor (Subramanian et al., 1969; Subramanian and Davis, 1970; Sabol et al., 1971; Dubnoff and Maitra, 1971) and to be contained in native small subunits. It was postulated that the limiting amount of dissociation factor present in the cell determined the number of free subunits available for the formation of an initiation complex. The dissociation factor was found to be active at low concentrations of Mg^{++} ions and, on the basis of the observation that the dissociation of

ribosomes by the factor could be reversed by increasing the Mg^{++} concentration, some authors suggested that the factor might act reversibly (Subramanian et al., 1969). The picture of the ribosome cycle emerging from these studies is as follows. After termination of its polypeptide chain the ribosome exists in the cell as a monomer until it is attacked by the dissociation factor. The resulting subunits can undergo either of two fates: 1) they may become part of an active polysome via the participation of the small subunit in an initiation complex; in this case, at some point after formation of the initiation complex, the dissociation factor is detached from the small subunit; or 2) the small subunit may lose the dissociation factor, without forming an initiation complex, and reassociate with a large subunit to again form an inactive monomer. According to this scheme, in the cell there is a rapid exchange of subunits within a pool of inactive monomers, from which subunits are recruited into polysomes through the formation of initiation complexes. In fact, Subramanian and Davis (Subramanian and Davis, 1971) reported a subunit exchange between inactive ribosomes in vitro, under conditions where initiation of protein synthesis was negligible, and suggested that trace amounts of dissociation factor, present in the medium and acting reversibly, account for this exchange.

A different view of the mechanism of the ribosome subunit exchange cycle in bacteria emerges from recent studies of Kaempfer, who found that the event of polypeptide chain termination is concomitant with a dissociation of ribosomes into subunits (Kaempfer, 1970), and that the dissociation factor acts in preventing the reassociation of subunits generated from polysome read-out, not in dissociating inactive monomers (Kaempfer, 1971). Therefore, a small subunit, generated from polysome read-out, either combines with dissociation factor, or immediately reassociates with a large subunit to form a stable monomer, that is, a monomer which is not efficiently attacked by the dissociation factor. The conclusion from these studies is that inactive monomers do exist in the cell, but that they are not recruited efficiently into active polysomes.

At least part of the discrepancy between the views taken by Kaempfer and by the group in Davis' laboratory might be explained by the different ionic conditions at which the experiments were conducted. Further work is clearly required for the complete elucidation of the details of the mechanism of subunit exchange.

Less extensive studies have been carried out on the ribosome dissociation-association cycle in eukaryotic cells. However, subunit exchange has also been demonstrated in eukaryotic cells (Kaempfer, 1969; Ceccarini, Campo and Andronico, 1970) and cell free systems (Jacobs-Lorena and Baglioni, 1970; Howard, Adamson and Herbert, 1970; Falvey and Staehlin, 1970). It has also been shown that the 40 S subunit forms the initiation complex (Heywood, 1970; Burgess and Mach, 1971; Heywood and Thompson, 1971) and a dissociation factor has been extracted from yeast (Pêtre, 1970) and reticulocyte ribosomes (Lubsen and Davis, 1972). In the case of rat liver polysomes it has been reported that initiation of synthesis of polypeptide chains is not required for subunit exchange (Falvey and Staehlin, 1970). In agreement with the view that inactive monomers are not efficiently recruited into polysomes (Kaempfer, 1971), some authors have reported an extremely sluggish equilibration of ribosomal monomers with active polysomes both *in vivo* (Joklik and Becker, 1965; Baglioni, Vesco and Jacobs-Lorena, 1969; Kabat and Rich, 1969) and *in vitro* (Howard *et al.*, 1970), as well as the absence of subunit exchange between inactive monomers (Falvey and Staehlin, 1970).

The small subunit exchange that we observed occurs under conditions thought to approximate the physiological one and, at sufficiently low concentrations of Mg^{++}, requires only a macromolecular fraction of a high speed supernatant and, possibly, a nucleoside triphosphate. The effect of the high speed supernatant fraction in promoting exchange is as yet difficult to interpret, because the sedimentation properties of the small subunits are changed during incubation with microsomes in the absence of the high speed supernatant fraction. Since the exchange occurs in a minimal medium, where the protein synthetic apparatus is not operative, it is clear that it is a net result of polypeptide chain

release and does not depend on the process of initiation. However, we have not yet determined whether 1) the exchange occurs subsequently to polypeptide chain release, involving the cyclic dissociation-association of inactive membrane-bound monomers--in agreement with the concept supported by Subramanian and Davis (Subramanian and Davis, 1971)--or 2) whether the exchange occurs only concomitantly with polypeptide chain release--in agreement with the idea supported by Kaempfer (Kaempfer, 1970 and 1971).

