ANALYTICAL STUDY OF MICROSOMES AND ISOLATED SUBCELLULAR MEMBRANES FROM RAT LIVER

VI. Electron Microscope Examination of Microsomes for Cytochrome b_5 by Means of a Ferritin-Labeled Antibody

JOSÉ REMACLE, STANLEY FOWLER, HENRI BEAUFAY, ALAIN AMAR-COSTESEC, and JACQUES BERTHET

From the Institute of Cellular Pathology, Université de Louvain, Brussels, Belgium. Dr. Remacle's present address is Facultés Universitaires Notre Dame de la Paix, Namur, Belgium. Dr. Fowler's present address is The Rockefeller University, New York 10021

ABSTRACT

The distribution of cytochrome b_5 in rat liver microsomes, and in two microsomal subfractions isolated by density equilibration in a linear sucrose gradient, was studied under the electron microscope by means of a ferritin-labeled hybrid anti-cytochrome b_5/anti-ferritin antibody. Results of this study show that cytochrome b_5 is present in essentially all microsomal vesicles derived from endoplasmic reticulum (ER), whether rough or smooth. Thus, the dissociation of ER constituents into two groups (*b* and *c*), achieved by subfractionating microsomes by isopycnic centrifugation (Beaufay, H., A. Amar-Costesec, D. Thines-Sempoux, M. Wibo, M. Robbi, and J. Berthet. 1974. *J. Cell Biol.* **61**:213–231), does not reflect the association of each group with distinct microsomal particles but reflects rather an enzymatic heterogeneity of the ER: the ratio of group *c* to group *b* enzymes increasing with the density and ribosome load of the particles.

Analytical subfractionation of the microsomal fraction from rat liver by means of isopycnic and differential density gradient centrifugation has revealed that the enzymes associated with this fraction fall into a number of distinct groups having significantly different centrifugal behavior patterns (1, 3, 7). Three such groups could be attributed to membrane elements distinct from the endoplasmic reticulum (ER)[1] proper, including plasma membranes (5′-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase), parts of the Golgi apparatus (galactosyl- and other glycosyltransferases), and a special set of membranes possibly related to outer mitochondrial membranes (monoamine oxidase). Two groups of enzymes, however, gave evidence of being associated with true ER elements. These are group *b*, which includes cytochromes b_5 and P-450, together with a number of oxidoreductase activities related to these hemoproteins, and group *c*, which includes glucose 6-phosphatase, several microsomal hydrolases, and glucuronyltransferase. On an average, group *b* differs from group *c* by a

[1] *Abbreviations used in this paper:* ab_5/aF-ferritin, antigen-antibody complex between ferritin and the anti-cytochrome b_5/anti-ferritin hybrid antibody; ER, endoplasmic reticulum; IgG, purified gamma globulin.

smaller sedimentation coefficient and a lower equilibrium density in sucrose and other density gradients, but both groups display a great dispersion with respect to these two properties. In particular, although group *b* enzymes are more abundant in the smoother subfractions, and those of group *c* in the rougher subfractions, careful quantitative analysis has made clear that this is not an absolute distinction. It has been shown that the ribosome load is the main factor which determines the equilibrium density of elements derived from ER (17), and since the two groups overlap extensively in the gradients it appears that enzymes of groups *b* and *c* occur in both smooth and ribosome-studded elements (3, 7).

Two alternative explanations have been proposed to account for these observations (1, 7) and are presented schematically in Fig. 1. According to the *multiplicity* explanation, there are two distinct sets of microsomal vesicles derived from the ER, one containing the *b* and the other the *c* group of enzymes. The latter set would bear on an average a greater number of ribosomes per unit mass. According to the *enzymatic heterogeneity* explanation, all microsomal vesicles derived from the ER contain both sets of enzymes, but in a ratio which increases in favor of group *c* with increasing ribosome load. It is, of course, of primary importance to our understanding of the organization of the ER that we be able to distinguish between these two possibilities. As shown in Fig. 1, this can be done morphologically with the help of a specific marker for each group. The histochemical studies of Leskes et al. (11) have provided evidence that glucose 6-phosphatase (a constituent of group *c*) is distributed in both the rough and smooth ER of parenchymal liver cells. These results argue against the multiplicity hypothesis described above. Nonetheless, the possibility remained that two sets of ER vesicles exist, one containing constituents of group *b* and the other not. To examine this question further, it was necessary to study the distribution of a specific marker of group *b*. In the present work, we have used for this purpose a hybrid anti-cytochrome b_5/anti-ferritin antibody (8). Our results snow that cytochrome b_5, a member of group *b*, is present in essentially all microsomal vesicles derived from ER, thus providing strong support in favor of the heterogeneity hypothesis. A brief report of these results has been published before (13).

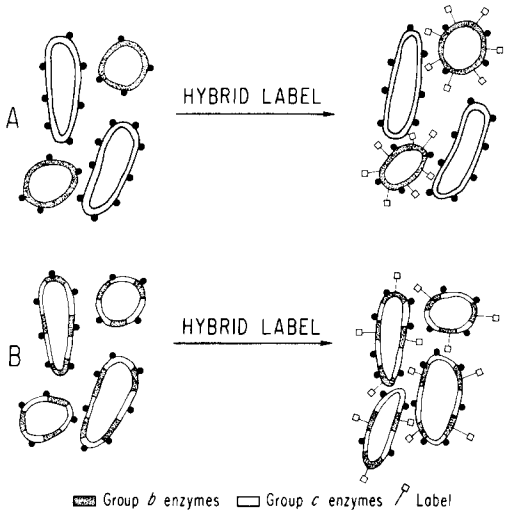

FIGURE 1 Two models of enzyme distributions in the microsomal vesicles derived from the ER. Based on the results of biochemical analysis of density equilibrium subfractionation of microsomes (1, 7), the organization of proteins on the membranes of the microsomes can be envisioned in two ways. Multiplicity model (A): distinct structures in the microsomes which are enzymatically homogeneous; heterogeneity model (B): a single microsomal membrane enzymatically heterogeneous. After reacting with the hybrid-ferritin complex, microsomal vesicles will show different distributions of label (Ψ) depending on which of the two distributions prevails.

MATERIALS AND METHODS

Tissue Fractionation and Biochemical Methods

Rat liver microsomes (P fraction) were prepared and subfractionated by means of isopycnic centrifugation in a linear gradient of sucrose, and the subfractions were analyzed for proteins and a number of enzymes, exactly as described previously (2, 6, 7).

Removal of Ribosomes

As a precaution against possible steric hindrance, ribosomes were removed from the microsomes and microsomal subfractions by treatment with pyrophosphate (3, 15). The fractions were passed through a column of Biogel A-150 m (Biorad Laboratories, Richmond, Calif.) equilibrated with 0.25 M sucrose containing 25 mM sodium pyrophosphate, pH 7.4, elution being performed with the same medium. Microsomal vesicles treated in this manner appear unaltered morphologically except for the loss of ribosomal profiles (3). Biochemical studies indicated that more than 90% of the ribosomal RNA is removed from the membranes by this procedure. No significant loss in cytochrome b_5 was noted after combined pyrophosphate treatment and gel chromatography.

