Agranular Membranes in Free Polysome Preparations and their Possible Interference in Studies of Protein Biosynthesis

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1. Phospholipid-rich membranous contaminants are present in free polysomes from rat liver isolated on discontinuous sucrose gradients. 2. Electron-microscopic studies indicate that the membranous contaminants are mainly agranular with very occasional granular membranes. This is confirmed by the study of their sedimentation behaviour and their initial rate of labelling with radioactive glucosamine *in vivo*. 3. Conventional ribosome-purification methods fail to remove the contaminants, whereas deoxycholate effectively solubilizes the membranous contaminants with little breakdown of polysomes. 4. Amino acid-incorporation studies show that these membranous contaminants may seriously interfere in assessment of the functional capacity of free polysomes in protein biosynthesis *in vivo*.

Free polysomes from rat liver have been separated from those bound to the endoplasmic reticulum membranes on continuous (Campbell, Serck-Hanssen & Lowe, 1965) or discontinuous (Bloemendal, Bont & Benedetti, 1964; Webb, Blobel & Potter, 1964) sucrose gradients. The membranous component of bound polysomes contains abundant phospholipid; this, however, should be absent from pure free polysomes (Hallinan, Murty & Grant, 1968a). Bloemendal, Bont, De Vries & Benedetti (1967) found very little phospholipid in their free-polysome fractions, but others (Tsukada & Lieberman, 1965) have found substantial amounts and have even suggested on the basis of their disaggregation by lipase and phospholipase that free polysomes are stabilized by phospholipids.

We recently confirmed the presence of substantial amounts of phospholipid in free-polysome fractions prepared on discontinuous gradients (Hallinan *et al.* 1968a). Hence an integrated biochemical and electron-microscopic study of free-polysome preparations was initiated to ensure that the phospholipid was not due to extensive contamination by bound polysomes and to eliminate interference in studies of polypeptide and glycoprotein biosynthesis caused by the phospholipid-rich components.

MATERIALS AND METHODS

Materials. These were obtained as follows: L-[U-¹⁴C]-leucine, D-[1-¹⁴C]glucosamine and [Me-³H]choline from The Radiochemical Centre, Amersham, Bucks.; ribo-

nuclease A (5 × crystallized) from Sigma Chemical Co., St Louis, Mo., U.S.A.; sodium deoxycholate from E. Merck A.-G., Darmstadt, Germany; anthrone from British Drug Houses Ltd., Poole, Dorset; iso-octane (spectroscopic grade), from Univar, Auburn, N.S.W., Australia; bovine serum albumin from General Biochemical Corp., Chagrin Falls, Ohio, U.S.A.

Animals. Male Wistar rats or Swiss white mice of an inbred strain maintained by the University of Sydney were normally starved for 16–24 hr. before use. Some animals were injected intraperitoneally with L-[U-¹⁴C]leucine (311 mc/m-mole) or D-[1-¹⁴C]glucosamine (3.1 mc/m-mole) or [Me-³H]choline (200 mc/m-mole), the last-named together with 10 mg. of casein hydrolysate (Nagley & Hallinan, 1968), and killed at selected times. Exact conditions are given in the appropriate Tables and Fig. 1.

Preparation of free polysomes. Two discontinuous-gradient techniques were used for the preparation of free-polysome fractions from the postmitochondrial supernatant of rat liver. The first was that described by Webb *et al.* (1964), in which postmitochondrial supernatant buffered with TMK [50 mM-tris-HCl (pH 7.6)–5 mM-MgCl₂–25 mM-KCl] was centrifuged for 4 hr. at 105 000 *g*_{av}. on a two-step 0.5–2.0 M-sucrose–TMK buffer gradient in the Spinco no. 40 rotor. The second method was that of Bloemendal *et al.* (1967), in which the postmitochondrial supernatant was centrifuged for 3 hr. on a two-step 1.5–2.0 M-sucrose–TMK buffer in the Spinco no. 50 rotor at 150 000 *g*_{av}. Free polysomes sediment through the 2 M-sucrose layer to the bottom of the tube. The surface of the pellet was rinsed and it was resuspended in 0.25 M-sucrose–TMK buffer after any traces of membranous material adhering to the wall of the tube had been carefully removed.