If the first alternative is correct, a small amount of dissociation factor present in the supernatant might be required. The low concentration of Mg^{++} and incubation at 37° C required for exchange, are consistent with properties reported for bacterial (Subramanian et al., 1969) and mammalian (Lubsen and Davis, 1972) dissociation factor. A stimulatory activity of ATP or GTP (Subramanian et al., 1969) as well as of GTP specifically (Gonzales, Bade, and Algranati, 1969; García-Patrone et al., 1971) on bacterial dissociation factor was also reported. However, some authors now attribute this effect to chelation of Mg^{++} by the nucleoside triphosphates (Subramanian and Davis, 1970).

On the other hand, if the second alternative is correct, that is, if dissociation into subunits occurs obligatorily upon chain release and the exchange we have observed occurs only concomitantly with this event, a dissociation factor might be unnecessary, and the function of the high speed supernatant fraction would be limited to its action in protecting the integrity of small subunits.

The inefficiency of the large subunit exchange in our in vitro system suggests that a reaction involving the detachment and reattachment of large subunits to membranes after release of their nascent chains does not occur within the time interval studied. Nevertheless, the possibility should be considered that added large subunits have lost their capacity to bind to membranes during the in vitro treatment, but that microsomal large subunits did indeed detach and reattach. We regard this latter alternative as unlikely, since added large subunits

were capable of exchanging with free polysomes and of combining with the small subunits released from microsomes. Moreover, microsomal large subunits remained bound to membranes at dilutions of microsomal suspensions sufficient to produce a puromycin induced detachment of 40% of the small subunits. However, it should be emphasized that the results *in vitro* may simply reflect the absence of factors or conditions present in the cell which are necessary for detachment of large ribosomal subunits upon chain termination or for the subsequent reattachment of added subunits to sites made available on microsomal membranes.

The implications of our data with respect to possible models of the assembly of the polysome-membrane complex will be discussed later (section 4.5).

4.3. Ribosome Attachment to Stripped Membrane Fractions in vitro

The binding of ribosomes to membranes was investigated, using ribosomes lacking nascent polypeptide chains, obtained by puromycin-KCl treatment of rough microsomes, and microsomal membranes, stripped of their ribosomes, also by the puromycin-KCl procedure. The binding of ribosomes to membranes *in vitro* has been studied by other workers in bacterial (Aronson, 1966; Coleman, 1969), reticulocyte (Burka and Schickling, 1969) and rat liver systems (Williams and Rabin, 1969; James *et al.*, 1969; Sunshine *et al.*, 1971; Blyth *et al.*, 1971; Roobol and Rabin, 1971; Süss *et al.*, 1966; Ragland *et al.*, 1970; Shires *et al.*, 1971 a and b; Khawaja and Raina, 1970). In the rat liver system, the binding of polysomes, obtained by detergent treatment of a postmitochondrial supernatant, to rough microsomes stripped of their ribosomes by aflatoxin (Williams and Rabin, 1971) or by chelating agents and ribonuclease (Shires *et al.*, 1971a) has been studied.

To demonstrate binding of ribosomes to membranes, most groups have carried out incubations at temperatures around 30° C. However, the group in Pitot's laboratory, working with a rat liver system, has found that attachment of polysomes to ribonuclease-EDTA treated rough

microsomes occurs readily at 0° C (Shires et al., 1971a). We have also found that binding of ribosomes to stripped RM occurs at 0° C; however, binding occurred more rapidly at 37° C, and for this reason we chose incubation at the latter temperature for 10 minutes as a standard assay condition.

When increasing amounts of ribosomes were incubated with a fixed amount of stripped RM, saturation of available membrane sites was attained with 30 to 40% of the input RNA bound to the membranes. Saturation at this level and the fact that the reconstituted ribosome-membrane complex could be isolated by centrifugation, suggest a large value for the binding constant of ribosomes to membranes under the conditions used ($\sim 10^8$ M^{-1}).

