EVIDENCE THAT THE TEMPERATURE-DEPENDENT ASSOCIATION OF RIBOSOMES WITH SMOOTH MICROSOMES DETERMINED BY CENTRIFUGAL SEPARATION MAY BE AN ARTEFACT

P. R. McINTOSH*, R. P. CLARK and B. R. RABIN,
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, UK

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1. Introduction

It has been reported that incubation of ribosomes with smooth microsomes from rat liver causes the formation of membrane–ribosome complexes at 25°C or 37°C, but not at 3–4°C [1,2]. This association was considered to involve specific binding of ribosomes to sites on the membrane surface of the microsomal vesicles created by the 'conditioning' of the membrane on incubation at the higher temperatures. The assay procedures for complex formation were based on centrifugal separation, and alternative methods of assay using the activity of a disulphide rearranging enzyme detected no complex formation between smooth microsomes and ribosomes in the absence of steroids at 35°C [3]. We have recently demonstrated [4] that exposure of post-mitochondrial supernatants to temperatures above 20°C generates ribosomal aggregates in which both free and bound ribosomes may be involved. This suggested that the complex formation observed in other laboratories [1,2] could have been an artefact due to interactions between the added ribosomes and the small number of bound ribosomes pre-existing in preparations of smooth microsomes. Ribosome–ribosome interactions of this kind could also explain the observation that rough microsomes appear to have an affinity for ribosomes at 37°C [5]. In this communication we show that ribosomal aggregates are formed during the incubation of microsomes and monoribosomes at 25°C and 37°C, and present data which indicates that the association that we observe between ribosomes and smooth microsomes is a methodological artefact of a complex origin in which ribosome–ribosome interactions play a role. We also show that the usual centrifugal methodology used to separate unassociated ribosomes from microsomes, which involves prolonged centrifugation of the mixture over a cushion of 1.8 M or 2.0 M sucrose solutions, fails to achieve complete separation when the ribosomes have been exposed to higher temperatures and are in the form of aggregates.

*Present address: Chemistry Branch, National Cancer Institute, N.I.H., Bethesda, Maryland 20014, USA.

2. Materials and methods

2.1. *Preparation of ^{14}C-monoribosomes*

Male albino rats (180 g) of the Sprague–Dawley strain were injected interperitoneally with 100 μCi of [6-^{14}C]orotic acid (The Radiochemical Centre, Amersham, Bucks, UK) in 0.5 ml of 0.9% saline 24 h before sacrifice in order to radiochemically label liver RNA. The animals were starved over this period but allowed drinking water ad libitum. After death by cervical dislocation, 6 g of liver was rinsed briefly with ice-cold preparative buffer, STKM. The composition of this buffer was 0.25 M sucrose (ribonuclease-free grade from Cambrian Chemicals Ltd., Croydon, UK), 50 mM tris(hydroxymethyl) aminomethane (A.R., from B.D.H.Chemicals Ltd., Poole, 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 quantity such as 1.8 M as in 1.8 M STKM, the

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prefix refers to an adjusted concentration of sucrose. TKM refers to this buffer without sucrose.

The excised liver was chopped finely with scissors and homogenised in a Potter–Elvejhem 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 passes were used to effect homogenisation. Mitochondria, cell debris, nuclei, etc. were removed by centrifugation of the homogenate in a fixed angle 8 × 50 ml rotor at 12 500 rev/min for 20 min in an MSE Angle 18 centrifuge at 4°C.