Chemical determinations. RNA was extracted and determined by a modified Schmidt–Thannhauser method (Hallinan & Munro, 1965a), with *E*₂₆₀^{1%} 312 (Munro & Fleck, 1966). Protein was determined by the method of Lowry,

Table 1. *Composition of rat liver free polysomes*

Isolation of free polysomes and estimation of RNA, protein and phospholipid were carried out as described in the Materials and Methods section.

Method	RNA (mg./g. of liver)	Protein (mg./g. of liver)	Phospholipid (mg./g. of liver)	RNA/ protein ratio	Phospholipid/ protein ratio
Webb <i>et al.</i> (1964)	0.74	0.81	0.124	0.91	0.15
Bloemendal <i>et al.</i> (1967)	0.67	0.83	0.101	0.80	0.12

Table 2. *Composition of liver free polysomes of rats starved for various periods*

Two pairs of matched rats (body wt. 200 ± 5 g.) were starved for 24 and 40 hr. respectively and were killed and free polysomes were isolated by the method of Bloemendal *et al.* (1967). Determinations of RNA, protein and phospholipid were carried out as described in the Materials and Methods section.

Time of starvation (hr.)	RNA (mg./g. of liver)	Protein (mg./g. of liver)	Phospholipid (mg./g. of liver)	RNA/ protein ratio	Phospholipid/ protein ratio
24	0.56	0.69	0.085	0.81	0.12
40	0.61	0.82	0.199	0.74	0.24

Rosebrough, Farr & Randall (1951) with bovine serum albumin as the standard. Phospholipid was extracted and washed by a modified Folch method (Hallinan, Duffy, Waddington & Munro, 1966) and phosphorus was determined by the method of Chen, Toribara & Warner (1956); the amount of phospholipid was obtained by multiplying the phosphorus value by 25 (Hallinan & Munro, 1965b). Glycogen was isolated from whole liver and free-polysome preparations exactly as described by Luck (1961) and was determined by the anthrone method of Seifter, Dayton, Novic & Muntwyler (1950).

Measurement of radioactivity in glycoproteins, phospholipids and proteins. Samples for measuring radioactivity in glycoproteins and phospholipids (0.1 ml.) were plated on glass-fibre disk and precipitated with 0.2 M-perchloric acid-1% phosphotungstate in the presence of 1 mg. of the appropriate 'chaser'/ml. (e.g. unlabelled glucosamine hydrochloride for glycoproteins). The disks were washed six times with 0.2 M-perchloric acid-1% phosphotungstate plus the 'chaser'. The acid was then neutralized by exposing the disks to ammonia vapour for 1 hr. and the disks were dried and counted in a Packard Tri-Carb model 3375 liquid-scintillation spectrometer. The efficiency of counting ^{14}C and ^3H under described conditions was 80% and 30% respectively. For measuring incorporation of radioactive leucine into protein, samples were acid-precipitated in centrifuge tubes and washed six times with 0.2 M-perchloric acid containing 1 mg. of unlabelled L-leucine/ml. RNA and lipid were removed by digesting for 60 min. at 37° with 0.3 M-NaOH (Hallinan & Munro, 1965a). The protein was then reprecipitated at a concentration of 0.2 M-perchloric acid and washed twice with 0.2 M-perchloric acid before being dissolved in 0.1 M-NaOH. Samples (0.1 ml.) were plated on glass-fibre disks and were carefully dried and counted as described above for glycoproteins and phospholipids.

Electron microscopy. Free polysomes isolated by both techniques were fixed in 1% osmium tetroxide in veronal-

acetate buffer, pH 7.4, for 4 hr., dehydrated in ethanol and embedded in Araldite. Thin sections (500–600 Å) were cut on an LKB Ultratome I, stained with lead acetate and examined in a Siemens Elmiskop I electron microscope.

