Polyribosomal Attachment to Rat Liver and Hepatoma Endoplasmic Reticulum *in vitro*

A METHOD FOR ITS STUDY

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A system for study and measurement of the attachment *in vitro* of exogenous polyribosomes to membranes has been presented. Its main features are use of low temperature, post-microsomal supernatant, pyrophosphate and citric acid to remove ribosomes from the surface of rough endoplasmic reticulum, and a method for quantitative separation of unattached from membrane-associated polyribosomes. The following were found. (1) Rough endoplasmic reticulum, from which ribosomes had been removed by treatment with pyrophosphate and citrate, bound over 50% of added polyribosomes, whereas the untreated (or control) rough and smooth endoplasmic reticulum and the smooth endoplasmic reticulum treated with pyrophosphate-citrate did not bind polyribosomes. (2) The polyribosome-binding capacity of rough endoplasmic reticulum stripped of its ribosomes decayed upon storage of the membranes at 0-4°C. The half-life of this decay was about 6 days whereas that of the polyribosome-binding capacity of hepatoma stripped rough endoplasmic reticulum was about 1.5 days. (3) Preparations of stripped rough endoplasmic reticulum after reassociation with polyribosomes *in vitro* were quite similar to preparations of native rough endoplasmic reticulum as viewed with the electron microscope. Evidence is presented to support the contention that association of polyribosomes with membranes was the result of polyribosomal reattachment to the membranes rather than trapping of the polyribosomes between vesicles of the membranes.

The functional significance of free and membrane-bound polyribosomes in eukaryotic cells has not been unequivocally determined (Campbell, 1970). Evidence has been presented suggesting that free polyribosomes synthesize protein for intracellular functions and bound polyribosomes synthesize protein for export (Redman, 1969; Takagi, Tanaka & Ogata, 1969; Hicks, Drysdale & Munro, 1969). Studies with serine dehydratase indicate that this enzyme is synthesized by polyribosomes bound to endoplasmic reticulum (Pitot & Jost, 1968). NADP-cytochrome *c* reductase, destined for inclusion into membranes of the endoplasmic reticulum, was found to be synthesized by both free and bound polyribosome fractions (Ragnotti, Lawford & Campbell, 1969). On the whole, however, there appear to be no serious arguments against the uniqueness of bound polyribosomal populations in their ability to produce exported proteins, at least in parenchymal cells of the liver and pancreas (Campbell, 1970).

Understanding how polyribosomes are attached to membranes would facilitate clarification of their function. One approach to this problem has been

recombination *in vitro* of separately isolated polyribosome and membrane fractions from bacteria (Aronson, 1966; Coleman, 1969), reticulocytes (Burka & Schickling, 1970), and rodent liver (Suss, Blobel & Pitot, 1966; James, Rabin & Williams, 1969; Williams & Rabin, 1969). All but one of these studies employed incubation of polyribosomes with smooth-membrane fractions (including plasma membranes) at 25-37°C. In the single exception, Suss *et al.* (1966) carried out association studies at 0-4°C, at which temperature only membranes derived from rough endoplasmic reticulum and treated with pyrophosphate or citric acid would accept polyribosomes. The present study extends the techniques and observations of Suss *et al.* (1966) and reports on a technique for reproducibly measuring interaction between polyribosome and membrane fractions.

MATERIALS AND METHODS

Animals. Experiments were performed with 190-240g male albino rats of the Holtzman strain (Holtzman Co., Madison, Wis., U.S.A.). The Morris hepatomas, 7800,

7794A and 5123C (Morris, 1965), were carried by intramuscular implantations in male albino rats of the Buffalo strain. In several tumour experiments membranes were also prepared from normal male Buffalo rats weighing 200–250 g. All rats were maintained on Purina chow diet with water *ad libitum*.