Immunocytochemical Labeling

Labeling was carried out with an anti-cytochrome b_5/anti-ferritin hybrid antibody-ferritin complex (ab_5/aF-ferritin) as described before (8). Nonspecific adsorption, which was particularly marked with microsomal vesicles, was greatly reduced by the use, for the formation of the ab_5/aF-ferritin complex, of a ferritin preparation previously treated with ribosome-free microsomes. Microsomes were suspended in 0.25 M sucrose-25 mM sodium pyrophosphate, pH 7.4, collected by centrifugation, and resuspended in 0.15 M NaCl, to a concentration of 20 mg of microsomal protein per milliliter. Ferritin was added to this suspension to a final concentration of 5 mg/ml, the microsomes were removed by centrifugation (105,000 *g* for 40 min), and the supernate was used for incubation with the hybrid antibody. The efficiency of this step in decreasing nonspecific adsorption suggests that some of the ferritin molecules in the preparation had a special affinity for microsomal vesicles and were preferentially removed by the treatment.

Incubation conditions providing maximal labeling with minimal nonspecific adsorption were selected on the basis of trial experiments. The pyrophosphate-treated fractions were incubated for 12 h at 4°C with ab_5/aF-ferritin complex in a medium containing 0.25 M sucrose, 3 mM imidazole-HCl buffer, pH 7.4, 0.15 M NaCl, 0.5% Triton WR-1339,[2] bovine serum albumin (13 mg/ml), and 0.05% NaN_3 (to inhibit bacterial growth). The concentration of microsomes was adjusted to 1 μg of membrane-bounded cytochrome b_5 per milliliter, and that of ab_5/aF-ferritin complex to provide a sixfold excess of cytochrome b_5 binding capacity. The amount of microsomal suspension used varied between 0.20 and 1.0 ml, and the total incubation volume varied between 3 and 4 ml, depending on the fraction. Controls were made by preincubating the ab_5/aF-ferritin complex in a 12-fold excess solution of cytochrome b_5 antigen for 2 h at 0°C. This mixture was then added to the microsome preparations and treated in the same manner as the tests. In both controls and tests, unreacted ferritin-hybrid antibody was separated from the membranes by chromatography on a column (2.5 × 10 cm) of Biogel A-150 m pre-equilibrated with the sucrose medium used for incubation.

Electron Microscopy

Samples of the labeled membranes were prepared for electron microscopy by the Millipore filter method of Baudhuin et al. (5) as modified by Wibo et al. (17), except for these two changes: (*a*) bovine serum albumin in an amount equal to that of the proteins of the membrane preparation was added before fixation, to produce better dispersion of the vesicles in the pellicle; and (*b*) staining with uranyl acetate (0.5% in 0.05 M maleate buffer, pH 5) was done *en bloc* for 2 h.

Quantitative Analysis

For quantitative analysis, all pictures were first enlarged to a final magnification of 100,000. All the profiles were then classified according to size by means of a plexiglas plate on which were drawn five concentric circles corresponding to radii of 20, 40, 60, 80, and 100 nm. Most of the membrane profiles were nearly circular, and could be easily and rapidly assigned to a size class by this method. Invaginations of the vesicles were not taken into consideration since ferritin label was very seldom found within them. The number of ferritin images associated with each profile was then determined. (The association of a ferritin label with a particular membrane profile was judged on the basis of closest contact.) The results were expressed for each size class in terms of the absolute number of profiles bearing 0, 1, 2, . . . ferritin images per profile. Section thickness was precisely determined by the method of Gillis and Wibo (9). All counts were adjusted to a standard thickness of 55 nm.

Nonspecific adsorption did not follow Poisson's law, and was corrected by an empirical method, based on the assumption that nonspecific adsorption and specific labeling are two entirely independent phenomena. According to this assumption, within each size class, the subclass in the test that contains zero ferritin images is under-counted, because some of the profiles which should belong to this subclass are "labeled" nonspecifically. To correct this count, we have assumed that the contribution of such profiles to other subclasses is in the same proportion as seen in the corresponding control. These contributions are calculated by simple proportionality rule. They are added to the 0 ferritin-subclass to provide the corrected number of profiles bearing no ferritin, and they are subtracted from the appropriate subclasses. The same process is then applied in turn to each subsequent subclass after subtraction of the nonspecific contributions to it by preceding subclasses. In practice, this is done as follows.

Let a_i be the ratio of the number of profiles bearing i ferritin images to that of profiles bearing no ferritin in the control; K be the ratio of the total number of profiles to that of profiles bearing no ferritin in the control

$$\left(K = \sum_{i=0}^{n} a_i\right);$$

X_i be the number of vesicles bearing i ferritins in the test.

Then a_iX_0 represents the number of profiles belonging to the *zero*-subclass artificially transferred to the i-subclass by nonspecific adsorption; $K X_0$ is the true number of profiles in the *zero*-subclass; $X_i' = X_i - a_iX_0$ = the number of profiles in the i-subclass corrected for artificial transfer from the *zero*-subclass ($i \geq 1$).

[2] The results obtained with the incubation of hybrid antibody and microsomes in the presence of Triton WR-1339 were similar to those obtained without Triton. Preliminary experiments indicated, however, that this high molecular weight surfactant did decrease nonspecific adsorption of ferritin.

By reiteration of the same procedure: $a_{i-1}X_1'$ becomes the number of profiles belonging to the *1*-subclass artificially transferred to the *i*-subclass by nonspecific adsorption ($i \geq 2$); $K X_1'$ is the true number of profiles in the *1*-subclass; $X_i'' = X_i' - a_{i-1}X_1'$ = the number of profiles in the *i*-subclass corrected for artificial transfer from the *zero-* and *1*-subclass ($i \geq 2$). The procedure is repeated until every subclass has been fully corrected.

RESULTS

Biochemical Properties

Fractionation of the liver by differential centrifugation and subfractionation of the microsomes by isopycnic centrifugation in a sucrose gradient were performed with satisfactory recoveries for all components, and yielded results similar in every respect to those published previously (2, 7). Table I gives the biochemical composition of the microsomal fraction. Fig. 2 shows the distributions of key components after density equilibration in a sucrose gradient and identifies the subfractions (*5* and *12*) that were selected for analysis. More complete details on the composition of these subfractions are given in Table I. The low density subfraction *5* is typically enriched in cholesterol and phospholipid, in group *b* enzymes, and in enzymes associated with non-ER elements; it is relatively depleted of glucose 6-phosphatase (group *c*), and especially of RNA. The high density subfraction *12* is correspondingly rich in RNA and glucose 6-phosphatase, and poor in group *b* enzymes; it is practically devoid of non-ER contaminants.

Table II provides an estimate of the contribution of different cell parts to the total protein of the microsomal fraction and subfractions. It is seen that subfraction *12* is fairly purified in elements derived from ER, whereas about one-third of the membrane elements in subfraction *5* are of non-ER origin. Correcting for these smooth non-ER elements, we find that the average RNA content per mg protein of true ER elements in this subfraction is a little more than one-fourth the RNA content in subfraction *12*.

