A Sub-Population of Rat Liver Membrane-Bound Ribosomes that are Detached *in vitro* by Carcinogens and Centrifugation

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The chemical-carcinogen-induced detachment of ribosomes from rat liver endoplasmic reticulum was studied *in vitro*. Incubation of postmitochondrial supernatant with 0.2 mM-diethylnitrosamine or *N*-2-acetylaminofluorene removed approx. 16% of membrane-bound ribosomes, measured as differences in RNA/protein values of membrane separated from unbound ribosomes by flotation. These ribosomes are also detached by exposure to high centrifugal forces (160 000*g*) and are among those removed by NADPH-catalysed lipid peroxidation. Extensive lipid peroxidation prohibits any measurement. The ribosomes (polyribosomes) removed are not those detached from the membrane by exposure to high KCl concentrations (loosely bound) or high KCl concentrations in the presence of puromycin (tightly bound). It is concluded then that centrifugally labile and carcinogen-sensitive ribosomes represent a previously unreported sub-population of membrane-bound ribosomes.

Carcinogen treatment causes a number of ultra-structural changes in susceptible tissues. One consistently observable effect is an apparent detachment of ribosomes from the endoplasmic reticulum [see, for example, Svoboda & Higginson (1968) and Flaks (1972)]. This apparent ribosome detachment, or degranulation, may be particularly significant, since it appears to be a direct effect of the carcinogen or a metabolite. Degranulation has been demonstrated *in vitro* when isolated microsomal membranes are treated with carcinogens (Williams & Rabin, 1971). Such degranulation is normally dependent on added NADPH and a functional hydroxylase, suggesting that an activated metabolite of the carcinogen is the degranulating agent (Williams & Rabin, 1971).

An important aspect of carcinogen-induced degranulation *in vitro* is that it has so far proved impossible to achieve greater than about 20% loss of membrane-bound RNA. It is thus important to know if this RNA lost represents a particular class of membrane-bound ribosomes, and, if so, how this class relates to other subpopulations.

A variety of treatments appear to detach particular subclasses of membrane-bound ribosomes *in vitro*. Treatment with ribonuclease (Tanaka & Ogata, 1972), mild EDTA treatment (Rosbash & Penman, 1971) and treatment with high salt concentrations (Adelman *et al.*, 1973) all release up to 30% of membrane-bound ribosomes *in vitro*, and these are classified as loosely bound ribosomes. Tightly bound ribosomes are removed by puromycin treatment in the presence of a high salt concentration, whereas approx. 15% of bound ribosomes remain refractory to this treatment (Adelman *et al.*, 1973). Differences

have been found in the proteins synthesized on polyribosomes bound with these different affinities (Tanaka & Ogata, 1972; Zauderer *et al.*, 1973; Shafritz, 1974).

Centrifugation, sometimes in combination with osmotic shock, has also been shown to remove membrane-bound ribosomes. This apparent centrifugal degranulation is concomitant with changes in protein synthesis (Bont *et al.*, 1972). The released fraction includes the polyribosomes responsible for histone synthesis (Zauderer *et al.*, 1973) and appears to have a different buoyant density from those that remain bound (Dissous *et al.*, 1974). This suggests a distinct centrifugally labile class of ribosomes. These ribosomes have not been related to the previously mentioned classes.

Because lipid peroxidation causes degranulation (Arstila *et al.*, 1972), inhibition of microsomal transformations (Kamataki & Kitagawa, 1973) and is NADPH-catalysed (Pederson & Aust, 1975), its effect on the measurement of carcinogen-induced degranulation had to be considered before an investigation of the relationship between this phenomenon and the above classes of membrane-bound ribosomes could proceed.

Experimental

Chemicals

N-2-Acetylaminofluorene was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K., diethylnitrosamine from Schuchart G.m.b.h., München, Germany, and puromycin dihydrochloride

from Sigma Chemical Co., St. Louis, MO, U.S.A. Ribonuclease-free sucrose was obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K., and used throughout. All other chemicals were of an analytical grade.

