

THE ASSOCIATION OF CHOLESTEROL, 5'-NUCLEOTIDASE, AND ALKALINE PHOSPHODIESTERASE I WITH A DISTINCT GROUP OF MICROSOMAL PARTICLES

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When a microsome fraction is prepared from rat liver by the method of de Duve et al. (11), it contains about 20% of the proteins of the homogenate, 75% of characteristic microsomal hydrolases (such as glucose 6-phosphatase, nucleoside diphosphatase, esterase), almost the same proportion of typical microsomal oxidoreductases (NADPH and NADH: cytochrome *c* reductases, cytochrome *b₅*, aminopyrine demethylase, cytochrome P₄₅₀), and 40-50% of the homogenate content in cholesterol, 5'-nucleotidase, and alkaline phosphodiesterase I (acting on *p*-nitrophenyl 5'-thymidylate). The latter three components exhibit a "nucleo-microsomal distribution" in the sense that the amount recovered in the nuclear fraction is much higher than that expected for particles of microsome size or observed for typical microsomal constituents. Since the high activity of 5'-nucleotidase and the abundance of cholesterol are characteristic properties of purified plasma membrane preparations isolated from low speed sediments (for a review see ref. 5), the obvious interpretation for the presence of these components in the nuclear fraction is that they belong to plasma membrane fragments of large size that sediment together with the nuclei and large cell debris.

In line with this interpretation, which is widely accepted, it is tempting to assume that small fragments of plasma membrane sedimenting together with the fragments of the endoplasmic reticulum are responsible for the microsomal localization of most of the remaining cholesterol, 5'-nucleotidase, and alkaline phosphodiesterase I. Another possible interpretation is that the microsomal plasma membrane constituents belong to membranous entities that do not originate directly from the plasma membrane, but are related to it either as biosynthetic precursors or as derivatives (pinocytotic vesicles, for instance). Finally, it is also possible that they are entirely unrelated to the plasma membrane and are associated with the main components of the microsomal fraction, i.e., with true elements of the endoplasmic reticulum. Owing to lack of definitive experimental data, most workers

have kept a noncommittal attitude on this subject, but there is a tendency to believe that at least the cholesterol of the microsomal fraction belongs largely to the endoplasmic reticulum proper (10). The results described in this note argue against this view and add further support to our earlier contention that the plasma membrane constituents found in the microsomal fraction, including most, if not all, of its cholesterol content, are associated with a trace component different from those that bear the typical microsomal hydrolases and oxidoreductases (1, 2).

We have previously reported how various enzymes are distributed after equilibration of the microsome fraction in a number of different density gradients. (Some examples of the distribution patterns observed in a sucrose-H₂O gradient are given in Fig. 1). These results may be summarized as follows: All components equilibrate over a wide range of density and their distribution patterns overlap to a large extent. There are, however, significant differences which allow the assayed components to be classified into four distinct groups of increasing median density: (a) Monoamine oxidase, 5'-nucleotidase and cholesterol; (b) microsomal oxidoreductases; (c) microsomal hydrolases; (d) RNA and some enzymes presumably adsorbed on ribosomes. The distribution of the proteins is almost superposable to that of the hydrolases, while that of the phospholipids is very close to that of the oxidoreductases.

When the composition of the density gradient is changed, the last three groups retain their identity, but 5'-nucleotidase and monoamine oxidase do not behave in the same manner and are thus presumably linked to different particles. More recent (unpublished) observations have shown that 5'-nucleotidase and monoamine oxidase can also be distinguished from each other and from the bulk of the microsome fraction by their rate of sedimentation in shallow stabilizing sucrose density gradients: 5'-nucleotidase sediments faster, and monoamine oxidase sediments more slowly, than do most other microsomal enzymes. We have

