Heterogeneous Distribution of Enzymes among Plasma-Membrane Fragments Sedimenting with the Microsomal Fraction of Rat Liver

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Plasma-membrane fragments recovered in the microsomal fraction of rat liver homogenates were shown to be heterogeneous in density. It was demonstrated that 5'-nucleotidase, the most commonly used plasma-membrane marker, is concentrated in the lightest subfraction. Two of the published procedures for the isolation of plasma-membrane fragments from the microsomal fraction (Touster *et al.*, 1970; Hinton *et al.*, 1971) are shown to give products which are not representative of all the plasma-membrane fragments of microsomal size, and it is argued that a third procedure (House & Weidemann, 1970) is likely to give a similar product.

After homogenization, the plasma membranes of liver cells are recovered in fragments of two distinct types, large membranous sheets and small vesicles. Methods have long been available for purifying the large sheets, which sediment into the crude nuclear fraction (Hinton, 1972; de Pierre & Karnovsky, 1973). When these sheets of membrane are further disrupted by vigorous rehomogenization, subfractions differing in density and enzymic composition may be separated (Evans, 1970; Thines-Sempoux, 1973). It is only recently that methods have been developed for separating plasma-membrane vesicles from the microsomal fraction by isopycnic banding on sucrose or Ficoll gradients (House & Weidemann, 1970; Touster *et al.*, 1970; Hinton *et al.*, 1971). As the plasma-membrane fragments found in the microsomal fraction are of similar size to the fragments prepared by further disrupting large sheets of membrane it is clearly possible that they will show a similar heterogeneity in density and enzymic composition and hence a fraction separated by isopycnic banding may not be representative. There has been evidence (Evans *et al.*, 1973) for such heterogeneity in the non-uniform distribution of insulin-binding sites between plasma-membrane vesicles separated by the method of Touster *et al.* (1970) and plasma-membrane sheets separated from the crude nuclear fraction. We now present evidence that the plasma-membrane vesicles recovered in the crude microsomal fraction are themselves heterogeneous in enzyme content and that the most commonly used plasma-membrane marker, 5'-nucleotidase, is not distributed uniformly among plasma-membrane elements of microsomal size.

Materials and Methods

Enzyme substrates were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Gradients were prepared from Mineral Water Sucrose (Tate and Lyle Ltd., London S.E.6, U.K.). The sucrose was usually treated with activated charcoal (Steele & Busch, 1967, see p. 104) to remove trace impurities.

Hooded rats of the University of Surrey strain were used in all experiments. The livers were homogenized in 0.25M-sucrose containing 2mM-Tris-HCl, pH 8.0, by using five strokes of a Potter-Elvehjem homogenizer with a pestle rotated at 2400 rev./min and a clearance of 0.35 mm between pestle and vessel, so that 3 ml of the resultant homogenate contained material from 1 g of liver. The homogenate was centrifuged for 15 min at 38000g in an MSE High-Speed 18 centrifuge. The supernatant was centrifuged for 90 min at 130000g in an MSE Super-Speed 65 ultracentrifuge to pellet the microsomal fraction. Plasma-membrane fragments were separated from the pellets either by an adaptation of the method of Touster *et al.* (1970) or by a method developed in our laboratory (Hinton *et al.*, 1971). When the method of Touster *et al.* (1970) was used, the livers (20-25g) were perfused *in situ* with 0.25M-sucrose containing 5mM-Tris-HCl, pH 8.0. A microsomal fraction was prepared as above and resuspended in about 35 ml of 2M-sucrose by using two strokes of the Potter-Elvehjem homogenizer. The suspension was loaded under a linear sucrose gradient ranging in density from 1.077 to 1.257 g/ml in an MSE B-XIV zonal rotor, and the rotor was finally filled with

2.2M-sucrose. All gradient solutions contained 5mM-Tris-HCl, pH8.0. After centrifugation for 16h at 45000rev./min, the gradient was displaced and 20ml fractions were collected.

The alternative method we used depends on the increase in the density of endoplasmic-reticulum fragments after pretreatment by sonication in the presence of small amounts of lead and has been described more fully elsewhere (Hinton *et al.*, 1971). Increasing the concentration of $\text{Pb}(\text{NO}_3)_2$ from the 0.5mM recommended by Hinton *et al.* (1971) to 0.75mM was found to give more reliable results.