Preparation of fractions of the endoplasmic reticulum. Except for the tumour-bearing rats, all animals were starved for 16–18 h before being killed at 9:00 a.m. Tumour-bearing rats were not starved. Rats were decapitated and their livers quickly removed and rinsed in ice-cold 0.44 M-sucrose in TKM buffer (0.05 M-tris-HCl buffer, pH 7.5, at 5°C; 0.025 M-KCl; 0.005 M-MgCl₂). Sucrose solutions were treated with 5 g of bentonite/litre (Fischer Scientific Co., 1458 N. Lamar Avenue, Chicago, Ill., U.S.A.), stirred overnight at 0–4°C, centrifuged at 78 500 *g_{av.}*, and filtered through a 1.2 µm-pore-size Millipore filter (Millipore Corp., Bedford, Mass., U.S.A.). All subsequent procedures were carried out at 0–4°C. The livers were blotted, weighed, minced with scissors in 2 vol. of 0.44 M-sucrose in TKM buffer and homogenized with 10 strokes in a Potter-Elvehjem homogenizer. A post-mitochondrial supernatant (S₂) was prepared by centrifuging the homogenate for 10 min at 17 000 *g_{max.}*. The S₂ fraction was adjusted to 1.3 M-sucrose in TKM buffer by the addition of 1.47 vol. of 2 M-sucrose in TKM buffer. A 20 ml portion of the adjusted S₂ fraction was layered over 10 ml of 2 M-sucrose in TKM buffer and overlaid with a 5 ml layer of 0.44 M-sucrose in TKM buffer. This discontinuous gradient was centrifuged in a Beckman type 50.1 rotor at 192 000 *g_{av.}* for 7 h or a Beckman type 30 rotor at 78 500 *g_{av.}* for 18 h. Membranes derived from rough endoplasmic reticulum (RER) floated at the boundary between the 2 M- and 1.3 M-sucrose layers, and those from smooth endoplasmic reticulum (SER) floated on top of the 1.3 M-sucrose layer. In experiments where it was unnecessary to prepare membranes free of contaminating unbound polyribosomes, membranes were separated by centrifugation in the above gradient at 192 000 *g_{av.}* in a Beckman type 50.1 rotor for 2 h. The membrane layers were collected after sectioning the tubes. The pooled SER and RER fractions were diluted with 2 vol. of TKM buffer, homogenized by 10 strokes in a Potter-Elvehjem homogenizer and sedimented in a Beckman type 30 rotor at 78 500 *g_{av.}* for 30 min. The membranes were washed by resuspending in 0.44 M-sucrose in TKM buffer (3 ml/g of tissue), followed by homogenization in a Potter-Elvehjem homogenizer (10 strokes) and then centrifuged into a pellet in a Beckman type 30 rotor at 78 500 *g_{av.}* for 30 min. The SER fraction from the livers of five rats was resuspended in about 200 ml of 0.44 M-sucrose in TKM buffer, homogenized by 10 strokes in a Potter-Elvehjem homogenizer and stored in an ice bucket. The RER fraction from the livers of five rats was resuspended in about 100 ml of 0.44 M-sucrose in TKM buffer and treated in the same manner.

Rough endoplasmic reticulum, 'stripped' of its ribosomes (RER_{st}) was prepared in the following manner. Suspensions of RER were diluted with an equal volume of a solution of 0.1 M-citric acid and 0.1 M-sodium pyrophosphate in 0.44 M-sucrose, pH 7.4. The solution had been treated with bentonite and filtered as described for the sucrose. After 30 min of gentle stirring at 0–4°C the membranes were centrifuged into a pellet at 78 500 *g_{av.}* for

30 min. Pellets of this preparation from five rats were homogenized in 70 ml of 0.44 M-sucrose in 0.05 M-tris-HCl buffer, pH 7.5, at 0°C and diluted with an equal volume of the citrate-pyrophosphate solution. The membranes were then sedimented by a 30 min spin at 78 500 *g_{av.}*, resuspended in 0.44 M-sucrose in 0.05 M-tris-HCl buffer, pH 7.5, at 0°C, and sedimented again. These washed pellets of RER_{st} from five livers were suspended in 100 ml of 0.44 M-sucrose in TKM buffer, homogenized with 10 strokes in a Potter-Elvehjem homogenizer and stored in an ice bucket. An SER fraction submitted to the same procedures as those used in preparation of RER_{st} was termed SER_{st}.

