The Selectivity and Stoichiometry of Membrane Binding Sites for Polyribosomes, Ribosomes and Ribosomal Subunits *in vitro*

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Differences in the binding sites for polyribosomes, template-depleted ribosomes and large ribosomal subunits were found in microsomal derivatives of the rough endoplasmic reticulum. 1. The stoichiometry of polyribosome and ribosome interaction *in vitro* with membranes was shown to be influenced by the relative concentration of interactants and the duration of their mixing. Large ribosomal subunits required a more prolonged mixing schedule to achieve saturation of membranes than did polyribosomes. 2. By using a procedure which minimized the effects on binding by the stoichiometric variables, competition between populations of polyribosomes, ribosomes and subunits for membrane sites showed that subunits, and to a lesser extent ribosomes, failed to block polyribosome attachment. 3. Polyribosomes isolated from liver, kidney and hepatoma 5123C entirely bound to a common membrane site, but some polyribosomes from myeloma MOPC-21 bound to other sites, perhaps influenced by their unique nascent proteins. 4. Subunit-binding sites appear on rough membranes only after endogenous polyribosomes have been removed, but no evidence that resulting changes in surface constituents are responsible was found. Large-subunit binding was largely abolished by lowering $MgCl_2$ concentration to 0.1 mM, whereas under the same conditions polyribosome binding was undiminished. 5. The large-subunit site appears to be distinct from the polyribosome site not only in the restriction of its affinity for particles but also spatially, to the extent that bound subunits do not hinder access of polyribosomes to their sites.

A number of investigators have reported that polyribosomes will form stable complexes with biological membranes *in vitro* and that these complexes are retrievable from sucrose gradients (for review, see Shires & Pitot, 1973b). Particular attention has focused on the rough microsomal membrane, whose ability to bind polyribosomes is greatly enhanced by removing the endogenous polyribosomes (i.e. 'conditioning') by any of a number of procedures, including treatment with LiCl (Scott-Burden & Hawtrey, 1969, 1971, 1972), KCl-puromycin (Adelman *et al.*, 1973; Rolleston, 1972), pyrophosphate-citrate (Ragland *et al.*, 1971) and RNAase†-EDTA (Shires *et al.*, 1971; Jothy *et al.*, 1973). Interest in these preparations stems from their evident presentation of binding sites that formerly held the endogenous polyribosome population, and which, when artificially complexed with exogenous polyribosomes, will form a reconstituted rough membrane.

Evidence that the sites exposed by conditioning procedures are those that once held the endogenous population has been presented by Shires *et al.* (1971, 1973) and extended by the experiments of Scott-

Burden & Hawtrey (1972). The problem of whether the integrity of the rough membrane is threatened by the conditioning procedure has been considered for the KCl-puromycin procedure by Adelman *et al.* (1973) and for the RNAase procedure by Shires *et al.* (1974b), who have investigated the compositional and enzymic character of the membranes before and after conditioning. Both procedures, by these assessments, appeared to preserve the membrane; however, membrane alteration is indicated after the use of a third conditioning procedure, namely pyrophosphate-citrate (Shires & Pitot, 1973a).

Conditioned rat liver rough membranes will quantitatively bind rat kidney polyribosomes (Shires *et al.*, 1974b) and large ribosomal subunits (Rolleston, 1972; Ekren *et al.*, 1973) to about the same extent as they will rat liver polyribosomes. Within the liver polyribosome population, polyribosomes from the membrane-bound group bound as well as those from the free group (Shires *et al.*, 1973; Rolleston & Mak, 1973). Only small ribosomal subunits (Rolleston, 1972; Ekren *et al.*, 1973) and ^{32}P -labelled polyribosomes, degraded by endogenous radiolysis (Shires *et al.*, 1971), have been reported to bind to

† Abbreviation: RNAase, ribonuclease.

liver membranes less extensively than do liver polyribosomes. This performance by liver membranes is not necessarily a non-discriminating one, unless it can be shown that all the particulate populations binding to the membranes, in fact, bind to the same sites on the membranes. The present paper describes an approach to this question in which known sites (those that bind liver polyribosomes) are blocked from accepting one population of polyribosomes by prior binding of another population.

Such an approach required a method for measurement of binding that allowed a large number of simultaneous assays. The previously described flotation-assay procedure (Ekren *et al.*, 1973) was chosen. During its adaptation for the blockage studies, a number of factors essential for accurate stoichiometric measurement of polyribosome-membrane interaction *in vitro* were investigated.

Materials and Methods

Preparation of polyribosomes and membranes

Male Holtzman rats (Holtzman, Madison, Wis., U.S.A.) were maintained in wire cages on Purina rat chow and water *ad libitum* with a diurnal light cycle of 12h dark (18:00–06:00h) and 12h light. Before death (generally in the morning) the animals were starved for approx. 24h, and those from which polyribosomes were to be isolated were injected with 200 μ Ci of [3 H]orotic acid (sp. radioactivity 20–25 Ci/mmol; from Amersham/Searle, Chicago, Ill., U.S.A.) 15–18h before death. Rough and smooth microsomal fractions and polyribosomes were isolated from postmitochondrial supernatant as previously described (Ragland *et al.*, 1971; Shires *et al.*, 1971). Postmicrosomal supernatant (S3), used for its RNAase-inhibitory activity, was obtained from a postmitochondrial supernatant centrifuged in a Beckman 50 Ti rotor at 50000 rev./min for 2.5h. RNAase inhibitor from rat liver was purified essentially as described by Gribnau *et al.* (1969) as modified by Ikehara & Pitot (1973).

Hepatoma 5123C was carried in male Buffalo rats (Simonsen, Minneapolis, Minn., U.S.A.) as described by Moyer *et al.* (1970). Tumours weighing 5–10g were excised from decapitated starved animals. Neoplastic tissue was dissected free from necrotic material and connective tissue, and a postmitochondrial supernatant prepared as described by Moyer *et al.* (1970). 3 H-labelled polyribosomes were obtained from hepatoma tissue by the same procedure as for normal liver. Separation of rough and smooth microsomal fractions was carried out by the procedure of Moyer *et al.* (1970).

The solid transplantable myeloma MOPC-21 was the generous gift of Dr. Melvin Cohn (Salk Institute, La Jolla, Calif., U.S.A.), and was carried in the gluteal region of Balb/c-mice (Microbiological

Association, Rockville, Md., U.S.A.). Tumours were harvested 2–3 weeks after transplantation. Animals were maintained in a light/dark-cycled room on Purina mouse chow.

The procedure for polyribosome isolation from myeloma MOPC-21 was similar to that previously used for kidney (Shires *et al.*, 1974b). [3 H]Orotic acid (50 μ Ci) was injected intraperitoneally into tumour-bearing mice 15–18h before death. Tumour tissue was removed, weighed and suspended in 2ml/g wet wt. of a normal rat liver postmicrosomal supernatant. The tissue was homogenized with 10 strokes of a motor-driven Potter–Elvehjem homogenizer, and a postmitochondrial supernatant made in the Sorvall SS34 rotor at 11000 rev./min for 10min. Sodium deoxycholate was added to a final concentration of 0.5%, and the resulting mixture incubated for 1h at 3°C. It was then layered over a two-step gradient of 1.35M- and 2.0M-sucrose– $T_{50}K_{25}M_5$ buffer [50mM-Tris–HCl (pH 7.4 at 0°C)–25mM-KCl–5mM-MgCl₂] and centrifuged in a Beckman 50 Ti rotor at 50000 rev./min for 2.5h.

