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A GEL FILTRATION APPROACH TO THE STUDY OF RIBOSOME-MEMBRANE INTERACTIONS

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Summary

1. Gel filtration on agarose can be used to investigate ribosome-membrane interactions without exposing the materials to the high, and possibly perturbing, hydrostatic pressures experienced during centrifugation procedures to separate free ribosomes from membrane vesicles.

2. After treatment of microsomes with degranulating agents, degranulated membranes are isolated from Sepharose 2B columns at the void volume, while displaced ribosomes elute at the total column volume. This provides a convenient method for monitoring degranulation in vitro.

3. Centrifugation of rough microsomes or ribosomes into dense pellets or layers, followed by resuspension, leads to preparations which will not pass rapidly or quantitatively through Sepharose 2B columns.

4. Methods are described for the isolation of degranulated microsomes and ribosomes which are eluted rapidly from Sepharose 2B at the void volume and total column volume, respectively. These materials are suitable for the investigation of ribosome-membrane binding in vitro, using a gel filtration separation to monitor binding.

5. Incubation of ^{3}H-labelled ribosomes with degranulated microsomes in vitro, leads to specific binding, demonstrated by the elution of the bound ribosomes at the void volume.

Introduction

The interaction of ribosomes with membranes of the endoplasmic reticulum is significant for the initial phases of synthesis and segregation of secreted proteins and probably in the regulation of protein synthesis. The nature of the interaction [1] and its functional role [2] have both been reviewed recently.

Although much current work emphasises the role of the nascent polypeptide chain in the attachment of ribosomes to membranes [3], it is still important to be able to define the components of the ribosome and membrane directly involved in their interaction. Accordingly, many groups have studied the binding of free or detached ribosomes to microsomal membranes in vitro. These studies have employed a variety of centrifugation methods for separating free ribosomes from membranes and membrane-bound ribosomes [4—11].

In this context, centrifugation methods suffer two disadvantages. Firstly, they are based on the rather small differences in density between free ribosomes and ribosomes attached to membranes. Secondly, to exploit these small density differences, centrifugation at high *g* for long periods is necessary and it is possible that the high hydrostatic pressures generated may perturb the ribosome-membrane interaction which is the subject for study. Numerous interactions between macromolecules or subcellular components have been shown to be sensitive to the hydrostatic pressures generated in ultracentrifugation conditions [12,13] and there have been several reports of ultracentrifugation-induced displacement of bound ribosomes from rough microsomes (see e.g. Ref. 14).

To avoid such problems we have investigated the possibility of using gel filtration to separate free from bound ribosomes. Agarose gels are available with exclusion limits in the range $10—50 \cdot 10^6$. Such gels should, in principle, exclude microsomal membrane fragments but retard ribosomes, ribosomal subunits and small polysomes. Tangen et al. [15] showed that when rat liver post-mitochondrial supernatant is applied to a Sepharose 2B column, microsomes are eluted at the void volume well separated from soluble proteins. We here show that the method can separate free from membrane-bound ribosomes and can be used to study in vitro both displacement of ribosomes from membranes (degranulation) and the association of free ribosomes with membranes.

Materials and Methods

Sepharose 2B was obtained from Pharmacia (GB) Ltd.,London,W5, U.K. Radiochemicals, [5-^{3}H]orotate (1.0 Ci/ml and 23 Ci/mmol) and [^{3}H]puromycin dihydrochloride (1.0 Ci/l and 3.5 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. PCS Solubiliser was obtained from Hopkins and Williams, Chadwell Heath, Essex, U.K.

The Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. provided the following: bovine serum albumin fraction V and fatty acid-free fraction V, neuraminidase from *Clostridium perfringens* type V (80% protein, 0.5 U/mg protein), phospholipase A from *Naja naja* venom (70% protein, 1000 units/mg protein) and phospholipase C from *Bacillus cereus* (50% protein, 120 U/mg protein).

All other Chemicals were Analar grade from BDH Chemicals Ltd., Poole, Dorset, U.K.

Sprague-Dawley male albino rats, body weight 100—200 g were used throughout.

Buffer solutions. All buffers were based on Tris brought to pH 7.5 at 20°C with HCl. The S-Tris buffer was 50 mM Tris-HCl containing 0.25 M sucrose,

STKM buffer was 50 mM Tris-HCl containing 0.25 M sucrose, 25 mM KCl and 5 mM $MgCl_2$; HTKM buffer was 100 mM Tris-HCl containing 100 mM KCl and 5 mM $MgCl_2$.

