MEMBRANE–RIBOSOME INTERACTIONS: ARTEFACTS RESULTING FROM THE TEMPERATURE-DEPENDENT FORMATION OF RIBOSOMAL AGGREGATES

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1. Introduction

Studies of the binding of ribosomes to smooth microsomes [1] and of in vitro protein synthesis by rough microsomes [2] have involved the incubation of subcellular fractions containing ribosomes at temperatures greater than 20°C. We report here that exposure to physiological temperatures can result in the generation of a mutual affinity between ribosomes that may lead to the formation of large aggregates. Both free and membrane-bound ribosomes appear to be able to participate in such aggregates, and the cross-reaction of such species in mixed systems can bring about an apparent and artefactual increase in microsomally-associated RNA.

2. Materials and methods

The animals used were male albino rats of the Sprague–Dawley strain of body weight 180 g. To radiochemically-label the liver RNA, each rat was injected in the peritoneal cavity 24 h before sacrifice with 50 μCi of [6-^{14}C]orotic acid (The Radiochemical Centre, Amersham, Bucks., UK) dissolved in 0.5 ml. of 0.9% saline. The animals were allowed food and water ad libitum, except where specifically stated.

2.1. *The preparation of post-mitochondrial supernatant (PMS)*

Death was by cervical dislocation. Livers were excised immediately and rinsed briefly in ice-cold preparative buffer, STKM. The composition of this buffer was 0.25 M sucrose (RNase-free grade from Cambrian Chemicals Ltd., Croydon, UK), 50 mM Tris(hydroxymethyl)amino methane (A. R., from B.D.H. Chemicals, Poole, Dorset, UK), 25 mM KCl and 5 mM $MgCl_2$, adjusted to pH 7.5 with A.R. hydrochloric acid at 20°C. When STKM is prefixed by a denomination such as 2.0 M STKM, the denomination refers to an adjusted concentration of sucrose. TKM refers to this buffer without sucrose.

After rinsing the excised liver, 2 ml of fresh STKM was added for every 1 g of liver. The liver was then chopped finely with scissors and homogenised in a Potter–Elvjehem apparatus using a motor-driven Teflon pestle rotated at 3500 rev/min in a glass vessel with a clearance of 7/1000 inch (0.18 mm). 20 passess were used to effect homogenisation.

Mitochondria, cell debris and nuclei were removed from the preparation by centrifugation of the homogenate in a fixed angle MSE 8 × 50 ml rotor at 12 500 rev/min for 20 min on the MSE Angle 18 centrifuge at 4°C.

2.2. *Scintillation counting*

Scintillation counting was performed in the Packard Tri-Carb Liquid Scintillation Spectrometer model 3375. The samples of various volumes were first made up to 5 ml by the addition of distilled water and then shaken with 5 ml of the commercial scintillation cocktail Instagel (from Packard Instruments International, Zurich). A ^{14}C-isotope quench curve was constructed by use of *n*-[1-^{14}C]hexadecane of very accurately known specific radioactivity (purchased from The Radiochemical Centre, Amersham). Operational counting efficiency was in the region of 75–80%.

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3. Results

When PMS was incubated at different temperatures for a period of 90 min prior to being submitted to centrifugation by the 'flotation' method illustrated in fig.1, in which membranous vesicles are displaced upwards from the 2.0 M STKM layer whilst free ribosomes are retained, pronounced differences were noted in the distribution of RNA between the microsomal 'bound' and the 'free' fractions of the spun tubes. Thus, the proportion of acid-insoluble [^{14}C]RNA associated with the combined microsomal fractions, the bound RNA, was considerably increased after the PMS had been incubated at 37°C relative to that observed after incubation at 0°C or 20°C (fig.2, table 1). A marked difference in the appearance of the spun tubes was also evident, the rough microsomal region being more intense in those tubes containing 37°C-treated PMS.

There are two fairly obvious possible explanations of these findings: (i) that free ribosomes became attached to microsomal vesicles during the course of incubation at 37°C but not at the lower temperatures; (ii) that the removal of microsomal vesicles from the 2.0 M STKM was more complete after incubation of the PMS at the higher temperature, with more rough vesicles remaining in the 2.0 M STKM in those tubes containing PMS incubated at 0°C or 20°C. This second possibility was rejected because phospholipid analysis of the 2.0 M STKM region of a tube containing PMS preincubated at 20°C did not detect a significant level of microsomal phospholipid, which would have been expected if the incomplete recovery of vesicles was involved.