Preparation of template-depleted ribosomes

Template-depleted ribosomes were obtained from freshly isolated 3 H-labelled rat liver polyribosomes incubated in a cell-free amino acid-incorporating system in the presence of puromycin, as described by Lawford (1969). The incubation medium contained (per ml) 1 μ mol of ATP, 0.5 μ mol of GTP (P-L Biochemicals, Milwaukee, Wis., U.S.A.), 10 μ mol of phosphoenolpyruvate (tricyclohexylamine salt; Sigma Chemical Co., St. Louis, Mo., U.S.A.), 50 μ g of pyruvate kinase (type II, Sigma), 0.5ml of freshly prepared postmicrosomal supernatant (Shires *et al.*, 1973), 100 μ g of freshly prepared polyribosomes, 1 μ mol of GSH (Calbiochem, La Jolla, Calif., U.S.A.), 0.5 μ mol of puromycin (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.) and 0.44M-sucrose– $T_{50}K_{25}M_5$ buffer. The incubations were for 45 min at 37°C. After being chilled, 20ml of the incubation mixture was layered above 15ml of 1.5M-sucrose– $T_{50}K_{25}M_5$ buffer and centrifuged for 12h in a Beckman 50.1 rotor at 50000 rev./min. The pellets were suspended by homogenization in a buffer containing 0.25M-sucrose, 0.5M-NH₄Cl, 50mM-Tris–HCl (pH 7.5 at 5°C) and 10mM-MgCl₂, and centrifuged for 3h in a 50 Ti Beckman rotor to give a second pellet. This was repeated once more and followed by a rinse in 0.44M-sucrose– $T_{50}K_{25}M_{0.1}$ buffer.

The template-depleted ribosomes thus obtained show a diminished endogenous incorporative capacity for amino acids *in vitro*, but experienced a 5–6-fold stimulation in activity on addition of poly(U) (Shires & Narurkar, 1970). The density of these ribosomes in CsCl gradients was 1.575, and, compared

with the original polyribosomes, they lost an average of 75.6% of their peptidyl- or aminoacyl-tRNA (Shires & Narurkar, 1970).

Preparation of ribosomal subunits from rat liver polyribosomes

^3H -labelled or unlabelled liver polyribosomes were incubated in triethanolamine-HCl buffer (50 mM, pH 7.5) with 2 mM-MgCl₂, 500 mM-KCl and 5 mM-puromycin for 15 min at 0°C followed by 15 min at 37°C. Subunit preparations were carried out either in a Beckman B XIV zonal rotor at 37000 rev./min or in a swinging-bucket rotor (Beckman SW 41: 41000 rev./min, 80 min; or Beckman SW 27: 25000 rev./min, 10 h). For the latter, mixtures were layered on top of isokinetic 0.35–1.54 M-sucrose gradients in 50 mM-triethanolamine buffer–2 mM-MgCl₂–500 mM-KCl. The zonal rotor was loaded with 347 ml of 2 M-sucrose, 250 ml of a linear 0.6–1.4 M-sucrose gradient and 25 ml of 0.6 M-sucrose, over which 12 ml of polyribosome mixture was layered followed by 50 ml of triethanolamine-HCl buffer containing 2 mM-MgCl₂ and 500 mM-KCl. The same buffer was present in all sucrose layers.

Conditioning of rough microsomal fractions for polyribosome attachment

The stripping of polyribosomes from the surfaces of rough microsomal vesicles ('conditioning') is required for the membranes to be able to accept exogenous polyribosomes at 3–5°C (Ragland *et al.*, 1971; Shires *et al.*, 1971, 1973). This was accomplished with rough-membrane preparations from rat kidney, liver and hepatoma 5123C by using RNAase (bovine pancreatic, five times recrystallized; Sigma) in the presence of dilute EDTA as described by Shires *et al.* (1971), or by the pyrophosphate-citrate method of Ragland *et al.* (1971).

Polyribosome-membrane interaction: methods of separating membrane-associated polyribosomes from the free non-associated polyribosomes after interaction in vitro

(1) The sedimentation assay (Ragland *et al.*, 1971), one of two used, entails mixing of conditioned rough microsomal membrane with ^3H -labelled polyribosomes, the layering of this mixture (final volume 3 ml) over 0.9 ml of 1.8 M-sucrose- $\text{T}_{50}\text{K}_{25}\text{M}_5$ buffer, and centrifuging in a Beckman SW 56 rotor for 8–10 h at 56000 rev./min. Membranes, together with the exogenous polyribosomes that have bound to them, accumulate at the interface with 1.8 M-sucrose, whereas polyribosomes which have not attached are pelleted. The tubes are cut below the membrane layer, and the contents above the cut decanted into an

11.5 ml tube. The SW 56 rotor tube is rinsed with $\text{T}_{50}\text{K}_{25}\text{M}_5$ buffer and the rinses are pooled with the membranes to a final volume of 11.5 ml. The membranes are then pelleted in a 50 Ti rotor by centrifuging for 2.5 h at 50000 rev./min. The membrane supernatant is decanted, and its volume measured. The sedimentation-assay method thus resolves the original binding mixture into three compartments: a polyribosome pellet (together with the small amount of 1.8 M-sucrose overlying it), a membrane pellet and the supernatant from the membrane fraction. Recovery of the total applied radioactivity from the three compartments added together is about 95% in all experiments.

(2) The flotation-assay procedure (Ekren *et al.*, 1973) involves mixing polyribosomes and conditioned rough membranes in a final volume of 1.9 ml of 0.44 M-sucrose- $\text{T}_{50}\text{K}_{25}\text{M}_5$ buffer. This mixture is then made 1.8 M with respect to sucrose in a final volume of 7 ml, transferred to a Beckman polyallomer tube and overlaid with 0.44 M-sucrose- $\text{T}_{50}\text{K}_{25}\text{M}_5$ buffer (Fig. 1). The tubes are centrifuged in a Beckman 50 Ti rotor at 50000 rev./min and cut approx. 2 cm from the bottom. The contents above the cut are decanted into a clean centrifuge tube and combined with the $\text{T}_{50}\text{K}_{25}\text{M}_5$ buffer used to rinse the first tube. Additional $\text{T}_{50}\text{K}_{25}\text{M}_5$ buffer is added to give a final volume of 11.5 ml. The contents below the cut, containing 'intermediate-zone material' (Fig. 1), are also decanted into a clean tube and mixed with $\text{T}_{50}\text{K}_{25}\text{M}_5$ buffer to a final volume of 11.5 ml. Both the 11.5 ml tubes are then spun in a 50 Ti rotor for 2.5 h at 50000 rev./min. The flotation assay thus divides the original polyribosome-membrane mixture into five fractions (Fig. 1): P-1, polyribosomes unattached to membranes and pelleted at the bottom of the tube;

Fig. 1. Scheme for the flotation separation of non-binding polyribosomes from membrane-attached polyribosomes

For details see the Materials and Methods section.