13.5 ml of the resulting post-mitochondrial supernatant was then treated with 1.5 ml of a pancreatic ribonuclease solution (0.25 mg·ml^{-1} in STKM; Sigma Type II RNase, 53 Kunitz units·mg^{-1}) for 1 h at 0°C. These conditions have been shown to bring about the complete disaggregation of polysomes to monoribosomes [6]. After incubation, one quarter vol of a 5% solution of sodium deoxycholate (in double-distilled water) was added in order to release bound monoribosomes from the membranes. The ^{14}C-monoribosomes were separated from the other components by centrifugation over a discontinuous gradient of 6 ml 2 M STKM, 1 ml 1.35 M STKM and 2 ml of 20% sucrose STKM in 14 ml tubes in an MSE Swing-Out 40 Ti Rotor for 4 h at 40 000 rev/min (195 000 g_{av}) at 4°C. The monoribosomes were removed by aspirating the 1.35 M STKM layer. TKM was added to this fraction to give a final sucrose concentration of the suspension of 24.8% w/w. The suspension was centrifuged for 45 min at 50 000 rev/min (195 000 g_{av}) in an MSE Swing-Out 65 Rotor (3 × 6.5 ml) to remove any contaminants which sediment more rapidly than monoribosomes. The supernatant was used as the ^{14}C-monoribosome preparation. Direct measurements of absorbance at 260 nm, using a Unicam SP 8000 spectrophotometer, indicated that the RNA concentration of the suspension was 0.95 mg RNA ml^{-1}. (1 ODU at 260 nm was taken to be equivalent to 42 μg RNA ml^{-1}).

2.2. *Preparation of unlabelled smooth microsomes*

Unlabelled post-mitochondrial supernatant was prepared as above from an untreated fed rat. 1 ml of the post-mitochondrial supernatant was made up to 2.0 M STKM by addition of 4.4 ml of 2.4 M STKM, and this solution was overlayed with 2 ml of 1.35 M STKM and 1 ml of 1 M STKM in 14 ml tubes. The discontinuous gradient was centrifuged in an MSE Swing-Out 40Ti Rotor at 40 000 rev/min (195 000 g_{av}) for 4 h at 4°C. The 1.0 M/1.35 M STKM interface of smooth microsomes was removed and resuspended in a volume of TKM such that measurements of refractive index indicated a sucrose concentration of 18.4% w/w. This suspension was used directly. The concentration of membrane protein was determined by the method of Lowry et al. [7] to be 1.1 mg·ml^{-1}.

2.3. *Preparation of unlabelled post-microsomal supernatant*

4 ml of unlabelled post-mitochondrial supernatant was layered over 2.2 ml. of 2 M STKM in a 6.5 ml tube and centrifuged at 50 000 rev/min (205 000 g_{av}) for 45 min in an MSE Swing-Out 60 Rotor at 4°C. The clear supernatant was taken as post-microsomal supernatant.

3. Results and discussion

To determine whether ribosomes associating with smooth microsomes after incubation at 25°C or 37°C are bound through ribosome–membrane or ribosome–ribosome interactions, ^{14}C-monoribosomes have been used in this study. If the only interactions involved are of the ribosome–membrane type, any bound ^{14}C-monoribosomes should be released from the complexes by detergent as monoribosomes (fig.1A). Conversely, if the only interactions which occur are of the free ribosome-bound ribosome type, none of the released bound ^{14}C-ribosomal material should be monoribosomal (fig.1B). However, if some aggregation between *free* ^{14}C-monoribosomes occurs during incubation at higher temperatures, it is possible that the resulting free ^{14}C-ribosomal aggregates may become attached to smooth vesicles by subsequent ribosome-membrane interactions (fig.1C). Provided a substantial proportion of the free ribosomal species (fraction X) remain as monoribosomes and these are bound with a similar affinity as the aggregates, the released bound ribosomal material (fraction Y) should also contain appreciable quantities of monoribosomes. If no ribosome-membrane binding occurs (e.g. fig.1D), fraction Y should not contain any monoribosomes.

