The Association *in vitro* of Polyribosomes with Ribonuclease-Treated Derivatives of Hepatic Rough Endoplasmic Reticulum

CHARACTERISTICS OF THE MEMBRANE BINDING SITES AND FACTORS INFLUENCING ASSOCIATION

By T. K. SHIRES, L. NARURKAR* AND H. C. PITOT

McArdle Laboratory for Cancer Research, University of Wisconsin Medical School,
Madison, Wis. 53706, U.S.A.

(Received 12 March 1971)

1. Pancreatic ribonuclease in dilute EDTA has been shown to condition rough-microsomal membranes from adult rat liver to accept exogenously added rat liver polyribosomes *in vitro* at 0-4°C. Treated smooth membranes would not significantly interact with polyribosomes. 2. The conditioning process decreased the membrane RNA content and removed polyribosomes from vesicle surfaces as viewed electron-microscopically. 3. Binding to these conditioned membranes was shown to be uninfluenced by changes of temperature (0-37°C) and pH (6.9-7.8) or the presence of cell sap, but was inhibited by increasing the concentration of potassium chloride. 4. Possession of a polyribosome-binding capacity by conditioned rough membranes was not dependent on adventitious materials that could be dislodged by high ionic strengths. 5. Trypsin treatment under mild conditions destroyed the binding capacity of ribonuclease-conditioned rough membranes. 6. A 2-10S residual RNA was recovered from ribonuclease-conditioned membranes, but its partial removal had no effect on the capacity of membranes to accept polyribosomes. However, some role for this residual RNA in attaching polyribosomes could not be discounted. 7. Evidence is considered that polyribosome-binding sites are intrinsic features of conditioned membranes isolated from rough-microsomal fractions, and that long-range ionic bonding is a primary factor in polyribosome interaction with these binding sites.

Interactions *in vitro* between separately isolated polyribosomes and membranes from a variety of tissues are well documented. The interactions are divisible into three types, the first of which has thus far exclusively involved structures from rodent liver (Suss, Blobel, & Pitot, 1966; Khawaja & Raina, 1970; Ragland, Shires & Pitot, 1971). In this type of interaction, native microsomal membranes would not accept ribosomes unless conditioned by chelating agents (pyrophosphate and citric acid; Suss *et al.* 1966; Ragland *et al.* 1971) or high salt concentrations (4M-lithium chloride; Khawaja & Raina, 1970). Conditioning for acceptance of exogenously added ribosomes was successful only for the treated rough-microsomal fraction, and not the treated smooth-microsomal membranes.

Interactions restricted to these conditioned rough membranes contrast with the second type of interaction that has been described for a more diverse series of membranes, all classifiable as smooth,

including washed reticulocyte membrane preparations (Burka & Schickling, 1970) and bacterial membranes (Aronson, 1966; Coleman, 1969). The major difference was in the incubation temperature required for interaction. Demonstrations of the second type of interaction were carried out at 25-37°C, whereas the first type of binding occurred at 0-4°C.

A third type of polyribosome-membrane interaction found *in vitro* is distinguished from the first two types by the methodology used. Interaction is measured indirectly as a function of the disulphide interchange enzyme activity, instead of by a direct demonstration of the interaction by ultracentrifugation (James, Rabin & Williams, 1969; Williams & Rabin, 1969; Sunshine, Williams & Rabin, 1971; Blyth, Freedman & Rabin, 1971). Smooth membranes from rat liver bound polyribosomes after addition of sex steroids to the incubation medium, but interaction with putatively degranulated rough-membrane fractions had no steroid dependence. Binding to both membrane

* Present address: Bhabha Atomic Research Centre, Bombay, India.

preparations required incubation at 24–37°C, and the steroid-dependent complexes formed were not stable on centrifugation (Sunshine *et al.* 1971). Despite the differences of method, which make comparison between type-three interactions and types one and two difficult, there appears to be a similarity between type-three binding to degranulated rough membranes and type-one interactions.

The significance of polyribosome-membrane interactions *in vitro* rests on the assumption that binding sites demonstrable on the various membranes are functional *in vivo*. It has been contended that type-one polyribosome-membrane interactions involve binding sites present on rough-microsomal membranes at the time of isolation but not experimentally manifest until unmasked by ribosome displacement during conditioning (Ragland *et al.* 1971). This contention would be supported if it could be shown that procedures for conditioning rough membranes did not generate *de novo* the capacity of the membrane to bind polyribosomes, and if binding sites were demonstrable after any procedure that cleanly displaced endogenous ribosomes. Because of these questions, studies on polyribosome binding were performed on rough-microsomal membranes conditioned by a new procedure, considerably milder than any used previously, employing ribonuclease. Characteristics of the binding sites revealed by this procedure were then assessed for evidence that they constituted genuine features of the membranes as isolated.

MATERIALS AND METHODS

Fractionation of rat liver cells. Male Holtzman rats (Holtzman Co., Madison, Wis., U.S.A.) were decapitated by using a guillotine. The livers were rapidly excised, chilled, weighed and minced in 2 vol. of cold 0.44 M-sucrose in TKM medium (50 mM-tris-HCl-25 mM-KCl-5 mM-MgCl₂, pH 7.0 at 25°C). Buffer pH was determined with radiometer electrodes (The London Co., Yellow Springs, Ohio, U.S.A.). Sucrose (Density Gradient Grade; Mann Research Laboratories, New York, N.Y., U.S.A.) was free of ribonuclease activity measured by the method of Anfinsen, Redfield, Choate, Page & Carrol (1954). Minced livers were homogenized with a Potter-Elvehjem homogenizer by using ten strokes of a motor-driven Teflon pestle. Postmitochondrial supernatant was made by centrifuging the tissue homogenate at 20000 *g*_{max} for 10 min in a Sorvall SS-34 rotor (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.). The upper two-thirds of the postmitochondrial supernatant was recovered by pipette and the lower third was discarded.

Preparation of polyribosomes. Postmitochondrial supernatant was prepared from rats starved overnight (14–16 h) and injected 12 h before death with 0.5–1.0 mCi of carrier-free [³²P]orthophosphate (E. R. Squibb and Sons, Franklin Park, Ill., U.S.A.; or Schwarz BioResearch Co., Orangeburg, N.Y., U.S.A.) or with 200 μ Ci of [5-³H]-

orotic acid/100 g body wt. (Amersham/Searle, Des Plaines, Ill., U.S.A.). The radioactively labelled postmitochondrial supernatant was incubated for 1 h at 0–2°C with 1.25% (w/v) sodium deoxycholate (Matheson, Coleman and Bell, Inc., Chicago, Ill., U.S.A.) or with 2% (w/v) Triton X-100 (Calbiochem, Los Angeles, Calif., U.S.A.), and 4 ml was layered over a two-step gradient of 1.3 M-sucrose in TKM medium (4 ml) and 2.0 M-sucrose in TKM medium (3 ml). The polyribosome pellets obtained after sedimentation at 165000 *g*_{max} for 2–3 h in a Beckman 50Ti rotor were rinsed with several changes of TKM medium, and the tube was wiped free of labelled membrane fragments (Robinson, 1969). The average E_{260}/E_{280} ratios of polyribosomes extracted with deoxycholate or Triton X-100 were 1.73 (s.d. $\pm$ 0.13) and 1.35 (s.d. $\pm$ 0.10) respectively. Uniform labelling with ³²P or ³H of polyribosomes isolated as described above has previously been shown by Ragland *et al.* (1971).