Tumour-bearing animals were also killed by decapitation and the tumour tissue was dissected free of connective tissues and necrotic or haemorrhagic areas. Membranes were prepared from tumour tissue in the same manner as used for liver.

Preparation of polyribosomes. Male albino rats of the Holtzman strain (180–220 g) were injected intraperitoneally with 1.0 mCi of carrier free Na₃³²PO₄ (E. R. Squibb and Sons, 2645 Rose Avenue, Franklin Park, Ill., U.S.A.) or with 100 µCi of [5-³H]jorotic acid (Amersham/Searle, 2000 Nuclear Drive, Des Plaines, Ill., U.S.A.)/100 g body wt. and then starved for 18–20 h before they were killed at 9:00 a.m. Polyribosomes were prepared by a modification of the procedure described by Blobel & Potter (1967). One volume of a 6.25% (w/w) solution of sodium deoxycholate (Matheson Scientific Inc., 1735 N. Ashland Avenue, Chicago, Ill., U.S.A.) in water was added to 4 vol. of post-mitochondrial supernatant (S₂) prepared as described above. A 4 ml portion of sodium deoxycholate-treated S₂ fraction was layered over a two-layer discontinuous gradient, with 4 ml of 0.5 M-sucrose in TKM buffer on top of 3 ml of 2.0 M-sucrose in TKM buffer. The gradients were then centrifuged at 165 000 *g_{av.}* for 2 h in a Beckman type 50 Ti rotor. The surface of each pellet was washed several times with TKM buffer and the walls of the tubes were wiped to remove labelled membrane fragments and lipid material (Robinson, 1969). The polyribosome pellets were either used fresh or stored at –70°C for up to 5 days. The *E*₂₆₀/*E*₂₈₀ ratio of polyribosomes was 1.74 ± 0.10 (*n* = 49), and the *E*₂₃₀/*E*₂₆₀ ratio was 0.92 ± 0.07 (*n* = 17), indicative of little protein or ferritin contamination (Wilson & Hoagland, 1965). Profiles of the ³²P-labelled polyribosomes were prepared and monitored as described by Noll, Staehelin & Wettstein (1963). Fractions of the gradients were collected in glass vials, made up to 10 ml with Bray's solution (Bray, 1960) and the radioactivity was counted in a Packard Tri-Carb scintillation counter. Quench correction was made with the automatic external standard. Counting efficiency for ³²P was approx. 80% and for ³H 30%.

Polyribosome-binding to endoplasmic reticulum in vitro. The procedure utilized was a modification of that reported by Suss *et al.* (1966). Polyribosomes, prepared as described above, were suspended in 0.44 M-sucrose in TKM buffer (about 3 ml for each pellet), homogenized with 6–10 hand strokes in a 3 ml Potter-Elvehjem homogenizer and centrifuged for 10 min at 200 rev./min in an International Centrifuge (model PR-2) (Needham, Mass., U.S.A.). Polyribosomal RNA concentration was determined by the extinction at 260 nm on the basis of 25 µg of RNA/ml equals 1.000 extinction unit. Membrane fractions were centri-

fuged at 2000 rev./min just before use to remove vesicle aggregates. Protein concentration was then determined on classified preparations immediately before mixture with polyribosomes.

At 0–4°C, various amounts of membranes up to 0.5 mg of protein were mixed with 0.5 ml of liver 78500 g_{av} . post-microsomal supernatant (S_3) in 10 ml conical glass centrifuge tubes. To each tube was added 100 μ g of RNA of 32 P-labelled polyribosomes and the final volume was adjusted to 3.5 ml with 0.44 M-sucrose in TKM buffer. Each tube was vigorously shaken for 20 s and 3.0 ml of each mixture was layered over 0.9 ml of 1.8 M-sucrose in TKM buffer. The gradients were then centrifuged for 6 h at 312000 g_{av} in an International SB 405 rotor or for 7 h at 308000 g_{av} in a Beckman SW 56 rotor. A sucrose concentration of 1.8 M had been found (W. L. Ragland, T. K. Shires & H. C. Pitot, unpublished work) to allow maximal sedimentation of labelled polyribosomes from an overlying 0.44 M-sucrose layer while preventing sedimentation of RER under conditions of 400000 g_{max} . and greater.