It was concluded that incubation at 37°C had caused the association of a substantial proportion of the free ribosomes within the PMS with the microsomal vesicles. Electron microscopic examination of the PMS after incubation at 37°C revealed that all the ribosomes associated with the vesicles were bound in the form of very large aggregates (see fig.3A cf. 3B). 'Free' aggregates of ribosomes, unassociated with membrane, were also formed (fig.3C). It was observed also that microsomal vesicles in the PMS were often fused together through ribosome–ribosome bridges (fig.3D).

The formation of aggregates was also demonstrated by a further analysis. The membranes were dissolved in 1% Triton X-100 and the product analysed on a continuous sucrose gradient. This gradient was so designed that monoribosomes and polysomes smaller

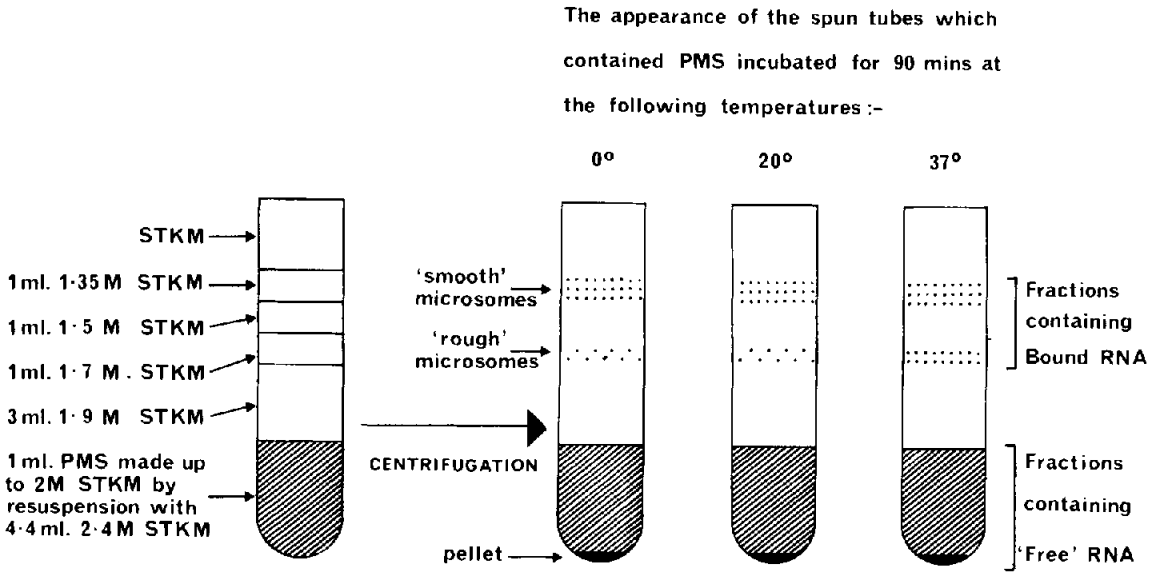

Fig.1. Separation by flotation of bound and free RNA. The 14 ml tubes were made up in duplicate and centrifuged for 22½ h in the MSE Swing-Out 40Ti Rotor at 35 000 rev/min (g_{av} = 150 000 g).