Preparations of ^{14}C-monoribosomes were incubated

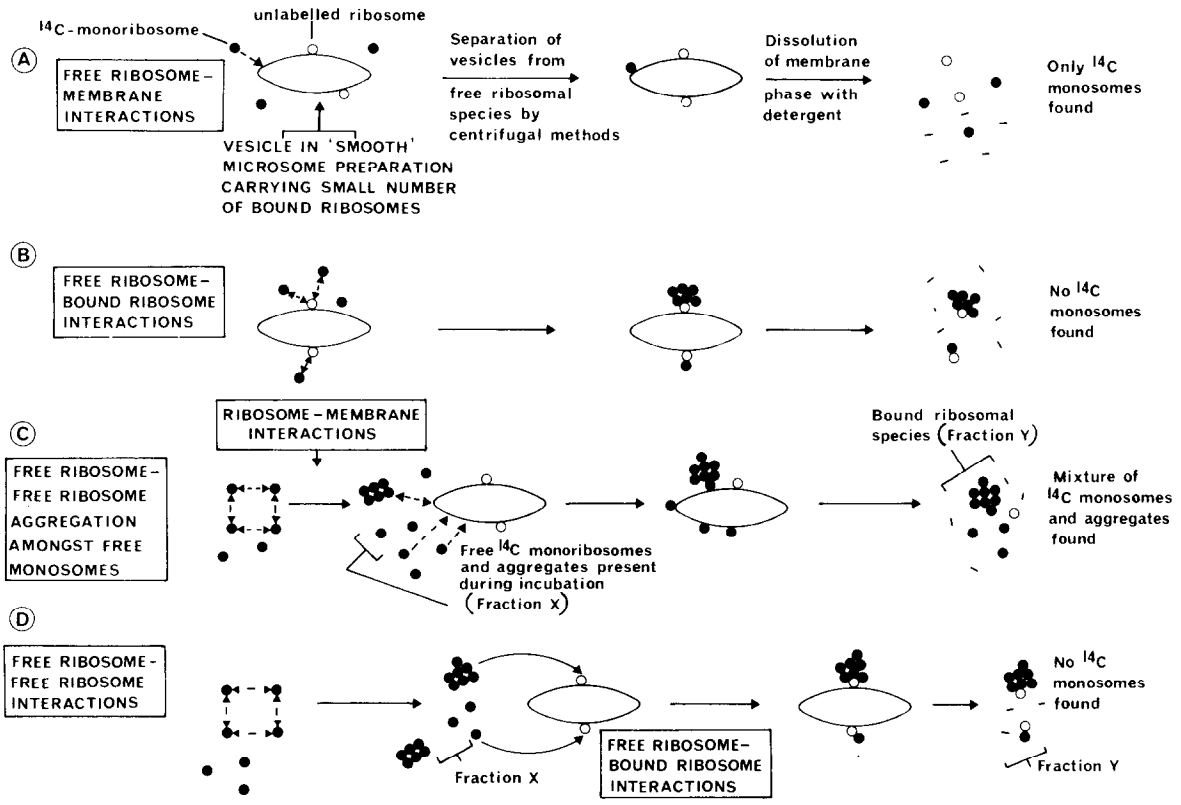

Fig.1. Possible sequences of interactions of account for the association of free ribosomes with smooth microsomes.

Table 1
^{14}C-DPM associated with the smooth microsomal fraction (1 ml) after centrifugation

Incubation	Temperature (°C)	Time (min)	^{14}C-DPM After Spin I	^{14}C-DPM After Spin II
B	0	60	2560 ± 15%	>400 ± 25%
C	25	30	5330 ± 10%	900 ± 20%
D	25	60	7600 ± 10%	1200 ± 20%
E	37	30	34 800 ± 5%	3100 ± 10%
F	37	36	48 300 ± 5%	2300 ± 15%

The microsomes were aspirated off as completely as possible from the tubes after Spin I and Spin II in a vol of 1 ml by use of a graduated syringe. 3 × 50 μl aliquots (after Spin II) were 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). Each sample was counted for 5 min in a Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375 and the resulting CPM converted to DPM by use of a predetermined quench curve. Operational counting efficiency was in the region of 75–80%. The error in counting each aliquot is quoted adjacent to the DPM.