After centrifugation, the tubes were cut with scissors just below the membrane layer floating at the interphase. When each tube had been cut approximately half way through, the tube was pinched so that the upper layer containing the membranes could be decanted without losing any of the bottom layer. The inner wall of the upper part of the tube and the scissor blades were rinsed with 4.5 ml of TKM buffer and the washings added to the upper membrane layer in a 12.5 ml centrifuge tube. The membranes were sedimented at 105000 g_{av} . for 35 min in a Beckman type 40 rotor. The supernatant solutions were decanted and 2.3 ml of each was transferred to a glass vial for radioactivity measurement. The membrane pellets were quantitatively transferred to glass counting vials by 3 portions of 0.2 ml of 0.1 M-NaOH. The 1.8 M-sucrose layers containing unbound polyribosomes were transferred to glass counting vials. The unbound polyribosomes in the pellet were added to the 1.8 M-sucrose layers by 3 washes of 0.2 ml of 0.1 M-NaOH. The volumes in the counting vials were adjusted to 10 ml with Bray's solution and the radioactivity was determined in a Packard Tri-Carb liquid-scintillation counter.

In each experiment there was one tube in the rotor that contained only polyribosomes. An average of 10% of the radioactivity floated above the 1.8 M-sucrose layer and subsequently sedimented as if it were membrane-associated. Therefore all radioactivity associated with the membrane layer was corrected for the counts remaining at the interphase in the tube containing only polyribosomes.

Electron microscopy. Pellets of various types of membranes, including RER $_{st}$, were immersed in 2% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, for 1 h and then washed 3 times with buffer. The samples were postfixed in 1% (w/v) osmium tetroxide in 0.1 M-cacodylate buffer for 1 h and then washed 3 times with buffer. The previous steps were carried out at 0–4°C. Embedding was carried out according to Mollenhauer (1964) by using Epon 812 and Araldite 502. Sections of the pellets were examined with a Hitachi HU-11B2 electron microscope at 75 kV.

Analytical methods. Protein was determined in various membrane fractions by the method of Lowry, Rosebrough, Farr & Randall (1951). The sucrose concentrations of the portions used for the assays were not large enough to

interfere with the colour reaction (Gerhardt & Beevers, 1968). The RNA contents of samples of the same fractions were determined by a modified Schmidt-Thannhauser procedure using $E_{1cm}^{1\%} = 312$ (32 mg/ml = 1.000 extinction units) (Fleck & Munro, 1962; Munro & Fleck, 1966).

RESULTS

Polyribosomes, uniformly labelled with 32 P (Fig. 1), associated with normal rat liver RER $_{st}$, conditioned with pyrophosphate and citrate, to the extent of 57% (Table 1). Similar experiments carried out with polyribosomes, labelled for 12 h with [5- 3 H]orotic acid, showed an average binding percentage of 61.8 ± 7.1 ($n = 7$). Binding of polyribosomes to smooth membranes treated with pyrophosphate-citrate (SER $_{st}$) as well as to the untreated smooth-membrane fraction (SER) was less than 10%. Binding of 32 P-labelled polyribosomes added to preparations of rough membranes (RER) also was not significant (Table 2).