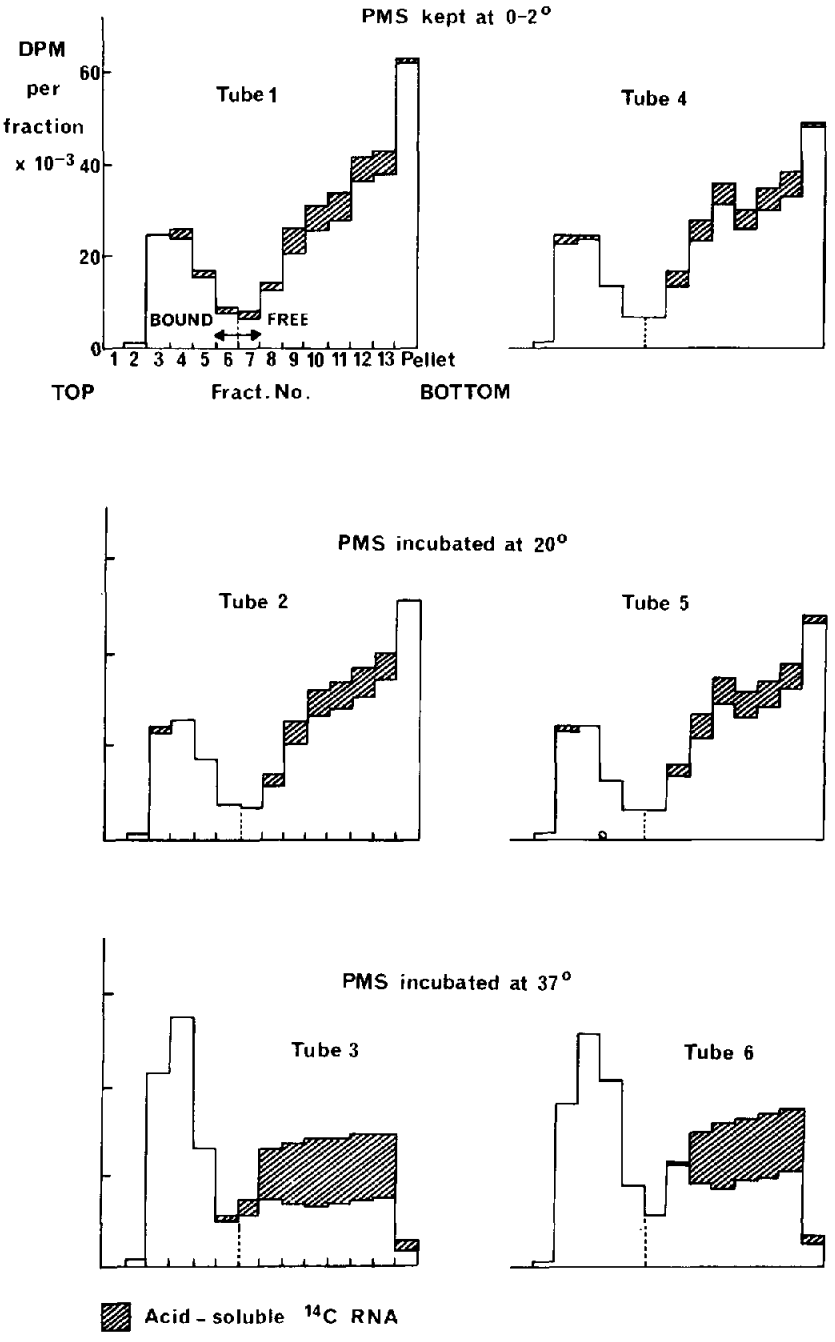

Fig.2. Distribution of [^{14}C]RNA in tubes containing various incubates of PMS submitted to the flotation procedure. 1ml fractions were carefully removed by aspiration with a small syringe from the top of the tubes after centrifugation as in fig.1. The pellets were removed with 5 × 1 ml. aliquots of double-distilled water and resuspended. Total DPM were estimated by counting aliquots of each fraction directly in the Instagel/water system. Acid-insoluble DPM were similarly estimated after precipitation of aliquots with ice-cold 5% TCA. The acid-soluble DPM in each fraction have been subtracted from the total DPM to yield the clear areas representing acid-insoluble DPM.