P-2, membranes with attached polyribosome; P-3, sedimentable material either incompletely pelleted or not having completed centrifugal or centripetal movement or material isopycnic to the sucrose ($d = \text{approx. } 1.3031$) of the intermediate zone; and S-1 and S-2, supernatants from P-2 and P-3 fractions respectively. Recovery of applied radioactivity was 86–91% when cellulose nitrate tubes were used to separate the supernatants, and 93–95% when polyallomer tubes were used for the same steps. Comparisons of experiments performed with both types of tubes showed no significant differences in the relative distribution of radioactivity.

The interaction of radioactive polyribosomes or particles is measured as the percentage of the total recovered radioactivity (d.p.m. of ^3H radioactivity) that is associated with the membrane fraction:

$$\beta' = \frac{P_2 CK}{P_1 + P_2 + P_3 + S_1 + S_2}$$

Values for P_1 , P_2 , P_3 , S_1 and S_2 are the d.p.m. recovered in fractions P-1, P-2, P-3, S-1 and S-2 respectively for a particular tube. Values for C are from β determined from a control tube which contains the same amount of labelled polyribosomes (and the same total radioactivity) as the experimental tubes, but no membranes. At least one control tube was centrifuged with every rotor, and that tube alone provides the value of C for the calculation of β' for the other tubes in the rotor. C for polyribosomes averages 0.01–0.02, for template-depleted particles and large subunits 0.03–0.04, and for small ribosomal subunits approx. 0.05–0.1. K is a factor for dissociation, which, according to Shires *et al.* (1971), is negligible. Support for this assumption is shown in Fig. 7.

Measurement of radioactivity

Pellets were prepared for liquid-scintillation counting by dissolving them in 0.5 ml of formic acid (Mallinckrodt; 99%) added directly to the pellets in the bottom of the tubes. After incubation at room temperature, the solution was decanted into counting vials and the tubes were rinsed with scintillant cocktail, the rinses being pooled with the digested material. Supernatant fractions were counted for radioactivity as 0.5 or 1.0 ml portions, the total radioactivity in individual fractions being obtained after measuring their total volume. Radioactivity counting was carried out in a Packard Tri-Carb liquid-scintillation counter in either Scintisol (Isolabs, Akron, Ohio, U.S.A.) or in 'PCS' (Amersham/Searle, Arlington Heights, Ill., U.S.A.). Values were converted into d.p.m. by using the automatic external standard and a modification of the FORTRAN program (AUTAES) of Spratt (1970) or by the Packard automatic radioactivity analyser.

Chemical and analytical determinations

Protein was measured by the Lowry method (Lowry *et al.*, 1951), corrected for sucrose interference as described by Shires *et al.* (1971), with bovine serum albumin (fraction V; Sigma) as a standard. The RNA content of membranes was determined by the procedure of Munro & Fleck (1966) as adapted by Ragland *et al.* (1971). Polyribosomes and ribosomal subunits were estimated by measuring E_{260} where $25 \mu\text{g}$ of polyribosomal RNA/ml = 1 E_{260} unit and where correction was made for ferritin contamination (Ragland *et al.*, 1971). RNA extraction and analysis of RNA on polyacrylamide gels were carried out as described by Shires *et al.* (1971). The procedure for cell-free amino acid incorporation has been detailed by Shires *et al.* (1971, 1973).

Results

Flotation assay

A number of characteristics of the flotation assay used by Ekren *et al.* (1973) have been encountered that influence results when it is used. The duration of centrifugation required for complete separation of unbound polyribosomes from membranes by the flotation assay was about 18 h. This unexpectedly long centrifugation was examined by studying the rate of pelleting of $100 \mu\text{g}$ of ^3H -labelled polyribosomes mixed with conditioned or unconditioned rough membranes and centrifuged by the flotation procedure. The results indicated that the presence of membranes retarded the pelleting of polyribosomes that do not attach to the membranes. In tubes from which membranes were omitted, nearly all polyribosomes were pelleted within 5 h, compared with less than 50% pelleted in tubes containing unconditioned (i.e. non-binding) membranes. In tubes containing either conditioned or unconditioned membranes, unattached polyribosomes continued to accumulate in the pellet for up to 18 h of centrifugation. Visually, membranes remained dispersed in the 1.8 M-sucrose of tubes spun for up to 6 h, but after 9–12 h of centrifugation they became compressed into a crescent-shaped zone on the inner face of the tube about two-thirds of the distance from the bottom. Centrifugation for more than 12 h dispersed this zone so that by 18 h the membranes were collected in a band at the interface of 1.80–0.44 M-sucrose. The long centrifugation time required to collect polyribosomes in membrane-containing tubes thus may be due to entrapment of polyribosomes in the upper portion of the 1.8 M-sucrose layer by aggregating membranes, followed by release of the polyribosomes as the aggregate is dispersed with continued centrifugation.

Initial studies on the extent of polyribosome-membrane interaction by the flotation method

Fig. 2. Effect of relative position of polyribosomes and membranes in centrifuge tubes in the sedimentation (S_I – S_{III}) and flotation methods (F_I – F_{III})

In the upper row, sucrose layers are pictured relative to the position of ^3H -labelled polyribosomes (100 μg), 0.5 mg of conditioned rough membranes (R_{RN}) and 0.5 ml of postmicrosomal supernatant (S_3 , from rat liver) before centrifugation for 24 h in a 50 Ti rotor (for 'F' experiments) or 10 h in an SW-56 rotor (for 'S' experiments). The bar graph indicates the percentage of polyribosomes (β) that associated with the membranes. Expts. F_I and S_I were carried out by previously described flotation and sedimentation procedures except that the initial polyribosome-membrane mixture of F_I was mixed on a vortex stirrer for 30 s, and the mixing for S_I was done by a single hand-inversion before layering.

(18–36 h of centrifugation) indicated lower extents of polyribosome binding than when the sedimentation assay was used. The cause for this discrepancy lies in the separation methods themselves. Fig. 2 shows the effect of the position of polyribosomes and membranes, relative to each other, in tubes prepared for sedimentation assay (Expts. S_{II} and S_{III}) and the flotation assay (Expts. F_{II} and F_{III}). In Expts. S_{II} and S_{III} , polyribosomes were layered under (Expt. S_{II}) or above the membranes. No mixing of interacting components occurred until centrifugation, and, where the membranes were uppermost (Expt. S_{II}), no stable binding of polyribosomes occurred. The converse arrangement (Expt. S_{III}) resulted in binding to about the same extent as occurred when no physical separation of reactants was present (Expt. S_I). Thus considerable binding of polyribosomes to membranes takes place during the separation spin, owing perhaps to the accumulation of the more rapidly sedimenting membranes at the 1.8 M-sucrose interface subsequent to centrifugal passage of the polyribosomes through this layer.