Protein, 5'-nucleotidase and glucose 6-phosphatase were assayed as described by Prospero *et al.* (1973). RNA was measured by the method of Fleck & Begg (1965). Alkaline phosphodiesterase and alkaline phosphatase were assayed by the automated method of Hinton & Norris (1972). ADPase* and ATPase were assayed in the presence of 0.1M-Tris-HCl, pH7.2 (37°C), 5mM-MgCl₂ and 5mM substrate. The assays were stopped and the phosphate was determined as in the other phosphatase assays (Prospero *et al.*, 1973). All enzyme and protein measurements were corrected for sucrose interference (Hinton *et al.*, 1969).

Results and Discussion

Flotation of a resuspended microsomal fraction through a sucrose gradient yields a zone at a density of about 1.14 which is enriched in 5'-nucleotidase (Fig. 1). The proportion of microsomal 5'-nucleotidase found in this region (approx. 55%) is comparable with that reported by Touster *et al.* (1970), who used a step gradient. However, alkaline *p*-nitrophenyl phosphatase, alkaline phosphodiesterase, ADPase and ATPase activities were all associated with material banding at a density of about 1.165, coincident with smooth endoplasmic-reticulum fragments as indicated by glucose 6-phosphatase activity. Little activity of these four enzymes was found in the region of rough endoplasmic reticulum fragments, indicated by the RNA peak banding at a density of 1.22. As discussed below, there is strong evidence from other sources that all four enzymes are, in fact predominantly located in the plasma membrane. To ascertain whether the differences in pattern between 5'-nucleotidase and the other enzymes were due to variations in the permeability of plasma membrane vesicles, we repeated the assays in the presence of 1% Triton X-100. The distribution patterns were identical with those obtained in the absence of detergent.

If the microsomal fraction is treated with $\text{Pb}(\text{NO}_3)_2$ to a final concentration of 0.75mM, then aggregation occurs which results in the clumping of all membranous material (Leskes *et al.*, 1971; Norris, 1974).

*Abbreviations: ADPase, adenosine diphosphatase; ATPase, adenosine triphosphatase.

However, if the resuspended microsomal fraction is briefly sonicated after treatment with 0.75mM- $\text{Pb}(\text{NO}_3)_2$ then, after isopycnic banding on sucrose gradients, a fraction containing about 25% of the microsomal activity of 5'-nucleotidase can be separated and recovered at a density of 1.14. Most of the remainder of the 5'-nucleotidase activity, together with the endoplasmic-reticulum fragments, is found in the undisrupted aggregates which band at the dense end of the gradient. The distribution of alkaline phosphatase and alkaline phosphodiesterase differs considerably from the distribution of 5'-nucleotidase (Fig. 2), a much smaller proportion being recovered in the central part of the gradient, and this moiety bands at a somewhat higher density than the fragments containing 5'-nucleotidase.

The experiments described above demonstrate that 5'-nucleotidase does not have the same distribution among the vesicles present in the microsomal fraction as four other enzymes also supposedly located in the plasma membrane. There are three possible explanations for this observation. First, there may be variations in the accessibility of the enzymes to the substrate. 5'-Nucleotidase is known to be located on the outside of plasma membrane vesicles (El-Aaser *et al.*, 1973). If the other enzymes were inside the vesicles and the denser vesicles were either more easily penetrated by the substrate or were 'inside out' (Wallach, 1967) then our results would be explained. However, if permeability played any role one would expect to observe an equalization of the apparent distributions when detergent was included in the incubation medium. As detergent did not affect the distributions our results cannot be explained by variations in the accessibility of the enzymes. Two possible explanations remain. First, either 5'-nucleotidase or the other enzymes may be partly located in cell structures other than the plasma membrane. Secondly, plasma membrane fragments of microsomal size may not contain a uniform complement of enzymes.