Subcellular fractionation. Postmitochondrial supernatant was prepared from rat liver by the method of Blobel and Potter [16]. Rough microsomes were prepared by centrifuging postmitochondrial supernatant on a discontinuous sucrose gradient [17]. To prepare microsomes, postmitochondrial supernatants were either eluted on Sepharose 2B [15] or centrifuged at 100 000 $\times g_{av}$ for 90 min at 4°C, the pellet being gently resuspended in buffer using a ground glass hand homogeniser. Pellets of microsomes were stored frozen at −20°C.

Degranulation. Suspensions of microsomes in STKM were degranulated by the following methods: by incubation either with 0.5 M KCl and 0.5 mM puromycin [8], or with 2 M LiCl [18] or with 5 mM EDTA and ribonuclease [19], or by dialysis against 10 mM EDTA [20].

Radioactive labelling and counting. Ribosomal RNA was labelled in vivo by giving intraperitoneal injections of approx. 100 μCi [5-^{3}H]orotate in 0.5 ml 0.9% saline to rats of 100 g body weight. The rats were allowed free access to food and water and were killed after 17—22 h.

For counting, radioactive samples were diluted to 1 ml with buffer and mixed vigorously with 9 ml PCS solubiliser. Duplicate samples were counted once on a Packard Tricarb Liquid Scintillation Spectrometer model 3375. The results were corrected for background and showed an efficiency for ^{3}H of 25—30%.

Gel filtration on Sepharose 2B. Sepharose 2B, supplied as a thick suspension in distilled water was diluted with buffer and equilibrated at room temperature with several changes of buffer for 24 h. After packing, columns were equilibrated at 4°C and all further work was performed at this temperature. Columns were obtained from Wright Scientific Ltd., Kenley, Surrey, U.K. and were of two sizes. Small columns (15 × 1.0 cm) were packed to a height of 12.5 cm and were used exclusively for analytical studies of the binding of ribosomes following incubation with membrane fractions. Samples of up to 2 ml were eluted at 2—3 ml/h under suction (Watson-Marlow No. 2 peristaltic pump) and 0.5 ml fractions were collected. Large columns (45 × 1.6 cm) were packed to a height of 30 cm and were used for all other studies (preparation of samples, degranulation experiments). Samples of up to 12 ml were eluted at approximately 5 ml/h using a constant hydrostatic pressure, or under suction, and 1.0 ml fractions were collected. An LKB Ultro-Rac 7000 was used for fraction collection. Large columns were re-used several times, but the small columns were re-packed with fresh gel after each run because of the difficulty of removing radioactivity completely from the gel.

Preparation of materials for ribosome-binding experiments. (a) Elution procedure for preparation of degranulated microsomal membranes. Fresh pellets of microsomes were resuspended in STKM and then degranulated using KCl and puromycin [21]. The suspension was diluted with a half volume of 50 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 5 mM $MgCl_2$, 2.25 M KCl and 3 mM puromycin. The microsomal concentration was adjusted so that the incubation mixture gave A_{260} = 50—75, and the mixture was stirred gently at room temperature for 90—120 min. The mixture was then applied to a large

Sepharose column equilibrated with HTKM and eluted in this buffer. Maximal loading volume was applied in order to maximize the concentration of eluted fractions. The most concentrated turbid void peak fractions were combined to give a stock suspension of degranulated microsomes. It was important that the microsomal pellets were homogenised gently by hand and incubated with KCl/puromycin immediately after preparation; otherwise aggregation of the material was observed on the column, as is common after pelleting of material (see below). For control experiments, fresh pellets of microsomes were resuspended directly in HTKM to give a control preparation of untreated microsomes. Both degranulated and control microsomal suspension were stored at 4°C with gentle stirring and were used within one week.