Methods

Preparation of postmitochondrial supernatant. Male Sprague-Dawley rats (200g) were killed by cervical dislocation and, unless otherwise stated, their livers were then perfused with 20 ml of ice-cold 0.9% NaCl solution before removal. The livers were then chopped finely and washed three times with ice-cold 0.25M-sucrose in TKM(E) medium (50mM-Tris/HCl, 25mM-KCl, 5mM-MgCl₂, pH 7.4, and 1mM-EDTA unless otherwise stated). After washing, the livers were homogenized with twice their wet weight of ice-cold 0.25M-sucrose/TKME medium in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 9500 rev./min (11 000g) in an 8 × 50 ml rotor in an MSE 18 centrifuge for 20 min at 4°C. The postmitochondrial supernatant was removed by decantation. The postmitochondrial supernatant used in the assay of lipid peroxidation was prepared in the same way, but by using 80mM-KCl rather than sucrose as the osmolyte in all solutions, since sucrose interferes with the assay reaction.

Incubations. Incubations of postmitochondrial supernatant with carcinogens were carried out as follows. Postmitochondrial supernatant was diluted 1:1 with 0.25M-sucrose/TKME medium. The carcinogens were dissolved in dimethyl sulphoxide at a concentration of 20mM, and samples were added to the diluted postmitochondrial supernatant. Dimethyl sulphoxide was also added to the controls containing NADPH. The samples were then incubated for 2½ h at 26°C in a shaking water bath.

The method of Borgese *et al.* (1974) was followed for puromycin/KCl degranulations; 1 ml of postmitochondrial supernatant was diluted with either 0.25M-sucrose/TKME medium, puromycin and/or KCl dissolved in 0.25M-sucrose/TKME such that the final concentrations were 0.25M-sucrose/TKME, 0.5M-KCl, 0.5mM-puromycin and the final volume was 2 ml. Incubation was for 1 h on ice, followed by 15 min at 26°C.

Flotations. To 2 ml of the incubated samples in a centrifuge tube 6 ml of 2.6M-sucrose/TKME was added, and the layers were completely mixed to give 8 ml of homogeneous 2.0M-sucrose/TKME. For puromycin/KCl and KCl degranulations the 2.6M-sucrose/TKME also contained 0.5M-KCl. Then 4 ml of 0.25M-sucrose/TKME was carefully overlaid and the tubes were centrifuged in a 6 × 14 ml MSE SW40 Ti rotor in an MSE 65 or 75 ultracentrifuge. Flotation was at 4°C, at 15000 rev./min (40 000–28 000g) for 16 h unless otherwise stated.

The membrane that floated to the interface was removed with a syringe, and diluted 5:1 (v/v) with cold TKME medium. After thorough mixing the membrane was harvested at 18000 rev./min (14000g_{av.}) at 4°C for 3 h in a 50 Ti angle Beckman rotor in a Beckman L centrifuge. The membrane pellet was then drained, the tube and pellet were washed with 0.25M-sucrose solution, 4 ml of 0.25M-sucrose was added, the pellet loosened with a Pasteur pipette and then resuspended by sonication with an MSE 100W ultrasonicator.

Assays. Protein concentrations were assayed by the method of Lowry *et al.* (1951), with human serum albumin as a standard. RNA determinations were carried out by alkaline hydrolysis (Fleck & Begg, 1965). Phospholipid was determined by the method of Folch *et al.* (1957). Lipid peroxidation was assayed by monitoring malondialdehyde formation in postmitochondrial supernatants prepared without sucrose, by using thiobarbituric acid (Ottolenghi, 1959).