Preparation of membranes. Postmitochondrial supernatant prepared from starved rats not treated with labelled compounds was adjusted to a sucrose concentration of 1.36 M, and the procedure of Moyer, Murray, Khairallah, Suss & Pitot (1970) was employed to obtain smooth-microsomal and rough-microsomal fractions. The postmitochondrial supernatant in 1.36 M-sucrose was centrifuged between layers of 2 M-sucrose in TKM medium and 0.44 M-sucrose in TKM medium at 192000 *g*_{av} in a Beckman 50.1 rotor for 3.5 h. Upper smooth-microsomal and lower rough-microsomal layers were separated with a tube cutter and the fractions were diluted with TKM medium [2:1 (v/v) for rough-microsomal and 1:1 (v/v) for smooth-microsomal preparations] before centrifugation at 78500 *g*_{av} for 45 min in a Beckman 30 rotor.

Conditioning of membranes. Smooth-microsomal and rough-microsomal pellets were suspended in 3 ml of 0.44 M-sucrose in TKM medium (50 mM-tris-HCl-25 mM-KCl, pH 7.0 at 25°C)/g of liver equivalent with 20 strokes of the homogenizer. Bovine pancreatic ribonuclease (5 $\times$ crystallized; Sigma Chemical Co., St Louis, Mo., U.S.A.) or ³H-labelled ribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) and EDTA (Fisher Scientific Co., Chicago, Ill., U.S.A.) were added to final concentrations of 15 μ g/ml and 5 mM respectively, and incubated with the membranes for 1 h at 2–4°C with constant slow stirring. Washing of the conditioned membranes was repeated one to three times by centrifugation at 78500 *g*_{av} for 45 min, followed by resuspension with 20 homogenizer strokes in fresh 0.44 M-sucrose in TKM medium. Unless otherwise specified, membranes were used for binding experiments immediately after preparation.

Association of polyribosomes with membranes and its measurement. Labelled polyribosomes were mixed with membranes and incubated as described by Ragland *et al.* (1971). Included in the mixture for ribonuclease inhibition was a preparation of rat liver postmicrosomal supernatant (Blobel & Potter, 1966, 1967b). The separation of labelled membrane-associated material from free material was performed by layering the polyribosome-membrane mixture in 0.44 M-sucrose in TKM medium over 1.8 M-sucrose in TKM medium (0.9 ml) and centrifuging for 7–10 h at more than 400000 *g*_{max} (Ragland *et al.* 1971). Tubes were cut below the sucrose layer interface. Membranes, which together with their attached ribosomes

rode above the interface, were decanted into clean tubes and centrifuged at $226000g_{\max}$ for 2 h. Supernatant above the membranous pellet was saved for assessment of the amount of unsedimented radioactive material, which usually represented 2–7% of the total amount applied. Unbound ribosomes occurred as pellets at the bottom of the 1.8M-sucrose layer. Ribosomal and membrane pellets were individually hydrolysed in formic acid (99%) and suspended in Scintisol-GP (Isolab Inc., Elkhart, Ind., U.S.A.) for scintillation counting on a Packard Tri-Carb scintillation spectrometer equipped for quench correction with automatic external standards or the automatic activity analyser.

Calculation of the percentage of applied ribosomes becoming membrane-bound (β) was made from membrane-associated radioactivity (M) floating on the 1.8M-sucrose layer, and the radioactivity of unbound ribosomes (P) in the pellet below the 1.8M-sucrose layer, by the equation $\beta = 100(M-C)/(M+P)$ where C is the radioactivity at the 1.8M–0.44M-sucrose interface of a control tube containing only postmicrosomal supernatant and an amount of labelled ribosomes equal to that in the experimental tubes. The necessity for C values was indicated by the 5–8% of total ribosomal radioactivity ($5.51 \pm 1.93\%$ for ^{32}P and $8.3 \pm 0.7\%$ for ^3H) remaining unsedimented above 1.8M-sucrose after 8–10 h of centrifugation at $405000g_{\max}$ in control tubes. The value for β for individual experimental tubes was determined from the value of C for the control tube accompanying them in each rotor. It should be noted that β is a percentage of the total sedimentable radioactivity and is independent of the amount of label not in the pellet.

RNA extraction and analysis. Membrane RNA was extracted by the following procedure. Frozen membrane pellets were suspended in 10mM-sodium acetate buffer, pH 5.1, to which sodium dodecyl sulphate (Matheson, Coleman and Bell, Inc., Norwood, Ohio, U.S.A.) was added to a final concentration of 1% (w/v) before mixture with bentonite (Fisher Scientific Co., Fairlawn, N.J., U.S.A.) and cold redistilled phenol (2 vol. of a saturated solution to 1 vol. of suspension). The suspension was centrifuged at 20000 rev./min in an SS-34 Sorvall rotor for 30 min, and the aqueous overlayer was removed with a pipette and stored on ice. The acetate buffer without bentonite was added to the underlayer, and the mixture was recentrifuged for 15 min. Sodium dodecyl sulphate and bentonite were added together with an equal volume of phenol to the aqueous overlayer resulting from this centrifugation and the mixture was centrifuged for 15 min. The aqueous phase was collected and pooled with earlier aqueous phases, and the nucleic acid was precipitated with 2 vol. of ethanol.

RNA extracted from membranes was analysed either on polyacrylamide gels or sucrose gradients. The 5 ml 10–40% (w/v) sucrose gradients (isokinetic and exponential; McCarty, Stafford & Brown, 1968) contained 10mM-sodium acetate buffer, pH 5.1, and were spun with sample for 2.75 h at $300000g_{\max}$ in a Beckman SW 50.1 rotor. Tubes were punctured at the bottom and 10-drop fractions collected. Polyacrylamide gels (2.6%) (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) cross-linked with bisacrylamide (Eastman) were prepared, and electrophoresis was performed by the method of Melera, Chet & Rusch (1970). The buffer consisted of 36 mM-

tris-HCl–30 mM- NaH_2PO_4 –1 mM-EDTA (disodium salt)–2% (w/v) of sodium dodecyl sulphate, pH 7.6 at 25°C. Gels were pre-run for 45 min before the sample was applied. Electrophoresis was performed at 6 mA/gel for 2.5 h, and the gels were scanned at 260 nm with a Gilford model 2400 spectrophotometer.