(b) Sucrose gradient procedure for preparation of displaced ribosomes. Pellets of microsomes from [5-^{3}H]orotate-labelled animals were stored at −20°C and thawed on ice, before being degranulated by the large-scale KCl/puromycin procedure described in the previous paragraph. The freezing and thawing promoted microsomal aggregation and optimized the subsequent separation of ribosomes from the degranulated microsomes [21]. Samples of the incubation mixture (0.75 ml) were layered onto 4.4-ml linear sucrose gradients consisting of 15—30% sucrose (w/v) in buffer (50 mM Tris-HCl, pH 7.5 containing 5 mM $MgCl_2$ and 750 mM KCl). The gradients were centrifuged at 20°C in a 6 × 5.5 ml swing-out rotor on an MSE Prepspin centrifuge at 40 000 rev./min (150 000 × g_{av}) for 100 min. Each gradient was withdrawn by suction from the bottom of the tube, using a peristaltic pump (Watson-Marlow No. 7), and passed through an LKB 8300 Uvicord II analyser, set at 254 nm and connected to an LKB 6520 six-channel chopper-bar recorder. Fractions (0.2 ml) were collected on an LKB UltroRac 7000 and those containing high concentrations of ribosomal material (monomers and subunits) were combined and dialysed overnight at 4°C against 500 ml HTKM. The dialysed suspension was stored at 4°C with gentle stirring and was used within a week. The yield was approximately 7 ml, $A_{260} = 6.8$, $A_{260} : A_{280} = 1.6—1.7$ and cpm/$A_{260} = 7000$. Modifications introduced in later experiments improved the recovery. Samples (3 ml) of incubation mixture were loaded onto 20-ml linear gradients (15%—30%) and the tubes were spun in a 3 × 25 ml swing-out rotor on an MSE SS65 centrifuge at 30 000 rev./min (95 000 × g_{av}) for 150 min. Each gradient was displaced from the top of the tube and analysed at 260 nm, using a density gradient scanner model 2480 attached to a Gilford 240 spectrophotometer. The yield was approximately 8 ml, $A_{260} = 8—10$, $A_{260} : A_{280} = 1.8—1.9$.

Ribosome-binding incubations. Membranes, prepared as in (a) above, and ^{3}H-labelled ribosomes, prepared as in (b) above were mixed, diluted to 2.2 ml with HTKM if necessary, and were then incubated with gentle stirring at 4°C for 30 min. A 2.0 ml sample was eluted on a small Sepharose column for analysis of binding and the remainder was retained for chemical and radioactive determinations.

'Carrier' ribosomes. In some cases, retarded fractions containing displaced ribosomes were collected from the gel filtration runs used for the preparation of degranulated microsomes. These unlabelled ribosomes were pooled and used as 'carrier' ribosomes in some experiments to dilute ^{3}H-labelled ribosomes

isolated by the linear sucrose gradient procedure.

Enzyme treatments of microsomes. Phospholipases A and C were dissolved in 50 mM Tris-HCl, pH 7.5 containing 2 mM $CaCl_2$ and pretreated to inactivate proteolytic impurities [22,23]. The treated enzyme solutions were stored at −20°C until required. Degranulated microsomes were incubated with neuraminidase, phospholipase A or phospholipase C in the presence of defatted bovine serum albumin. This was included to provide a preferential substrate for any remaining contaminating proteases [6] and to bind fatty acids released by phospholipase action [22]. The serum albumin was dissolved directly in the suspension of microsomes in HTKM (A_{280} = 12.4) at a concentration of 25 mg/ml. Enzyme was then added to give defined final enzyme concentrations. Each incubation mixture was stirred gently at 37°C for 30 min, before being cooled to 4°C for ribosome-binding incubation. No further action was taken to inactivate the enzymes.

Electron microscopy. Microsomal pellets were treated with 2.5% glutaraldehyde in 0.85 M cacodylate-HCl buffer, pH 7.4 for 2 h, washed with the same buffer for 30 min and then fixed in 1% osmium tetroxide solution for 2 h [24]. Pellets were dehydrated in solutions of increasing ethanol concentration, with three changes in absolute ethanol. Pellets were left overnight in 30% resin in absolute ethanol [25], followed by two changes of 100% resin for 24 h each. Finally, pellets were immersed in fresh 100% resin which was polymerised at 60°C for 18 h. Silver sections were cut on an LKB Ultratome and stained with uranyl acetate and lead citrate. Stained sections were examined in an AEI 801A electron microscope at 60 kV.

Assays. Protein was estimated by the Lowry procedure [26] using crystalline bovine serum albumin as standard. The ribosomal content of microsomes was estimated from the RNA : protein ratio [27], using the procedures and data described in [17]. An approximate estimate of microsomal concentration was obtained from absorption at 280 nm; a plot of A_{280} against concentration derived from Lowry assay gave A_{280} = 7—9 for 1 mg/ml protein. So microsomal quantity could be expressed in A_{280} units, i.e. the product of the measured A_{280} and the volume in ml. Similarly, ribosomal concentration was estimated from A_{260} and quantity was expressed in A_{260} units.

Results

(i) Use of gel filtration to monitor degranulation

In a previous short communication [20] it was shown that, if rough microsomes were treated with EDTA and then fractionated on Sepharose 2B, degranulated membranes could be obtained well-separated from displaced ribosomes. However, it was noted that considerable material aggregated on the top of the column and reduced the flow rate. We have subsequently tried to reduce this by minimizing the handling and storage of rough microsomes, but we find that any preparation based on pelletting of rough microsomes or spinning them on to a concentrated sucrose cushion to form a packed layer, subsequently leads to aggregation and poor column performance.

