BINDING OF CYTOCHROME $b_5$ TO MEMBRANES OF ISOLATED SUBCELLULAR ORGANELLES FROM RAT LIVER

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ABSTRACT

The in vitro incorporation of a well-characterized integral protein cytochrome $b_5$ into membranes of various subcellular organelles was investigated by biochemical and immunochemical methods. Microsomes, peroxisomes, and outer mitochondrial membranes, all containing endogenous cytochrome $b_5$, incorporated large amounts of the hemoprotein in such a way that it was reducible by an inherent NADH cytochrome $b_5$ reductase. Lysosomal membranes did not incorporate cytochrome $b_5$. Inner mitochondrial and Golgi membranes, which do not naturally contain cytochrome $b_5$, bound it in vitro but it was not reduced in the presence of NADH. These results show some discrepancies between the natural localization and the in vitro binding of cytochrome $b_5$. They confirm one aspect of the fluid membrane theory and bring new elements to our understanding of the maintenance of the specific features of the membranes of subcellular organelles with respect to the cell dynamism.

KEY WORDS cytochrome $b_5$ · subcellular organelles · membrane-protein interaction · membrane specificity

The notion of the specific membrane proteins was first proposed by de Duve when he introduced the general concept of specific enzyme markers for the characterization of subcellular entities (13, 14). Some of these enzymes were bound to membranes, others were in soluble form either in the cytoplasm or inside the organelles. Later, enzymes were discovered to be associated with several subcellular compartments, so that some exceptions to this concept were introduced, but in fact most of the bound enzymes were found to be associated with only one subcellular membrane (15, 16, 2).

Cytochrome $b_5$ is in fact one of these exceptions since it is associated with both the endoplasmic reticulum (ER) (64, 2) and the outer mitochondrial membrane (62). Its presence in the peroxisomal membrane was also proposed but it is absent from the Golgi, lysosomal, inner mitochondrial, and plasma membranes (17, 23).

Cytochrome $b_5$ is a very much-studied, amphipathic, integral protein (61). It contains only one peptide chain but is organized, on a spatial level, into two distinct parts: a hydrophilic part contain-
ing the heme peptide core which extends out of the membrane, and a hydrophobic part embedded in the lipid bilayer (63). The hydrophilic part of the cytochrome (T-cytochrome \( b_s \)) can be easily purified after digestion of the microsomes by trypsin (46, 66); on the other hand, the entire molecule (D-cytochrome \( b_s \)) can also be purified after extraction with detergents (63).

D-cytochrome \( b_s \) can be incorporated in vitro into the ER and into the outer mitochondrial membranes (65). Its binding to the inner mitochondrial membrane has also been suggested (65). The exogenous hemoprotein behaves in the same way as the endogenous cytochrome and is fully active. The binding to the membrane is due to the interaction between the membrane lipids and the hydrophobic segment of the protein (19, 54, 65). The hemoprotein can also be set in artificial membranes (18, 27, 50, 56, 67).

In this paper, we question the reasons for the maintenance of cytochrome \( b_s \) on specific subcellular membranes. The absence of cytochrome \( b_s \) from some membranes could be the result of certain characteristics which prevent the protein from being inserted into the membrane, or it could be related to more complex phenomena connected, for example, to the biogenesis of cytochrome \( b_s \) and to the movements of membrane proteins between intracellular compartments.

Our approach to the problem was to investigate the binding of D-cytochrome \( b_s \) to different subcellular membranes and to compare the normal localization of the protein with its in vitro incorporation. In a preceding work, we investigated the binding of cytochrome \( b_s \) to the plasmalemma and have shown that binding occurred in vitro on the cytoplasmic face of the membrane (53). In this work, the results clearly show that, except for the lysosomes, D-cytochrome \( b_s \) binds in vitro to the membranes of all the subcellular organelles.

**MATERIALS AND METHODS**

**Preparation of Subcellular Membrane Fractions**

Using methods already published, we prepared microsomes (1), outer mitochondrial membranes (23), Golgi apparatus (71), and lysosomes (69). Lysosomal membranes were obtained by diluting the lysosomes 10 times with \( H_2O \) and by washing them in 0.5 M NaCl to remove adsorbed enzymes. Peroxisomes were purified following the method described by Leighton et al. (33) but with a few modifications: the \( \lambda \) fraction was washed twice by centrifugation at 25,000 rpm in rotor n°40 (Ultracentrifuge model L2-65, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) and with a time integral of the squared angular velocity (\( W = \frac{\alpha d t}{t} \)) of \( 1.7 \times 10^8 \) rad\(^2\) s\(^{-1}\). The fluffy layer was removed with the supernate to reduce contamination of the fraction by microsomal vesicles. The pellet, obtained from 4 g of liver, was resuspended in 0.5 ml of a 41.2% (wt/wt) sucrose solution (\( p = 1.19 \)) and the suspension was layered on 4.5 ml of sucrose gradient whose density increased uniformly from 1.19 to 1.27. The gradient was centrifuged at 39,000 rpm for 2 h (\( W = 118 \times 10^8 \) rad\(^2\) s\(^{-1}\)) in an SW65 rotor. The material that equilibrated at a density higher than 1.235 was the peroxisomal preparation.