Electron microscopically, pyrophosphate-citrate-treated rough membrane fractions displayed no ribosomal images (Plate 1a). The membrane vesicles tended to be irregular in shape, often flattened, and usually accompanied by some amorphous intervesicular debris. These changes in appearance of stripped fractions compared with those untreated control preparations seemed

Fig. 1. Profile of rat liver polyribosomes in a 15–30% sucrose density gradient. The polyribosomes were labelled *in vivo* for 18 h with 32 P. The top of the tube is on the right. —, Extinction at 260 nm. The values over the peaks indicate the number of ribosomal units in the polyribosomes of each peak. ●, 32 P Radioactivity in 10-drop fractions of the gradient.

Table 1. Comparison of the binding of 100 μ g of RNA of 32 P-labelled polyribosomes to 0.5mg of protein of 18h-old stripped rough endoplasmic reticulum with and without homogenization before sedimentation of the polyribosome-membrane complex

See the text for details of the binding experiments. After polyribosomes and membranes had been mixed they were centrifuged over 1.8M-sucrose to sediment polyribosomes and float membranes. The membrane fractions were collected and diluted with buffer. Duplicate samples of these membranes were either sedimented by centrifugation without previous homogenization or were homogenized with 5-10 hand strokes in a Potter-Elvehjem homogenizer before sedimentation.

Source of membranes	% binding	
	Without homogenization	With homogenization
Liver	57.4	56.2
Hepatoma 5123C	49.6	44.9

Table 2. Percentage binding of 100 μ g of RNA of 32 P-labelled polyribosomes to 0.5mg of protein of endoplasmic reticulum stored for 1 and 4 days at 0-4°C

Preparative procedures are given in the text. The values given are means $\pm$ S.E.M. with the number of observations noted in parentheses.

Membrane fraction	% binding	
	1-day-old membranes	4-day-old membranes
RER _{st}	56.0 $\pm$ 1.4 (3)	34.6 $\pm$ 2.1 (3)
RER	4.7 $\pm$ 3.0 (5)	3.7 $\pm$ 1.4 (7)
SER	4.9 $\pm$ 4.1 (5)	4.4 $\pm$ 1.9 (7)
SER _{st}	7.1 $\pm$ 3.4 (5)	2.4 $\pm$ 1.4 (7)

attributable to the number of homogenization and sedimentation steps, generally eight in number, to which the membranes had been submitted.

The absence of ribosomes from stripped membrane preparations by morphologic criteria was reflected in RNA/protein ratios of the membranes (Fig. 2). After incubation of RER fractions with the chelating agents, the ratio declined by a factor of almost 4. Washing the preparations only once was sufficient to lower the ratio to its minimum (Fig. 2). Treatment of smooth-membrane fractions (SER) with pyrophosphate-citrate depressed their RNA/protein ratios from 0.03 to 0.01. Ultrastructurally, SER_{st} was similar to RER_{st}.

Rough membranes reconstituted by incubation of RER_{st} and polyribosomes resembled native RER (Plate 1b). Ribosomal images were closely opposed

Fig. 2. Removal of ribosomes from RER by pyrophosphate-citrate solution. The RNA/protein ratios of RER before and after treatment with the pyrophosphate-citrate solution and subsequent washing in 0.44M-sucrose in TKM buffer are indicated by $\circ$. See the text for methods.

to membrane surfaces. Some apparently unattached ribosomes were encountered. Their presence was predicted by the amount of applied radioactivity in the control tubes (without membranes) that remained unsedimented above the 1.8M-sucrose at the end of 6-7h centrifugation at 400000g_{max}. Vesicle surfaces in the reconstituted rough-membrane preparations showed sizeable areas free of any attached ribosomes. Some vesicle profiles had no ribosomal appendages whatsoever. In some vesicles, ribosomes were found attached to the inner membrane surface. No vesicle was observed with ribosomes attached to both inner and outer membrane surfaces.