Analytical methods. Protein in membrane fractions was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin (fraction V; Sigma Chemical Co.) as standard. Sucrose interference with the colour reaction (Gerhardt & Beevers, 1968) was controlled by using 0.44M-sucrose in TKM medium in the blanks. The RNA content of polyribosomes or membranes was determined by the methods of Fleck & Munro (1962) and Munro & Fleck (1966) by using $E_{260}^{1\%}$ at 260 nm = 312. Polyribosomal RNA was also estimated by measuring E_{260} , where 25 mg of polyribosomal RNA/ml = 1.000 E_{260} unit (Ragland *et al.* 1971) and correction for the absorption at 260 nm by ferritin contaminating the polyribosomal suspensions was made as described by Wilson & Hoagland (1965).

Electron microscopy. Pellets or suspensions of membranes were fixed in cold 2% (v/v) glutaraldehyde in either 0.1M-sodium cacodylate buffer, pH 7.6, or 0.1M-potassium phosphate buffer, pH 7.6, for 1 h. After three washes in fresh buffer, the samples were post-fixed in 1% (w/v) osmium tetroxide in the cacodylate or the phosphate buffer. Both buffers contained 5% (w/v) of sucrose and 5 mM- MgCl_2 . All steps were performed at 0–4°C. Embedding was carried out by using the mixture of Epon 812 and Araldite 502 described by Mollenhauer (1964).

Amino acid incorporation in vitro. The incubation mixture contained 0.44M-sucrose–TKM (pH 7.4 at 37°C); 1 μmol of ATP and 0.25 μmol of GTP (Miles Laboratories, Inc., Kankakee, Ill., U.S.A.); 10 μmol of phosphoenolpyruvate and 30 μg of pyruvate kinase (Sigma Chemical Co.); 19 amino acids (0.05 μmol each) from Calbiochem; 0.05 μmol of [^{14}C]phenylalanine 350 mCi/mmol from New England Nuclear Corp., Boston, Mass., U.S.A.; 6 μmol of β -mercaptoethanol; 100 μg of membranes (measured as RNA); and 4 ml of freshly prepared postmicrosomal supernatant. The reaction mixtures were incubated for 30 min at 37°C in a total volume of 1 ml. The reaction was stopped with 2 ml of 10% (w/v) trichloroacetic acid. The precipitate was reprecipitated two times with 5% trichloroacetic acid, extracted two times with acetone–ether (3:1, v/v), and incubated for 30 min at 90°C in 5% trichloroacetic acid. All operations were performed in 12 ml centrifuge tubes. Precipitates were collected after each step by centrifuging at 2000 rev./min for 15 min. Formic acid (0.3 ml) was used to hydrolyse the pellets before scintillation counting.

RESULTS

Conditioning of rough-microsomal membranes with ribonuclease produced a preparation that accepted exogenously added polyribosomes (Fig. 1). A decrease in the membrane RNA/protein ratio accompanied the conditioning (Table 1), and, electron-microscopically, profiles of the conditioned rough microsomes showed no ribosomal images (Plate 1a). The binding of ribosomes to untreated

rough-microsomal or smooth-microsomal fraction or to ribonuclease-treated smooth-microsomal fraction was considerably less, at all membrane concentrations, than to conditioned rough-microsomal preparations (Fig. 1). High concentrations of ribonuclease-treated smooth-microsomal fraction non-specifically retained large numbers of particles, both because of particle trapping and because of rough-

Fig. 1. Binding of ribosomes at constant concentration with increasing amounts of membrane taken from four different preparations. ●, Ribonuclease-treated rough-microsomal membranes; ○, ribonuclease-treated smooth-microsomal membranes; ▲, rough-microsomal membranes; △, smooth-microsomal membranes. In all experiments membranes were treated with 100 μ g of polyribosomes in the presence of postmicrosomal supernatant at constant volume. Interval markings around each point represent the s.d. for ten or more experiments.

microsomal contamination of smooth-microsomal fractions, shown by the presence of RNA (Fig. 5). In Table 2 the results of a quantitative assessment of polyribosome trapping by conditioned rough membranes show that it is not a significant explanation of ribosome-membrane association.

The polyribosome-membrane complex did not dissociate during 10h. Complexes centrifuged on 1.8M-sucrose in TKM medium for 7–10h after their formation at 406000g_{max}. did not lose appreciable amounts of radioactivity. The RNA/protein ratios of these complexes (formed by interacting 0.5mg of membranes with 100 μ g of polyribosomes) were approx. 0.14, a value less than the average RNA/protein ratio of untreated rough-microsomal preparations (Table 1).

Electron microscopy performed on membrane-ribosome complexes confirmed that exogenously added polyribosomes were attached to the surfaces of ribonuclease-treated rough-microsomal membranes (Plate 1b–d). The material examined was

EXPLANATION OF PLATE I

Electron micrographs of rough-microsomal fractions treated with ribonuclease-EDTA before (a) and after (b–d) addition of exogenous polyribosomes. The membranes in (a) were fixed as pellets after two washes in 0.44M-sucrose-TKM medium to remove ribonuclease. Membranes (0.5mg) in (b–d) had been mixed with 100 μ g of polyribosomes in the presence of postmicrosomal supernatant, layered over 1.8M-sucrose in TKM medium and centrifuged at 408000g_{max}. for 8h. The membrane fraction above the heavy sucrose layer was fixed directly in glutaraldehyde. Thick arrows indicate unattached polyribosomes and the thin arrows point to polyribosomes only partially attached to the membranes. Magnification $\times 70775$.

Table 1. *Effects of removing residual RNA from conditioned membranes on their capacity to bind exogenous polyribosomes*

Binding was carried out with 0.5mg of membranes and 100 μ g of polyribosomes, and the values shown are means $\pm$ s.d. for four to ten experiments. RNA and protein concentration of the membrane preparation was measured before mixture with polyribosomes. Conditioning with pyrophosphate-citrate buffer was accomplished by the method of Ragland *et al.* (1971). Treatment of ribonuclease-conditioned rough membranes with pyrophosphate-citrate buffer or potassium citrate was carried out by resuspending pellets of conditioned membranes in 20–40 vol. of these media (pH adjusted to 7.1) with 20 homogenizer strokes.