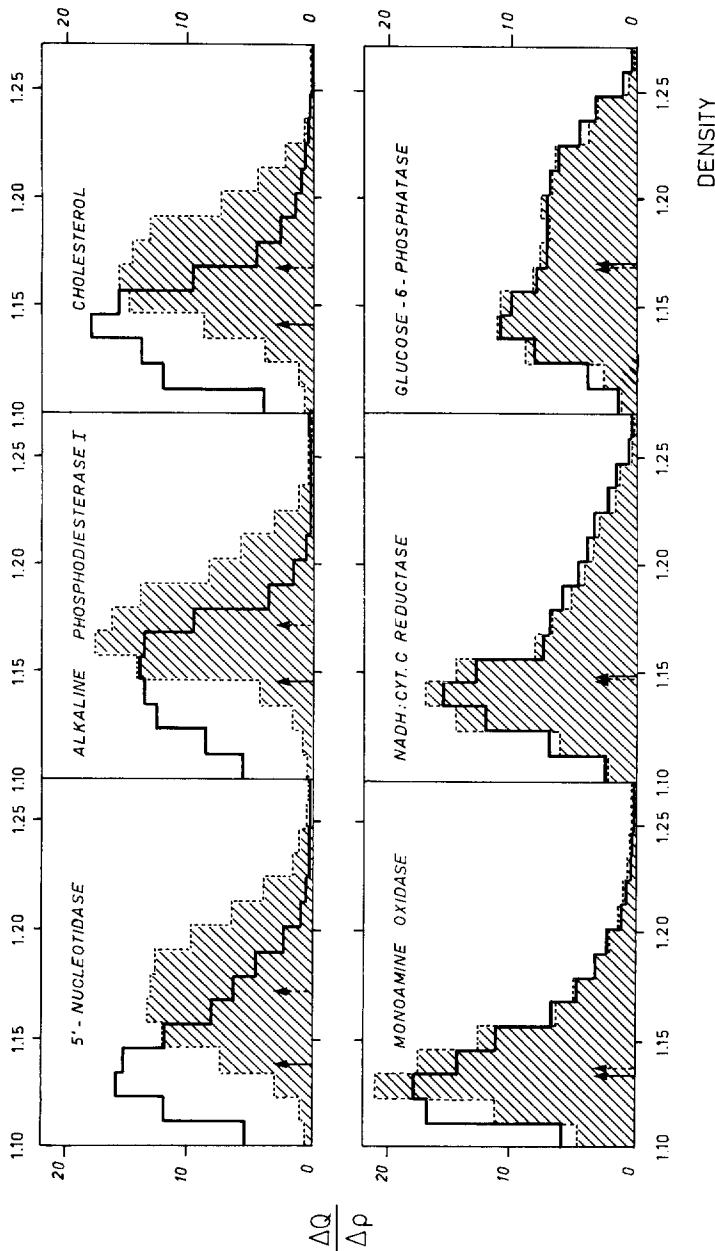


FIGURE 1 Influence of a pretreatment with digitonin on the distribution of some microsome components in sucrose density gradients.

The microsome fraction was prepared according to de Duve et al. (11) using 0.25 M sucrose, 3 mM imidazole buffer pH 7.4. In the control experiments (solid lines), the particles were washed once and finally suspended in 0.25 M sucrose-3 mM imidazole buffer. A 10 ml sample of this suspension, containing the microsomes from 8 g of liver, was layered over a 38 ml sucrose density gradient extending to the density 1.34 over a radial distance of 1 cm. The rotor used in these experiments has been described by Beaufay (4). Except for minor modifications, the filling and emptying of the rotor was performed as described previously (15). Almost complete density equilibration was achieved by centrifuging 180 min at 35,000 rpm. For preparing digitonin treated microsomes (shaded areas), the washing of the first microsome pellet was replaced by the following treatment: The microsome pellet was resuspended in 3 ml per g of liver of 0.25 M sucrose-3 mM imidazole buffer pH 7.4, containing 0.25 per cent digitonin. After standing for about 15 min at 0°, the suspension was centrifuged for 30 min at 40,000 rpm in the N° 40 rotor of the Spinco preparative ultracentrifuge. The pellet was resuspended in 0.25 M sucrose-3 mM imidazole buffer (without digitonin) and processed as the control. The ordinate of the graph is the ratio of the fractional activity recovered in a given density range divided by the density increment in this range. The arrows indicate the position of the median densities.