In flotation experiments F_{II} (Fig. 2), membranes (R_{RN}) were layered on top of the dense sucrose and, in Expt. F_{III} , in the reverse position. The extent of polyribosome-membrane binding was much decreased in both situations compared with that where polyribosomes were pre-mixed and adjusted to 1.8 M-sucrose (Expt. F_I). The contrasting results of Expt.

F_{III} with Expt. S_{III} suggest that in the absence of tight entrainment of membranes, as in Expt. S_{III} , little stable interaction was demonstrable. The centrifugally migrating membranes and the centrifugally migrating polyribosomes may apparently pass each other without binding. In Expt. F_{III} it might be expected that the membranes are relatively dispersed in the 1.8 M-sucrose at the time that most of the polyribosomes are collecting in the pellet (4–5 h), and it is this dispersal that is thought to decrease the chance of interaction during the centrifugal passage of most of the polyribosomes. Consequently when the flotation procedure is used, polyribosomes must be mixed and binding brought to completion before the start of the separation procedure.

Fig. 3 shows the results of a controlled mixing of interactants followed by separation of unbound polyribosomes by the flotation method. The extent of interaction reached a maximum after about 30 min of mixing, and prolongation of mixing resulted in a slight decline in binding (calculated as β). This decline seems to coincide with heightened supernatant radioactivity (Fig. 3). After 2 h of rotational mixing, the percentage of total polyribosomal radioactivity that has been converted into non-sedimentable radioactivity approached 15%, and that decline in β nearly 10%. Although there was a decrease in β of 2–3% between 30 and 60 min, control experiments with polyribosomes alone (not shown) revealed no

increase in supernatant activity on prolonged mixing, indicating a role of membranes in the production of non-sedimentable radioactivity.

Controlled mixing of template-depleted ribosomes with conditioned rough membranes showed that, like polyribosomes, ribosome-membrane interaction was maximal by about 30 min, and that mixing for longer than 1 h led to a pronounced rise in the percentage of radioactivity in the combined supernatant fractions. Large ribosomal subunits differed in requiring twice the mixing time to fill sites as did polyribosomes (Fig. 4a). Small subunits likewise need a much longer mixing time than do polyribosomes, but their stability during mixing was much lower than that of large subunits, polyribosomes and template-depleted ribosomes, as judged by their high supernatant radioactivity accrued during mixing (see Ekren *et al.*, 1973). Both template-depleted ribosomes and large ribosomal subunits showed little interaction with rough microsomal membranes that still bore endogenous polyribosomes (Ekren *et al.*, 1973; Rolleston, 1972).

Mg²⁺ concentration has a pronounced effect on the extent of large-subunit interaction with membranes (Fig. 4b). Decreasing the Mg²⁺ concentration to 0.1 mM almost eliminated subunit interaction. Prolonging the mixing time to 90 min does not increase

Fig. 3. Effect of mixing time on polyribosome binding

³H-labelled polyribosomes (100 µg) were added to 1.0 mg of conditioned rough membranes in a final volume of 1.9 ml of 0.44 M-sucrose-T₅₀K₂₅M₅ buffer. The tubes were mixed on a Kraft rotator (Kraft Apparatus, Brooklyn, N.Y., U.S.A.). Both mixing and flotation separation were carried in the absence of RNAase inhibitor. The bar graph refers to the percentage of the recovered radioactivity found in the combined supernatant fractions (S₁ and S₂). All points and bars are the averages of two to four tubes for which the standard deviation of the mean was in all cases less than 1%. All results were obtained by using a single freshly prepared batch of conditioned rough membranes, and all steps were carried out in a cold-room (2–4°C).

Fig. 4. Effect of mixing time on the binding of ribosomal subunits assayed by the flotation procedure

(a) Interaction of 100 µg of large ribosomal subunits (○) or small subunits (□) with 0.5 mg of conditioned rough membranes compared with the interaction found with polyribosomes (Δ). T₅₀K₂₅M₅ buffer was used. The bar graph refers to the percentage of supernatant radioactivity found in the combined fractions S₁ and S₂. The bar on the right at each time-point refers to small subunits and that on the left to the large subunits. (b) Effect of Mg²⁺ concentration on membrane interaction with polyribosomes (Δ, 5 mM-Mg²⁺; ▲, 0.1 mM-Mg²⁺) or with large ribosomal subunits (○, 1.0 mM-Mg²⁺; ●, 0.1 mM-Mg²⁺). The bar graph to the left at each time-point refers to the percentage of supernatant radioactivity found with large subunits at 1 mM-Mg²⁺ and that to the right with 0.1 mM-Mg²⁺. Mixing was carried out as described in Fig. 3.

the extent of interaction. Polyribosome binding was unaffected by Mg^{2+} concentration over the range 0.1–5.0 mM.

Use of the flotation procedure under conditions that include mixing of interactants for 30 min and centrifugation for 18–24 h is sufficient to measure the extent of polyribosome binding to conditioned rough membranes and, under these conditions, the assay reproduces the extent of binding obtained by using the sedimentation assay (Ragland *et al.*, 1971; Shires *et al.*, 1971). The interaction of polyribosomes and rough microsomal membranes that have not been conditioned is minimal as measured by both the flotation and the sedimentation assay (Ekren *et al.*, 1973; Shires *et al.*, 1971); as was found with the sedimentation assay, attachment of polyribosomes required conditioning of rough membranes to displace the endogenous polyribosomes, thus making the binding sites available for interaction with an exogenous population (Ragland *et al.*, 1971; Shires *et al.*, 1971).

The membrane-bound polyribosomes collected from the flotation-assay tube after 18 h of centrifugation were active in cell-free amino acid incorporation. Polyribosomes and membranes were mixed for 30 min, and half of the preparation was submitted to a sedimentation assay and half to a flotation assay. The reconstituted rough membranes were collected separately from the tubes of each procedure, diluted with $T_{50}K_{25}M_5$ buffer, pelleted at 105 000g for 1 h, and resuspended in incorporation buffer (Shires *et al.*, 1973). Comparison of the capacity of these rough membranes to incorporate amino acids (per 100 μ g of RNA) with that of rough microsomal membranes showed the membranes from the sedimentation assay to be about equal to microsomal membranes in this function, whereas those from the flotation assay were one-half to one-third as active as rough microsomal membranes.