Some stoichiometry of the association between polyribosomes and RER_{st} is shown in Fig. 3. When the amount of polyribosomes was kept constant in the binding reactions (100 μ g) and the amount of RER_{st} varied from 0.05 to 0.4mg, radioactivity associated with the membranes increased proportionally. The membrane preparations SER, SER_{st} and RER, displayed little change at different membrane concentrations in their status as essentially non-binding membranes. The distribution of 32 P radioactivity from 100 μ g of polyribosomes interacted with 0.05-0.4mg of RER_{st} is shown in

EXPLANATION OF PLATE I

- (a) Electron micrograph of isolated vesicles of stripped rough endoplasmic reticulum. Magnification $\times 48\,000$.
(b) Electron micrograph of vesicles of stripped rough endoplasmic reticulum after addition of polyribosomes and centrifugal treatment to remove non-binding polyribosomes. Magnification $\times 64\,000$. Polyribosomes attached to the outer membrane surface are indicated by the dark arrow and those on the inner surface by the light arrow.

Fig. 3. Binding of polyribosomes *in vitro* to RER stored for 4 days at 0–4°C. Polyribosomes labelled with ^{32}P (100 μg of RNA) were mixed with various amounts of membranes (measured as protein) in the presence of liver ribonuclease inhibitor. Time of storage of the endoplasmic reticulum was the interval between decapitation and the mixing of endoplasmic reticulum with polyribosomes. The polyribosome-membrane mixture was centrifuged over a dense sucrose layer to float the membranes and sediment the unbound polyribosomes. The percentage of the radioactivity associated with the membranes has been plotted against the amount of membrane. Membrane fractions used were RER ($\square$), rough endoplasmic reticulum that had been stripped of its ribosomes (RER_{st} , $\bullet$), smooth endoplasmic reticulum (SER, $\times$) and smooth endoplasmic reticulum that had been subjected to the same stripping procedure as RER_{st} (SER_{st} , $\triangle$).

Fig. 4 for non-membrane-associated and bound fractions. Variation in the total applied radioactivity of all experiments was 10%. The 8% of unsedimentable activity was virtually constant over the entire range of membrane concentration.

Based on experiments like that of Fig. 3, the maximum possible amount of binding can be estimated. If the amounts of polyribosomes (based on RNA determination) and membranes (based on proteins) in the binding reaction mixture are known, and given a percentage of the polyribosomes that comes to be associated with RER_{st} , the RNA/protein ratio of the reconstituted rough membranes can be calculated. This calculation omits consideration

Fig. 4. Distribution of radioactivity between unbound polyribosomes, membrane (RER_{st}), and supernatant fractions after mixing a constant amount of ^{32}P -labelled polyribosomes (100 μg of RNA) with various amounts of membranes (0.05–0.4 mg of protein). Methods of stripping membranes and separating the three fractions are given in the text. The time between decapitation and mixing of membranes with polyribosomes was 4 days. The percentages of total radioactivity in the unbound polyribosome fraction ($\circ$) and in the membrane fraction ($\bullet$) were added to obtain the amount of sedimentable radioactivity (—) (approx. 92%). The percentage of solubilized counts is represented by the space above the solid line (i.e. 8%).

of the small residual RNA left on RER_{st} after pyrophosphate-citrate treatment. The RNA/protein ratio of reconstituted membranes increases with increasing amounts of RER_{st} reacting with 100 μg of polyribosomes. At slightly greater than 0.5 mg of membranes, the ratio begins to decline regardless of the amount of binding. This point of maximum RNA/protein ratio represents saturation of the capacity of RER_{st} to bind polyribosomes. The best RNA/protein values obtained experimentally were about 0.114 by using slightly less than saturation proportions, i.e. 100 μg of polyribosomes with 500 μg of membranes.

The tightness with which added polyribosomes fasten to RER_{st} was investigated by submitting the reconstituted rough membranes to homogenization. As shown in Table 1, the percentage of bound polyribosomes measured after homogenization indicates