Results

Of the methods available to measure the effects of various agents and treatments on ribosome-membrane interactions, flotation of total membrane from postmitochondrial supernatant was chosen. Flotation has been shown to give the most satisfactory separation of free and bound ribosomes (Ekren *et al.*, 1973). Postmitochondrial supernatant is the freshest fraction amenable to the analysis required, as its preparation takes only 30 min. In the absence of lipid peroxidation it also has an adequate recycling capacity for 1mM-NADPH *in situ*. This was shown by following the absorbance at 376 nm, a wavelength that gives both an on-scale absorbance value (1.1) for 1mM-NADPH, and a minimum in the absorbance of the postmitochondrial supernatant. After 2½ h at 26°C, 90% of the absorbance remained. On addition of 1mM-NADP⁺ to the postmitochondrial supernatant, the absorbance quickly rose to, and remained at, a value consistent with complete reduction.

The data in Table 1 show the effects of lipid peroxidation on the measurement of carcinogen (diethylnitrosamine)-induced degranulation. With no inhibition of peroxidation the difference between RNA/protein ratios of recovered membrane from identical experiments is very large, approx. ±25% of the mean value. In comparison, the error in measurement of individual RNA/protein ratios is less than ±3%.

Two precautions were taken to avoid lipid peroxidation. The livers were perfused with cold 0.9% NaCl to remove as much iron as possible, as iron catalyses peroxidation (Wills, 1969). EDTA (1mM) was also used as an inhibitor of peroxidation

Table 1. *Effects of liver perfusion and EDTA on the measurement of microsomal-membrane degranulation*

Postmitochondrial supernatants were prepared, in the presence and absence of 1 mM-EDTA, from unperfused livers or livers perfused with cold 0.9% NaCl. Preparations, incubations and flotations were as described in the Experimental section. RNA/protein ratios of the membrane recovered after 14 h flotations at 15000rev/min (40000–28000g) are expressed as a percentage of the control value $\pm$ S.E.M. Reproducible results could not be obtained in the absence of both perfusion and EDTA. The mean absolute control values are 0.116 ± 0.004 , 0.103 ± 0.009 and 0.101 ± 0.004 for columns 1, 2 and 3 respectively. Protein values are percentages of the membrane protein recovered compared with the control values. The mean absolute control values were 2.32 ± 0.05 , 1.97 ± 0.17 and 2.08 ± 0.31 mg of membrane protein recovered per ml of incubation mixture for columns 1, 2 and 3 respectively. The numbers of experiments are in parentheses.

	1 EDTA, not perfused		2 Perfused, no EDTA		3 EDTA, perfused	
	RNA/protein	Protein	RNA/protein	Protein	RNA/protein	Protein
Control	100 (4)	100 (4)	100 (3)	100 (3)	100 (12)	100 (12)
NADPH (1 mM)	71.8 ± 4.6 (4)	98.3 ± 6.8 (4)	85.0 ± 2.5 (3)	96.4 ± 3.6 (3)	99.6 ± 1.2 (12)	98.4 ± 6.3 (12)
NADPH (1 mM)+ diethylnitrosamine (0.2 mM)	76.4 ± 5.4 (4)	97.8 ± 5.8 (4)	—	—	87.3 (1)	101 (1)

Table 2. *Recovery of microsomal RNA and protein after flotation for different times at different g values*

Incubations and flotations were carried out as described in the Experimental section, with the times and speeds of flotation varied as indicated. The proteins are expressed as mg of membrane protein recovered/ml of postmitochondrial supernatant mixed for flotation, $\pm$ S.E.M. for the numbers of experiments given in parentheses. All RNA/protein values are expressed as percentages of the value for the membrane recovered after 1 h of flotation at 15000rev./min, which had a mean absolute value of 0.101 ± 0.002 .