We believe that the first explanation is untenable for the following reasons. Cytochemical evidence indicates that 5'-nucleotidase, ADPase and alkaline *p*-nitrophenyl phosphatase are all located mainly in the plasma membrane (Goldfischer *et al.*, 1964), although some 5'-nucleotidase activity can be demonstrated in lysosomes and ADPase activity in the Golgi apparatus. ATPase is located in plasma membranes and mitochondrial inner membranes (Sabatini *et al.*, 1964), but measurement of succinate dehydrogenase activity showed that a negligible proportion of the latter was recovered in the microsomal fraction. Cell-fractionation experiments confirm the localization of ADPase (Wattiaux-de Coninck & Wattiaux, 1969) and *p*-nitrophenyl phosphatase (Emmelot *et al.*, 1964) in the plasma

Fig. 1. Distribution of enzymes after flotation of a rat liver microsomal preparation for 16h at 45000 rev./min in a B-XIV zonal rotor

The microsomal fraction from 20g of rat liver was resuspended in 32ml of 2M-sucrose and layered under a linear sucrose gradient extending from a density of 1.077 to a density of 1.21. (a) —, protein; $\square$, glucose 6-phosphatase; $\bullet$, RNA; ----, density (5°C). (b) $\circ$, 5'-Nucleotidase; ∇ , alkaline phosphatase; $\bullet$, alkaline phosphodiesterase. (c) $\blacksquare$, ADPase; $\blacktriangle$, ATPase. ADPase, ATPase, 5'-nucleotidase and glucose 6-phosphatase activities are expressed as μmol of phosphate formed/min, alkaline phosphatase activity is expressed as μg of *p*-nitrophenol phosphate formed/min.

membrane and suggest that this structure is also the source of the alkaline phosphodiesterase (Prospero *et al.*, 1973). Both the latter enzymes have, in fact, been used as markers for plasma-membrane fragments in the subfractionation of the microsomal fraction (Touster *et al.*, 1970). Some 5'-nucleotidase (Bergeron *et al.*, 1973) and ADPase (Cheetham *et al.*, 1970) activities have been found in the Golgi apparatus, but the experiments of Fleischer & Fleischer (1969, 1970) show that the activity of 5'-nucleotidase in the Golgi apparatus must be small compared with the activity in the plasma membrane. In addition, these authors find that fragments of the Golgi apparatus band at a much lower density than plasma-membrane fragments. As we find ADPase-rich fragments band at higher densities than fragments rich in 5'-nucleotidase, our results cannot be explained by supposing that Golgi fragments make any significant contribution to the ADPase activity of the microsomal

fraction. The results which we have presented also indicate that the reported 5'-nucleotidase in rough endoplasmic-reticulum fragments (Widnell, 1972) can only be responsible for less than 10% of the 5'-nucleotidase of the microsomal fraction.

Hence one must conclude that the plasma-membrane fragments recovered in the microsomal fraction are heterogeneous. This conclusion is strengthened because a very similar heterogeneity is found when sheets of plasma membrane separated from the crude nuclear fraction are rehomogenized, to give fragments of microsomal size (Thines-Sempoux, 1973). Very recently it has been demonstrated that the plasma-membrane vesicles separated from the microsomal fraction by the method of Touster *et al.* (1970) are highly enriched in hormone-binding sites as compared with the sheets of plasma membrane separated from the crude nuclear fraction and hence probably derive largely from the sinusoidal

Fig. 2. Distribution of enzymes after centrifugation of a rat liver microsomal preparation treated by sonication and incubation at 0°C with 0.5 mM-Pb(NO₃)₂ as described in the text

Centrifugation was carried out for 150 min at 47000 rev./min in a B-XIV zonal rotor. (a) $\square$, Glucose 6-phosphatase; —, protein; ----, density (5°C). (b) $\circ$, 5'-Nucleotidase; ∇ , alkaline phosphatase; $\bullet$, alkaline phosphodiesterase. 5'-Nucleotidase and glucose 6-phosphatase activities are expressed as μmol of phosphate formed/min. Alkaline phosphatase is expressed as μg of *p*-nitrophenol phosphate formed/min.

surface of the liver cells (Wisher & Evans, 1974). Similar results were obtained by House & Weidemann (1970) in plasma-membrane vesicles separated by their method. However, when plasma-membrane sheets isolated from the crude nuclear fraction are disrupted, hormone-binding activity is concentrated in vesicles of density 1.16–1.18 (Evans *et al.*, 1973) which would not be concentrated in fractions separated by the method of Touster *et al.* (1970). Hence

it is difficult to make any conclusions on the origins of the subfractions separated in the present experiments. However, it is clear that all three methods that have been proposed for the separation of plasma-membrane fragments from the microsomal fraction give similar products which, however, are not typical of all the plasma-membrane fragments that are recovered in the microsomal fraction. Our results also indicate that 5'-nucleotidase should not,

as has been the case up to now, be used as the sole plasma-membrane marker, as it is not evenly distributed among plasma-membrane fragments of microsomal size.

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