Fig. 5. Effects of post-microsomal supernatant on polyribosome profile in the presence of membrane (RER). Polyribosomes and membranes were mixed with or without 1–2 ml of post-microsomal supernatant in a final volume of 4 ml of 0.44 M-sucrose-TKM buffer. The mixtures were layered over a discontinuous gradient containing lower and middle layers of 2 M-sucrose-TKM buffer and 0.5 M-sucrose-TKM buffer, respectively. Polyribosomes of the mixture were sedimented at $226000g_{\max}$ for 2 h, resuspended in TKM buffer, and $75\mu\text{g}$ of polyribosomes was spun through a 10–40% isokinetic sucrose density gradient for 80 min at $201000g_{\text{av}}$ in a Beckman SW41 rotor. —, Polyribosomes plus RER alone; ----, polyribosomes with RER plus post-microsomal supernatant. The top of the gradient is on the left.

that only a small amount of label was sheared from the membrane fraction. Compared with reconstituted membrane fraction that had not been homogenized, the homogenized membranes lost declined by only 1.2%.

An advantage of the experimental system for measuring polyribosome-membrane interaction *in vitro* seemed the inclusion of post-microsomal supernatant with its ribonuclease-inhibitory activity. As shown in Fig. 5, the presence of non-binding membranes (SER) was deleterious to polyribosomes, shifting the polyribosome profile toward smaller forms (compare with Fig. 1). The period of experimental contact between polyribosome and membranes was about 2 h. Addition of post-microsomal supernatant to the mixture of polyribosomes and membranes tended to discourage, but not prevent, the shift (Fig. 6). Shires & Narurkar (1970) and Shires, Narurkar & Pitot (1970) showed the interaction between polyribosomes and membranes is probably complete after a few minutes and that post-microsomal supernatant is not an absolute requirement for interaction. Thus it appears the advantage of including post-microsomal supernatant with the mixture of polyribosome and membranes is to maintain the sizes of polyribosomal units during the short period before their adherence to the membrane.

Fig. 6. Log percent of polyribosomal ^{32}P ($100\mu\text{g}$ of polyribosomal RNA) associated with 0.5 mg of protein of RER_{st} that had been maintained at $0-4^\circ\text{C}$ for various periods of time. Membranes from liver (●) and from well-differentiated Morris hepatocellular carcinomas 7800, 7794A and 5123C (○) were used. The slopes were determined by the method of least squares.

An unavoidable aspect of the binding system *in vitro* was a time-dependent deterioration of the acceptance capacity of RER_{st} . In Fig. 6, the radioactivity associated with 0.5 mg of 1-day-old RER_{st} was 57%, compared with 37% for 4-day-old RER_{st} . Table 2 shows similar results for other experiments. Aging of non-binding membranes (SER, SER_{st} and RER) did not influence their general inability to interact with polyribosomes. The half-life of polyribosome-binding capacity of normal liver RER_{st} , shown graphically in Fig. 6, was 5.5–6 days.

An estimate of the extent to which reconstituted rough membranes approximate native RER could be gained by examining the Y-intercept (61%) of the normal RER_{st} decay plot in Fig. 6. The RNA/protein ratio of membranes at this theoretical point would be 0.12. Table 3 shows that the RNA/protein ratio of native RER, from which RER_{st} had been derived, was 0.16.

Since polyribosomes would associate only with the specific membrane preparation RER_{st} , studies

Table 3. Comparison of estimated and measured RNA/protein ratios of liver stripped rough endoplasmic reticulum-polyribosome complex

The methods of protein and RNA analysis are given in the text. The estimated value is based on the decay slope in Fig. 6.

Age of membranes	RNA/protein ratio	
	Estimated	Measured
0 days (Y-intercept)	0.12	0.16 (native)
3 days	0.084	0.080

were extended to include RER_{st} from other than normal liver, and binding of normal rat liver polyribosomes to RER_{st} from three different Morris minimum-deviation hepatomas was investigated. Table 1 shows that polyribosome association with RER_{st} from hepatoma 5123C was only slightly less than that with normal liver membranes. Once bound to tumour membranes, polyribosomes were not appreciably subject to detachment by homogenization (Table 1). Further study with hepatomas 5123C, 7800 and 7794A disclosed differences in polyribosome acceptance that were age dependent. Fig. 6 shows the plot of tumour-membrane age *in vitro* against polyribosome-binding capacity. Results from all three tumours fit the same line, showing that all three displayed a similar rapid loss in polyribosome-binding capacity. The half-life of tumour-membrane binding capacity was 1–1.5 days. Extrapolation of the tumour-membrane binding line in Fig. 5 to zero time gives a Y-intercept fairly close to that of normal liver RER_{st}. Binding capacities of tumour and normal adult liver membranes are seen as similar in the native state and differ only in the rate at which their capacities disappear.