Time of flotation	1 15000rev./min (40000–28000g)		2 30000rev./min (160000–112000g)	
	Protein	RNA/protein	Protein	RNA/protein
1 h	0.73 ± 0.04 (2)	100 (2)	1.02 ± 0.03 (2)	96 ± 2 (2)
2½ h	0.87 ± 0.006 (2)	99 ± 1.2 (2)	1.12 ± 0.06 (2)	82.2 ± 2 (2)
20 h	1.46 ± 0.006 (2)	96.5 ± 1.5 (2)	2.16 ± 0.08 (2)	80.7 ± 4.5 (2)

(Wills, 1969), and was present in all solutions. It was chosen in preference to free-radical traps, since these may interrupt the electron-transport chain necessary for carcinogen activation. At this concentration EDTA has no degranulating effect of its own in the presence of 5 mM-Mg²⁺.

Either of these treatments independently leads to reproducible results, but allows NADPH-induced degranulation that masks any effect of diethylnitrosamine (Table 1, columns 1 and 2). When perfusion and EDTA addition are used in combination, NADPH-dependent degranulation is completely inhibited and diethylnitrosamine-dependent degranulation can be observed (Table 1, column 3). It was also found that unincubated controls gave the same values as incubated controls; thus temperature-dependent ribosome aggregation, as has been reported (McIntosh *et al.*, 1975), does not occur under these conditions.

The extent of lipid peroxidation allowed by these various treatments was measured as malondialdehyde formation, assayed with thiobarbituric acid

(Ottolenghi, 1959). Parallel preparations and incubations, with KCl rather than sucrose to maintain the osmolarity, were used, since sucrose interferes in the malondialdehyde assay reaction. It was found that both perfusion and EDTA are necessary to inhibit completely NADPH-induced lipid peroxidation, as expected if NADPH-induced degranulation is a consequence of peroxidation. Lipid peroxidation was not detected under any of the subsequent incubation conditions.

The effects of time and speed of centrifugation on the yield and RNA/protein ratio of the floated membranes was investigated before examination of the effects of centrifugation on the measurement of carcinogen-induced degranulation. The data in Table 2 show the consequence of different times of centrifugation at both 15000 and 30000rev./min (the membrane floating from 40000 to 28000g and from 160000 to 112000g respectively). As expected, not all the microsomal membrane floated to the interface in a 20 h centrifugation at 15000rev./min. Changing the yield of recovered membrane at this

Table 3. *Effect of different centrifugal forces on the measurement of N-2-acetylaminofluorene-induced degranulation*
 The RNA/protein ratios of membrane floated from incubated postmicrosomal supernatant after a 14h centrifugation at the speeds indicated are expressed as a percentage of the 15000 rev./min control value, $\pm$ S.E.M. The mean absolute control value was 0.108 ± 0.009 . Protein values are a percentage of the membrane protein recovered compared with the control values at each speed. The mean absolute control values were 1.92 ± 0.4 and 2.48 ± 0.64 mg of membrane protein recovered per ml of incubate at 15000 rev./min and 30000 rev./min respectively. The number of experiments is given in parentheses.

	1 15000 rev./min (40000–28000g)		2 30000 rev./min (160000–112000g)	
	RNA/Protein	Protein	RNA/Protein	Protein
Control	100 (5)	100 (5)	83.6 ± 1.8 (2)	100 (2)
NADPH (1 mM)	99.6 ± 0.8 (5)	98.2 ± 6.6 (5)	84.1 ± 2.4 (2)	97.5 ± 7.5 (2)
NADPH (1 mM)+ N-2-acetylaminofluorene	87.5 ± 1.4 (4)	99.1 ± 2.6 (5)	83.7 ± 2.8 (2)	100 ± 6 (2)
N-2-Acetylaminofluorene (0.2 mM)	104.8 ± 3.1 (5)	103 ± 4.3 (5)	85.1 ± 4.2 (2)	99.1 ± 6.8 (2)

speed does not, however, change its RNA/protein ratio (Table 2, column 1). At the higher speed of 30000 rev./min the RNA/protein ratio of recovered membrane is consistently lower, again regardless of yield (Table 2, column 2).