The RNA to protein ratio of the "reconstituted" RM was equal to 0.125, corresponding to ~65% of the value observed in untreated RM. This valued is close to that observed by other workers in RM reconstituted from "conditioned" rough microsomes (i.e. rough microsomes treated with chelating agents) and detergent prepared polysomes (Ragland et al., 1971; Shires et al., 1971a). Phospholipid, however, is a better denominator to represent membranes than protein, since the various stripping procedures remove membrane proteins in addition to ribosomes. When our results are expressed on a phospholipid basis, the RNA content of reconstituted RM is ~55% that of untreated RM. The reasons for the low RNA to phospholipid ratios found in reconstituted RM are not yet clear, but binding sites might be inactivated during preparation or storage of the membrane fraction. However, there was no difference in the capacity of stripped RM stored for 24 hours or two weeks to bind ribosomes.

In order to investigate the specificity of the ribosome-membrane interaction, we examined the binding of ribosomes to rat liver smooth microsomes and to human erythrocyte ghosts, both treated by the same stripping procedure as the rough microsomes. The time course of ribosome binding to smooth membranes was similar to that for rough microsomes. However, on a phospholipid bases binding of RNA to smooth microsomes was only half that observed for stripped RM.

Other groups have also investigated the binding of polysomes to smooth microsomes from rat liver. The group in Rabin's laboratory has postulated that association of ribosomes to membranes can be followed indirectly, by measuring the activity of the microsomal enzyme which catalyzes disulfide exchange (James et al., 1969; Williams and Rabin, 1969; Sunshine et al., 1971). It is thought by these authors that this enzyme is located in microsomal membranes at ribosome binding sites, so that the activity cannot be assayed with added substrates if the ribosomes are not removed (Williams, Gurari and Rabin, 1968). The authors report that, in the presence of steroid hormones, smooth microsomes can acquire as many ribosomes as rough microsomes (James et al., 1969; Williams and Rabin, 1969) and, moreover, that the action of the hormones in promoting the binding is sex specific (Sunshine et al., 1971; Blyth et al., 1971). However, since it has not been possible to isolate the presumptive ribosome-membrane complex, which is said not to withstand centrifugation, and since the correlation between ribosome binding and masking of the enzyme activity is not well established, the interpretation of these results should be regarded as tentative.

The group in Pitot's laboratory has reported a temperature dependent binding of ribosomes to untreated smooth microsomes (Shires et al., 1971b). According to these authors, smooth membranes incubated with polysomes at 37° C attained an RNA to protein ratio comparable to that attained by EDTA-RNase treated rough microsomes ("conditioned" RM) incubated with polysomes at 0° C. However, in contrast to "conditioned" RM, there was virtually no binding of ribosomes to untreated smooth microsomes at 0° C. On the other hand, analysis of the data with RNase-EDTA conditioned smooth microsomes presented by the same authors shows considerable binding at 0° C, equal to about one-third of that obtained for "conditioned" RM (Shires et al., 1971a).

Our results with smooth microsomes cannot be unambiguously interpreted until the extent of contamination of this membrane fraction by plasma membrane fragments, Golgi components and other membrane types is assessed. Clearly, the contamination by rough microsomes (20 to 25%),

estimated by the RNA content of SM before stripping (Table I), is not sufficient to entirely account for the observed binding. The binding of ribosomes to smooth microsomes may, therefore, reflect the presence of ribosome binding sites on these membranes, although in smaller number than in rough microsomes.

Binding of ribosomes to erythrocyte ghosts treated for stripping was virtually nil at both temperatures examined, in agreement with a previous report on the binding of reticulocyte polysomes to reticulocyte membranes or untreated erythrocyte ghosts (Burka and Schickling, 1970). The absence of ribosome binding to erythrocyte ghosts, as well as the sevenfold reduction in binding to heat treated stripped RM, suggest specificity in the in vitro ribosome-membrane association reaction. In the future, we plan to test other membrane fractions, such as rat liver plasma or Golgi membranes, in their capacity to bind ribosomes.