Analysis of free polysomes on sucrose gradients. Linear 10–30% (w/v) sucrose gradients (20 ml.) were prepared above a cushion of 7 ml. of 50% (w/v) sucrose in Spinco tubes. The sucrose for the gradients and cushion contained TMK buffer. The free-polysome suspension (in 0.5 ml. volume containing approx. 0.5 mg. of RNA) was layered on top of the gradient and centrifuged in the SW 25.1 rotor at $64000g_{av}$ for 2 hr. at 0° . The bottom of the tube was then punctured with a G19 needle, 2 ml. fractions were collected and the E_{260} value of each fraction was measured. Radioactivity was determined in 0.1 ml. samples from each fraction as described above.

RESULTS

It is apparent from the results in Tables 1 and 2 that the free polysomes sedimenting through the 2 M-sucrose step in both discontinuous-gradient methods for preparing them are significantly contaminated. The relatively high phospholipid content in free-polysome preparations suggests that the contaminants are probably phospholipid-

EXPLANATION OF PLATES 1 AND 2

Electron micrographs of free-polysome preparations. Polysomes were fixed in osmium tetroxide and stained with lead acetate. Magnification: Plate 1, $\times 84600$, and Plate 2, $\times 100000$, showing membranous elements (arrowed) in addition to ribonucleoprotein particles. Most of the membranes are vesicular in appearance and of agranular type.

Table 3. *Resistance of membrane contaminants to removal by conventional ribosome purification techniques*

(A) Free polysomes were isolated as described in the Materials and Methods section. (B) A portion of fraction A was re-centrifuged over 3.0 ml. of 1.0 M-sucrose plus TMK buffer for 2 hr. at 105 000g_{av.} (Munro *et al.* 1964). (C) Another portion of fraction A was re-centrifuged over 3.0 ml. of 1.0 M-sucrose plus TMK buffer containing 0.23 M-NaCl, for 2 hr. at 105 000g_{av.} (Peterman & Pavlovic, 1963).

Fraction	RNA (mg./g. of liver)	Protein (mg./g. of liver)	Phospholipid (mg./g. of liver)	RNA/ protein ratio	Phospholipid/ protein ratio
A	0.59	0.72	0.187	0.81	0.26
B	0.40	0.53	0.112	0.75	0.21
C	0.38	0.37	0.081	1.03	0.21

rich membranes. Indeed the phospholipid/protein ratio of polysomes prepared by the method of Webb *et al.* (1964), which ranges as high as 0.26 in some preparations, is up to 70% of that found for granular endoplasmic reticulum (bound polysomes). Increasing the period of starvation up to 40 hr. in one experiment did not reduce the phospholipid/protein ratio of the free polysome preparations (Table 2). Rather the polysomes prepared from two rats starved for 40 hr. had double the phospholipid/protein ratio of polysomes from two weight-matched rats starved for 24 hr. Polysomes prepared by the method of Bloemendal *et al.* (1967) contain comparatively less contaminants, but these are still sufficient to interfere seriously in some studies. Free polysomes isolated from mouse liver by the method of Bloemendal and co-workers were found to be similarly contaminated. Electron microscopy and sucrose-gradient analysis have identified the contaminants as membranous components.

Electron microscopy of free-polysome fractions. Electron micrographs of fixed sections of free-polysome pellets isolated by both methods show that these preparations were contaminated with numerous membranous elements, mostly vesicular in appearance (Plates 1 and 2). Many of the vesicles were small, often 40 mμ in diameter, and appeared to contain material of increased electron opacity. Most of the membranes were agranular, though rare granular forms were observed and many of the agranular membranes gave the impression of being ribosome-studded except on close examination, owing to the close proximity to the numerous ribosomes in the fraction (arrowed in Plates 1 and 2).