Tests for the effect of dilution on polyribosome-membrane interaction

The dependence of binding on the relative dilution of interactants is expressed by the following equation, whereby the starting amount of polyribosomes (P , in μ g of RNA), the amount of those polyribosomes bound to membranes (P_b), and the membrane concentration (M , in μ g of protein) are related:

$$\frac{(P - P_b)(M - P_b)}{P_b} = K \quad (1)$$

$(P - P_b)$ represents the amount of unbound polyribosomes, and $(M - P_b)$ the unfilled hypothetical receptor sites. Rearranging eqn. (1) gives:

$$\frac{P_b}{P} = \frac{M}{K} - \frac{P_b M}{K P} - \frac{P_b}{K} + \frac{(P_b)^2}{K P} \quad (2)$$

$P_b/P \times 100$ is the percentage membrane-bound polyribosomes of the total polyribosomes (P) applied, and a plot of P_b/P versus M at constant P/M should describe a curve whose descending limb passes through the origin if relative dilution of reactants affects their binding. Conversely, if concentration is irrelevant to the extent of interaction, a plot of P_b/P versus M should be horizontal. It is assumed that in all cases the volume in which the interactants are suspended is constant, as is the factor K .

In Fig. 5, plots from the results of experiments carried out with three different P/M ratios show that in no case is horizontal linearity maintained. The plot at $P/M \approx 0.2$ gave the most pronounced curvature. Comparison of the supernatant radioactivities from tubes at the various points on the curves revealed that the percentage of non-sedimentable radioactivity was higher in tubes with lower polyribosome concentration (Fig. 5). Further, the

Fig. 5. Effect of interactant dilution on the extent of polyribosome attachment to conditioned rough membranes

3H -labelled polyribosomes and membranes were mixed for 30 min in a constant volume of 1.9 ml of 0.44 M-sucrose- $T_{50}K_{25}M_5$ buffer. The left-hand ordinate refers to the curves and the right to the bars. All points on a single curve have the same ratio of the amount of polyribosomes to membranes: $\bullet$, 0.05; $\circ$, 0.1; $\blacksquare$, 0.2. At each polyribosome concentration, the bars refer to the percentage of total recovered radioactivity in the supernatant fractions ($S_1 + S_2$) from experiments for which points on the overlying curve are derived. In each case, the left-hand bar refers to the point on the 0.2-ratio curve, the middle the 0.1-ratio curve and the right, the 0.05-ratio curve. All points and bars are the average of two or three experiments, the standard deviations for which were 3.6%. All the data were obtained by using a single batch of freshly prepared conditioned rough membranes, and all assays of polyribosome binding were carried out simultaneously by the flotation procedure.

deviation of the plots of each P/M ratio from horizontal appears roughly to correlate with the percentage of radioactivity in the supernatant. The dependence of P_b/P on relative dilution of interactants, indicated by the results in Fig. 5, would seem to stem from the quantitative influence of the supernatant radioactivity on the calculation of P_b/P , and therefore on the calculation of β in the assays. The appearance during the flotation assay of supernatant radioactivity from polyribosomes labelled from [^3H]orotic acid has previously been interpreted as the result of limited RNA degradation (Ekren *et al.*, 1973). In the present study, the use of rat liver RNAase inhibitor, either in semipurified form or in postmicrosomal supernatant preparation (S3), decreased the percentage of supernatant radioactivity in binding mixtures containing subunits, template-depleted particles or polyribosomes by as much as one-half of that when no inhibitor was included. RNAase inhibitor has not been used as a routine in flotation assay, however, because the higher supernatant radioactivity in tubes not supplied with inhibitor was more accurately measured than the lower amounts found in tubes containing inhibitor.

The data of Fig. 5 do not indicate whether supernatant radioactivity originates from polyribosomes before or after binding or from non-interacting polyribosomes. Inspection of Fig. 3, however, shows that it increases with the duration of mixing and therefore that much of it probably originates during that step. This is strengthened by comparison of the percentage of supernatant radioactivity in identical assay tubes centrifuged for 18, 24, 32 and 36 h, in which no differences were found, suggesting that little new supernatant radioactivity is formed during the centrifugation itself. The presence of membranes in the binding-assay mixture is essential for the appearance of supernatant radioactivity. Tubes containing polyribosomes, but no membranes, which were spun for 18–36 h yield a percentage of radioactivity in the supernatant generally less than 1%.

Approximately the same relationship between supernatant radioactivity and the P/M ratios involving large ribosomal subunits or template-depleted ribosomes were observed as were seen with polyribosomes, and approximately the same percentages of supernatant radioactivity emanating from subunits or ribosomes were encountered at a given subunit or ribosome concentration for each P/M ratio as appeared with polyribosomes (results not shown).

Analysis of further studies carried out exactly as in Fig. 5 but with additional P/M ratios showed that the percentage supernatant radioactivity could be reproducibly confined within narrow limits for polyribosomes, large subunits and the template-depleted ribosomes. Minimization of supernatant radioactivity to a range of 8–12% could be achieved by

using 25–100 μg of polyribosomes at a P/M ratio of 0.05, or by using 100 μg of polyribosomes with 0.25–2.0 mg of membranes. These latter concentrations have been used in all our previous binding studies (Ragland *et al.*, 1971; Shires *et al.*, 1971, 1973, 1974b). By avoiding ratios other than these and by limiting mixing times to no more than 1 h, it has been possible to maintain supernatant radioactivity within the same limited range.

Stoichiometry

Earlier studies on the stoichiometry of polyribosome-membrane interaction were founded on the observation that the binding capacity of membranes was saturable (Ragland *et al.*, 1971; Shires *et al.*, 1971, 1973; Borghese *et al.*, 1972). By using the flotation assay and controlled mixing, the stoichiometry of interactions has been re-examined. The method of Ackerman & Potter (1949) for determining 'pseudo-irreversible' binding of enzyme inhibitor has been adapted for polyribosome binding (Fig. 6). Membrane attachment of labelled polyribosomes was blocked by prior binding of 25–50 μg of unlabelled polyribosomes. The amount of labelled polyribosomes was constant in all tubes, and the amount of membranes varied. The 'pseudo-irreversibility' of polyribosome interaction with membranes is indicated by the parallel lines obtained with different amounts of blocking polyribosomes. The points at which each plot reaches the abscissa represents the amount of membrane whose binding capacity for labelled polyribosomes was completely blocked (Fig. 6). On the basis of the intercept values in Fig. 6, 1 mg of membrane can interact with 112–122 μg of polyribosomes. Taking into account additional experiments, the overall range of interaction is 109–128 μg .