In the following work, therefore, unfractionated microsomes have been prepared without pelleting by eluting postmitochondrial supernatant through

TABLE I

DEGRANULATION OF TOTAL MICROSOMES BY VARIOUS TREATMENTS

Suspensions of total microsomes were degranulated (see Methods for details of the treatments) and then eluted in STKM, and the turbid void peak fractions were combined for analysis. Four control samples were incubated in STKM alone. Assuming RNA: protein ratios for ribosomes and for fully degranulated membranes of 0.36 and 0.03, respectively [17], the above control treatment produces 18% degranulation, while the treatments with EDTA and with KCl puromycin produce approx. 70% degranulation.

Treatment	RNA/protein	
	Before treatment	After treatment
EDTA	0.151	0.077
KCl/puromycin	0.115	0.058
Lithium chloride	0.151	0.093
EDTA/ribonuclease	0.115	0.107
Control (average values)	0.133 ± 0.021	0.114 ± 0.014

Sepharose 2B and collecting the turbid void peak fractions [15]. The resultant suspension re-eluted consistently without aggregation or reduction in flow rate, and was used as starting material in all further degranulation experiments.

Degranulation of this 'total' microsomal suspension was studied, monitoring degranulation by chemical assay, displacement of ^{3}H-labelled RNA and electron microscopy. The microsomal suspension isolated by gel filtration was subjected to test degranulations in various conditions and then re-eluted from Sepharose 2B for analysis. Material eluting at the void peak was pooled, and RNA : protein ratios determined. Electron microscopy of this pooled material confirmed the chemical evidence of degranulation.

Four methods of degranulation were compared. Table I shows RNA : protein ratios for microsomal suspensions and for void peak 'degranulated' microsomes following the various treatments. For every experiment a control non-degranulating incubation was carried out. Most degranulation was shown by treatment with EDTA and with KCl/puromycin. The latter treatment gave the biggest difference compared to its individual control and so was used thereafter for all degranulation treatments.

The effective degranulation and separation of degranulated membranes from displaced ribosomes were confirmed by analyses of the elution profiles using ^{3}H-labelled ribosomes. Rats were injected with [5-^{3}H]orotate, microsomes were isolated, treated with KCl/puromycin and fractionated on Sepharose 2B (Fig. 1a). Whereas protein (A_{280}) was equally distributed between void peak and retarded material, most of the radioactivity was associated with the retarded material, presumably as free ribosomes or subunits. By contrast when the same microsomes were incubated with buffer alone, the bulk of radioactivity was in the void peak corresponding to ribosomes bound to membrane vesicles. The RNA : protein ratio of the void peak after KCl/puromycin treatment was 0.055, whereas it was 0.107 after control treatment.

These findings show that gel filtration on Sepharose 2B can be used to monitor degranulation of microsomes and to prepare degranulated microsomes free from displaced ribosomes.