Application of conventional ribosome purification techniques to the removal of free-polysome contaminants. Peterman & Pavlovic (1963) and Munro, Jackson & Korner (1964) have shown that ribosomes can be purified of proteinaceous contaminants by exposure to high ionic strengths or by re-centrifugation through a layer of 1.0 M-sucrose. Hence these methods were tried separately and in combination for purifying free polysomes. Table 3

Table 4. *Removal of membrane contaminants of free polysomes with deoxycholate*

Free polysomes were prepared as described in the Materials and Methods section, and the samples equivalent to 1.531 mg. of RNA were treated with different concentrations of deoxycholate separately. They were sedimented by centrifugation for 2 hr. at 105 000g_{av.} and the polysomes were analysed for RNA, protein and phospholipid as described in the Materials and Methods section.

Concn. of deoxycholate used (%)	RNA recovered (%)	Phospholipid/ protein ratio	RNA/ protein ratio
0	100	0.182	0.70
0.25	99.1	0.080	0.85
0.5	98.3	0.027	0.97
1.0	99.8	0.016	—

shows that though some removal of phospholipid and protein is effected there is also a loss of RNA and the phospholipid/protein ratio is not lowered to an acceptable value. However, high ionic strengths obviously remove much extraneous protein, since the RNA/protein ratio of the fraction is increased from 0.8 to over 1.0.

Deoxycholate has been widely used for preparing membrane-free ribosomes by dissolving the membranous components of microsomes. However, Burka (1967) has reported that deoxycholate causes a preferential concentration-dependent breakdown of free reticulocyte ribosomes, compared with those co-sedimenting with the reticulocyte membranes, and suggested that all free ribosomes might be similarly susceptible to deoxycholate breakdown. Hence a range of deoxycholate concentrations was studied to test whether it could be usefully applied to the purification of free polysomes. The results in Table 4 clearly establish that deoxycholate effectively solubilizes the membranous contaminants associated with the free polysome fraction. Further, the loss of RNA was negligible over the entire concentration range of deoxycholate employed. In some experiments

Table 5. *Distribution of ^{14}C radioactivity between deoxycholate-treated polysomes and membranes*

Two pairs of matched rats were injected with $10\mu\text{C}$ of L-[^{14}C]leucine and one pair was killed at 10 min. and the other at 60 min. Free polysomes were isolated by the method of Bloemendal *et al.* (1967). Polysomes were re-suspended and a portion was treated with deoxycholate to solubilize membranes (final sodium deoxycholate concn. 1%) and the purified polysomes were sedimented by centrifugation. Chemical and radiochemical determinations on the untreated polysomes, pellet and deoxycholate-treated supernatant were performed as described in the Materials and Methods section.

Time of labelling (min.)	Fraction analysed	Sp. radioactivity (counts/min./mg. of protein)	% of radioactivity in the fraction
10	Whole free polysomes	1980	
	Membranes	1115	22
	Purified polysomes	2290	78
60	Whole free polysomes	518	
	Membranes	740	72
	Purified polysomes	294	28

greater losses of RNA, up to 20% from free-polysome fractions, was observed with deoxycholate treatment, but the losses never exceeded those experienced with polysomes associated with the endoplasmic reticulum.

Interference by the membranous contaminants in studies of protein biosynthesis by free polysomes. In a previous investigation it was found that deoxycholate-soluble, phospholipid-rich, membranous contaminants were responsible for virtually all of the incorporation of [^{14}C]glucosamine into the glycoproteins of free polysome fractions and that purified free polysomes were inactive, though their activity in the crude state sometimes exceeded that of bound polysomes (Hallinan *et al.* 1968a). Therefore the extent of interference by membranes in studies of polypeptide labelling by [^{14}C]leucine *in vivo* was also determined. The results in Table 5 indicate that 10 min. after intraperitoneal injection of tracer, deoxycholate-soluble membranous contaminants contain 22% of the total radioactivity but the purified free polysomes exhibit the greater specific radioactivity. However, after 60 min. labelling most of the radioactivity is in the membranous components, and these even exhibit over twice the specific radioactivity of purified free polysomes.