The inner mitochondrial membranes were prepared by the method of Colbeau et al. (12) with slight modifications: a mitochondrial (M) fraction was prepared by the fractionation procedure of de Duve et al. (14). The mitochondria were slowly resuspended in a 20 mM phosphate buffer, pH 7.5, solution containing 0.2 mg/ml bovine serum albumin. The suspension was homogenized by 25 strokes of the tight pestle in a Deunce homogenizer (Kontes Co., Vineland, N. J.) and, after 10 min, centrifuged at 55,000 rpm for 20 min in rotor n°65 (\( W = 33.5 \times 10^8 \) rad\(^2\) s\(^{-1}\)). The pellet was also homogenized, centrifuged as described above, and finally resuspended by 25 strokes of the homogenizer in a small volume of phosphate buffer. 1.5 ml of the membrane suspension was layered on a sucrose gradient starting with a density of 1.25 ml of 52.2% (wt/wt) sucrose \( p = 1.25 \), 1.1 ml of 48.6% (wt/wt) sucrose (\( p = 1.22 \)), 1 ml of 39.3% (wt/wt) sucrose (\( p = 1.18 \)), 0.5 ml of 16.6% (wt/wt) sucrose (\( p = 1.07 \)). Equilibration was obtained after centrifugation in an SW65 rotor for 2 h at 39,000 rpm (\( W = 118 \times 10^8 \) rad\(^2\) s\(^{-1}\)). The material at the 1.22-1.25 interface of density was used as the inner mitochondrial membrane preparation.

**Biochemical and Immunological Assays**

Cytochrome \( b_s \) measurements were carried out on a Unicam SP 1800 spectrophotometer (Pye, Unicam Ltd., Cambridge, England) using the differential spectrum of the oxidized and reduced form. The hemoprotein was converted from the oxidized to the reduced form, either by the endogenous cytochrome \( b_s \) reductase in the presence of 63 \( \mu M \) NADH, or by addition of Na\( _2 \)S\( _2 \)O\( _4 \). A \( \Delta \varepsilon_{424-409} \) of 160 cm\(^{-1}\) mM\(^{-1}\) was used to express the results in millimoles. A molecular weight of 12,900 was also considered for the T-cytochrome \( b_s \) (30). Enzymic and other chemical constituents of subcellular fractions were assayed as presented in detail by Beaujou and al. (4).

The activity of the anti-cytochrome \( b_s \) antibodies was estimated by their capacity to bind T-cytochrome \( b_s \) (23). 1 U of anti-cytochrome \( b_s \) activity was expressed as the amount of antibody needed to bind 1 \( \mu g \) of T-cytochrome \( b_s \).
Antigen and Antibody Preparations

T- and D-cytochrome b5 preparations were purified by methods already described (46, 63). T-cytochrome b5 was used to produce rabbit anti-cytochrome b5 antibodies. The antibodies were purified by affinity chromatography using Sepharose 4B as the matrix. The purified preparation showed an activity of 44 μg cytochrome b5 bound per mg protein and resulted from a 200-fold purification compared to the antiserum. The purities of the antigen and the antibody preparations were previously established (23). The antibodies reacted with both T- and D-cytochrome b5 if judged by the identity reaction of the two antigens in a double immunodiffusion test.

F(ab) fragments were obtained by papain digestion (51) with a yield of 94%; this was established by gel chromatography on Sephadex G100. Anti-cytochrome b5 F(ab) were no longer able to precipitate T-cytochrome b5 in an immunodiffusion test. Depending on the differences established (23), the antibodies were purified by affinity chromatography on Sephadex G100. Anti-cytochrome b5 F(ab) were no longer able to precipitate T-cytochrome b5 if judged by the identity reaction of the two antigens in a double immunodiffusion test.

The anti-F(ab)/ferritin conjugates were prepared by reacting ferritin and affinity purified IgG goat anti-rabbit F(ab) with glutaraldehyde (3). After the coupling, free IgG, ferritin aggregates, and the largest part of the free ferritin were separated from the IgG/ferritin conjugates by chromatography on Bio-gel A5m (Bio-Rad Laboratories, Richmond, Calif.) (42).

Materials

Ferritin, crystallized six times, came from Fluka, A. G. Buchs, Switzerland. Chemicals were purchased from Merck A. G., Darmstadt, W. Germany. Sephadex (G-25, G-75) and Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden.