Further understanding of the binding of ribosomes to membranes in vitro will derive from a comparison of the abilities of different kinds of ribosomes to bind to stripped RM. For example, it will be of interest to compare the binding capacity of ribosomes obtained from free and bound polysomes by high salt-puromycin treatment, as well as that of free polysomes and polysomes obtained from rough microsomes by detergent treatment. Some preliminary experiments on this problem have been conducted in our laboratory, however, clear differences have not yet been established. Shires et al. (Shires et al., 1971a) found that free polysomes and polysomes obtained from rough microsomes by detergent treatment bound to "conditioned" RM to the same extent. On the other hand, the same authors have reported that polysomes obtained from rough microsomes by detergent treatment have a higher affinity for smooth microsomes than do free polysomes (Shires et al., 1971b). Recently, it has been reported in a preliminary communication that large ribosomal subunits have a higher affinity for RNase-EDTA or 2 M LiCl treated rough microsomes than do small ribosomal subunits (Ekren and Shires, 1972).

It must be emphasized that all the binding studies reported have been conducted at ionic strengths considerably lower than physiological conditions. An incapacity of the membranes to discriminate between different kinds of ribosomes may, therefore, be attributable to the low ionic strength of the assay medium. However, it has been reported that at ionic conditions thought to be closer to physiological conditions, the binding of ribosomes to membranes is much reduced (our own unpublished data; Coleman, 1969; Shires et al., 1971a). Presumably, then, the in vitro binding observed at low ionic strengths, results from the recognition by ribosomes of the same membrane binding sites which are utilized in vivo, but should not be interpreted as a process directly reflecting the ordered, regulated assembly of the polysome-membrane complex in the cell. This assembly may be closely connected to the process of protein synthesis and be related to the nature of the product manufactured. It should be expected, therefore, that a functional assembly of the polysome-membrane complex in vitro will require the availability of a system for the initiation of translation of specific mRNAs by ribosomal subunits.

4.4. Structural Studies on Free and Bound Ribosomes

Salt washed, puromycin treated ribosomes were used for the comparison of the structural properties of free and membrane-bound polysomes. These experiments, therefore, were designed to reveal difference between the two types of ribosomes attributable only to tightly bound structural components, not detachable by the high salt treatment.

Since no difference in the buoyant densities of free and bound ribosomes was revealed by CsCl density gradient centrifugation, it is unlikely that there are major differences due to extra protein(s) or lipoproteins(s) between these two types of ribosomes. Two optical density peaks, at densities of 1.54 and 1.61 g/cm^3, which were taken to correspond to small and large subunits respectively, were observed both in the case of free and bound ribosomes. The buoyant densities of small and large ribosomal subunits obtained by EDTA treatment have been

reported to be equal to 1.52 and 1.58 g/cm^3 respectively, in L cells (Perry and Kelley, 1966) and to 1.53 and 1.60 g/cm^3 respectively, in hepatocytes (Henshaw, 1968). The slightly higher values that we obtain may be explained by the removal of proteins from ribosomal subunits by the high salt treatment prior to formaldehyde fixation.

Rosbash and Penman (Rosbash and Penman, 1971b) have reported the existence of two classes of membrane-bound ribosomes, prepared by detergent treatment from a HeLa cell membrane fraction, one of which has a buoyant density equal to that of free ribosomes and the other a considerably lower density. Our data, however, are not directly comparable to the results of these authors, since their ribosomes were not treated by the KCl-puromycin procedure.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, which resolved as many as half of the proteins known to exist in mammalian ribosomes (King, Gould and Shearman, 1971; Martini and Gould, 1971), revealed close similarities between the protein complements of subunits of free and membrane-bound ribosomes. However, one more intense band, corresponding to a large polypeptide of approximate molecular weight of 50,000 daltons, was apparent in large subunits obtained from free polysomes. This protein band was also present, in lesser amounts, in large subunits obtained from bound polysomes. Since high salt washed rough microsomes are free of contaminating free ribosomes, the presence of a lower amount of this protein in bound ribosomes remains to be explained. It should also be investigated whether this polypeptide represents a tightly bound factor, not detachable by our washing procedure, or a structural protein.

Our results with free and bound ribosomes from rat liver are similar to those obtained by gel electrophoresis analysis of bacterial (Brown and Abrams, 1970) and chick embryo (Fridlender and Wettstein, 1970) ribosomes. Brown and Abrams found that free and bound ribosomes from Streptococcus fecalis have similar protein complements, with the exception of one extra band, corresponding to a polypeptide of molecular

weight equal to 45,000 daltons, in the large subunits of free ribosomes. This polypeptide could be detached from ribosomes by treatment with 1 M NH_4Cl. Fridlender and Wettstein, in addition to an extra protein band in chick embryo free polysomes, found one protein band present in bound polysomes and absent in free polysomes. On the other hand, major differences between the protein complements of free and bound ribosomes have been reported in pigeon pancreas (Székely et al., 1966) and reticulocytes (Burka and Bulova, 1971). However, in these cases no attempts were made to exclude that the extra proteins found in bound ribosomes were due to adventitiously adsorbed membrane proteins.