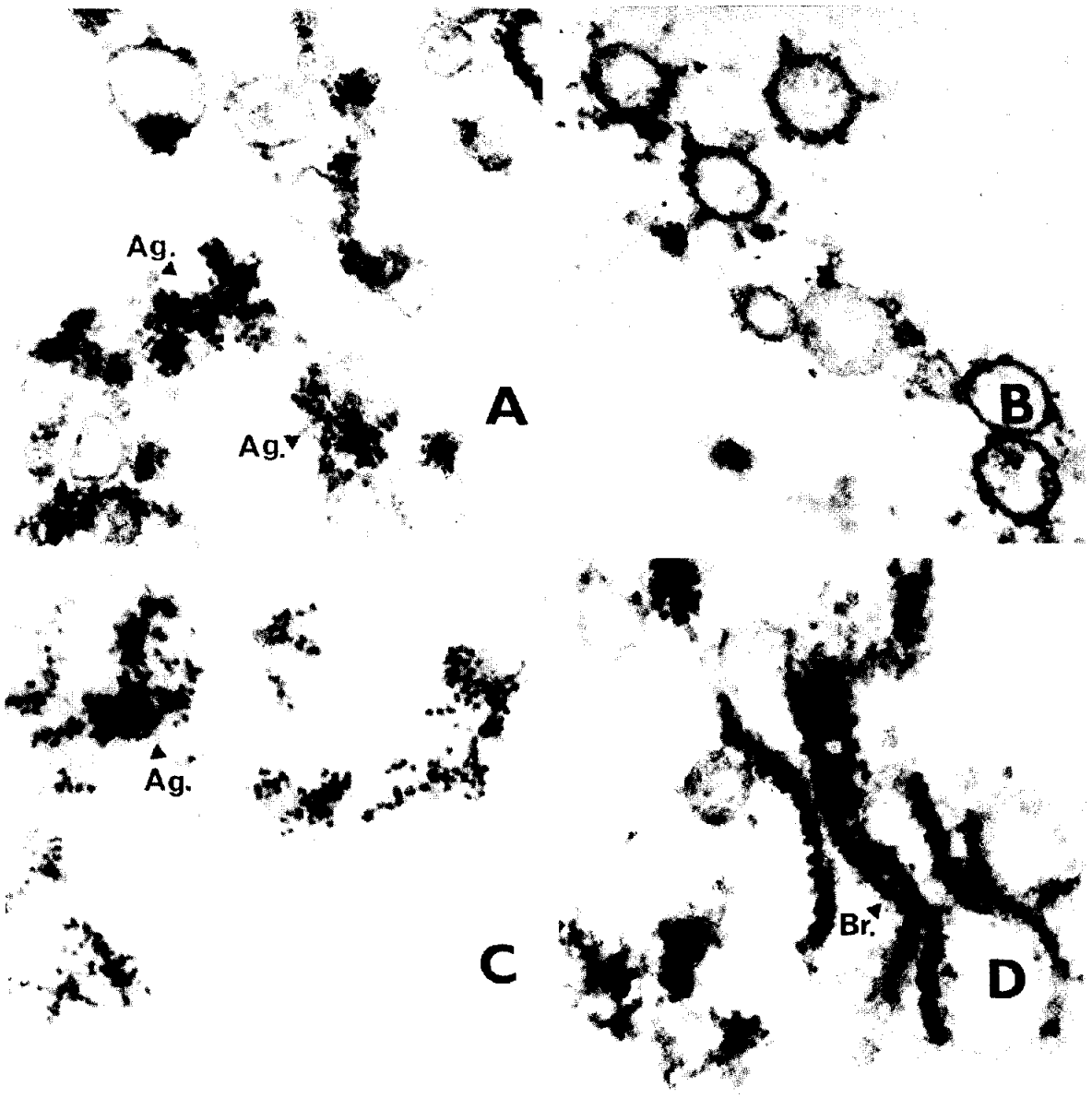

Fig.3. Electron micrographs of aggregated and control material. (A) PMS after incubation at 37°C for 90 min (× 50 000). (B) as in (A) incubated at 20°C (× 50 000). (C) The resuspended pellet from a spun tube containing 37°C-treated PMS (see fig.1) (× 64 400). (D) as (A) (× 72 000). [Ag.] refers to ribosomal aggregates. [Br.] refers to ribosome-ribosome bridges between fused vesicles. The samples were allowed to settle onto the surface of Millipore filters (0.22 μm) for 30 min in the cold. Excess suspension was discarded and the material adhering to the filters fixed with 4% aqueous OsO_4 for 30 min. After rinsing with distilled water and post-fixing with 12.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 30 min, the samples were left in cacodylate buffer overnight. They were then dehydrated in an ascending ethanol gradient and embedded into Spurr resin polymerised at 70°C overnight.

Table 1
Distribution of [^{14}C]RNA in the flotation gradients

Tube number	1	4	2	5	3	6
Temperature of incubation	←0°C→		←20°C→		←37°C→	
'Bound' RNA (F1–F6) (total microsomes)	24%	23%	25%	23%	57%	53%
'Free' RNA in 2M STKM (F7–F13)	55%	59%	57%	60%	41%	45%
'Free' RNA in pellet	21%	17%	18%	17%	2%	2%
Total 'free' (2 M STKM + pellet)	76%	76%	75%	77%	43%	47%

Results expressed as % of recovered acid-insoluble DPM
Data derived from the information expressed in fig.2.

than about 13 monomeric units in length (375S) are retained on the gradient and do not pellet. A marked shift to pelleting species was observed after incubation of the PMS at 37°C (table 2). Whereas monoribosomes and small polysomes were evident in PMS preparations incubated at 0°C or 20°C, they were almost entirely absent in the 37°C-treated PMS, demonstrating that virtually all the ribosomes in the PMS, both free and bound, participated in the aggregates.