Shires *et al.* (1971) reported that membrane vesicles of conditioned microsomal preparations sometimes could not accommodate all ribosomal members of a large polyribosome. Where large polyribosomes attached, electron microscopy has shown that only two or three of the ribosomal units of the polyribosome were apposed to the membrane surface, whereas the rest of the polyribosome was unattached and pendent. Since radioactivity in the pendent portions appeared to be membrane-bound, measurement of polyribosome binding as the percentage of membrane-associated radioactivity would tend to be high. This can be corrected by dividing the polyribosomes into shorter segments before binding. The procedure adopted in our experiments for decreasing the size of polyribosomes has been homogenization of the polyribosome suspension in a 2.5 ml Potter-Elvehjem homogenizer. The resulting preparation is like that described by Berdinskikh *et al.* (1971), in which monoribosomes and diribosomes

Fig. 6. Blockage of the binding of ^3H -labelled polyribosomes to conditioned rough membranes by unlabelled polyribosomes

Membranes were mixed with 25 or 50 μg of unlabelled polyribosomes and mixed for 30 min in 1.9 ml of 0.44 M-sucrose- $\text{T}_{50}\text{K}_{25}\text{M}_2$ buffer (25 μg , solid line with intercept of approx. 0.28; 50 μg , solid line with intercept of 0.56). Then, 100 μg of labelled polyribosomes was added and mixed for an additional 30 min. Flotation assay of the extent of binding by the labelled polyribosomes was then performed. The control tubes (solid line with intercept near origin) received no unlabelled polyribosomes but underwent the same mixing schedule as the experimental tubes. Experiments shown with the solid lines used polyribosomes (labelled and unlabelled populations) which, after isolation from rat liver, were homogenized with five strokes of a loose-fitting Potter-Elvehjem homogenizer, which was carried out in an ice-bath or in a cold-room ($2-4^\circ\text{C}$). Experiments shown with the dashed lines used polyribosomes that were not homogenized.

predominate and with a greatly diminished proportion of larger forms. No difference in the supernatant radioactivity in binding assays has been seen between homogenized and unhomogenized preparations. Although some large polyribosomal forms are still present in the preparation, use of unhomogenized polyribosomes gave markedly different results in the blockage study shown in Fig. 6. The pronounced curvature most apparent at high degrees of inhibitor blockage (with unhomogenized polyribosomes) appears to be due to the spurious association of radioactivity with membranes which was preventable when the shorter polyribosomal forms were used.

Because the intercepts on the abscissa in Fig. 6 are susceptible to influence by polyribosome chain

Fig. 7. Determination of the extent of ^3H -labelled polyribosome binding on membranes, 50% of whose binding sites have been blocked with unlabelled polyribosomes

The experiments were carried out as described in Fig. 6. Percentage inhibition was calculated from the binding (β') of labelled polyribosomes at a given inhibitor/membrane ratio compared with binding to membranes in the absence of inhibitor.

length, an alternative approach for determining binding capacity was used. An experiment like that of Fig. 6 was performed to examine how prior attachment of polyribosomes affects binding to remaining sites. As shown in Fig. 7, the ratio of inhibitor to membrane concentration was plotted against the percentage inhibition of binding, by the method of Reif & Potter (1953). The method allows assessment of the extent of site occupation at a middle range of inhibition. RNAase-conditioned liver membranes, by this method, bound 58 μg of polyribosomes per mg of membrane at 50% inhibition (Fig. 7), and the range of the binding capacity for these membranes was calculated to be 105–120 μg of polyribosomal RNA per mg of membrane protein. Similar studies with rough liver membranes conditioned with pyrophosphate-citrate showed a somewhat lower binding capacity of 70–78 μg of polyribosome per mg of membrane.

Experiments like those shown in Fig. 6 were carried out in the presence of RNAase inhibitor, either in its semi-purified form or in fraction S3. When the results of these experiments were plotted by the method of Reif & Potter (1953) the extent of binding of labelled polyribosomes at 50% inhibition was within the same range as that found when the experiment was performed in the absence of RNAase inhibitor.

Fig. 8. Inhibition slopes

(a) Δ , ^3H -labelled liver polyribosomes versus unlabelled liver polyribosomes binding to RNAase-conditioned liver membranes (slope = 0.46); $\square$, ^3H -labelled liver polyribosomes versus unlabelled liver polyribosomes binding to pyrophosphate-citrate-conditioned liver membranes (slope = 0.6). (b) Δ , ^3H -labelled hepatoma polyribosomes versus unlabelled normal rat liver polyribosomes on RNAase-conditioned liver membranes (slope = 0.45); $\circ$, labelled normal liver polyribosomes versus unlabelled hepatoma polyribosomes binding to conditioned liver membranes (slope = 0.44); $\square$, labelled liver polyribosomes versus unlabelled tumour polyribosomes binding to RNAase-conditioned hepatoma rough membranes. (c) $\square$, Labelled liver polyribosomes versus unlabelled myeloma polyribosomes binding to conditioned liver membranes (slope = 0.35); Δ , labelled liver versus unlabelled liver polyribosomes binding to conditioned liver membranes (slope = 0.45). (d) $\square$, Labelled liver polyribosomes versus unlabelled large ribosomal subunits from liver binding to conditioned liver membranes (slope = 0.12); $\circ$, labelled liver polyribosomes versus unlabelled template-depleted ribosomes binding to conditioned liver membranes (slope = 0.25); Δ , labelled versus unlabelled liver polyribosomes (slope = 0.48). All experiments were performed with 1 mg of membrane mixed in 1.9 ml of 0.44M-sucrose- $\text{T}_{50}\text{K}_{25}\text{M}_5$ buffer for 30 min initially with variable amounts of unlabelled polyribosomes or subunits, followed by mixing for 30 min with 100 μg of labelled polyribosomes. Flotation analysis of the binding of label was carried out and β' calculated. Determination of the amount of membrane-bound inhibitor was done by assessing β' for labelled polyribosomes obtained from the same population from which the unlabelled forms were derived in separate tubes mixed for 60 min. Results shown in each panel were obtained by using freshly isolated polyribosomes, subunits and membranes, and flotation analysis of both control and experimental tubes was carried out simultaneously. All polyribosome preparations were homogenized as described in Fig. 6.

Comparison of binding sites by comparative susceptibility to inhibition

Fig. 8 shows the linear relationship between the blockage of membrane binding sites by an unlabelled population of liver polyribosomes to the attachment of a second labelled population. The slope of the plot of unlabelled versus labelled liver polyribosomes binding to RNAase-conditioned liver membranes averaged 0.46 (range 0.44–0.48), thus not quite reaching the ideal inhibition slope of 0.5. Some experiments with RNAase-conditioned liver membranes have given slopes of approx. 0.6, and those conditioned with pyrophosphate-citrate have consistently given 0.59–0.68 (Fig. 8a). Slopes of greater than 0.5 could involve failure to complete binding of the labelled population, or be due to site 'instability' during the mixing. Consequently it seemed that results with RNAase-conditioned liver membranes involving slopes greater than 0.5 could be discarded as poorly executed experiments, but such an interpretation is not warranted for the pyrophosphate-

citrate membranes which may be altered by conditioning (Shires & Pitot, 1973a).

General experimental conditions for these inhibition studies have included use of (a) a range of polyribosome-membrane concentrations appropriate for near-constant supernatant radioactivity, (b) similar reaction volumes, mixing times and centrifugation times in all experiments and control tubes and (c) two types of control tubes: the standard control in which membranes are excluded as described in the Materials and Methods section, and a control containing membranes and labelled polyribosomes from the same population as the unlabelled inhibitor. This latter serves to determine the exact extent of inhibitor binding. Procedurally, inhibition studies involve two sequential mixing steps of a constant amount of membranes first, for 25–30 min, with unlabelled inhibitor, and followed by mixing for 25–30 min with a constant amount of labelled population, the latter being added directly to the mixture of inhibitor and membranes. Results with this sequential mixing

procedure may be reproduced with two alternative procedures. A varying amount of inhibitor may be added to a constant amount of labelled polyribosomes and membranes and mixed in a single step. This alternative entails a true 'competition', but control tubes establishing the extent of inhibitor binding are still required. The second alternative procedure uses a dual-labelled polyribosome population, the inhibitor having a ^{14}C label, thereby obviating inhibitor control tubes.