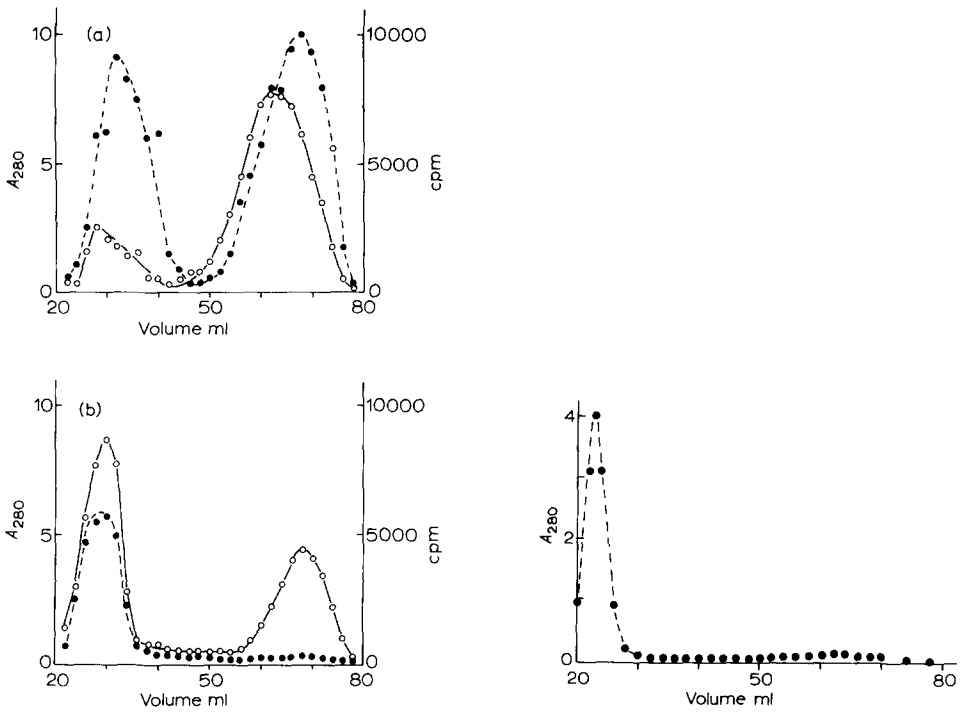

Fig. 1. Degranulation of ^{3}H-labelled total microsomes by KCl/puromycin treatment. ^{3}H-labelled total microsomes were incubated (a) with KCl/puromycin in STKM, and (b) in STKM alone. Aliquots containing 109 A_{280} units and $336 \cdot 10^3$ cpm were eluted in HTKM and monitored by A_{280} (●- - - - - -●) and cpm (○- - - - - -○).

Fig. 2. Elution behaviour of a stock preparation of degranulated microsomes. A stock suspension of degranulated microsomes was prepared by the large-scale gel filtration procedure described in Materials and Methods. An aliquot of the suspension containing 21 A_{280} units of degranulated microsomes was then re-eluted in HTKM; this elution was monitored by A_{280} (●- - - - - -●).

(ii) Preparation of materials for ribosome-binding studies

It was hoped that the gel filtration method could be extended to separate free ribosomes from membrane-bound ribosomes after 'reconstitution' incubations to characterize the ribosome-membrane interaction. This required ribosome and membrane preparations which were sufficiently concentrated, which flowed reproducibly on Sepharose 2B with membranes eluting at the void volume and ribosomes at the total column volume, and which were in buffer suitable for ribosome-binding studies (see below).

The basic degranulation procedure described in (i) gave preparations of degranulated membranes too dilute for this purpose. To obtain more concentrated stock degranulated microsomes, the initial gel filtration to isolate microsomes was replaced by centrifugation (see Materials and Methods). It was important to resuspend the microsome pellet and degranulate immediately, but with this precaution the material showed no visible aggregation on the column. Three preparations achieved greater than 95% degranulation with a final average RNA : protein ratio of 0.037. When such preparations of degranulated membranes were re-eluted immediately, 90% of the eluted material consistently

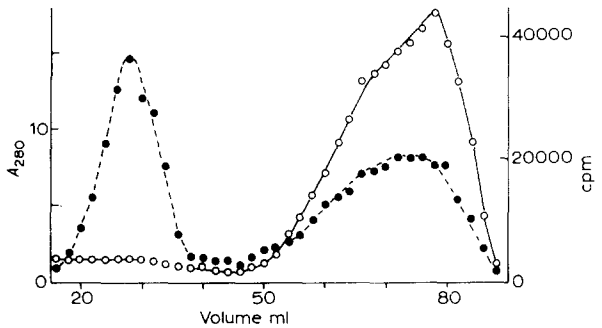

Fig. 3. Elution of total microsomes after treatment with KCl and [^{3}H]puromycin. A suspension of total microsomes was incubated with KCl and [^{3}H]puromycin in STKM as in a standard degranulation, and a sample containing 432 A_{280} units and 10.8 . 10^5 cpm was eluted in HTKM and monitored by A_{280} (●- - - - -●), and cpm (○- - - - -○).

appeared in the void volume (Fig. 2); there was minimal contamination with ribosomes or small proteins. Nor was there contamination with the puromycin used in degranulation; Fig. 3 shows that after degranulation with [^{3}H]puromycin, the label was almost entirely associated with the displaced material at the total column volume.

Degranulated microsomes could not be stored as frozen pellets because of subsequent aggregation [21] and we also found aggregation after storage of frozen suspensions. However, storage as a suspension at 4° C was satisfactory for periods up to a week and gave no visible aggregation in subsequent elutions. The procedure gave a good yield of extensively degranulated membranes whose high concentration, low contamination and consistent flow properties on Sepharose 2B columns made them suitable for ribosome-binding studies.

Displaced ribosomes isolated by gel filtration were also too dilute to be used in binding studies. Centrifugation of ribosomes through discontinuous sucrose gradients to yield a pellet could not be used since all resuspended ribosomal pellets showed considerable aggregation when re-eluted. This was avoided by isolating ribosomes by centrifugation on a linear sucrose gradient. Separation of ribosomes from degranulated microsomes was maximised by prior freezing and thawing of the microsomal suspension to promote aggregation of microsomal vesicles [21]. The complete procedure is described in Materials and Methods.