It was found that even short incubations of PMS at 37°C resulted in the formation of aggregates, but that further aggregation took place as a function of time up to 60 min (fig.4). The tendency to form aggregates increased very sharply between 22°C and 37°C (fig.5).

The aggregates were found to possess a buoyant density equivalent to 1.39 g·ml^{-1} of CsCl. This is considerably less than control ribosomal species, whose buoyant densities were equivalent to 1.51 g·ml^{-1} after preparation from PMS incubated at 0°C and 20°C respectively (fig.6).

Table 2
Distribution of [^{14}C]RNA in analytical sucrose gradients

Tube	Temp. of inc. of PMS	[^{14}C]RNA as % recovered DPM in gradient Non-Pelleting		Pelleting	
1,4	0°C	48%,	35%	52%,	65%
2,5	20°C	35%,	66%	65%,	34%
3,6	37°C	2%,	4%	98%,	96%

After incubation for 90 min at the stated temperature, 1 vol of PMS was added to 1 vol of a buffer containing 2% (w/w) Triton X-100, 275 mM KCl, 25 mM KCl and 5 mM $MgCl_2$ (pH 7.5 at 20°C). The samples were resuspended gently and kept on ice for 30 min. 0.5 ml was transferred onto a 15 ml 20–40% sucrose TKM gradient (containing 0.2% Triton X-100) and centrifuged at 16 000 rev/min (30 000 g_{av}) for 18.5 h. in a MSE Swing-Out 25 Al. Rotor. The tubes were fractionated into 30–35 fractions by aspiration from the base of the tube through a Pasteur pipette using a peristaltic pump. It was calculated that particles of sedimentation characteristic greater than 375S (equivalent to a polysome made up from 13 ribosomal units [3]) would have pelleted under these conditions. The table excludes consideration of all ^{14}C-material sedimenting so slowly as not to enter the top of the gradient (20% sucrose). Monoribosome peaks were observed in most profiles.

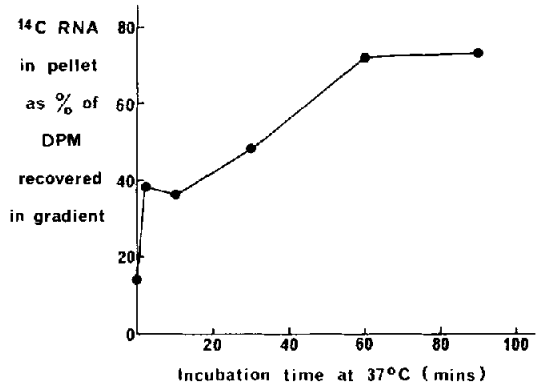

Fig.4. Aggregation of ribosomes in PMS incubated at 37°C as a function of time. After incubation for various times, the PMS samples were treated and centrifuged as described for table 2.

4. Discussion

The temperature-dependent aggregation of ribosomes is not a phenomenon restricted to post-mitochondrial supernatants, and has also been observed to occur when isolated polysomes or monoribosomes were incubated at 37°C with smooth microsomes [4]. Aggregation was not observed in the absence of smooth microsomes. Preparations of microsomes are known to contain large quantities of ribonuclease [4,5], and the formation of aggregates may depend upon the prior hydrolysis of rRNA. The removal by

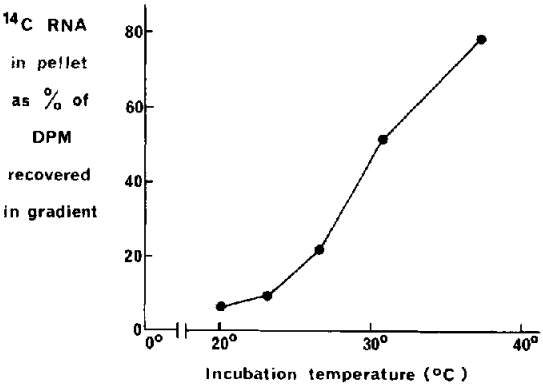

Fig.5. Aggregation of ribosomes in PMS incubated at different temperatures. After incubation for 60 min at the temperatures indicated, the PMS samples were treated and centrifuged as for table 2.