The $2\frac{1}{2}$ h spin at the higher g value is exposed to a ' $g \times \text{time}$ ' equivalent to a 10h spin at the lower speed, and the yield of recovered membrane reflects this. The RNA/protein value does not. There is no significant change of RNA/protein ratio between $2\frac{1}{2}$ and 20h of flotation, although the yield of recovered membrane doubles, again suggesting that a representative sample of the membrane is recovered long before quantitative flotation. Phospholipid/protein ratios were found to be the same for membranes recovered at both g values, showing that the difference in RNA/protein ratios is not a consequence of protein adsorption or loss. Thus we conclude that approx. 17% of the ribosomes bound to membrane prepared at 15000 rev./min are absent from membrane prepared at 30000 rev./min.

The effect of this centrifugal degranulation on the measurement of degranulation induced by the carcinogen N-2-acetylaminofluorene is shown in Table 3. The RNA/protein ratios at the lower g value show a clear NADPH-dependent degranulation (Table 3, column 1). No such effect is observable at high g value where all the RNA/protein ratios are the same and equal to that of carcinogen-treated membrane floated at low g value (Table 3, column 2).

This strongly suggests that the carcinogen-susceptible ribosomes constitute the same class as those that are centrifugally labile. To classify these ribosomes in terms of classically described loose, tight or refractory binding, a sequential method of ribosome detachment was used (Borgese *et al.*, 1974), and the results obtained at high and low g values were compared with carcinogen-induced and centri-

fugal degranulation. The results are shown in Table 4.

As expected, treatment with puromycin alone causes no degranulation additional to centrifugal degranulation. High-salt degranulation, of loosely bound ribosomes, is evident at both g values; so is the removal of both tightly and loosely bound ribosomes with puromycin and high salt concentration. Centrifugal degranulation is evident in addition to the detachment of both loosely and tightly bound ribosomes. This contrasts sharply with degranulation induced by the carcinogen, which removes only centrifugally labile ribosomes.

Refractory ribosomes, those not removed by puromycin and high-salt treatment, remain at the high g value. The apparently refractory ribosomes at low g include the centrifugally labile class. The proportion of the total bound ribosomes that are refractory at high g is higher than that found by previous workers (Adelman *et al.*, 1973). However, the RNA/protein ratios are in agreement with those found by Borgese *et al.* (1974) for stripped rough membrane. Rebinding at 0°C as reported (Adelman *et al.*, 1973) is likely to have occurred, as the experimental technique involves incubation of ribosomes and membranes at 4°C , before their separation. It should also be noted that the RNA may be overestimated, since at low RNA/protein ratios, absorbance corresponding to protein hydrolysis is more likely to interfere, whereas it does not for RNA/protein ratios of approx. 0.1.

The proportion of ribosomes removed during centrifugation does not increase significantly when g values higher than those obtained at 30000 rev./min are used, suggesting that the centrifugally labile ribosomes have all been removed at 30000 rev./min. As refractory ribosomes remain, centrifugally labile and refractory ribosomes are not the same class.

Table 4. *Influence of centrifugal conditions on the measurement of membrane degranulation by various agents*

Incubations and flotations for the various agents were carried out as described in the Experimental section. Flotations were for 14h at the speeds indicated. RNA/protein ratios are expressed as a percentage of the control value at 15000rev./min, $\pm$ S.E.M. The mean absolute control RNA/protein value was 0.105 ± 0.004 . Protein values are a percentage of membrane protein compared with the control values at each speed. The mean absolute control values were 1.42 ± 0.26 and 2.48 ± 0.64 mg of membrane protein recovered per ml of incubate at 15000rev./min and 30000rev./min respectively. The number of experiments is given in parentheses.