Ribosomes prepared thus were not contaminated with puromycin. Fig. 4 shows the preparative sucrose gradient after degranulation of unlabelled microsomes with [^{3}H]puromycin; the central peak of ribosomal material is well separated from the [^{3}H]puromycin near the meniscus. When [5-^{3}H]orotate-labelled microsomes were used the procedure gave a final, dialysed ^{3}H-labelled ribosome suspension of sufficient quantity and concentration to use in a series of reconstitution experiments.

When such a suspension of ^{3}H-labelled ribosomes was run alone on an analytical Sepharose 2B column, 90% or more of the eluted material was retarded, mainly appearing around the total volume column. However, total recovery from the column was low. Recovery of ^{3}H-labelled ribosomes could be

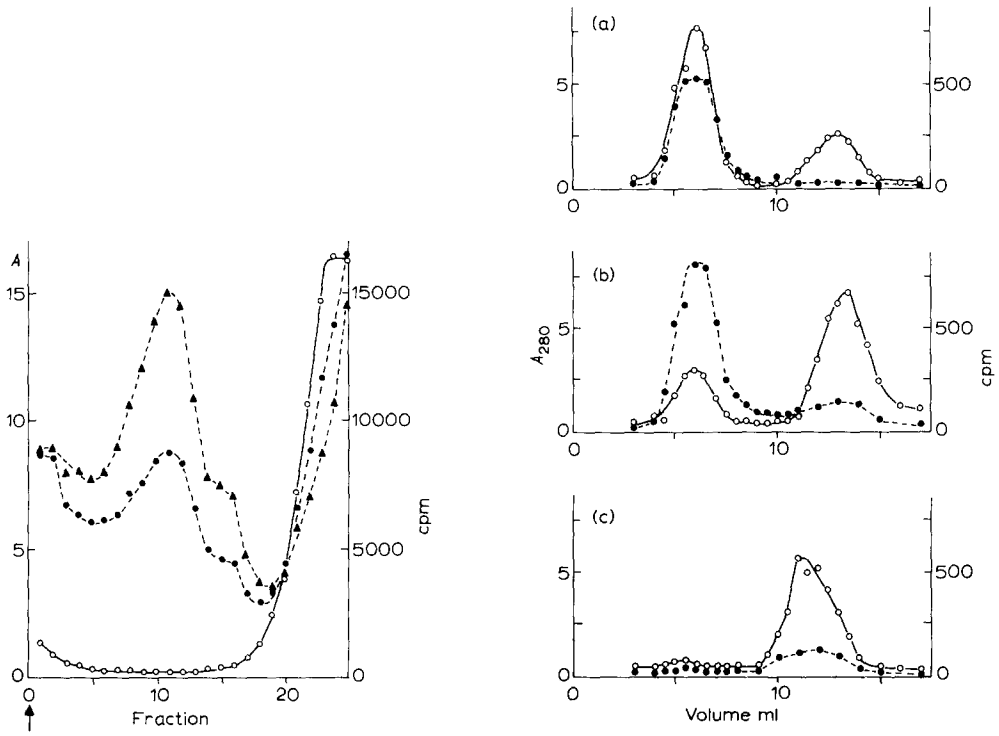

Fig. 4. Linear sucrose gradient after treatment of total microsomes with KCl and [^{3}H]puromycin. A suspension of total microsomes was incubated with KCl and [^{3}H]puromycin in STKM, and a sample containing 35 A_{280} units, 44 A_{260} units and 155 · 10^3 cpm was centrifuged on a 15—30% gradient in the same buffer. The gradient was then fractionated from the bottom of the tube and monitored by A_{260} (▲- - - - -▲), A_{280} (●- - - - -●) and cpm (○- - - - -○). The arrow indicates the position of the pellet.

Fig. 5. Analysis of 'reconstitution' incubations with ^{3}H-labelled ribosomes. Aliquots of a stock suspension of ^{3}H-labelled ribosomes were incubated with (a) degranulated microsomes; (b) total microsomes, and (c) carrier ribosomes. A sample of each incubation mixture was eluted in HTKM and monitored by A_{280} (●- - - - -●) and cpm (○- - - - -○).

increased by the addition before elution of cold 'carrier' ribosomes to increase the bulk of the sample.