TABLE I

Biochemical Properties of Microsomal Preparations (Submitted to Morphological Analysis)

	Fraction			
		Microsomal subfractions		
	Microsomes	*5*	*12*	Ratio *12/5*
		Density range		
	Full	1.123–1.140	1.245–1.267	
		Protein (mg/g liver)		
	44.9 (18.4%)	5.0 (2.0%)	2.0 (0.81%)	0.40
Constituent		Specific Content*		
Cytochrome b_5	6.9	8.5	3.8	0.45
Cholesterol	28	58	4	0.07
RNA	100	43	229	5.32
Phospholipid	0.47	0.64	0.28	0.44
Enzyme		Relative Specific Activity‡		
NADH cytochrome *c* reductase	3.39	5.08	1.64	0.32
Glucose 6-phosphatase	3.94	2.98	4.52	1.52
Alkaline phosphodiesterase I	2.44	2.76	0.04	0.015
Galactosyltransferase	3.87	13.19	0.14	0.011
Monoamine oxidase	1.16	3.88	0.08	0.021
Cytochrome oxidase	0.26	–	–	–
Acid phosphatase	0.70	0.78	0.25	0.32

* The specific content is expressed per mg of protein, in μg for cytochrome b_5 (mol wt 12,000), cholesterol and RNA, and in μmoles of phosphorus for phospholipid.

‡ Relative specific activity is the ratio of the amount of enzyme and the amount of protein expressed in percent of the total amount recovered. The recoveries for the enzymes and protein ranged between 85 and 110%.

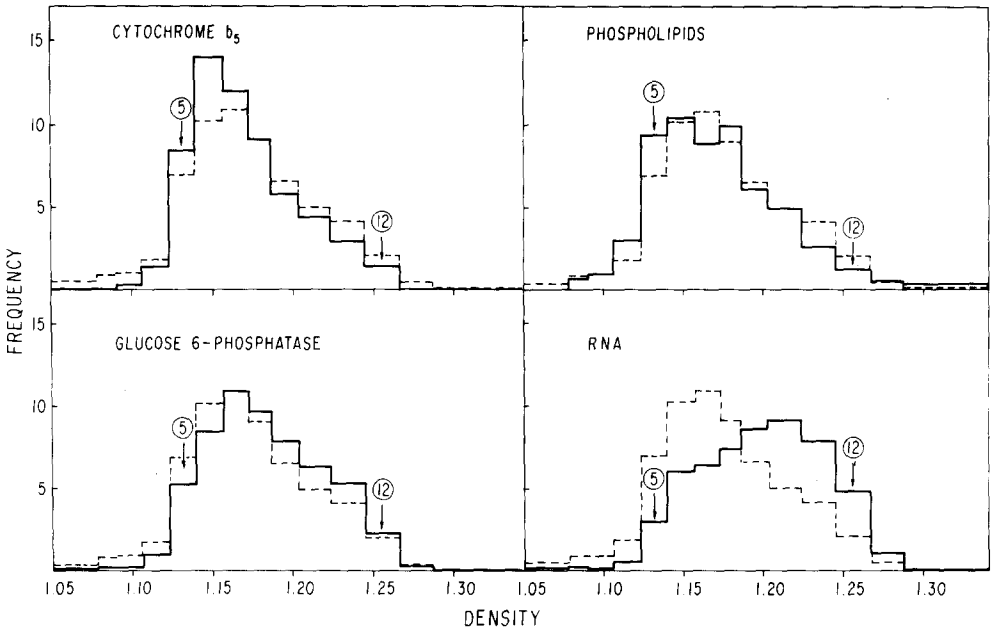

FIGURE 2 Subfractionation of microsomal fraction by isopycnic centrifugation through sucrose gradient. The distribution of protein is presented by dotted lines superimposed on each profile. The arrows indicate the two subfractions (*5* and *12*) chosen for detection of cytochrome b_5 under the electron microscope with the ab_5/aF-ferritin label.

TABLE II

Amount of Protein Related to Components Other Than ER in Microsomal Fraction and Subfractions

Component	Microsomes	Microsomal subfractions	
		5	*12*
Plasma membranes	7.0	7.9	0.1
Golgi complex	3.9	13.2	0.1
Lysosomes	1.0	1.1	0.4
Mitochondria, excluding outer membranes	4.3	–	–
Outer mitochondrial membranes	3.9	12.9	0.3
Total	20.1 [16.2]	37.1 [24.2]	0.9 [0.6]

Values are expressed in percent of total protein of the preparations. They have been computed from the ratio of the relative specific activity of marker enzymes in the fraction and in purified preparations of the subcellular components. The relative specific activity was taken to be 35 for alkaline phosphodiesterase I in plasma membranes, 100 for galactosyltransferase in Golgi elements, 70 for acid phosphatase in lysosomes, 5 for cytochrome oxidase of mitochondria and 30 for monoamine oxidase in outer mitochondrial membranes. The latter was excluded from the sum of values given between brackets since the outer membranes of mitochondria were found to contain cytochrome b_5 reacting with the ab_5/aF-ferritin complex (8).

Morphological Observations

The appearance of the initial microsomal fraction after incubation with ab_5/aF-ferritin complex is shown in Fig. 3. The difference between control and test is marked: the level of labeling in the test is much higher than in the control, and a large proportion of the test profile perimeter is involved in labeling. The ferritin label is found only on the outer face of the vesicle membrane, and in the test is often separated from it by a space of about 10–12 nm. Some profiles, mostly of large size, did not label at all.