with smooth microsomes at 0, 25 and 37°C, for a period of 30 min or 60 min. Each incubation contained 0.80 mg of smooth microsomal protein, 0.95 mg of monoribosomal RNA and 0.5 ml of unlabelled post-microsomal supernatant in a total vol of 2.5 ml 17% (w/w) sucrose-TKM. After incubation the experimental tubes were cooled in ice and the mixtures centrifuged as indicated in fig.2. This procedure

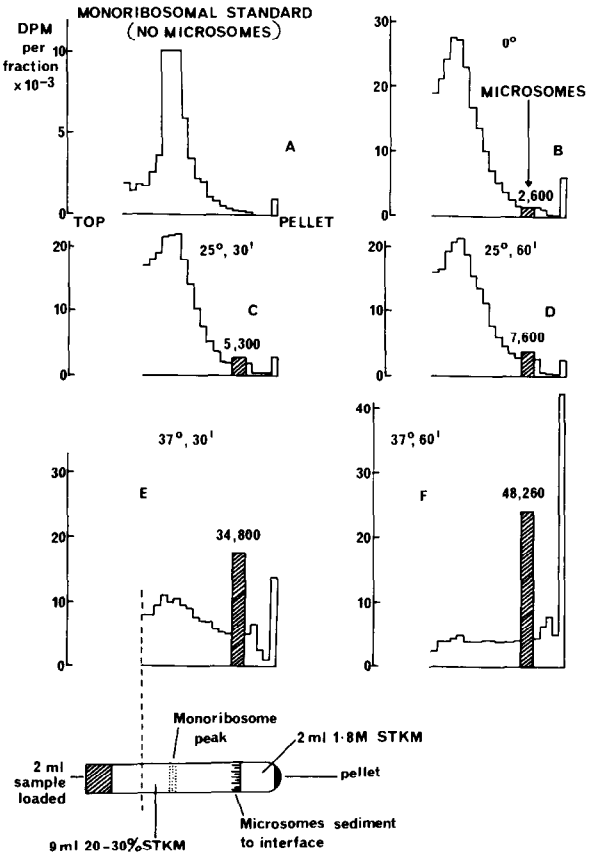

Fig.2. Spin I, the centrifugation of incubated smooth microsomes and ^{14}C-monoribosomes through a continuous sucrose gradient. The tubes were centrifuged for 2 h at 40 000 rev/min (195 000 g_{av}) in an MSE Swing-Out 40Ti (6 × 14 ml) Rotor at 4°C. The microsomes were removed as described for table 1 and the remaining contents of the tube except the upper portions were fractionated into 0.5 ml aliquots by aspiration from the base of the tubes through a Pasteur pipette using a peristaltic pump. ^{14}C-DPM were then estimated for each fraction by the addition of 4.5 ml of Instagel and treatment as for table 1. In the radioactivity profiles above, the heights of the microsomal fractions have been lowered to correct for their larger volume. The actual DPM recovered are recorded above.

is designed to remove, in particular, free monoribosomes and other slowly-sedimenting species from the microsomes. After incubation at 25°C, there is little specific accumulation of ^{14}C-activity in the microsome fraction when compared to the activity present in the *adjacent* non-microsomal fractions (fig.2C and D). This indicates that the increase in microsomally-associated ^{14}C-DPM relative to the control microsomes (table 1) is probably caused by a slight shift in the sedimentation profile of the free ribosomal material to more-rapidly-sedimenting species, due to free ribosome–free ribosome interactions between the monoribosomes. There is thus no evidence for an interaction between smooth membranes and ribosomes at 25°C. In contrast, after incubation at 37°C, there is an obvious and striking apparent 'specific' association of ^{14}C-DPM with the microsomes (fig.2E and F), and also a clear indication that the free monoribosomes have aggregated to a considerable extent, as shown by depression of the monoribosomal peaks and elevation in the levels of aggregated free ^{14}C-material in the 1.8 M STKM cushions. However, when the microsomal fraction from Spin I of the experiment at 37°C is centrifuged over 1.8 M STKM most of the radioactivity which is apparently specifically associated with the microsomes sediments as free species in the 1.8 M STKM (fig.3E and F). Since the levels of radioactivity in the microsomal fractions are no higher than those in the adjacent non-microsomal fractions, it is probable that the radioactivity remaining with the microsomes after Spin II is residual contaminating free material *not* specifically associated with the microsomes. There is thus no evidence for interactions between smooth membranes and ribosomes at 37°C.