(iii) Reconstitution studies, binding of ribosomes to membranes in vitro

The stock ^{3}H-labelled ribosome and membrane suspensions were prepared in HTKM buffer, and binding incubations were carried out in this buffer since artefactual ribosome-membrane binding has been reported at low K^+ concentrations [8,28]. Fig. 5 shows elution profiles of analytical column runs after incubation of ^{3}H-labelled ribosomes with various preparations. After incubation with degranulated microsomes (Fig. 5a) most of the labelled ribosomal material runs at the void peak together with the membrane protein. However, this is not observed when the incubation is carried out with control untreated microsomes (Fig. 5b); some labelled ribosomes run at the void peak but the majority are eluted at the total column volume. It should be noted that twice as much microsomal protein was taken for this control incubation than for that

TABLE II

ANALYSIS OF ELUTIONS AFTER BINDING INCUBATIONS

^{3}H-labelled ribosomes were incubated with degranulated microsomes (DGM) and the incubation mixture was then eluted in STKM. All the void peak fractions were combined for analysis, and the retarded peak fractions were treated likewise. In control incubations, ^{3}H-labelled ribosomes were incubated with total microsomes (TM) or non-radioactive carrier ribosomes (CR).

Membrane	Total A_{280} applied	Total cpm eluted		Void peak cpm/total cpm recovered (× 100%)
		Void	Retarded	
(a)				
DGM	8.4	3740	3390	52
TM	9.0	1320	4050	25
CR	9.7	240	2570	9
CR	9.9	360	5110	7
(b)				
DGM	21.1	2280	1010	69
DGM	19.1	5010	1110	82
DGM	19.6	3340	1460	70
TM	16.8	610	1140	35
TM	15.6	450	1660	21

with degranulated microsomes. When labelled ribosomes are incubated simply with a suspension of unlabelled ribosomes and then run on the Sepharose 2B column (Fig. 5c) practically all the labelled material runs close to the total column volume. These findings indicate that the gel filtration method can demonstrate specific binding of ribosomes to degranulated membranes. Free ribosomes alone run as small particles and are fully retarded by the column, and ribosomes incubated with untreated microsomes show a comparatively low level of binding of the ribosomes to vesicles which elute at the void volume. But this is evidently a nonspecific effect compared to the extensive binding to degranulated microsomes shown by the transfer of most labelled material from the total column volume to the void peak.

This transfer of labelled ribosomes from the total column volume to the void peak was used in further analysis of the binding phenomenon. The extent of ribosome binding can be expressed as the percentage of the total recovered radioactivity which is found in the void peak. Table II shows the results of several experiments in which labelled ribosomes were incubated with degranulated microsomes and control materials at various concentrations. Incubations with equivalent concentrations of degranulated microsomes, untreated microsomes and carrier ribosomes (Table IIa) show again preferential binding to the degranulated microsomes confirming the results of Fig. 6. The proportion of ribosomes bound increases when increased amounts of degranulated microsomes are included, whereas there is only a slight increase in the extent of binding to untreated microsomes (Table IIb). It seems clear that the gel filtration method can monitor the specific binding of ribosomes to sites unmasked on degranulated microsomes which are absent from untreated rough and smooth microsomes.

To test the method further, incubations were carried out with degranulated

TABLE III

BINDING INCUBATIONS WITH PRETREATED MEMBRANES

Aliquots of degranulated microsomes from a stock suspension were first incubated with an enzyme and then with an aliquot of ^{3}H-labelled ribosomes. After elution of the mixtures, the void and retarded peaks were analysed as in Table II. In the first experiment with degranulated microsomes no enzyme was present in the first incubation; in the other control experiment, an aliquot of ^{3}H-labelled ribosomes was incubated with non-radioactive carrier ribosomes only.

Treatment	Eluted cpm		Void peak cpm/total cpm recovered (× 100%)
	Void	Retarded	
No enzyme	1180	510	79
Neuraminidase (2 mg/ml)	1640	580	74
Phospholipase A			
4.4 μg/ml	260	810	24
44 μg/ml	1040	4800	18
Phospholipase C			
0.35 μg/ml	1880	740	72
3.5 μg/ml	650	900	42
Carrier ribosomes	60	1710	3

microsomes which had been pretreated in ways which might affect their ability to bind ribosomes. Aliquots of a stock suspension of degranulated microsomes were incubated with hydrolytic enzymes and then cooled to 4°C and incubated with added ^{3}H-labelled ribosomes; the mixtures were then eluted on analytical Sepharose 2B columns and analysed as before (Table III). Degranulated microsomes which had experienced no enzymic degradation bound 79% of the eluted ribosomal label. This high level of binding was only slightly reduced by neuraminidase treatment, but the phospholipase treatments significantly reduced the ability of degranulated microsomes to bind ribosomes, and this effect increased with the amount of phospholipase present in pretreatment. Jothy et al. [29], have also reported that phospholipase treatment reduces the ability of degranulated microsomes to bind ribosomes. In a control incubation with carrier ribosomes, only negligible amounts of labelled ribosomes appeared in the void peak, confirming that genuine binding of ribosomes to membrane vesicles was occurring in the other runs. These results show that the gel filtration method can be extended to analyse the requirements for ribosome-membrane interaction and the chemical nature of the components involved in the interaction.