Figs. 4 and 5 show subfractions *5* and *12* treated

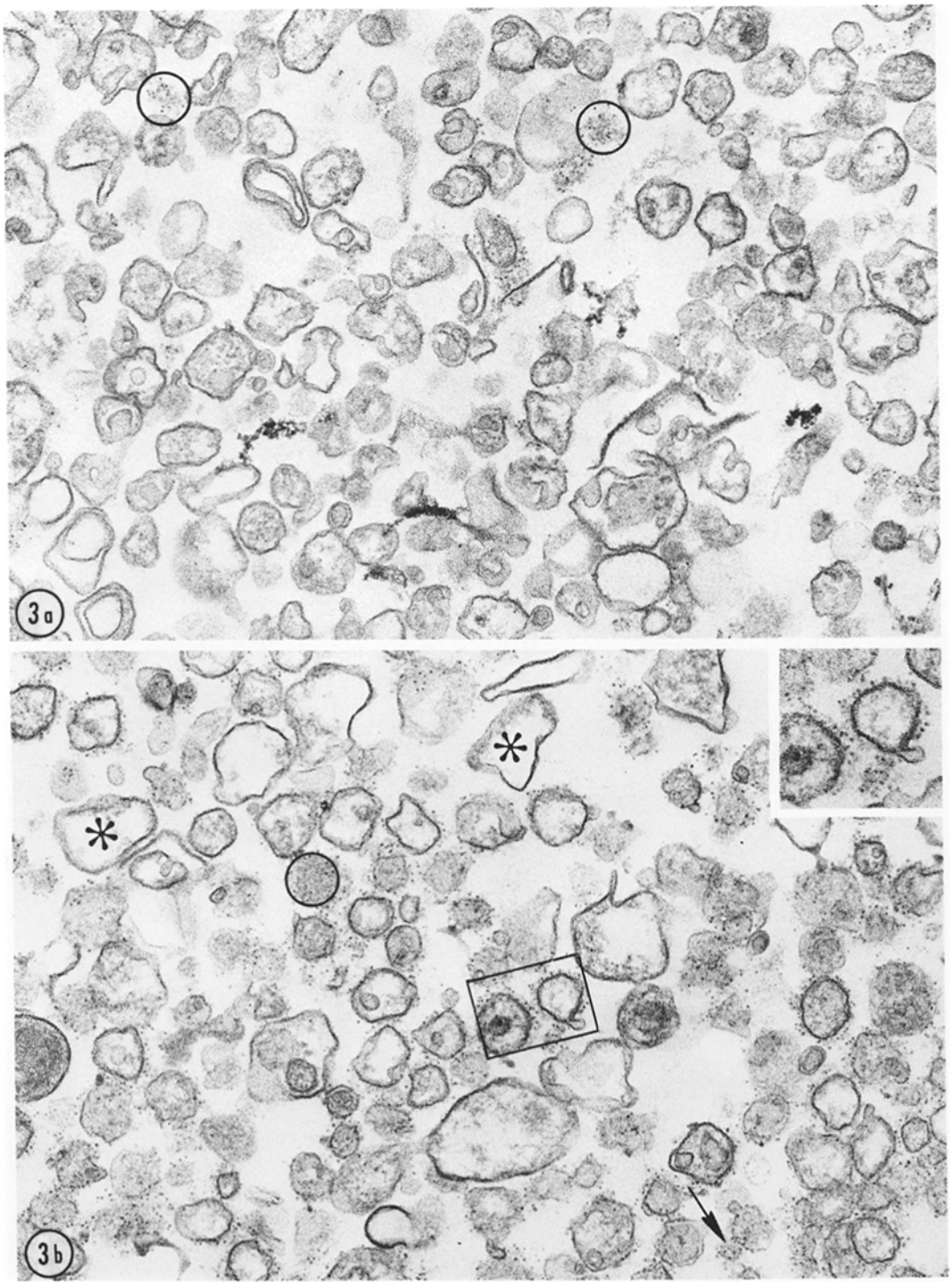

FIGURE 3 Electron micrographs of microsomal (P) fraction incubated with the ab_5/aF-ferritin complex after detachment of ribosomes by Na-pyrophosphate. In the control (*a*), most profiles have no ferritin on their surface. Occasional free aggregates of ferritin are seen (circles). In contrast, many vesicle profiles in the test (*b*) are labeled with ferritin. Some ferritin is observed on polar sections of vesicles (arrow). The rectangle indicates a microsome vesicle enlarged in the inset (× 75,000) to show ferritin attached to the membrane surface. The exceptional profiles not labeled are usually large in size (asterisks). × 50,000.

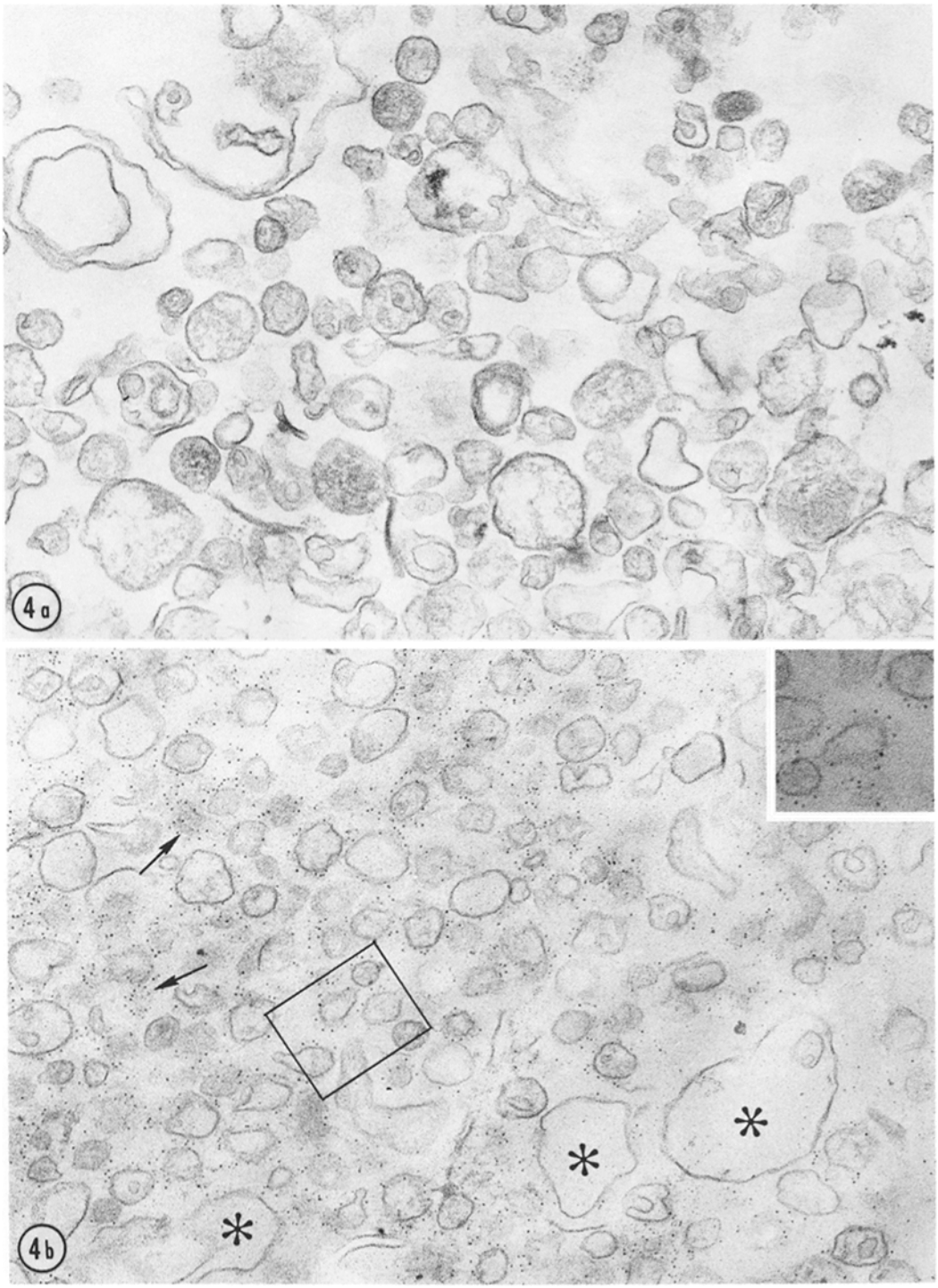

FIGURE 4 Result of incubation of light microsomal subfraction 5 with ab_5/aF-ferritin label after removal of ribosomes by Na-pyrophosphate. Most vesicles in the control (*a*) carry no ferritin label on their surface. In the test (*b*), large numbers of vesicles are labeled with the ferritin. Polar sections of some vesicles show ferritin on their surface (arrows). The rectangle indicates a microsome vesicle enlarged in the inset (× 75,000) to show ferritin attacted to the membrane surface. Some large profiles (plasma membrane fragments?) are less heavily labeled (asterisks). × 50,000.