4.5. Possible Mechanisms for the Assembly of the Polysome-Membrane Complex

Considerable evidence indicates that free and bound polysomes are responsible for the translation of different messenger RNAs in the liver cell, where their activities in the synthesis of apoferritin and serum proteins has been compared (Redman, 1969a and b; Takagi et al., 1969 and 1970; Hicks et al., 1969; Ganoza and Williams, 1969). It has also been reported that in the reticulocyte free polysomes are active in the synthesis of haemoglobin, while bound polysomes are responsible for the synthesis of other proteins (Bulova and Burka, 1970). In other systems direct evidence that free and bound polysomes translate different messengers is as yet lacking and it cannot be excluded that some proteins are made on both free and bound polysomes, as has been reported for immunoglobulins in myeloma cells (Lisowska-Bernstein, Lamm and Vassalli, 1970) and for the membrane-bound enzyme NADPH-cytochrome c reductase in hepatocytes (Ragnotti et al., 1969). It is possible that the membrane-bound condition of polysomes has some function in addition to the intracellular distribution of specific proteins, such as increasing the stability of mRNAs, as has been previously suggested (Pitot, 1969). However, for the purpose of this discussion, which is limited to the liver, we will assume that the main function of the polysome-membrane complex is the intracellular distribution of specific

proteins. The problem, therefore, is to understand how polysomes, active in the synthesis of specific proteins, recognize endoplasmic reticulum membranes. For the sake of simplicity, we will discuss in turn three general possibilities for the mechanism of assembly of the polysome-membrane complex: 1) free and bound ribosomes represent two non-interchangeable populations; 2) the large subunits of free and bound ribosomes are non-interchangeable, while the small subunits change between the free and the bound states; 3) both the large and the small subunits can change between the free and the bound states.

Possibility (1). For free and bound ribosomes to be non-interchangeable, they must either be structurally different or the polysome-membrane complex must be permanent, that is, ribosomes must not be detached from endoplasmic reticulum membranes upon termination of polypeptides. In the first case, binding occurs because one class of ribosomes, structurally distinguishable from the other class, and capable of translating specific mRNAs--such as mRNAs directing the synthesis of secretory proteins--, recognizes membranes of the endoplasmic reticulum. A recognition of specific mRNAs by one class of ribosomes is quite possible, especially in view of reports on the preferential translation of specific messengers by bacterial ribosomes (Lodish, 1969). In this case, the permanence or non-permanence of the polysome-membrane complex is not relevant to the relationship between free and membrane-bound ribosomes. In the case that the ribosome-membrane complex is permanent, the two classes of ribosomes need not be structurally different. Once a ribosome becomes membrane-bound, it remains so during its entire biological lifetime. It is, however, difficult to envisage how such a situation could be true for small subunits, unless protein synthesis in membrane-bound ribosomes did not involve the ribosome dissociation-association cycle described for free polysomes, or unless both ribosomal subunits were attached directly to the membrane.

The idea that free and bound ribosomes belong to two segregated populations has been advocated by Tata (Tata, 1967 a and b, 1968, 1970 and 1971). His reasons for supporting this possibility are based mainly

on his studies on the distribution and function of ribosomes in hepatocytes during hormone induced amphibian metamorphosis. Metamorphosis was observed to cause a coordinated synthesis of ribosomes and membrane phospholipid (Tata, 1967a and 1970), a recruitment of ribosomal monomers into polysomes, and a larger proportion of ribosomes to become membrane-bound (Tata, 1967a); moreover, the RNA in the newly synthesized polysomes appeared to be associated preferentially with the microsomal membranes (Tata, 1967a and 1968). The implications of this latter observation, however, are not unequivocal, since it was not established whether the newly synthesized RNA associated with membranes was ribosomal or messenger RNA. The data, therefore, are not at all incompatible with a model in which free and bound ribosomes are capable of exchanging.