	1 15000rev./min (40000–28000g)		2 30000rev./min (160000–112000g)	
	RNA/protein	Protein	RNA/protein	Protein
Control	100 (7)	100 (7)	86.9 ± 1.6 (7)	100 (7)
Puromycin (0.5mM)	101.8 ± 3.2 (2)	97.4 ± 3.1 (2)	89.6 ± 7.6 (2)	102.3 ± 5.6 (2)
NADPH (1mM)+ N-2-acetylaminofluorene (0.2mM)	87.5 ± 1.4 (5)	99.1 ± 2.6 (5)	83.7 ± 2.8 (2)	100 ± 6.0 (2)
KCl (0.5mM)	81.1 ± 7 (5)	87.1 ± 7.2 (3)	65.8 ± 4.9 (5)	93.6 ± 6.5 (3)
Puromycin (0.5mM)+ KCl (0.5M)	36.3 ± 1.3 (4)	89.7 ± 0.4 (3)	25.0 ± 1 (4)	97.8 ± 3.8 (3)

Discussion

The results presented above are best rationalized by postulating the existence of a previously unrecognized class of ribosomes that are bound to reticular membranes by centrifugally labile interactions. These ribosomes are also susceptible to carcinogen-induced degranulation and are included in those susceptible to degranulation induced by lipid peroxidation. They do not belong to any previously reported class of bound ribosomes.

It is also possible that the apparent centrifugal degranulation does not involve ribosomes at all, but is merely a consequence of ribosome dissociation and the loss of small subunits. Such centrifugal dissociation of ribosomes has been reported previously (Martin *et al.*, 1969; Infante & Graves, 1971; Infante & Krauss, 1971) and would be expected to occur in the range of *g* values and sucrose concentrations used. However, these workers showed that dissociation only occurs for ribosomes free of nascent polypeptide chains, and probably of mRNA.

In contrast, the previous observations of centrifugal degranulation involve the release of polyribosomes active in protein synthesis (Bont *et al.*, 1972; Zauderer *et al.*, 1973; Dissous *et al.*, 1974). The removal of both ribosomal subunits has also been implicated in carcinogen-induced degranulation (Williams *et al.*, 1968; Williams & Rabin, 1971). Thus it is unlikely that the postulated class of ribosomes is an artifact of ribosome dissociation.

However, the physical chemistry of centrifugal degranulation is not understood. It may be a consequence of high *g* itself or of the relatively high hydrostatic pressures developed. Centrifugal lability may be a property of a particular subclass of ribosomes (polyribosomes) or of ribosome-binding sites,

or both. The polyribosomes programmed for histone synthesis appear to be included in the centrifugally sensitive population (Zauderer *et al.*, 1973). This suggests that polyribosomes directing the synthesis of particular classes of proteins may be differently bound, a possibility requiring investigation.

By using centrifugal techniques it is impossible to separate free from membrane-bound ribosomes without the exposure to considerable centrifugal fields. Thus the full extent of the centrifugally labile population is not known. It may be considerably greater than the approx. 16% reported here. Since the extent of detachment of centrifugally labile ribosomes will vary with methods of separation, these ribosomes will have been regarded as part of the free or of the bound populations, depending on the conditions used; those traditionally regarded as free ribosomes will have been variably contaminated with centrifugally labile ribosomes, originally a bound population. Non-centrifugal methods of ribosome/membrane separation could be used to elucidate the extent of this problem.

The difficulties discovered with lipid peroxidation may not be universal, and may often have been fortuitously avoided. Susceptibility to peroxidation has been shown to vary with age (Grinna & Barber, 1973), diet (Corwin & Schwarz, 1960) and species (Kamataki & Kitagawa, 1974). The variability of lipid-peroxidation effects is probably enhanced by the autocatalytic nature of the process.

Another consequence of the findings presented here is that the measurement of carcinogen-induced degranulation requires careful design of centrifugal and incubation conditions. The interpretation of published data on the use of carcinogen-induced degranulation as a screening test for chemical

carcinogens (Purchase & Lefevre, 1975; Purchase *et al.*, 1976) needs to be reconsidered in the light of these findings.

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