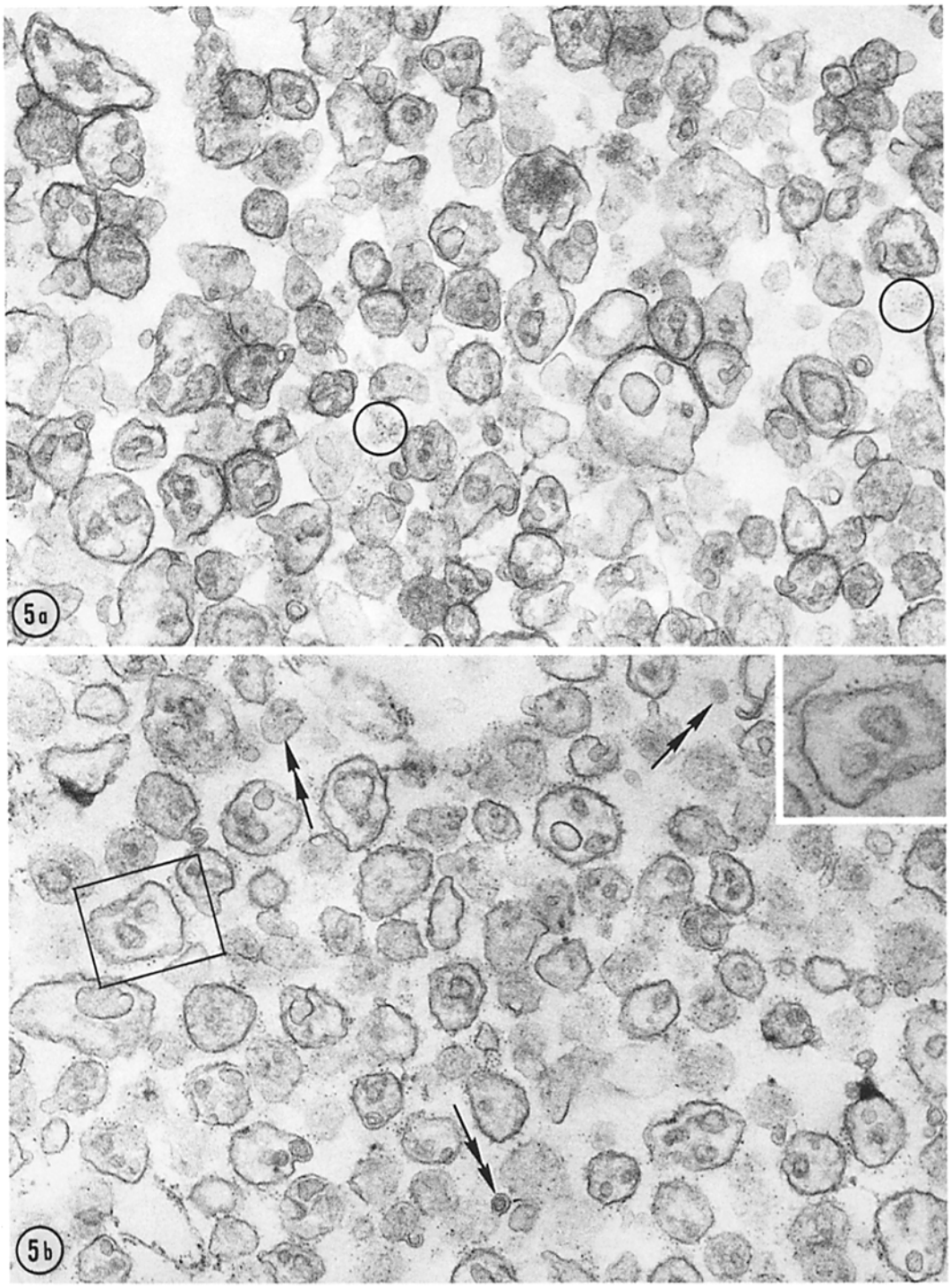

FIGURE 5 Appearance of heavy microsomal subfraction *12* after incubation with ab_5/aF-ferritin label. Before incubation, ribosomes were removed by Na-phosphate. Most profiles in the control (*a*) are free of ferritin labeling. The majority of ferritin images seen are present as aggregates (circles). Nearly all profiles in the test (*b*) contain ferritin on their surface. The rectangle indicates a microsome vesicle enlarged in the inset (× 75,000) to show ferritin attached to the membrane surface. Some very small vesicles do not appear to carry ferritin label (double arrows). × 50,000.

with ab_5/aF-ferritin complex. In general appearance, the images resemble that of the parent microsomal fraction. Their most notable aspect is the large number of profiles that are labeled with ferritin, especially in subfraction *12* (Fig. 5*b*), which contains only a few tiny unlabeled profiles.

In all three preparations examined, many vesicles showed internal vesiculation, presumably due to invagination of their membrane. These interiorized parts hardly ever showed any labeling, even when they appeared to be connected to the exterior by a channel (Fig. 5*b*). Perhaps these channels are too narrow for the ab_5/aF-ferritin complex to pass through.

In addition to the preparations shown here, many others were examined in the course of our search for optimal experimental conditions. Extensive labeling in the tests was observed in all cases; labeling in the control was the same regardless of whether the preparations were incubated with ab_5/aF-ferritin preincubated with cytochrome b_5 or with aF/aF-ferritin complexes. The results presented in Figs. 3–5 and 7–9, although obtained from single experiments, may be considered representative.

Quantitative Analysis

In Fig. 6 are shown the size distributions of the microsomal vesicles seen in the images subjected to quantitative analysis. Confirming the observation of Wibo et al. (17), we find that the vesicles in subfraction *12* tend to be larger, those in subfraction *5* smaller, than those in the parent microsomal fraction. An unexpected finding is that of a slight but systematic shift towards lower sizes of the test distribution with respect to the control. Possibly, binding of the antibody-ferritin complex may cause some shrinkage or breakage of the microsomal vesicles; or the presence of added cytochrome b_5 in the control may cause some swelling. This effect obviously does not appreciably alter the validity and significance of our observations.

The results of our quantitative measurements of the distribution of the ferritin label are presented graphically in Figs. 7–9. In each of these figures, we show side by side, for each size class, the raw data for test and control and the corrected distribution. It is noticed that the correction is important only for the lower subclasses.

As illustrated by Fig. 10, the average ferritin load of the labeled profiles is essentially proportional to the diameter of the profiles, indicating that the number of hybrid antibody-ferritin com-

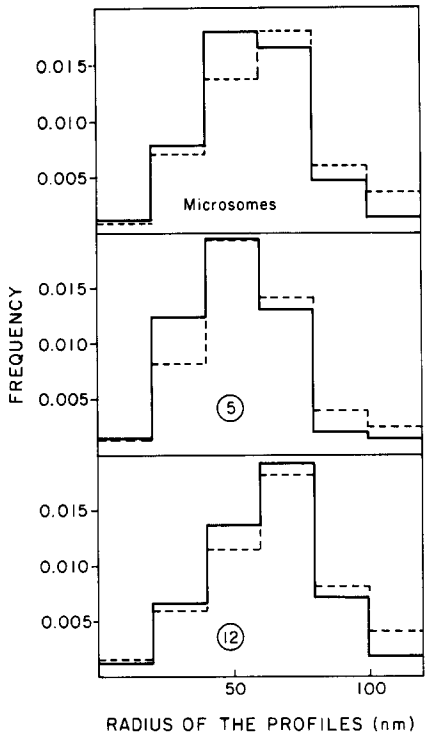

FIGURE 6 Distribution of profile radius seen in the images subjected to quantitative analysis. Test (——), Control (—). Frequency is defined as $\Delta N/N\Delta R$ where N is the total number of profiles and R is the profile radius. The upper radius limit has arbitrarily been set at 120 nm. The dimension of the frequency scale in nm^{-1}.

plexes bound per unit surface area of membrane is independent of the size of the vesicles. Furthermore, the average ferritin load is the same for all three preparations, which is surprising in view of the differences in their cytochrome b_5 content (see Table I). The results listed in Table III show that an eightfold increase in the amount of ab_5/aF-ferritin complex used raised the degree of nonspecific adsorption, but did not increase the degree of specific labeling. Therefore the immunochemical label cannot be limiting. It looks rather as though the degree of labeling that we reach corresponds to some sort of saturation. Indeed, if we convert the immunochemical results into number of ferritin-labeled hybrid antibodies bound per milligram protein, using the methodology of Baudhuin et al. (4) and Wibo et al. (17),[3] and compare the results

[3] This calculation is based on the amount of microsomes fixed and filtered, the surface area of the pellicle specimen examined, the thickness of the Epon section, and the magnification used.