Rough-microsomal preparations	Average RNA/protein ratio	Average percentage binding
No conditioning	0.202 $\pm$ 0.02	3.0 $\pm$ 0.2
Ribonuclease-EDTA conditioning	0.090 $\pm$ 0.04	79.6 $\pm$ 9.1
Pyrophosphate-citrate conditioning	0.032 $\pm$ 0.02	83.0 $\pm$ 0.5
Ribonuclease-EDTA conditioning then pyrophosphate-citrate (50 mM)	0.029 $\pm$ 0.05	69.9 $\pm$ 6.4
Ribonuclease-EDTA conditioning then 10% potassium citrate	0.025 $\pm$ 0.007	75.6 $\pm$ 10.8

Table 2. *Distribution of ^{32}P in fractions of reconstituted rough membranes after homogenization*

Initial distribution (A) involved the combination of 100 μg of polyribosomes and 0.5 mg of conditioned rough-microsomal membrane, mixed in the presence of postmicrosomal supernatant, and determination of radioactivity in the non-binding polyribosomal pellet, the bound 'membrane' fraction and the non-sedimentable 'supernatant' fraction. Procedure B involved resuspension of reconstituted rough membranes from procedure A with 20 homogenizer strokes and assessment of the displacement of radioactivity out of the membrane fraction into either the 'polyribosomal' or 'supernatant' fractions. Procedure C started with the reconstituted rough membranes that had gone through procedure B, which were homogenized with 20 strokes and then assessed for the distribution of radioactivity. The percentage of polyribosome radioactivity is the percentage of total label (c.p.m.) in each experiment that sedimented through 1.8 M-sucrose at 408 000 g_{max} after homogenization or as part of the initial distribution.

	Radioactivity (c.p.m.)				% of polyribosome radioactivity
	Total	Polyribosomal pellet	Membrane pellet	Supernatant	
(A) No homogenization	1685.9	219.6	1431.6	34.65	13.02
(initial distribution)	1663.3	254.4	1374.2	34.5	15.3
(B) First homogenization	1035.8	8.3	1014.9	12.6	1.2
(C) Second homogenization	713.4	43.8	672.6	0	6.1
	689.9	16.0	673.9	0	2.3

collected at the end of the 8 h centrifugation, which separated non-associating polyribosomes. To prevent distortion of the polyribosome-membrane combinations, membrane layers overriding the dense sucrose layer at the completion of centrifugation were transferred directly to fixative. In the sections inspected some polyribosomes were encountered only partially or segmentally attached to the membranes, giving the appearance of free loops or tails. Some polyribosomes appeared to be separate from membrane, and were probably the unsedimentable forms identified and assayed by the control binding tubes (see the Materials and Methods section). Perhaps because of compression during centrifugation, many membrane vesicles were seen to be collapsed into irregular shapes with extensive flattened surfaces. This permitted frequent surface views of membrane surfaces. Non-vesicular membranes were also observed in sections of conditioned rough membranes before their mixture with polyribosomes (Plate 1a), and thus these forms must arise during the process of conditioning with ribonuclease.

The stability of membrane binding sites themselves, in the absence of admixed ribosomes, was limited. A preparation of rough-microsomal fraction was treated with ribonuclease and stored at 0°C, and samples were withdrawn at various times for combination with ribosomes by using the standard binding procedures. The earliest exposure of sites to ribosomes was approx. 12 h after the livers were resected. The capacity of membranes to accept ribosomes declined at a rate the half-life of which was estimated from Fig. 2 to be 4.6 days. The RNA/protein ratio of the aging membrane

preparation remained at 0.06 throughout the 6 days of observation.

If membrane sites for polyribosome attachment were produced by membrane preparation or conditioning, then adventitious proteins of other substances isolated with membrane fractions, or added secondarily from postmicrosomal supernatant, were possible components of attachment sites. Therefore ribonuclease-conditioned rough membranes were treated with 150 mM-tris as described by Dallner, Siekevitz & Palade (1966) and Glaumann & Dallner (1968), and the capacity of the membrane to accept exogenous polyribosomes was tested as shown in Table 3. The protein content of these salt-treated membranes was approx. 81% of that of the parent ribonuclease-conditioned preparations and 43% of the rough-microsomal fraction. The capacity of these membranes to bind polyribosomes was similar to that found for conditioned membranes not treated with 150 mM-tris (Table 3). If 150 mM-tris was replaced by 500 mM-tris, binding to membranes was 74% of the exogenously added polyribosomes.

Inclusion of postmicrosomal supernatant in the binding mixture (Ragland *et al.* 1971) was designed to protect polyribosomes against ribonuclease attack. Supernatant also represented a possible source of factor(s) that could participate in polyribosome-membrane interaction. The percentage attachment of polyribosomes to membranes in the presence and in the absence of the supernatant is shown in Fig. 3. No supernatant requirement for interaction was demonstrable. Ribonuclease-conditioned membranes washed with 150 mM-tris were also able to accept polyribosomes to an extent that

Fig. 2. Aging rate at 0°C of the capacity of ribonuclease-conditioned microsomal membranes to accept ^{32}P -labelled ribosomes. From a large pool of conditioned membranes stored on ice, samples were withdrawn and their protein contents determined, and 0.5mg of the membranes was mixed with 100 μg of fresh polyribosomes in the presence of postmicrosomal supernatant. The percentage of accepted ribosomes was determined by using the standard procedures.

was not impaired by the absence of supernatant in the binding mixture (Table 3 and Fig. 3).

Since neither salt-removable factors nor any u.v.-absorbing material from postmicrosomal supernatant could be implicated in the polyribosome-binding capacity of conditioned membranes, the possibility that enzyme attack on the membrane by trypsin could alter this capacity was considered. Ribonuclease-conditioned rough membranes were incubated for 1h or 12h at 2–3°C with trypsin at concentrations (0.3mg/ml, or about 0.9mg/g of liver equivalent) in the range of those employed by others to release membrane-associated enzymes (Omura, Siekevitz & Palade, 1967;

Orrenius, Berg & Ernster, 1969; Kylenstierna, Nicholls, Hovmoller & Ernster, 1970), then washed with 0.44M-sucrose in TKM medium, and their capacity to bind polyribosomes tested. The results (Table 3) indicated an enzymic destruction of the binding sites. Trypsin treatment of unconditioned rough-microsomal fraction also damaged the sites, because it was found that subsequent treatment of the membranes with ribonuclease did not restore the binding capacity. Apparently the presence of ribosomes on the binding sites did not protect them against proteolysis.