Discussion

The interaction in vitro of ribosomes, polyribosomes and subunits with membranes derived from the endoplasmic reticulum has been studied extensively by centrifugation techniques. Reviews of the literature indicate that a wide variety of procedures has been used and that there is only a limited consensus about results [1,30]. In part the disagreements may arise from different artefacts affecting the results from different procedures; for example methods for separation of free ribosomes from ribosomes bound to membrane vesicles

by a rapid differential centrifugation may lead to considerable contamination of the vesicle pellet by physically entrapped 'free' ribosomes. On the other hand methods based on centrifugation to equilibrium in a density gradient have several drawbacks; the difference in density between ribosomes and the most dense rough membranes may be so small that a conventional layer of 1.8 M sucrose will not ensure complete resolution [8], the passage of slowly sedimenting dense ribosomes through layers of already equilibrated microsomes may allow artefactual binding in uncontrolled conditions [28] and it may be that partially degraded ribosomes equilibrate at densities where only membrane-bound ribosomes are expected. For example, McIntosh et al. [31,32] have shown clearly that incubation of ribosomes with smooth microsomes at 37°C leads to formation of ribosomal aggregates with density 1.39 g/ml which are not separated from smooth microsomes by conventional density gradient centrifugation and can thus lead to incorrect deductions about ribosome-membrane binding.

However, all these difficulties are in a sense technical and could possibly be overcome by careful controls and a correct choice of procedures. But centrifugation methods have the intrinsic drawback that the separation of free and bound ribosomes is carried out at high hydrostatic pressures which may influence the binding interaction. It has been shown that sea urchin ribosomes dissociate into subunits during ultracentrifugation [33]. The equilibrium is a function of pressure in the range 100—500 atm and the change in molar volume has been calculated. Harrington and Kegeles [13] quote a large number of macromolecular interactions which are known to be sensitive to pressure. Given the nature of the forces involved, the interaction between ribosomes and membranes would be expected to show a large change in molar volume and hence be sensitive to hydrostatic pressure. Several examples have been quoted of centrifugation-induced degranulation [14,34—37].

For this reason it is important to be able to check the results of studies on the ribosome-membrane interaction by a technique which avoids high pressures. Gel filtration using gels which exclude membrane vesicles but retard free ribosomes is an attractive approach. In a preliminary communication [20] we provided evidence that gel filtration on Sepharose 2B could be used to monitor degranulation of rough microsomes induced by EDTA. We have now confirmed and extended that conclusion. The problem of sample aggregation previously noted has been avoided by the use of microsomes themselves isolated by gel filtration [15]. Rough microsomes which have been densely packed by pelleting or by spinning on to a concentrated sucrose cushion are difficult to disperse into a fine homogeneous suspension; such conventional microsome suspensions invariably clog Sepharose 2B columns where the spaces between beads have a minimum diameter of approx. 10 μm. By contrast, microsomes prepared by gel filtration form a fine highly light-scattering suspension which flows reproducibly through such columns. Through the use of [5-^{3}H]orotate-labelled microsomes we have shown that this provides a reliable, convenient and artefact-free method for monitoring degranulation of microsomes in vitro.

The analysis of binding of ribosomes following in vitro incubation of ribosomes with membrane preparations presents more problems. Conventional methods for the preparation of degranulated membranes and ribosomes involve

isolation of the materials as pellets after centrifugation: such preparations do not flow freely on columns of Sepharose. The methods described here produce concentrated preparations of degranulated membranes and of displaced ribosomes without packing of the materials into dense pellets. The degranulated microsomes are themselves isolated by gel filtration and the ribosomes are isolated from the central region of a continuous sucrose density gradient centrifugation. The degranulated microsomes isolated thus elute reproducibly at the void volume from columns of Sepharose 2B. The ribosomes prepared by the technique described here appear only at the total column volume when eluted from Sepharose 2B, but the yield of material recovered from the column is variable and dependent on the presence of other components in the mixture loaded. This has made difficult extensive quantitative characterization of the ribosome-membrane binding phenomenon in terms of affinities, numbers of sites, etc. However the data obtained (Fig. 5a—c and Tables II and III) show that the method is capable of monitoring the binding of ribosomes to membranes in vitro and of detecting specific binding sites on degranulated microsomes. The method can therefore be used to investigate the structural requirements for binding and to check the results obtained using centrifugation methods to study the ribosome-membrane interaction.

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