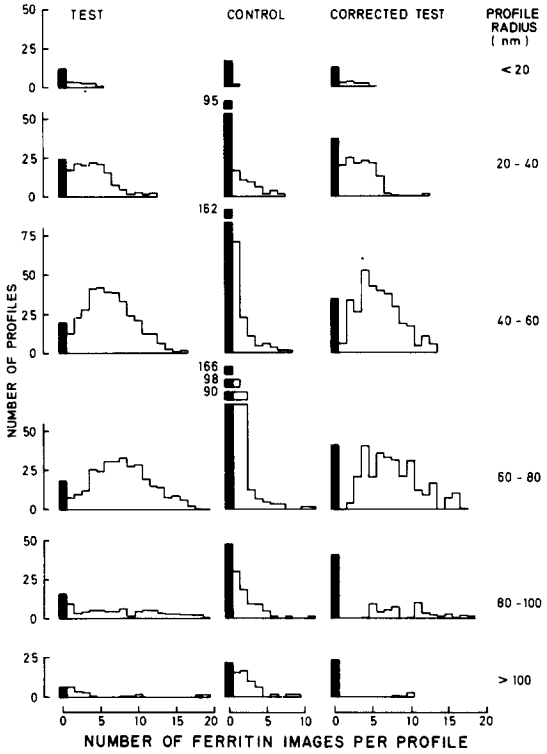

FIGURE 7 Distribution of number of profiles in relation to the ferritin load in the microsomal (P) fraction. Profiles were grouped according to size into six different classes, and the number of profiles bearing n ferritin images was determined. The analysis was performed on 1330 and 665 profiles, respectively, in the test and in the control. Values listed in the graph have been adjusted to 1,000 profiles. The mathematical procedure for correction of the test for nonspecific adsorption is presented in the text.

with those of the biochemical determinations, we find that we label only between 5 and 7.5% of the cytochrome b_5 molecules, depending on the preparation. This point will be examined further in the Discussion.

It follows from the above considerations that we cannot attach much significance to the actual number of hybrid antibodies bound. On the other hand, the number of profiles that are labeled is not likely to be overestimated once it has been corrected for nonspecific adsorption. Our results have been summarized in this fashion in Table IV. We see that subfraction *12* contains only 3.7% of unlabeled profiles. Even this small number may be overestimated, since the unlabeled profiles occur almost exclusively in the two smaller size classes, and therefore most likely belong to labeled vesicles of which the labeled portion was not included in the thickness of the section. A similar artifact probably affects also the results obtained on the two other fractions.

In Table V the results of Table IV have been multiplied by the corresponding average radius, and then normalized, to provide an estimate of the proportion of labeled and unlabeled membrane surface area. Comparing the totals of Table V with the values of Table II, we find that there is a rough agreement for each fraction between the proportion of unlabeled membrane surface area and the proportion of protein contributed by non-ER elements (excluding outer mitochondrial membranes, which are known to contain cytochrome b_5, and to bind the hybrid antibody-ferritin complex). The agreement would be even closer if account were taken of the fact that some of the small unlabeled profiles probably appear so as a result of unfavorable sectioning.

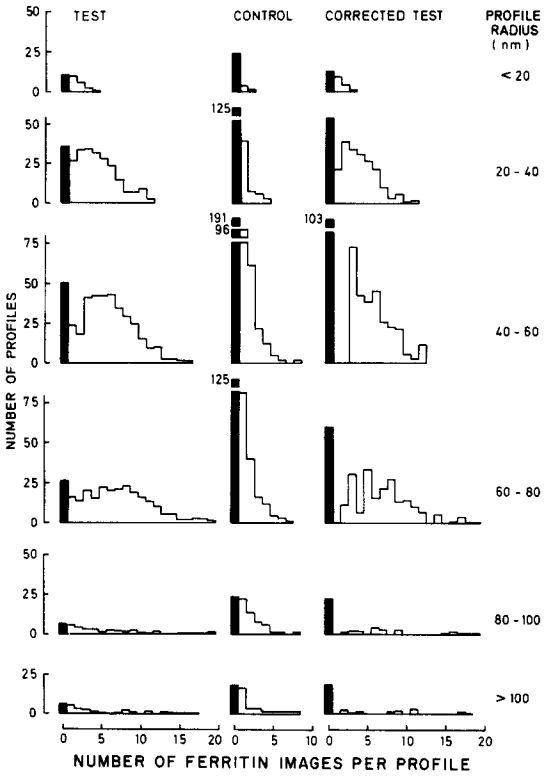

FIGURE 8 Distribution of number of profiles in relation to the ferritin load in the light microsomal subfraction *5*. The analysis was performed on 1,330 and 665 profiles in the test and in the control, respectively. Values presented in the graph have been adjusted to 1,000 profiles.

Influence of Antibodies on NADH Cytochrome c Reductase Activity

In order to assess the proportion of cytochrome b_5 molecules accessible to the ab_5/aF-ferritin complex, we compared the effectiveness of this complex to inhibit microsomal NADH cytochrome *c* reductase activity with that of the bivalent ab_5/ab_5 antibody. The results, represented in Fig. 11, show that the complex is less effective than the hybrid antibody alone, which itself is somewhat less effective than the symmetrical antibody. It appears, therefore, that the bound ferritin may prevent some of the complexes from interacting with their target. This effect is, however, much too small to account for the low labeling ratios mentioned above.