We think that the possibility that free and bound ribosomes belong to non-interchangeable populations is unlikely, because 1) many studies (Loeb and Howell, 1967; Talal and Kaltreider, 1968), including ours, point to the structural similarities between free and bound ribosomes and 2) our experiments on the exchange of small subunits of membrane-bound ribosomes indicate that at least the association of small subunits with the endoplasmic reticulum is not a permanent one.

Possibility (2). Again, non-interchangeability between free and bound large subunits could be due either to structural differences between the two classes of large subunits, and/or to the existence of a permanent large subunit-membrane complex. The association of small subunits with the endoplasmic reticulum, on the other hand, would be temporary, in that small subunits, upon termination of polypeptides, could dissociate from membrane-bound large subunits and participate in the formation of free polysomes. Conversely, small subunits released from free polysomes could form specific initiation complexes and then recognize membrane-bound large subunits. For example, specific initiation factors, which recognize messengers specifying secretory proteins, or these messengers themselves might direct small subunits to membrane-bound large subunits. If membrane-bound large subunits are structurally identical to free large subunits, then one must assume

that once a large subunit becomes membrane-bound it undergoes a conformational change so as to become distinguishable from free large subunits and recognizable by the proper initiation complex.

Another way in which polysomes containing the correct messenger RNA might become associated with the endoplasmic reticulum is via direct bonds between mRNA and microsomal membranes. The existence of a membrane-bound messenger RNA has been suggested by some authors (Shapot and Pitot, 1966; Pitot, 1968; Faiferman, Cordunella and Pogo, 1971; Lee et al., 1971). If the evidence is accepted that ribosomes are attached to membranes via their large subunits only, and that mRNA is bound to small subunits, then it is difficult to envisage how the mRNA could be in direct association with membranes throughout its entire length. It could, however, be partially attached to membranes, for example via one of the terminal segments (see also below).

A model according to which free and bound large subunits are non-interchangeable and small subunits are capable of changing from the free to the bound state, is compatible with our data on exchange, as well as with the possibility that one extra protein in free large subunits is the basis for a structural difference between free and bound large subunits. However, we do not yet know whether the more intense protein band found in the electrophoretic patterns of free large subunits represents a ribosomal structural protein or a tightly bound factor. Moreover, its presence in lesser amounts in bound large subunits remains to be explained. This kind of a model is also compatible with the recent report (Baglioni et al., 1971) that newly synthesized large subunits, but not small subunits, can bind to microsomal membranes in the absence of protein synthesis. However, because many studies indicate that the 28 S RNAs of free and bound ribosomes turn over at the same rate (Loeb and Howell, 1967; Tanaka et al., 1970), and because of the extensive similarities between free and bound large subunits, we believe that it is possible that bound large subunits are capable of joining a pool of free ribosomes. The rate of equilibration between free and bound large subunits may, however, be slower than that for small subunits and

require promotion by appropriate factors.

Possibility (3). The possibility that both ribosomal subunits can change from the free to the bound state entails a higher degree of flexibility in the utilization of the protein synthetic machinery, in that ribosomes could be recruited for the synthesis of proteins with different intracellular fates, according to the physiological needs of the cell, without the requirement for the synthesis of a new population of ribosomes.

Some ways in which this situation might be achieved are schematically represented in Fig. 29. Scheme A depicts a situation in which mRNA is directly attached to microsomal membranes via an untranslated segment at the 5' terminus, and provides the only link between the ribosomes and the membrane. In this case, there is movement of the ribosome with respect to the messenger-membrane complex. The existence of a class of membrane-bound ribosomes, detachable from microsomal vesicles by low concentrations of ribonuclease has been reported for tissue culture cells (Rosbash and Penman, 1971a; Lee et al., 1971). Since a similar ribonuclease snesitivity has not been observed in liver microsomes (Blobel and Potter, 1967b), we consider scheme A of Fig. 29 to be unlikely, at least in the hepatocyte.

Scheme B depicts a situation in which the messenger RNA is directly attached to the microsomal membranes at the 5' terminus, but ribosomes are also in direct contact with the membranes. If the ribosomes and the 5' terminus of mRNA are fixed with respect to other membrane components this kind of situation results in mechanical difficulties for initiation around a fixed point. On the other hand, attachment of messenger RNA to the membranes via the 3' terminus (not shown in Fig. 29) would result in inefficient utilization of mRNA. However, the possibility that bound ribosomes or bound mRNA are capable of moving along a fluid membrane should not be overlooked.