TABLE I
Median Densities of Microsomal Components in
Sucrose-H₂O Gradient

The experiments were performed as described in the legend of Fig. 1. Published methods were used (eventually with minor modifications) for the assays of 5'-nucleotidase (12), alkaline phosphodiesterase I (8), monoamine oxidase (3), NADH and NADPH: cytochrome *c* reductases (11), aminopyrine demethylase (17), cytochrome *b₅* (14), glucose 6-phosphatase (11), esterase (7), nucleoside diphosphatase (13), proteins (16), and RNA (18). Cholesterol was isolated as the digitonide after saponification and was assayed colorimetrically (19). Phospholipid phosphorus was determined on lipid extracts (6).

	Normal mi- crosomal fraction	Microsomal fraction treated with 0.25% digitonin
5'-nucleotidase	1.138	1.172
Alkaline phosphodi- esterase I	1.145	1.171
Cholesterol	1.141	1.167
Monoamine oxidase	1.134	1.137
NADH: cytochrome <i>c</i> reductase	1.149	1.148
NADPH: cytochrome <i>c</i> reductase	1.157	1.154
Aminopyrine demethylase	1.152	1.152
Cytochrome <i>b₅</i>	1.145	1.151
Phospholipids	1.150	1.158
Glucose 6-phosphatase	1.169	1.168
Esterase	1.166	1.164
Nucleoside diphosphatase	1.172	1.167
Proteins	1.164	1.166
RNA	1.201	1.195

also found that alkaline phosphodiesterase I behaves in many respects like 5'-nucleotidase.

While studying the influence of detergents on various properties of the microsome fraction, we noticed that digitonin has a peculiar influence on the equilibrium density of some particles. We used amounts of digitonin too low to disrupt most of the particles, as indicated by the following observations: It is known (9, 13) that nucleoside diphosphatase is almost completely latent in intact microsome particles, and that it can be rendered

active as well as soluble by treatment with detergents or by vigorous mechanical disruption. Under the conditions of our experiments, less than 10% of the enzyme was solubilized or activated, and less than 5% of the microsomal proteins were solubilized, at a digitonin concentration of 0.25%. As judged by these two criteria, most microsomal particles did not seem to suffer serious damage. When a microsome fraction treated with 0.25% digitonin was equilibrated in a sucrose density gradient the results presented in Fig. 1 and in Table I were obtained. The distributions of 5'-nucleotidase, alkaline phosphodiesterase I, and cholesterol were shifted towards higher densities, whereas those of all the other assayed components were not appreciably affected. As shown in Table I, the median density of the three first components was increased by some 0.03 density unit, which is highly significant (median densities are reproducible within 0.01 density unit). The median densities of all other components were not significantly altered.

The simplest interpretation of these results is that 5'-nucleotidase, alkaline phosphodiesterase I, and at least most of the cholesterol are bound to the same or similar particles, distinct from those that carry the other assayed components. Since total proteins are not shifted, and total phospholipids are shifted little if at all, by the digitonin treatment, the particles involved must represent a small proportion of the microsomal constituents. A surprising consequence of our findings is that cholesterol seems completely or largely associated with this special and relatively rare type of particles. This leads to the conclusion that the bulk of the endoplasmic reticulum contains little, if any, cholesterol. Obviously, the two distribution patterns shown in Fig. 1 overlap sufficiently to allow for the presence of some cholesterol that is not shifted by digitonin. But the magnitude of the shift, as well as the comparable shifts suffered by the two enzymes indicate that this unaffected fraction must be small.

Preliminary experiments on the mechanism of the digitonin effect suggest that digitonin is bound to the particles, presumably to their cholesterol, and that the increase in density results from the binding of the glycoside.

It seems most likely that the digitonin binding particles are related to the plasma membrane, since 5'-nucleotidase and cholesterol are quite concentrated in the latter. Unpublished results from our

laboratory also indicate that alkaline phosphodiesterase I has a high specific activity in plasma membrane preparations obtained by the method of Emmelot et al. (12). Further investigations are needed to establish whether the microsomal particles are small fragments, precursors, or derivatives of plasma membranes and whether the digitonin binding particles belong to a single or to more than one population.

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