DISCUSSION

It is clear from the results of our analysis that the great majority of true ER-derived vesicles are specifically labeled with the ab_5/aF-ferritin marker and thus contain cytochrome b_5 in their membranes. This finding is compatible with the multiplicity hypothesis only if one assumes that group *c* enzymes (such as glucose 6-phosphatase) are concentrated in a very small number of vesicles. The cytochemical studies of Leske et al. (12), however, indicate just the opposite, that glucose 6-phosphatase is associated with a large population of microsomal vesicles. One might argue that, if the cytochrome b_5 content of fractions *5* and *12* in Table I are normalized to phospholipid (in the absence of reliable values for membrane proteins) instead of total protein (some of which may be secretory or content protein), the amounts of hemeprotein in the light and heavy subfractions are similar.[4] In this case, the discrepancy between

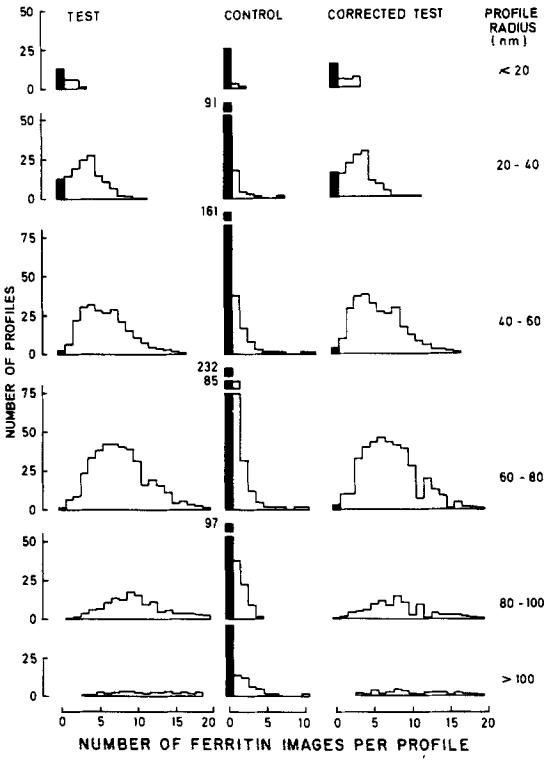

FIGURE 9 Distribution of number of profiles in relation to the ferritin load in the heavy microsomal subfraction *12*. The analysis was performed on 1,330 and 665 profiles in the test and in the control, respectively. Values presented in the graph have been adjusted to 1,000 profiles.

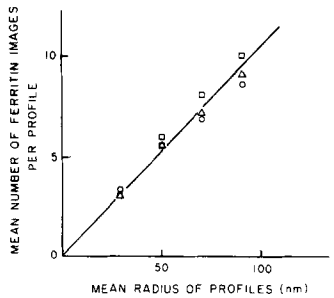

FIGURE 10 Relation between the average ferritin load of the profiles and their radius. Calculation of the average number of ferritin images per profile was done after correction for nonspecific adsorption as described in the text. The corrected *zero* subclass was excluded from the calculation, and adjustment to a section thickness of 55 nm was made. Microsomal fraction (□), subfraction *5* (○), subfraction *12* (△). The values of the three preparations are not significantly different.

TABLE III

Effect of Hybrid Concentration on the Labeling of Microsomes

	Average no. of ferritin images per profile		
Excess of hybrid*	Test	Control	Corrected test
5	9.5	2.4	7.1
40	12.1	5.5	6.6

* The excess of hybrid is expressed as the ratio of the cytochrome b_5 binding capacity of the ab_5/aF-ferritin label to the cytochrome b_5 content of microsomes.

[4] Normalization of the cytochrome b_5 content to phospholipids is also questionable since whole microsomes and especially subfraction *5* are contaminated with non-ER membranes that contribute phospholipids too.

TABLE IV

*Distribution of Labeled and Nonlabeled Profiles in the Different Microsomal Preparations**

	Microsomes		Microsomal subfractions			
			5		*12*	
Profile radius	Nonlabeled	Labeled	Nonlabeled	Labeled	Nonlabeled	Labeled
nm						
<20	1.3	1.1	1.3	1.6	1.5	1.1
20-40	3.7	12.6	5.3	19.2	1.6	12.0
40-60	3.4	32.1	10.3	28.9	0.3	27.3
60-80	4.1	29.5	6.0	20.1	0.2	38.7
80-100	4.1	5.2	2.3	1.9	0	13.9
>100	2.3	0.6	1.8	1.3	0	3.4
Total	18.9	81.1	27.0	73.0	3.6	96.4

* The results are expressed in percent of the total number of profiles of each preparation.

TABLE V

*Relative Surface Area of the Vesicles Labeled with Ferritin**

	Percent of total membrane surface area					
	Microsomes		Microsomal subfractions			
			5		*12*	
Average profile radius	Unlabeled	Labeled	Unlabeled	Labeled	Unlabeled	Labeled
nm						
10	0.2	0.2	0.2	0.3	0.2	0.2
30	1.9	6.5	3.0	10.9	0.8	5.8
50	2.9	27.7	9.8	27.4	0.2	22.2
70	5.0	35.6	8.0	26.7	0.2	44.0
90	6.4	8.1	3.9	3.3	0.0	20.3
(110)‡	4.4	1.1	3.8	2.7	0.0	6.1
Total	20.8	79.2	28.7	71.3	1.4	98.6

* The relative surface area is taken as the normalized product of the percent of vesicles of each class and the average radius.

‡ This value may be underestimated.

specific cytochrome b_5 content (uneven) and ferritin labeling (even or nearly so) is less striking. However, this reassessment does not argue against the heterogeneity hypothesis since the differences between the light and heavy subfractions in glucose 6-phosphatase activity normalized to phospholipids become even more pronounced. Taken together, our findings and those of Leskes et al. (12) thus provide strong evidence for the enzyme heterogeneity of the ER-derived microsomes.

One might attribute the results of both our studies and those of Leskes et al. (12) to a homogenization artifact during which different membranes of the ER become fused together. There are several pieces of evidence which suggest that this is not the case. First, mixing of labeled and unlabeled rough microsomes followed by homogenization does not lead to the formation of "hybrid" vesicles by fusing of two different particles (12). Secondly, histochemical studies of glucose 6-phosphatase reaction in thin sections of liver tissue showed the stain to be present throughout all the ER cisternae of adult liver cells (11). Thirdly, as mentioned above, the various enzymes of the ER distribute in a nonhomogeneous fashion in sucrose density gradients. Consideration of both the results of the sucrose density gradient experiments and the findings that both cytochrome b_5 and

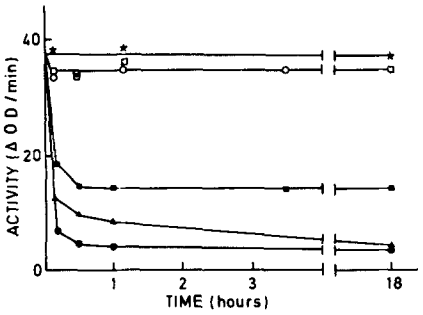

FIGURE 11 Inhibition of NADH cytochrome *c* reductase by anti-cytochrome b_5 antibodies. Before assaying for NADH cytochrome *c* reductase activity, microsomes (0.4 mg protein) were incubated for various lengths of time (abscissa) at 0°C in 0.25 M sucrose containing 25 mM phosphate buffer, pH 7.4, 0.15 M NaCl, 0.02% NaN_3 (to inhibit bacterial growth), and different preparations of proteins: nonimmune IgG (○); nonimmune IgG and ferritin (□): ab_5/ab_5 IgG (●); ab_5/aF hybrid IgG (▲); ab_5/aF-ferritin complex (■); none (*). The amount of hybrid antibody binding capacity present was six times higher than the content of membrane-bounded cytochrome b_5. The same amount of IgG protein was present in all tests.

glucose 6-phosphatase are present in most of the ER-derived vesicles leads us to think that many of the enzymes of the ER are dispersed throughout all the ER. We cannot answer the question raised previously (7) whether the biochemical heterogeneity of the ER-derived microsomes is intrinsic to the ER in each cell, or results from correlated differences in enzymatic activities and in ribosome load between different cells.