Scheme C depicts a situation in which the initiation complex, formed in the cell sap, recognizes a membrane-bound large subunit.

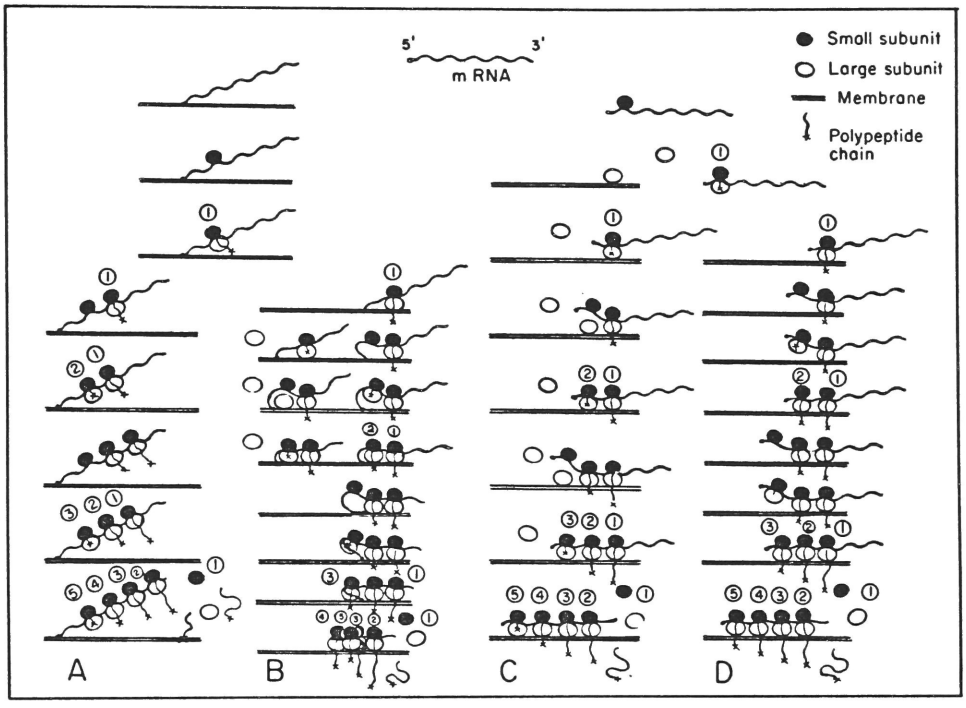


Figure 29. Schematic representation of possible modes of assembly of the polysome-membrane complex, assuming that free and bound ribosomes are interchangeable.

In this respect, it is similar to the model discussed for possibility (2), with the difference that the large subunit-membrane complex is unstable until it joins to the initiation complex.

In scheme D, initiation occurs in the cell sap. An initiation factor induces some change in the large subunit, which results in the binding of the ribosome to the membrane. Alternatively, the emerging amino terminal segment of the nascent polypeptide, perhaps carrying a messenger encoded modification, could be involved in the recognition of the membrane.

In all the schemes of this figure, ribosomes are shown to become detached from the membranes as ribosomal subunits, after termination of polypeptide chains. This does not imply that inactive ribosomes exist as subunits in the cell sap, but rather that either at, or following, the termination step, the subunits equilibrate with a pool of free ribosomes.

Reversible changes in the distribution of free and bound ribosomes, occurring in times shorter than the half-life of ribosomal RNA, have been reported to be induced by starvation in mouse sarcoma (Lee et al., 1971) and in Krebs tumour cells (Faiferman et al., 1971), and in mouse myeloma cells by NaF, which is an inhibitor of initiation of protein synthesis (Bleiberg, Zauderer and Baglioni, personal communication). It has also been reported that in HeLa cells cycloheximide blocks the entry of newly synthesized mRNA into the rough endoplasmic reticulum (Rosbash, personal communication). The newly synthesized mRNA is found in small polysomes free in the cytoplasm, some of which appear to be converted to membrane-bound polysomes upon removal of cycloheximide. These studies suggest that free and bound ribosomes may be interchangeable in the cell, and that the rough condition of the endoplasmic reticulum is acquired through the assembly of functional polysomes. However, further experimental data are required both in in vivo and in in vitro systems, to distinguish between the various possibilities and to elucidate the details of the process of assembly of membrane-bound polysomes.

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