During the procedure of conditioning rough-microsomal membranes to accept polyribosomes, the conditioning agents themselves, such as ribonuclease, could conceivably interact with membrane surfaces and serve as attachment points for polyribosomes. Conditioning involves suspension of rough-microsomal fraction in Mg^{2+} -free tris buffer containing 0.5mM-EDTA and ribonuclease (5–15 $\mu\text{g}/\text{ml}$ or 15–45 $\mu\text{g}/\text{g}$ of liver equivalent). Ribonuclease or EDTA acting alone did not produce membranes capable of accepting polyribosomes. Only when both were acting together on the membranes was a binding capacity produced. When ^3H -labelled ribonuclease (15 $\mu\text{g}/\text{ml}$) was used to condition rough membranes, approx. 4% (720ng/g of membrane protein) of the total applied radioactivity remained with the membranes through three washes in 0.44M-sucrose in TKM medium. Subsequent washing in 50mM-sodium pyrophosphate-citrate buffer, a procedure compatible with full binding capacity (Table 1), decreased the concentration of the added enzyme to 60ng/g of membrane protein. If the basically charged ribonuclease molecule positioned on the membrane played a role in attaching exogenous polyribosomes, use of increased amounts of enzyme for conditioning should be reflected in an increased percentage of bound polyribosomes. Fig. 4 shows that, even at very high enzyme concentrations (500 $\mu\text{g}/\text{ml}$), there was no marked increase in polyribosome attachment.

Although conditioning with ribonuclease cleared ribosomes from the surfaces of rough-microsomal membranes, some RNA remained in the preparations. Sucrose-density-gradient analysis of RNA from rough-microsomal and smooth-microsomal fractions (Fig. 5) showed the presence of 28S, 18S and 4–5S RNA species, which was corroborated by analysis on 2.6% polyacrylamide gels. RNA extracted from ribonuclease-conditioned rough-microsomal or smooth-microsomal membranes contained no 28S or 18S RNA, but instead a band in sucrose density gradients (Fig. 5) or polyacrylamide gels occurred in the approximate region of 2–10S. Neither analytical procedure resolved this band of small RNA species into any identifiable peaks. Differ-

Table 3. *Polyribosome-binding capacity and protein and RNA content of membranes treated with trypsin or high concentrations of tris buffer*

For evaluation of binding capacity, in all cases 0.5 mg of membranes was mixed with 100 μ g of 32 P-labelled polyribosomes in the presence of postmicrosomal supernatant (S3), unless otherwise specified. Binding values shown are the means $\pm$ s.d. Protein and RNA contents were determined on membranes before mixture with polyribosomes. Trypsin incubations were made at 2–3°C with 0.3 mg of enzyme/ml in a membrane suspension of 3 ml of 0.44M-sucrose in TKM medium/g of liver equivalent of membranes. For treatment with 150 mM-tris a pellet of conditioned membranes was suspended with 20–40 vol. of the tris (with 25 mM-KCl and 5 mM-MgCl₂) by 20 homogenizer strokes.

Rough-microsomal preparations	RNA content (mg/g of liver equivalent)	Protein content (mg/g of liver equivalent)	% attachment of 32 P-labelled polyribosomes
No treatment	1.801	9.32	9.3 $\pm$ 2.8
Ribonuclease-EDTA			
1 h	0.431	4.95	74.2 $\pm$ 8.6
12 h	0.512	5.83	79.0 $\pm$ 2.3
Trypsin (0.3 mg/ml)			
1 h	0.535	3.23	4.1 $\pm$ 0.1
12 h	0.451	1.33	5.8 $\pm$ 5.1
Ribonuclease-EDTA then trypsin			
1 h	0.554	3.35	2.8 $\pm$ 1.7
12 h	0.414	1.38	13.3 $\pm$ 3.8
Ribonuclease-EDTA and 105 mM-tris	0.426	4.03	69.1 $\pm$ 8.0 (with S3) 75.3 $\pm$ 3.2 (no S3)

Fig. 3. Influence of postmicrosomal supernatant on the association of polyribosomes and ribonuclease-conditioned (15 μ g/ml) rough-microsomal membranes. In all experiments various amounts of membrane were mixed with 100 μ g of either 32 P- or 3 H-labelled polyribosomes in a constant volume with (○) or without (●) postmicrosomal supernatant. Δ , Ribonuclease-conditioned membranes were washed once with 150 mM-tris buffer containing 25 mM-KCl and 5 mM-MgCl₂, and were mixed with polyribosomes in the absence of postmicrosomal supernatant. Each point represents an average of three to seven experiments, and the intervals above and below each point represent the s.d.

Fig. 4. Effects of ribonuclease concentrations used in the conditioning of rough-microsomal membranes on the binding of polyribosomes. Rough-membrane fractions were suspended with 20 strokes of a Potter-Elvehjem homogenizer at a concentration of 3 ml of 0.44M-sucrose in TK medium/g of liver equivalent (see the conditioning procedure in the Materials and Methods section). Ribonuclease, at a final concentration of 5 μ g/ml (---), 15 μ g/ml (—) or 500 μ g/ml (···), was added to membrane suspensions in the presence of 5.0 mM-EDTA. Incubation of all suspensions was performed for 1 h at 1–4°C with constant stirring. Polyribosomes were labelled with 32 P and were obtained from postmitochondrial supernatant by treatment with 1.25% sodium deoxycholate. The intervals above and below each point represent the s.d. of five experiments.

ences in specific radioactivities of 2–10S RNA derived from rough or smooth membranes, suggested in Fig. 5, were not reproduced on the gels.

Fig. 5. Profiles on sucrose density gradients of RNA extracted from rough-microsomal fractions (a); smooth-microsomal fractions (b); ribonuclease-conditioned rough membranes (c); ribonuclease-treated smooth membranes (d). See the Materials and Methods section for details. E_{260} (—); ${}^3\text{H}$ radioactivity (----).

If the 2-10S RNA retained by ribonuclease-conditioned rough membranes were playing a role in the interactions of the membranes with polyribosomes, its removal could lead to a decrease in the binding capacity of the membranes. The washing of ribonuclease-conditioned membranes with either 50mM-pyrophosphate-citrate buffer or 10% (w/v) potassium citrate decreased the residual RNA/protein ratio by more than one-third

(Table 1). The binding capacities of these membranes, however, were not appreciably diminished compared with unwashed ribonuclease-conditioned control membranes possessing much higher RNA/protein ratios (Table 1).