Exploitation of the inhibition technique to study comparative site affinity for polyribosome populations of differing origin necessitated the establishment of optimal mixing times and the relative supernatant radioactivity produced for each population to be tested. Polyribosomes from kidney, myeloma MOPC-21 and hepatoma 5123C, as well as template-free ribosomes from liver, were all found to have near-maximal binding with 30 min mixing and to be associated with a 5–9% loss of radioactivity in the supernatant fractions. Small ribosomal subunits had to be precluded from consideration because of their predisposition toward high supernatant radioactivity (Fig. 4).

Any interaction of competing populations in the binding mixture before members of the labelled population can bind was viewed as a potential difficulty, especially when ribosomal subunits or template-depleted ribosomes were involved. Evidence for any such interaction was checked by mixing the two populations in question (one of which was labelled) and analysing the sedimentation patterns, in a 10–40% sucrose gradient, of the unlabelled population by extinction and the labelled population by radioactivity distribution. Large ribosomal subunits (with ^3H label) mixed with unhomogenized liver polyribosomes (without label) sedimented in a 60S peak and were not retarded in the upper portions of the gradient, as would have been expected if aggregation with the larger polyribosomes had occurred. Similarly, template-free ribosomes, as well as shortened (homogenized) polyribosomes, appeared not to interact significantly with unlabelled unhomogenized polyribosomes. Considerable aggregation *in vitro* between large subunits and template-free ribosomes was demonstrated, and thus studies in which these populations compete for binding sites were considered to be unfeasible.

In comparing the binding of different polyribosomal populations to conditioned liver membranes, if the inhibition curve of competing liver and non-liver populations had the same slope as that of two competing liver populations, then the binding sites used by the non-liver population would be identical with those used by the liver population. In these terms, hepatoma 5123C polyribosomes (Fig. 8b) and kidney polyribosomes (see Pitot & Shires, 1973) appear to use the same binding sites as the

autologous liver populations. In contrast, competition of polyribosomes from myeloma MOPC-21 with those from liver (Fig. 8c) showed that the former interacted with liver membranes at points other than at the sites for liver polyribosomes since the slope with competing myeloma and liver populations (0.357) markedly differs from that where competition was between liver populations (slope = 0.457).

Inhibition studies with ribosomal subunits or with template-depleted ribosomes against the binding of liver polyribosomes gave slopes of 0.12 and 0.25 respectively, compared with a liver versus liver slope of 0.48 (Fig. 8d). A slope of 0.15 was obtained when unlabelled polyribosomes were the inhibitors of binding of labelled large subunits. Slopes of 0.12–0.25 suggest that although ribosomes and subunits may bind to liver membrane quantitatively as well as liver polyribosomes, neither appears to utilize extensively sites identifiable as polyribosome sites. Nonetheless, some polyribosome sites were used, indicating either that subunits and ribosomes interact at random or that there are differences within each population such that some members of the population recognize polyribosome sites and others recognize some other site.

The possibility that binding of subunits or depleted ribosomes to non-polyribosome sites might stem from some effect of RNAase conditioning of rough microsomal membranes, secondary to removal of endogenous polyribosomes, was considered. It has been shown that RNAase-conditioned membranes retain some RNA, averaging about 10S in size, which presumably originates from nuclease attack on the endogenous polyribosomal RNA (Shires *et al.*, 1971). Removal of this RNA by rinsing the membranes in a pyrophosphate-citrate solution did not significantly impair polyribosome binding (Shires *et al.*, 1971). However, the presence of this residual RNA might influence the attachment of large subunits or template-depleted ribosomes. Experiments were carried out in which RNAase-conditioned rough membranes were rinsed with buffered pyrophosphate-citrate as described by Shires *et al.* (1971). The binding of polyribosomes, template-depleted ribosomes and large subunits to these membranes was compared by using the flotation assay and optimal mixing conditions. It was found that all three bound to these membranes to the same extent. Supernatant radioactivity was within the same range for all assays. Thus the non-polyribosomal binding sites on RNAase-conditioned rough membranes cannot be ascribed to salt-desorbable substrates on the membranes.

Discussion

Assembly of rough membranes *in vitro* from polyribosomes and membranes has been shown to be critically dependent on the methodology of assembly.

The need for extensive mixing of interactants in order to obtain maximum interaction is not surprising considering their large multimolecular sizes. With controlled mixing, polyribosomes from all sources studied, as well as template-depleted ribosomes, required about the same extent of mixing for complete interaction, whereas the large ribosomal subunit required longer. The explanation for, and the generality of, these results is not apparent at the present time. In retrospect, the importance of mixing was not fully appreciated during our earlier studies of interaction *in vitro* (Ragland *et al.*, 1971; Shires *et al.*, 1971, 1973), largely because the sedimentation system was used for assay of interactions and for batch preparation of reconstituted rough membranes. Although the results of these earlier studies are not repudiated, it is now clear that, for all studies of polyribosome-membrane interaction *in vitro*, the stoichiometric significance of each step in assembly and assay of isolation of these complexes must be accounted for.

The influence of supernatant radioactivity on stoichiometry involves not only the identity of the binding particles but also the relative concentrations of interactants. By maintaining polyribosome (or subunit) concentration within prescribed ranges, supernatant radioactivity can be predictably held to a minimal range, and concentration dependence thereby overcome. For 68 assays between 100 μ g of polyribosomes and 0.5 mg of conditioned rat liver rough membranes, the mean value of 71.3% had a standard deviation of 10.8%. However, when the variation of values of triplicate assays in experiments using a single membrane preparation was examined the variability was usually less than $\pm 3\%$. Linearity of inhibition plots was obtained only when all points were obtained from a single membrane preparation. That the membranes are a source of the variability independent of their role in the appearance of supernatant radioactivity is suggested by the near-constancy of the percentage of supernatant radioactivity and the percentage recovery of applied radioactivity, with the use of constant mixing times, incubation conditions and medium. Still, supernatant radioactivity must introduce some error into the calculation of the extent of binding (β'), although the error involved can be no greater than the percentage of supernatant radioactivity from a given tube. Thus by deliberately limiting the amount of that radioactivity by choice of appropriate polyribosome/membrane ratios, any error encountered is reproducible within narrow limits.

The origins of the supernatant radioactivity are not clear. RNA extracted from the polyribosome complexes differs from polyribosomal RNA in the appearance of small E_{260} peaks in the 19–22S range in polyacrylamide gels (T. K. Shires, unpublished work). These peaks also appear in RNA from

freshly isolated rough microsomal membranes, but are less accentuated. The 19–22S peaks are similar to those described for rough microsomal RNA by Aaij *et al.* (1971), who attributed their appearance to degradation of 28S RNA. Whether supernatant radioactivity in our experiments originates from labelled 28S RNA alone is uncertain. Polyacrylamide-gel analysis of RNA extracted from polyribosome-membrane complexes is made difficult by the presence of low-molecular-weight RNA on the RNAase-conditioned rough membrane itself (Shires *et al.*, 1971).