DISCUSSION

It is difficult to understand the polyribosome-membrane association results other than in terms of direct interaction between the two structures. Physical trapping of polyribosomes by aggregation of membranes is not a tenable explanation as shown by the experiments in this paper. Homogenization of reconstituted membranes, a process expected to release trapped polyribosomes, failed to separate more than a small percentage of radioactivity from the reconstituted membranes. SER, RER and SER_{st} preparations, which should be as physically capable of trapping polyribosomes as RER_{st}, showed little radioactivity remaining associated with these membrane preparations. The polyribosome-binding capacity of membrane measured as a function of age

in vitro is also not consistent with a trapping phenomenon. Electron microscopy of the reconstituted membranes, as well as the homogenization studies shown in Table 1, indicate a close and stable conjunction of polyribosome and membrane.

Electron microscopy of the reconstituted membranes revealed areas bare of ribosomes although reconstitution experiments were carried out near saturation. The finding of ribosomes on the inner surface of some reconstituted vesicles, presumably due to inversion of the vesicles by homogenization, implicates similar events before reconstitution. Before exposure to polyribosomes, some RER_{st} vesicles may have been turned inside out and thus made unable to present a binding surface to the polyribosome-containing compartment of the binding suspension. The inversion of surfaces would evolve from the homogenization steps incumbent on RER_{st} preparation and might be facilitated by the pyrophosphate-citrate stripping step. Inversion would explain why entire vesicles failed to bind polyribosomes.

The time-dependent decay of the capacity of the membranes for polyribosome association represents an alternative explanation for non-binding regions on the surface of reconstituted membranes. James *et al.* (1969) have related the binding of polyribosomes to membranes at 37°C to the activity of an enzyme catalysing disulphide interchange. This enzyme was reported to decay as membranes were aged *in vitro*. The instability of this enzyme might form the basis for the decline in polyribosome-binding capacity in the present experiments.

The fact that tumour membranes lose the capacity to bind polyribosomes more rapidly than do liver membranes is not understood. It is quite possible the difference in stability of binding capacity is inherent within the membrane itself as has been postulated earlier (Pitot, 1969; Pitot *et al.* 1969; Wallach, 1968). This finding is also compatible with studies that demonstrated that in many of the highly differentiated hepatocellular carcinomas, including those utilized in this study, the number of free polyribosomes was markedly increased and the number of bound polyribosomes diminished (Webb, Blobel & Potter, 1964, 1965).

A degree of polyribosome predilection for certain membrane preparations and not others has been reconfirmed after Suss *et al.* (1966). Endoplasmic-reticulum derivatives classified as 'smooth' by density-gradient centrifugation, electron microscopy and chemical analyses (Murray, Suss & Pitot, 1967; Moyer, Murray, Khairallah, Suss & Pitot, 1970) will not bind significant amounts of polyribosomes *in vitro*, nor will membranes bearing large numbers of ribosomes (RER). Association occurred only after RER membranes had been treated with chelating agents. Sachs (1958) and Vogt

(1960) have shown that these agents remove ribosomes from RER surfaces. The present results support this contention and further imply that these chelating agents uncover sites on the membrane surface with which polyribosomes interact.

Whereas the aim of the present study was development and testing of a system for examining polyribosome-membrane association, it appears that product analysis of the reconstituted rough membranes is feasible after removal of unbound polyribosomes and non-binding membranes from the preparation. The zero-time extrapolation in Fig. 6 indicated that the RNA content of native RER and reconstituted RER was similar. At present, however, the biochemical equivalence of these reconstituted rough membranes with native RER has not been shown.

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