The influence of conditions, such as ionic composition, pH, temperature etc., in the incubation medium containing the mixture of polyribosomes and membranes requires some assessment. The

Fig. 6. Effect of Mg^{2+} and K^+ concentrations on the binding of polyribosomes to ribonuclease (15 μ g/ml)-conditioned membranes. In all experiments 0.5 mg of membranes was mixed with 100 μ g of 3H -labelled polyribosomes in a constant volume of 0.44 M-sucrose-tris buffer, pH 7.5 at 25°C. No postmicrosomal supernatant was present in the mixture. The K^+ and Mg^{2+} concentrations in the binding mixture were varied as indicated. The average binding of two to three experiments is shown for different K^+ concentrations with 5 mM- Mg^{2+} (●) and at 0.01 mM- Mg^{2+} (Δ). In an additional set of experiments polyribosomes were preincubated for 2 h at 2–3°C in various K^+ concentrations with a constant concentration of 5 mM- Mg^{2+} before mixture with conditioned membranes in media having the same ionic composition as that of the preincubation (○).

ionic environment normally employed for association studies *in vitro* was 50 mM-tris–25 mM-potassium chloride–5 mM-magnesium chloride. The result of altering K^+ and Mg^{2+} concentrations in the final binding mixture is shown in Fig. 6. The percentage of bound polyribosomes was virtually unchanged with K^+ concentrations of up to 100 mM, that of Mg^{2+} being constant at 5 mM. At higher K^+ concentrations the degree of polyribosome interaction with membrane diminished, reaching 12% at 500 mM. If the Mg^{2+} concentration was lowered to 0.01 mM, polyribosome binding was progressively diminished by K^+ concentrations up to 100 mM. With low Mg^{2+} concentration and 100 mM- K^+ only 14% of the exogenous polyribosomes were attached to membranes, compared with an average 81% at the same K^+ concentration and 5 mM- Mg^{2+} . The diminished polyribosome binding could have stemmed from ion-induced structural changes in the ribosomes rather than from an effect on interaction with the binding site. The nature of such an effect was examined in two different studies. In the first, polyribosomes were preincubated with

Table 4. Association at different temperatures of polyribosomes with ribonuclease-conditioned rough membranes

In all experiments 0.5 mg of membranes was made to react with 100 μ g of 3H -labelled polyribosomes in the presence of a constant amount of postmicrosomal supernatant. For incubations at 37°C the binding mixture without polyribosomes was initially placed in a 37°C water bath for 5 min and then polyribosomes were added. Incubation began at the time of addition of polyribosomes, and was terminated when tubes were transferred to an ice bath. Polyribosome association is given as the percentage bound.

Incubation time ... Temperature (°C)	Polyribosome association (%)			
	0 min	10 min	15 min	20 min
37	76.1	82.6	81.1 84.5	83.4
2–4	74.3	77.3	72.9	69.3

Table 5. Comparison of the incorporation of amino acids *in vitro* by rough-microsomal membranes and reconstituted rough membranes

Reconstituted rough membranes were assembled from conditioned rough membranes and polyribosomes (rate 5:1) in the presence of postmicrosomal supernatant and centrifuged over 1.8 M-sucrose for 14 h at 375 000 g_{max} . The membranes were homogenized in 1.36 M-sucrose, then centrifuged over 2 M-sucrose for 4 h at 375 000 g_{max} . Both reconstituted rough membranes and freshly prepared rough-microsomal membranes were added to the incorporation mixture as a suspension in 0.44 M-sucrose-TKM medium.

	RNA/protein ratio of membranes before incubation	Radioactivity (c.p.m./ μ g of RNA)
Fresh rough-microsomal fraction	0.191	181.5*
Reconstituted rough-membrane fraction	0.170	173.9*

* Average of 3 values.

different concentrations of K^+ for 2 h at 2–3°C, to reach approximate equilibrium with the various concentrations of potassium chloride, and then were transferred to a complete binding mixture at the same K^+ concentrations as those in the preincubation buffers. The binding of these preincubated polyribosomes to conditioned rough membranes was of the same magnitude as for the unconditioned preparations (Fig. 6). In the second

experimental approach, polyribosomes were pre-incubated in 50mM-tris-880mM-potassium chloride-12mM-magnesium chloride for 30min at 0°C, which converted them predominantly into mono-ribosomes (T. K. Shires, L. Narurkar, T. Ekren & H. C. Pitot, unpublished work). Material collected from this monoribosomal peak was mixed with membranes in the presence of 25mM-potassium chloride and 5mM-magnesium chloride and 45% of the particles were bound. This percentage, although below values obtained with polyribosomes in standard TKM medium, was well above values obtained with 500mM-potassium chloride (Fig. 1).

Temperature and pH were assessed as factors in polyribosome-membrane interaction by their direct variation in the binding mixture. The effect of altering the pH was studied in the range pH 6.9-7.8. The percentage of polyribosomes associated with conditioned rough membranes was 67.3-75.2% (polyribosome/membrane ratio, 1:5). The extent of interaction obtained between polyribosomes and membranes when incubated together at 2-4°C and 37°C is shown in Table 4. Neither the higher temperature nor the duration of incubation at 37°C had any demonstrable influence on association, compared with the degree of association found at 2-4°C.

To see if the ribosome-membrane products of combination *in vitro* resemble native rough membranes, the ability of the reconstituted rough membranes to synthesize polypeptide was measured. Because of the presence of some unattached and partially attached polyribosomes (Plate 1b-d), preparations of reconstituted rough membranes were homogenized and spun in dense sucrose to simultaneously remove unattached particles and membrane vesicles to which no particles had attached (Table 5). Comparison of the incorporative activity of freshly prepared rough-microsomal membranes and reconstituted rough membranes was based on their RNA content. The incorporative activities of the reconstituted and freshly prepared rough membranes was comparable (Table 5), indicating that the exogenously added polyribosomes carried their endogenous template to ribonuclease-conditioned rough membranes during binding and that adherence to the membrane surface did not interfere with their incorporative function.

DISCUSSION

Conditioning of rough-microsomal membranes with ribonuclease for acceptance of polyribosomes appears to be caused by ribonuclease action on membrane-bound ribosomes. Ribonuclease alone, incubated with rough membranes from rat liver, does not successfully generate a polyribosome-

binding capacity for the membranes, nor does it lead to release of endogenous ribosomal particles (Blobel & Potter, 1967a; Stahl & Bielka, 1966). Successful conditioning required ribonuclease treatment with low concentrations of Mg^{2+} , a situation described as heightening the susceptibility of intraribosomal RNA to nuclease attack (Weller, Schechter, Musgrave, Rougvie & Horowitz, 1968; Cox, 1969) and resulting in a more thorough degradation (Cox, 1969). Release of ribosomal proteins may be expected by ribonuclease action on rRNA (Santer & Smith, 1966; Cox, 1969). Electron-microscopic studies show that conditioning with ribonuclease removes all particulate material from the surfaces of rough-membrane preparations and have demonstrated that no particulate material sedimentable through sucrose at 308000g_{av.} for 8h (indicative of the degradation of endogenous ribosomes) is released by the conditioning procedure (T. K. Shires, L. Narurkar, T. Ekren & H. C. Pitot, unpublished work).