The small amount of ^{14}C-ribosomal material associated with the microsomal fractions after Spin II (fraction V) was analysed on continuous sucrose gradients. The microsomes were first treated with one volume of 2% Triton X-100 buffer (275 mM KCl, 25 mM Tris base, 5 mM $MgCl_2$; pH 7.5 at 20°C) to dissolve the membranous components, and then submitted to centrifugation as in fig.4. In the experiment at 37°C almost all of the radioactivity is found in the resulting pellet, indicating that the 'bound' ribosomes are aggregated (fig.4E and F). This is consistent with a mechanism of apparent 'association' as illustrated in figure 1D, in which the only interactions involved are of the ribosome–ribosome type. A small number of

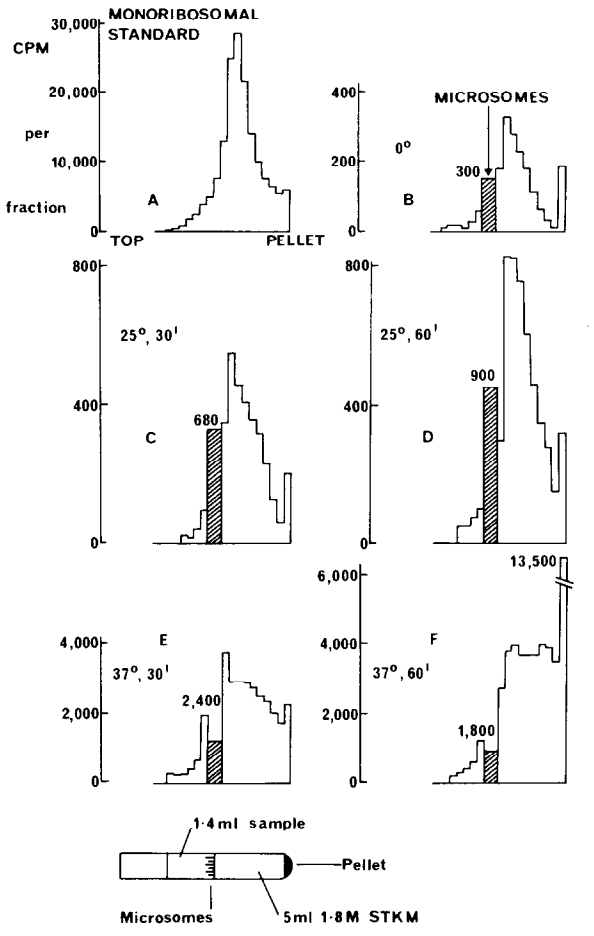

Fig.3. Spin II, the microsomal material removed from Spin I is centrifuged over 1.8 M STKM. The tubes were centrifuged at 40 000 rev/min (195 000 g_{av}) for 10.8 h in an MSE Swing-Out 40Ti Rotor (6 × 14 ml) at 4°C. The tubes were then fractionated as described in fig.2. In the radioactivity profiles above, the heights of the microsomal fractions have been lowered to correct for the larger volume of this fraction. The discontinuities between the heights of the microsomal fraction and the adjacent fractions particularly evident in profiles E and F may be attributable both to inaccuracies involved in counting low levels of radioactivity (see table 1) and the difficulty of precisely rematching the position of this removed fraction with the continuously-fractionated residual tube contents.