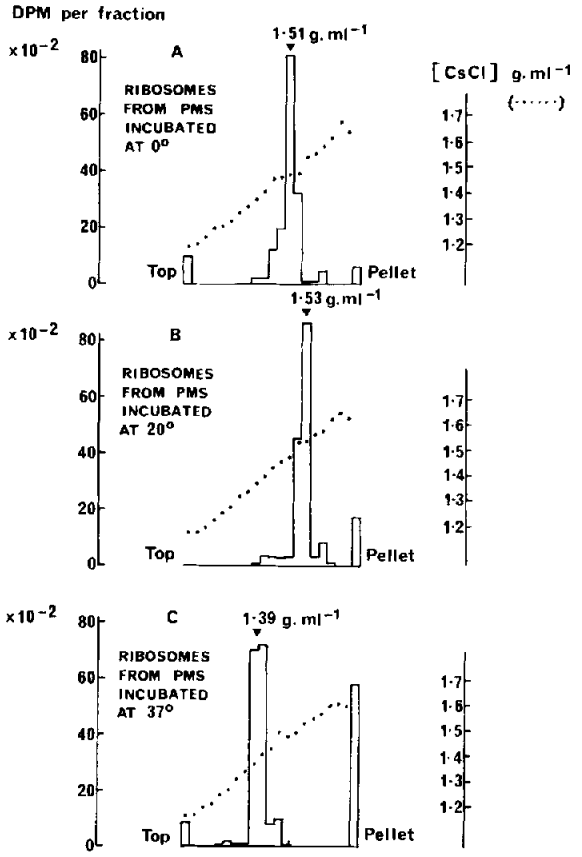

Fig.6. Centrifugation of aggregated and control ribosomes over a preformed continuous CsCl gradient. PMS from a rat starved overnight was prepared as usual and incubated for 1 h at (A) 0°C, (B) 20°C and (C) 37°C. The total ribosomes present were separated from the membranous and soluble components of the PMS by the addition of 1 vol of 2% Triton X-100 buffer (see table 2) and centrifugation of 5 ml of the resulting solution over 4 ml of 1.8 M STKM in the MSE 8 × 14 ml. Angle Rotor at 50 000 rev/min (160 000 g_{av}) for 16 h. The upper contents of the tubes including the top 2 mm of the 1.8 M STKM layer were aspirated off and discarded. The remaining contents of the tube were diluted with TKM and harvested by recentrifugation at 50 000 rev/min for 1 h. The pellets were resuspended in 15 ml of TKM and vigorously resuspended. 1 ml of the suspensions were then fixed with 0.2 ml of formaldehyde solution (1 vol of 40% formaldehyde + 1 vol of 2 M Tris adjusted to pH 7.9 with KOH) and kept on ice for 2 h before loading onto the CsCl gradients. These had been preformed by centrifuging 2.5 ml of 1.3 g·ml^{-1} CsCl over 2.5 ml of 1.7 g·ml^{-1} CsCl in the MSE 3 × 6.5 ml Swing-Out Rotor at 35 000 rev/min (100 000 g_{av}) for 80 h. The samples were spun for 16 h at 35 000 rev/min. The tubes were fractionated into about 20 fractions as for table 2.

hydrolysis of substantial amounts of rRNA may also explain the observed decrease in the buoyant density of the ribosomes forming the aggregates. The marked affinity for ribosomes displayed by rough microsomes incubated at 37°C in other studies [6] may be explained in terms of the mutual interaction of free and bound ribosomes caused by exposure to higher temperatures.

A consideration of the aggregation phenomenon is clearly of importance in all studies involving the exposure of ribosomes to conditions favourable for the generation of aggregates. The conclusion drawn by Shires and co-workers [1], that the incubation of smooth microsomes at 37°C 'conditioned' the surfaces of the vesicles to create specific ribosome binding sites needs to be re-examined. The observed increase in membrane-associated RNA could have been caused by either or both of the following phenomena unconnected with membrane–ribosome interactions: (i) Formation of membrane-bound aggregates from free ribosomes and the small numbers of residual ribosomes on the membrane; (ii) Failure of the subsequent separation methods used to achieve the complete removal of aggregates of free ribosomes from the smooth vesicles because of the decreased buoyant density of the ribosomes. This suggestion may explain at least in part the discrepancies in the literature on the interaction of smooth membranes with ribosomes. Such interactions were claimed to have been detected by direct procedures [1] but were not found by an indirect methodology [7].

The mechanism of formation of aggregates is not understood, but does not appear to involve the formation of disulphide bonds [4]. It is possible that the phenomenon is analogous to the 'precipitation' of isolated ribosomes on exposure to temperatures higher than 37°C [8–10]. Of interest in this connection is the observation of Cox [10], that pretreatment with ribonuclease caused ribosomes to precipitate spontaneously at 45°C. Untreated ribosomes did not precipitate until the temperature had been raised to 75°C.

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