Although conditioning with ribonuclease effectively destroys the ribosomes as particles, a spectrum of low-molecular-weight RNA remains associated with the membrane. Much of this residual RNA can be removed without effect on the membrane binding capacity. Rough membranes conditioned with pyrophosphate-citrate (Ragland *et al.* 1971) or 4M-lithium chloride (Scott-Burden & Hawtrey, 1969) also are associated with residual RNA of small size. Some of the membrane RNA remaining after digestion by ribonuclease has been identified as small resistant fragments of rRNA (Ganguli, 1970). One specific type of RNA has been identified from residual nucleic acid, notably a DNA-like RNA collected from pyrophosphate-citrate-conditioned membranes (Lamar, Prival & Pitot, 1966). The extent to which residual RNA may be removed without destruction of the binding capacity was indicated by the work of W. L. Ragland (personal communication), who obtained membrane RNA/protein ratios of less than 0.01 by using repeated pyrophosphate-citrate washing; this is an RNA content well below that of the smooth-membrane fractions (Ragland *et al.* 1971; Moyer *et al.* 1970). Moreover, membranes, the binding capacity of which has been destroyed by trypsin, still have considerable amounts of RNA. However, although much of the residual RNA apparently has little significance for the acceptance capacity of conditioned membranes, some type of RNA could be imagined whose position on the membrane is trypsin-sensitive but salt-resistant, and which could provide ligands for polyribosomes. Binding sites on ribonuclease-conditioned membranes were stable at the high ionic strengths of 150-500mM-tris buffers, 50mM-pyrophosphate-

citrate buffer or 10% potassium citrate, just as were the sites on rough-microsomal membrane fractions conditioned with 50mM-pyrophosphate-citrate buffer (Suss *et al.* 1966; Ragland *et al.* 1971) or with 4M-lithium chloride (Khawaja & Raina, 1970). Consequently, there is no evidence that adventitious material, such as ribonuclease from the conditioning process or the postmicrosomal supernatant of the binding mixture, comprises an indispensable constituent of the binding site. On the other hand, the binding capacity of conditioned rough membranes was destroyed by incubation with trypsin, an attack that under the conditions employed involves membrane surface components (Lust & Drochmans, 1963; Omura *et al.* 1967; Ito & Sato, 1969; Orrenius *et al.* 1969; Kuylenstierna *et al.* 1970). Thus polyribosome binding sites may be considered as surface features, the trypsin-sensitivity of which was caused either by attack on protein immediately accessible to the enzyme or by some other binding substance bound to the conditioned rough membrane by accessible protein.

The observation that substances such as adsorbed proteins or removable fragments of RNA, that are adventitious to conditioned rough membranes, have no demonstrable role in attaching polyribosomes is evidence that the binding sites are not generated by the conditioning procedures. Instead, the sites must be considered as pre-existing on rough-microsomal membranes at the time of isolation. In displacing ribosomes from rough membranes, the conditioning process uncovers the sites, making them available for interaction with exogenous polyribosomes. Especially important to this view was the observation that the polyribosome-binding capacity was not characteristic of untreated rough-microsomal fraction or conditioned smooth-microsomal preparation, but only of rough-microsomal fraction stripped of endogenous ribosomes.

Successful conditioning has been accomplished by a variety of agents (e.g. 50mM-pyrophosphate-citrate buffer, 4M-lithium chloride and ribonuclease) that probably have quite different individual modes of action in clearing rough-membrane surfaces (Ragland *et al.* 1971; Scott-Burden & Hawtrey, 1969). Where direct comparison is possible, the binding characteristics of rough membranes conditioned with different agents are quantitatively similar with respect to their capacity to accept polyribosomes and the lability of their binding sites (see Ragland *et al.* 1971). Ribosomal interaction with rough-microsomal fraction treated with aflatoxin or EDTA has been reported under incubation conditions and assay methods different from those used by us (Williams & Rabin, 1969; James *et al.* 1969). Previously described bind-

ing capacities of bacterial and eukaryotic smooth membranes (Aronson, 1966; Coleman, 1969; Burka & Schickling, 1970), including those needing the presence of steroid (James *et al.* 1969; Williams & Rabin, 1969; Blyth *et al.* 1971; Sunshine *et al.* 1971), differ from those on conditioned rough membranes of liver in reportedly requiring incubation at 25–37°C for interaction with ribosomes to be achieved. The significance of this difference is unknown.

The influence of ionic conditions, but not of pH or temperature, on polyribosome binding suggests an electrostatic rather than an enzymic mechanism of interaction. Other investigators have also obtained evidence for the importance of an ionic component in ribosome-membrane interaction. The attachment of polyribosomes *in vitro* to membranes isolated from *Bacillus amyloliquifaciens* was shown by Coleman (1969) to be inhibited by higher concentrations of K⁺. In Coleman's (1969) system binding was decreased to about 5% with 100mM-K⁺, whereas in the system containing liver membranes 500mM-K⁺ was required to decrease binding to 15%. Although the higher K⁺ concentrations used in Coleman's (1969) experiments did not alter bacterial polyribosome profiles in sucrose density gradients, rat liver polyribosomes do display a conversion into smaller sizes in K⁺ concentrations that gave a maximum decrease in binding (Martin & Wool, 1969; Lawford, 1969; T. K. Shires, L. Narurkar, T. Ekren & H. C. Pitot, unpublished work). This change alone does not explain K⁺ inhibition of binding to conditioned rough membranes from rat liver, since ribosomal particles obtained from rat liver polyribosomes by 880mM-K⁺ were bound nearly as well as polyribosomes when incubation with membranes was carried out in a medium containing 25mM-K⁺ (T. K. Shires, L. Narurkar, T. Ekren & H. C. Pitot, unpublished work).

Schlessinger, Marchesi & Kwan (1965) and Khawaja & Raina (1970) have demonstrated a role of Mg²⁺ and/or spermidine in the interaction of ribosomes with bacterial and rat liver conditioned rough membranes. In our studies decreases in Mg²⁺ concentrations did not themselves lead to major decreases in binding. Such a decrease was achieved only when the K⁺ concentration was also increased, indicating the importance to binding of the K⁺/Mg²⁺ concentration ratio, rather than the absolute concentration of either ion. Some aspects of polyribosome and ribosome structure also depend on the relative concentrations of K⁺ and Mg²⁺ (Bonanou, Cox, Higginson & Kanagalingam, 1968; Coleman, 1969; Lawford, 1969; Martin & Wool, 1969; Nolan & Arnstein, 1969), but whether this dependence is involved in attachment to conditioned rough membranes has not been ascertained.