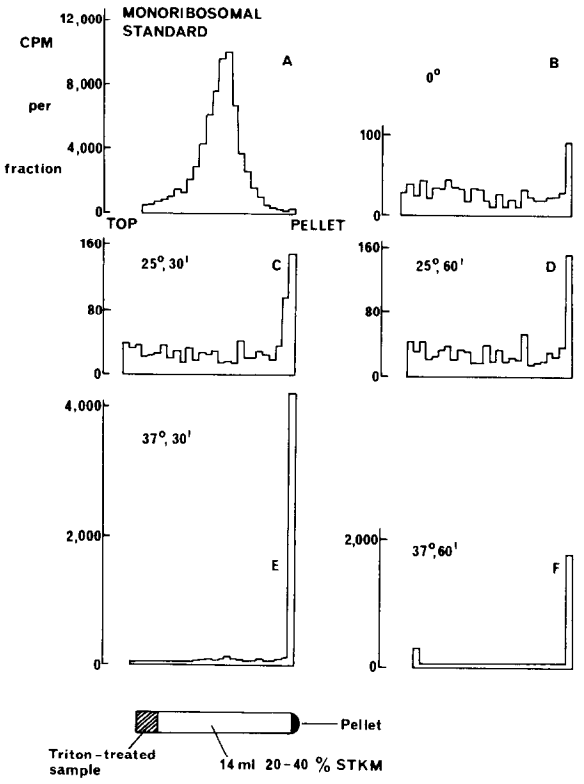

Fig.4. Analysis of ^{14}C-ribosomal material apparently remaining associated with the microsomal fraction after Spin II. The tubes were centrifuged at 40 000 rev/min (195 000 g_{av}) for 3.75 h in an M.S.E. Swing-Out 40Ti Rotor (6 × 14 ml) at 4°C. 0.5 ml fractions were removed by aspiration from the base of tubes through a Pasteur pipette using a peristallic pump. ^{14}C-DPM were estimated in the Instagel system as for fig.2.

monoribosomes is associated with fraction Y after incubation at 37°C for 30 min (fig.4E). Since the bulk of the free ^{14}C-ribosomal species (fraction X) is still monoribosomal under these conditions (fig.2E) it is probable that the monoribosomes in fraction Y are residual contaminating free monoribosomes. After treatment at 25°C, aggregates were also located in fraction Y, but the low levels of radioactivity associated with the microsomes after Spin II make interpretation difficult and it is not clear whether monoribosomes are also present in this fraction (fig.4C and D).

These findings show that free ribosome–free ribosome interactions take place during the incubations at 25 and 37°C and that free ribosome–bound ribosome interactions could in part be responsible for the apparent association of the added free ^{14}C-ribosomes with the smooth microsomes (table 1). However, it seems clear that most of the aggregated ribosomes in fractionY from the incubated microsomes were not actually

bound to the vesicles at all. The separation procedures used are not capable of complete resolution of microsomes from ribosomal aggregates because of the reduction in the buoyant density of aggregated ribosomes [4]. This is probably due to the removal of some RNA by nuclease action during incubation at 37°C.

We conclude that there is no evidence for the interaction of the membrane component of smooth microsomes with free ribosomes in the system studied.

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References

[1] Shires, T. K., Narurkar, L. M. and Pitot, H. C. (1971) Biochem. Biophys. Res. Commun. 45, 1212.
[2] Rolleston, F. S. and Mak, D. (1973) Biochem. J. 131, 851.
[3] Roobol, A. and Rabin, B. R. (1971) FEBS Lett. 14, 165.
[4] McIntosh, P. R., Clark, R. P. and Rabin, B. R. (1975) FEBS Lett. this issue 349–353.
[5] Borgese, N., Mok, W., Kreibich, G. and Sabatini, D. D. (1974) J. Molec. Biol. 88, 559.
[6] McIntosh, P. R. (1975) Ph. D. Thesis, University of London.
[7] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265.