The amino acid-incorporation capacity of membrane-bound polyribosomes collected from flotation-assay tubes was less than that from the sedimentation-assay tubes, even though the interactants came from a common preparation. The basis for the difference may lie in the dense sucrose solution used for the flotation assay, in the longer centrifugation time used for the flotation assay or in a combination of both factors. Leslie & Mansbridge (1970) reported that centrifugation of microsomal membranes through dense sucrose inhibited incorporative capacity of the membranes, but they ascribed this to degradation of mRNA occurring during the centrifugation. This explanation cannot be ignored, especially since in our experiments no RNAase inhibitor was supplied to the assay tubes. The small change in some of the rRNA seen with the flotation assay seems not to be an explanation, since extensive degradation of intraribosomal rRNA has been reported to have little effect on protein-synthetic capacity (Huvos *et al.*, 1970; Grove & Johnson, 1973).

An alternative explanation of the lower amino acid-incorporation activity by reconstituted rough membranes from the flotation procedure involves their membranes. T. K. Shires (unpublished work) has observed that lipoperoxidation of the membrane of rough microsomal preparations inhibits their protein-synthetic activity *in vitro*. Endogenous lipoperoxidation by rough membranes stored at 3–5°C under atmospheric O_2 became perceptible after about 24 h, judged by malonyl dialdehyde production. Membranes at the completion of the flotation assay are about 48 h old. The extra 9–10 h of aging *in vitro* compared with the membranes from the sedimentation system might be a sufficient difference in lipoperoxidation to affect incorporative capacity. In that light, the difference in synthetic activity between membranes from the two isolation systems would pose no serious problem with regard to the quantitative accuracy in measuring polyribosome-membrane interaction, since the membrane changes appear after interaction and do not involve detachment of polyribosomes from the membrane. However, where isolation of batches of reconstituted rough membranes is a goal, the sedimentation type of isolation system is clearly preferable to the flotation system.

In the inhibition studies, sites on conditioned membranes attaching liver polyribosomes were shown to differ from those attaching most of the large ribosomal subunits and some of the template-depleted ribosomes. Since neither of these two particles appreciably interacted with rough microsomal membranes still bearing their endogenous polyribosomes, it seems that the sites for subunit-ribosome attachment have appeared as a result of conditioning. Because there is no evidence that in the cells of the liver, rough endoplasmic reticulum carries large amounts of large subunits or ribosomes (Kwan *et al.*, 1968; Webb & Morris, 1969), it cannot be argued that conditioning has uncovered pre-existing sites for these particles, as could be done for polyribosomes (Shires *et al.*, 1971). On the other hand, a strong case that subunits and ribosomes interact with artificial sites that appear because of the conditioning process cannot be sustained at present either. Large-subunit binding has been reported to occur on rough membranes conditioned with a variety of agents (Ekren *et al.*, 1973; Shires & Pitot, 1973b; Rolleston, 1972); and the binding obtained bears both quantitative and qualitative similarities to that with polyribosomes. On RNAase-conditioned rough membranes, residual RNA on the membranes after conditioning seems to be a possible ligand for subunits (Shires *et al.*, 1971), yet its removal by washing the membranes with pyrophosphate-citrate failed to diminish the binding of either subunits or template-depleted ribosomes (Ekren & Shires, 1972).

Sites for subunit and ribosome binding, in addition to being physically separate from polyribosome sites, differ in yet two other ways: filling of the subunit (but not ribosome) sites requires a more prolonged duration of mixing, and binding to the subunit ribosome sites is more sensitive to Mg^{2+} concentration (at 25mM-KCl) than is binding to the polyribosome sites. The importance of both bivalent and univalent cations in attachment and detachment of polyribosomes and subunits is well accepted (Adelman *et al.*, 1973; Rolleston, 1972; Shires *et al.*, 1971). The difference between subunit and polyribosome binding *in vitro* that occurs at 0.1 mM- Mg^{2+} and 25mM-KCl disappears if the KCl concentration is increased to 100mM, at which concentration polyribosome binding is nearly completely inhibited (Shires *et al.*, 1971). Thus polyribosome and subunit binding differ in degree but not in the ionic character of their interaction. The particular interpretational problem with this ionic character is the uncertainty whether the ion effect acts on the complex assembly directly, or indirectly by dissociating integral constituents of either interactant, or through possible conformation changes of either interactant.

The region of the membrane attaching a polyribosome has been viewed as having either a static or

an active form (Shires *et al.*, 1974a). In the static view, binding points on the surface for ligands on the polyribosome reside on specific membrane components inserted at appropriate points along the membrane co-extensive with the polyribosome. In the active view, binding points for polyribosomal ligands are the domain of a co-operative arrangement of surface components, disorganization of which would disturb functional attachment. Among the implications of the first view are (1) that specific constituents having high affinity for polyribosomal ligands may be isolatable from the membranes, and (2) since the polyribosome attaches to the membranes, in part, through the large subunit (Adelman *et al.*, 1973), sites recognizing ligands on the large subunit should attach both polyribosome and large subunit *in vitro*. An implication of the second view is that given the apparent compositional similarity of membranes from rough and smooth microsomal preparations [excluding plasma membrane and possibly Golgi membranes (Shires & Pitot, 1973b)], some smooth membranes might, under appropriate non-degradative stimuli, be induced to bind polyribosomes *in vitro*.

Some evidence is available to help choose between these two hypothetical forms of the binding site. With regard to the second view of an active form of binding site, smooth microsomal membranes have been shown by several laboratories to accept polyribosomes, although little attention has been given to the condition of the membranes during and after interaction (see Shires & Pitot, 1973b).

The first view of a static binding site is currently not well supported. Large subunits bind predominantly to sites different from those for polyribosomes, and these polyribosome sites bind polyribosomes from a variety of sources. Further, no unequivocal evidence is presently available for a high-affinity membrane constituent. However, D. J. Bailey, R. K. Murray & F. S. Rolleston (unpublished work) have obtained a protein unique to rough membranes which is of non-ribosomal origin. The results with template-depleted ribosomes and myeloma polyribosomes are also a cause for hesitancy in rejecting the static binding-site theory, since neither bind exclusively to the polyribosome binding sites.

The binding of myeloma polyribosomes to non-polyribosomal binding sites is interesting in the light of the suggestion by Milstein *et al.* (1972) that myeloma protein may be synthesized in stages, initially on polyribosomes in the cytoplasm and later by membrane-bound polyribosomes. A possibly similar but more complex process may occur for immunoglobulin in lymphocytes (Lisowska-Bernstein *et al.*, 1970; Vassalli *et al.*, 1971). It may be that nascent myeloma proteins interact with the membrane surface in a manner different from that of chains on other polyribosome populations.

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