It would be particularly interesting to know whether cytochrome b_5 is distributed randomly in the membranes as the data of Rogers and Strittmatter (14) suggest or whether it occurs as small patches. Unfortunately, our technique does not allow a more detailed topographic study to be carried out, for we have found that as little as 5% of the molecules of cytochrome b_5 in our preparations are labeled with the hybrid-ferritin complex. This finding cannot be simply that a portion of the cytochrome b_5 is unavailable for reaction because it is buried in the interior of the membrane. Mild proteolytic digestion of vesicles has proven that almost all of the measurable cytochrome b_5 of microsomes is present on the outer surface of the membrane (10). However, several other factors could affect the extent to which the hybrid-ferritin complex reacts with cytochrome b_5. One factor is that a portion of the vesicle membrane is invaginated and the cytochrome b_5 within appears to be inaccessible to the hybrid. We estimate, using the morphometric technique of Weibel et al. (16) for analysis, that the invaginations comprise about 20% of the total membrane surface of the vesicles. Table III and Fig. 10 strongly indicate that, under the conditions we have employed, the labeling of the membranes is saturated. One explanation is that perhaps many of the cytochrome b_5 molecules on the membrane are prevented from reaction by steric interference from nearby hybrid-ferritin complexes attached to the membrane. Fig. 11 suggests that steric hindrance does indeed occur but that the effect is not sufficient to account for all the cytochrome b_5 which is not labeled. Another consideration is the fact, as shown in the preceding paper (8), that about 18% of the hybrid preparation bearing anti-cytochrome b_5 activity consists of antibodies of the type ab_5/ab_5 or ab_5/aX whose presence would remain undetected in the electron micrographs. We believe from our previous work (8) that the number of hybrid molecules associated as a complex with one ferritin molecule is in the range of one to three. Some ferritin-hybrid labeling therefore may include reaction with more than one molecule of cytochrome b_5. Finally, there is also the possibility that, during chromatographic processing, ferritin molecules may become dissociated from the ab_5/aF-ferritin complex, leaving the hybrid unlabeled and thus undetected. We feel that, because of these numerous factors, the ferritin-hybrid label, although entirely satisfactory to qualitatively distinguish membranes on the basis of their cytochrome b_5 content, is of limited use for determining more precise details of the topographic distribution of cytochrome b_5 on membranes.

The authors wish to thank Dr. C. de Duve for many valuable discussions and for his continuous interest in this study. The authors also express their gratitude to Miss A. Trouet and Dr. M. Wibo for their collaboration in several aspects of this work. The valuable assistance of H. Shio in the preparation of electron micrographs and the technical assistance of J. Nackers is also gratefully acknowledged.

This work has been supported by grants from the Belgian Fonds National de la Recherche Scientifique (F.N.R.S.), Fonds de la Recherche Fondamentale Collective and Ministère de la Politique et Programmation Scientifique. Dr. J. Remacle was an Aspirant of the F.N.R.S. Dr. S. Fowler was supported during this work by the Helen Hay Whitney Foundation.

Received for publication 4 March 1975, and in revised form 17 June 1976.

REFERENCES

1. AMAR-COSTESEC, A., H. BEAUFAY, E. FEYTMANS, D. THINES-SEMPOUX, and J. BERTHET. 1969. Subfractionation of rat liver microsomes. *In* Microsomes and Drug Oxidations. J. R. Gillette, A. H. Conney, D. J. Cosmides, R. W. Estabrook, J. R. Fouts, and G. J. Mannering, editors. Academic Press, Inc., New York. 41–58.
2. AMAR-COSTESEC, A., H. BEAUFAY, M. WIBO, D. THINES-SEMPOUX, E. FEYTMANS, M. ROBBI, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. *J. Cell Biol.* **61**:201–212.
3. AMAR-COSTESEC, A., M. WIBO, D. THINES-SEMPOUX, H. BEAUFAY, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. IV. Biochemical, physical and morphological modifications of microsomal components induced by digitonin, EDTA and pyrophosphate. *J. Cell Biol.* **62**:717–745.
4. BAUDHUIN, P., and J. BERTHET. 1967. Electron microscopic examination of subcellular fractions. II. Quantitative analysis of the mitochondrial population isolated from rat liver. *J. Cell Biol.* **35**:631–648.
5. BAUDHUIN, P., P. EVRARD, and J. BERTHET. 1967. Electron microscopic examination of subcellular fractions. I. The preparation of representative samples from suspensions of particles. *J. Cell Biol.* **32**:181–191.
6. BEAUFAY, H., A. AMAR-COSTESEC, E. FEYTMANS, D. THINES-SEMPOUX, M. WIBO, M. ROBBI, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. I. Biochemical methods. *J. Cell Biol.* **61**:188–200.
7. BEAUFAY, H., A. AMAR-COSTESEC, D. THINES-SEMPOUX, M. WIBO, M. ROBBI, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. III. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. *J. Cell Biol.* **61**:213–231.
8. FOWLER, S., J. REMACLE, A. TROUET, H. BEAUFAY, J. BERTHET, M. WIBO, and P. HAUSER. 1976. Analytical study of microsomes and isolated subcellular membranes from rat liver. V. Immunological localization of cytochrome b_5 by electron microscopy: methodology and application to various subcellular fractions. *J. Cell Biol.* **71**:535–550.
9. GILLIS, J.-M., and M. WIBO. 1971. Accurate measurement of the thickness of ultrathin sections by interference microscopy. *J. Cell Biol.* **49**:947–949.
10. ITO, A., and R. SATO. 1969. Proteolytic microdissection of smooth-surfaced vesicles of liver microsomes. *J. Cell Biol.* **40**:179–189.
11. LESKES, A., P. SIEKEVITZ, and G. E. PALADE. 1971. Differentiation of endoplasmic reticulum in hepatocytes. I. Glucose-6-phosphatase distribution *in situ*. *J. Cell Biol.* **49**:264–287.
12. LESKES, A., P. SIEKEVITZ, and G. E. PALADE. 1971. Differentiation of endoplasmic reticulum in hepatocytes. II. Glucose-6-phosphatase in rough microsomes. *J. Cell Biol.* **49**:288–302.
13. REMACLE, J., S. FOWLER, H. BEAUFAY, and J. BERTHET. 1974. Ultrastructural localization of cytochrome b_5 on rat liver microsomes by means of hybrid antibodies labeled with ferritin. *J. Cell Biol.* **61**:237–240.
14. ROGERS, M. J., and P. STRITTMATTER. 1974. Evidence for random distribution and translational movement of cytochrome b_5 in endoplasmic reticulum. *J. Biol. Chem.* **249**:895–900.
15. SACHS, H. 1958. The effect of pyrophosphate on the amino acid incorporating system of rat liver microsomes. *J. Biol. Chem.* **233**:650–656.
16. WEIBEL, E. R. 1969. Stereological principles for morphometry in electron microscopic cytology. *Int. Rev. Cyt.* **26**:235–302.
17. WIBO, M., A. AMAR-COSTESEC, J. BERTHET, and H. BEAUFAY. 1971. Electron microscope examination of subcellular fractions. III. Quantitative analysis of the microsomal fraction isolated from rat liver. *J. Cell Biol.* **51**:52–71.