Relationship between polysomes and membranes. Tsukada & Lieberman (1965) indicated that the phospholipid components of free-polysome fractions may function in some way to stabilize the polysome structures. Hence free-polysome preparations were analysed on sucrose gradients and the relative positions of the membranes and the polysomes were determined. Deoxycholate, which solubilizes the membranes, was then tested for its effect on the integrity of polysomes. Polysomes were also disaggregated with ribonuclease and the effect of this on the sedimentation behaviour of the membranes was investigated. Membranes were located

on the gradients by labelling their lecithin with [$\text{Me-}^3\text{H}$]choline. Studies in this Laboratory have shown that 97% of the radioactivity incorporated from choline enters membrane lecithin and sphingomyelin, provided that a 'chase' of unlabelled amino acids is administered simultaneously with the choline to prevent minor incorporation of its metabolites into protein (Nagley & Hallinan, 1968).

A typical sedimentation profile for free polysomes with very few contaminating monomers and dimers is shown in Fig. 1(a). The position of the 80s monomer peak in our experiments is consistent with that found by Munro *et al.* (1964), who analysed rat liver polysomes under similar conditions. Membranes are distributed throughout the gradient with a noticeable concentration towards the bottom. Fig. 1(b) shows ribonuclease-disaggregated polysomes with a typical large monomer peak. However, the membranes are still distributed throughout the gradient and concentrated towards the bottom much as in the control sample. This demonstrates that most of the membranes are not associated with the polysomes as they do not accompany the ribosomes into the monomer peak nor the messenger-RNA fragments to the top of the gradient. It also shows that very few of the membranes represent bound polysomes as almost all of the E_{260} peak moves into the monomer peak after ribonuclease treatment (Fig. 1b) that does not detach bound ribosomes from their membranes (Blobel & Potter, 1967). The effect of treatment with 1% sodium deoxycholate is shown in Fig. 1(c). The membranes have been effectively solubilized by this treatment with more than 80% of the radioactivity moved to the top of the gradient. However, almost all of the polysomes remained intact and no noticeable increase in monomers and dimers was observed though there was a marked shift towards a lower apparent aggregate size. Hence membranes or phospholipids do not appear to be essential for the

Fig. 1. Sucrose-density-gradient analysis of choline-labelled free polysomes. Rats were given $100 \mu\text{C}$ of [$M\epsilon$ - ^3H]choline intraperitoneally and killed after 35 min. Free polysomes were prepared by the technique of Bloemendal *et al.* (1967). These were resuspended in 0.25M-sucrose in TMK buffer and treated as follows before centrifugation on 10–30% sucrose gradients overlaying a 50% sucrose cushion. Both gradients and cushion contained TMK buffer. (a) Untreated free polysomes; (b) incubated with $1 \mu\text{g.}$ of ribonuclease/ml. for 5 min. at room temp. (Wettstein, Staehelin & Noll, 1963); (c) treated with deoxycholate (final sodium deoxycholate concn. 1%). ●, E_{280} ; ○, radioactivity.

stability of free polysomes, though they may influence the distribution of larger polysomes on sucrose gradients, perhaps indicating weak interactions between polysomes and membranes.

Radiochemical evidence confirming the agranular nature of the membranous contaminants. Injected radioactive glucosamine is incorporated into glycoproteins in the livers of intact rats as *N*-acetylhexosamine and sialic acid (Molnar, Robinson & Winzler, 1965; Lawford & Schachter, 1966). This incorporation can be measured accurately by counting the accumulation of radioactivity in acid-washed precipitates of liver subcellular frac-

Table 6. Incorporation of [^{14}C]glucosamine by free-polysome, microsome and submicrosomal membranes *in vivo*

A rat was injected intraperitoneally with $28 \mu\text{C}$ of D-[^{14}C]glucosamine and killed 5 min. later. Free polysomes were prepared by the method of Bloemendal *et al.* (1967). Total microsomes, and granular and agranular microsomal membranes, were prepared according to the methods of Hallinan & Munro (1965b). All the fractions were made to 1% with respect to sodium deoxycholate and the solubilized membranes were separated from the ribosomes by centrifugation for 2 hr. at $105000g_{\text{av.}}$. Chemical and radiochemical determinations were made on the supernatant as described in the Materials and Methods section.