The ionic interaction between ribosomes and conditioned rough membranes observed *in vitro* very probably is not the sole mode of connexion between these two structures. Adelman, Blobel & Sabatini (1970) have suggested an ionic linkage between ribosomes and membranes from hepatic rough endoplasmic reticulum because of their separation in high concentrations of potassium chloride. However, the separation was considerably facilitated by addition of puromycin to the potassium chloride buffer, suggesting a second, possibly non-ionic, interaction between the aminoacyl-polypeptide and the membrane. Rat liver polyribosome preparations utilized in the present binding studies *in vitro* contained both nascent polypeptide chains as well as template activity (T. K. Shires, L. Narurkar, T. Ekren & H. C. Pitot, unpublished work). Either one of these ribosomal components could be involved in attachment *in vitro* without their role being apparent. Nonetheless a scheme may be warranted whereby new ribosomes are guided to their membrane sites by long-range electrostatic attraction, and thereafter, during translation, develop secondary links with the membrane through ribosome-associated molecules.

Evidence of the distribution of binding sites on conditioned membranes from the rough-microsomal preparations was seen in electron micrographs of the polyribosome-membrane complexes. Salient features of the ultrastructure of the complexes were the occurrence of polyribosome chains partially attached to membrane surfaces and the extensive cross-sectional surfaces apparently uncombined with ribosomes. Widespread absence of sites would explain the extensive areas of membrane surface not interacting with ribosomes, and also explain why the RNA/protein ratios of membrane-polyribosome complexes do not reach the ratio for rough-microsomal membranes. Site removal could result from physical damage during preparation, aging *in vitro* or inversion of membrane surface (Ragland *et al.* 1971). The final distribution of sites demonstrable *in vitro* is probably also determined by the original patterned distribution of sites on rough endoplasmic reticulum *in vivo* (Pitot, 1969). During the procedure of deriving microsomal vesicles from rough endoplasmic reticulum, disruption of the native topographic patterns of binding sites could be expected, and it would be expected that new patterns on the membrane remnants would be unable to accommodate whole chains of many larger polyribosomes.

This work was supported in part by grants from the National Cancer Institute (CA-07175), the National Institutes of General Medical Sciences (5-T01-GM-00130) and the American Cancer Society (P-314), and by a fellowship from the International Atomic Energy Agency to L.N.

REFERENCES

- Adelman, M. R., Blobel, G. & Sabatini, D. (1970). *J. Cell Biol.* **47**, 3a.
- Anfinson, C. B., Redfield, R. R., Choate, W. L., Page, J. & Carrol, W. B. (1954). *J. biol. Chem.* **207**, 201.
- Aronson, A. (1966). *J. molec. Biol.* **15**, 505.
- Blobel, G. & Potter, V. R. (1966). *Proc. natn. Acad. Sci. U.S.A.* **55**, 1238.
- Blobel, G. & Potter, V. R. (1967a). *J. molec. Biol.* **26**, 293.
- Blobel, G. & Potter, V. R. (1967b). *J. molec. Biol.* **28**, 539.
- Blyth, C., Freedman, R. B. & Rabin, B. R. (1971). *Nature, Lond.*, **230**, 137.
- Bonanou, S., Cox, R. A., Higginson, B. & Kanagalingam, K. (1968). *Biochem. J.* **110**, 87.
- Burka, E. R. & Schickling, L. F. (1970). *Biochemistry, Easton*, **9**, 459.
- Coleman, G. (1969). *Biochem. J.* **112**, 533.
- Cox, R. A. (1969). *Biochem. J.* **114**, 753.
- Dallner, B., Siekevitz, P. & Palade, G. E. (1966). *J. Cell Biol.* **30**, 97.
- Fleck, A. & Munro, H. N. (1962). *Biochim. biophys. Acta*, **55**, 571.
- Ganguli, P. K. (1970). *Revue can. Biol.* **29**, 19.
- Gerhardt, B. & Beevers, H. (1968). *Analyt. Biochem.* **24**, 337.
- Glaumann, H. & Dallner, G. (1968). *J. Lipid Res.* **9**, 720.
- Ito, A. & Sato, R. (1969). *J. Cell Biol.* **40**, 179.
- James, D. W., Rabin, B. R. & Williams, D. J. (1969). *Nature, Lond.*, **224**, 371.
- Khawaja, J. A. & Raina, A. (1970). *Biochem. biophys. Res. Commun.* **41**, 512.
- Kuylenstierna, B., Nicholls, D. G., Hovmoller, S. & Ernster, L. (1970). *Eur. J. Biochem.* **12**, 419.
- Lamar, C., Prival, M. & Pitot, H. C. (1966). *Cancer Res.* **26**, 1909.
- Lawford, G. R. (1969). *Biochem. biophys. Res. Commun.* **37**, 143.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Lust, J. & Drochmans, P. (1963). *J. Cell Biol.* **16**, 81.
- McCarty, K. S., Stafford, D. & Brown, O. (1968). *Analyt. Biochem.* **24**, 314.
- Martin, T. E. & Wool, I. (1969). *J. molec. Biol.* **43**, 151.
- Melera, P., Chet, I. & Rusch, H. P. (1970). *Biochim. biophys. Acta*, **209**, 569.
- Mollenhauer, H. H. (1964). *Stain Technol.* **39**, 111.
- Moyer, G. H., Murray, R. K., Khairallah, L. H., Suss, R. & Pitot, H. C. (1970). *Lab. Invest.* **23**, 108.
- Munro, H. N. & Fleck, A. (1966). *Analyst, Lond.*, **91**, 78.
- Nolan, R. D. & Arnstein, H. R. V. (1969). *Eur. J. Biochem.* **10**, 96.
- Omura, T., Siekevitz, P. & Palade, G. E. (1967). *J. biol. Chem.* **242**, 2389.
- Orrenius, S., Berg, A. & Ernster, L. (1969). *Eur. J. Biochem.* **11**, 193.
- Pitot, H. C. (1969). *Archs Path.* **87**, 212.
- Ragland, W. L., Shires, T. K. & Pitot, H. C. (1971). *Biochem. J.* **121**, 271.
- Robinson, G. B. (1969). *FEBS Lett.* **4**, 190.
- Santer, M. & Smith, J. R. (1966). *J. Bact.* **92**, 1099.
- Schlessinger, D., Marchesi, V. T. & Kwan, B. C. K. (1965). *J. Bact.* **90**, 456.

- Scott-Burden, T. & Hawtrey, A. O. (1969). *Biochem. J.* **115**, 1063.
- Stahl, J. & Bielka, H. (1966). *Acta biol. med. germ.* **17**, K5.
- Sunshine, G. H., Williams, D. J. & Rabin, B. R. (1971). *Nature, Lond.*, **230**, 133.
- Suss, R., Blobel, G. & Pitot, H. C. (1966). *Biochem. biophys. Res. Commun.* **23**, 299.
- Weller, D. L., Schechter, T., Musgrave, D., Rougvie, M. & Horowitz, J. (1968). *Biochemistry, Easton*, **7**, 3668.
- Williams, D. J. & Rabin, B. R. (1969). *FEBS Lett.* **4**, 103.
- Wilson, S. H. & Hoagland, M. B. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 600.