Source of membranes	Glycoproteins (counts/min./mg. of protein)	Phospholipid/ protein ratio
Free polysomes	70	0.23
Total microsomes	34	0.53
Agranular microsomal membranes	61	0.54
Granular microsomal membranes	18	0.55

tions (Lawford & Schachter, 1966; Hallinan, Murty & Grant, 1968b). These studies also showed that after injection of [^{14}C]glucosamine, the microsome fraction is labelled much more quickly and to a greater extent than is whole liver, nuclei, mitochondria, lysosomes or the post-microsomal supernatant (Molnar *et al.* 1965; Lawford & Schachter, 1966). Subsequently it was found that if labelling periods of less than 10 min. after intraperitoneal injection of [^{14}C]glucosamine were used, agranular microsomal membranes exhibit a much higher specific radioactivity (expressed as counts/min./mg. of protein) than do any other membranous components of the microsome fraction, including granular endoplasmic reticulum and plasma membrane (Hallinan *et al.* 1968b). Hence rapid initial labelling with [^{14}C]glucosamine is a useful marker for agranular microsomal membranes, distinguishing them from all other major types of cellular membranes.

Therefore the initial rates of labelling with radioactive glucosamine (expressed as counts/min./mg. of protein) were compared in membranes from free-polysome preparations, granular and agranular microsomal membranes and in membranes from total microsomes, all prepared from a single rat injected intraperitoneally 5 min. earlier with [^{14}C]glucosamine. It can be seen from Table 6 that the labelling of the membranous components from free polysomes closely resembles that of the agranular microsomal membranes, but is four times greater than that exhibited by granular microsomal membranes and twice as great as that of total

microsome membranes. Hence the radiochemical evidence wholly confirms that obtained by electron microscopy and from the study of sedimentation behaviour, in identifying the membranous components of free polysomes as agranular membranes.

DISCUSSION

Free polysomes are separated on discontinuous sucrose gradients from membrane-bound polysomes and from free monomer ribosomes and ribosome sub-units by virtue of their large size and high specific gravity. This allows them to sediment through the 2.0M-sucrose step with its specific gravity of about 1.26 and its very high viscosity at 0°. Hence it is most surprising to find them contaminated with agranular membranes, which are often very small and should have a specific gravity not exceeding 1.18 (Rothschild, 1963).

It seems unlikely that the membranes were carried through 2.0M-sucrose in association with free polysomes, as in general the polysomes and the membranes did not co-sediment when free-polysome preparations were analysed on sucrose gradients. However, Luck (1961) observed that agranular membranes associated with glycogen granules (sp.gr. 1.5–1.6) could sediment through 2.1M-sucrose in 1–3hr. centrifugation. Despite the absence of obvious glycogen granules in any of the electron micrographs examined, chemical analysis revealed substantial amounts of glycogen in free-polysome preparations (sometimes accounting for over 20% of the dry weight). Furthermore the amounts of membranous material in our free polysomes (0.1–0.2mg. of phospholipid/g. of liver equivalent of fraction) correspond with the amounts observed by Luck (1961) in his glycogen pellets, which, incidentally, also contained free polysomes. Hence co-sedimentation with glycogen may be regarded as a likely explanation for the presence of membranes in free polysome fractions. In this case the presence of membranous material in our free polysomes and its absence from the free-polysome preparations of Bloemendal *et al.* (1967) could be explained simply in terms of the well-known variation among rat strains in their rates of liver glycogen depletion during starvation (Reid, 1967). Against this, however, we found that starvation for up to 40hr. did not decrease the membrane content of free polysome preparations, but rather increased it.

Our results show that amino acid-incorporation studies *in vivo* into crude free polysomes may be misleading, especially over longer periods of labelling, owing to the large amount of incorporation into deoxycholate-soluble, presumably membranous, material. Calculations from the data of Bloemendal *et al.* (1967) indicate that in their

system also a large proportion of the radioactivity in polypeptides after 60 and 120min. of labelling *in vivo* is associated with the deoxycholate-soluble fraction, though they failed to find significant membranous contamination of their free polysomes.

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