Cytochrome b5 Incorporation

Specific centrifugation and incubation conditions were used for the different membrane preparations. They are summarized in Table I. The general protocol of the experiment was as follows: the preparations (1 mg of protein) were incubated for 30 min at 37°C in 0.5 ml of medium which contained 23 nmol (30 for microsomes and outer mitochondrial membranes) of D-cytochrome b5; T-cytochrome b5 was used in the controls. The membranes were spun down and washed twice with 10 ml of medium. Cytochrome b5 assays were carried out on half the washed preparations; the rest of the membranes were saved and used for antibody labeling, i.e., they were first incubated for 1 h at 4°C in 1 ml of medium containing 20 mg of bovine serum albumin with anti-cytochrome b5 F(ab) which had a binding activity two times greater than the amount of cytochrome b5 present. For the control experiments, normal F(ab) was used in place of anti-cytochrome b5 F(ab). The membranes were washed twice and collected by centrifugation. They were then treated to react with the anti-F(ab)/ferritin conjugate. The amount of conjugate introduced corresponded to two ferritin molecules per cytochrome b5 present in the preparation. Incubation was carried out for 15 min at 4°C in 0.5 ml of medium containing 40 mg of bovine serum albumin. The membranes were sedimented by centrifugation at 25,000 rpm for 15 min, washed three times, resuspended in 0.5 ml of medium, fixed with 1.5% (wt/vol) glutaraldehyde, then collected on Millipore filters (Millipore Corp., Bedford, Mass.) before embedding in Epon (70).

RESULTS

Analysis of the Immunochemical Material

Anti-cytochrome b5 antibodies inhibited the microsomal NADH cytochrome c reductase. IgG antibodies and F(ab) fragments gave similar inhibi-
bition curves (Fig. 1). The F(ab) fragments were obtained by papain digestion (92% yield) of the IgG tested in the same experiment. In the antibody:antigen ratio (2:1) used for the immunochromical labeling of subcellular fractions, the F(ab) fragments produced a 55% inhibition of the NADH cytochrome c reductase activity. As the maximum inhibition was 90%, we estimate that 61% of the cytochrome b₅ reacted with the anti-cytochrome b₅ F(ab).

The binding of the anti-F(ab)/ferritin conjugates to F(ab) fragments was analyzed after centrifugation in a density gradient (Fig. 2). In the control, we found that the conjugates had sedimented to the lower part of the gradient (Fig. 2a) while the F(ab) fragments remained at the top (Fig. 2b). After reaction with the anti-F(ab) antibodies, part of the [³H]F(ab) fragments were recovered in the heavy fractions (below 2.65 ml). Concerning the latter, the molar ratio of the F(ab) fragments to the ferritin molecules was 0.51. Similar results have already been published on the reactivity of anti-IgG/ferritin conjugates (39). On the other hand, small precipitates were formed when increasing amounts of F(ab) fragments were incubated with anti-F(ab)/ferritin (Fig. 3). Maximum precipitation was obtained when 16 µg of F(ab) fragments were added to 420 µg of anti-F(ab)/ferritin conjugates, this being a molar ratio of one F(ab) for two ferritin molecules. At the equivalence, the precipitate contains one F(ab) molecule for 0.57 ferritin. Moreover, in excess of antibody, Fig. 3 shows that the amount of F(ab) recovered in the precipitate was proportional to the F(ab) added, but with a low yield (33%). Obviously, only part of the antibody/ferritin precipitated the F(ab) fragments.

Both analyses of the immunochromical material indicate that in toto the number of F(ab) frag-

![Figure 1](attachment:image1.png)

**Figure 1** Inhibition of NADH cytochrome c reductase by anti-cytochrome b₅ antibodies. Microsomes (7.6 µg of protein and 0.05 µg of cytochrome b₅) were incubated for 1 h at 0°C in 0.5 ml PBS containing 2.5 mg/ml bovine serum albumin with increasing amounts of anti-cytochrome b₅ IgG (•) and F(ab) fragment (○) obtained by papain digestion. The NADH cytochrome c reductase activity was then tested. The normal serum and the PBS-albumin solution did not show any activation or inhibition of the enzyme activity and were used as controls.

![Figure 2](attachment:image2.png)

**Figure 2** Distribution pattern of three preparations analyzed after centrifugation in density gradients. Three samples containing (a) 1.7 mg of anti-F(ab) IgG labeled with ferritin, (b) 0.7 mg of F(ab) labeled with [³H]acetic anhydride (31), and (c) the mixture of the two former preparations were incubated for 1 h at 4°C in 0.6 ml of PBS solution and loaded on 4.5 ml of 10-60% (wt/wt) sucrose density gradients. Centrifugation was performed at 60,000 rpm for 150 min (w = 35 × 10⁶ rad s⁻¹) in an SW 65 rotor. The pellets at the bottom of the tubes were resuspended in 0.5 ml of PBS (hatched blocks). The colored complex formed between the iron and the αα'-dipyridyl was used to assay for the iron. The average iron content of the ferritin was 0.12 mg per mg protein. The [³H]acetylated F(ab) was measured on an Isocap/300 (Searl Analytic, Inc. Des Plaines, Ill.) scintillating counter. The specific radioactivity of the [³H]acetylated F(ab) was 216,000 cpm per mg protein. The material recovered in the gradient accounts for 90-100% of the initial preparation. Protein (——), iron (....), [³H] (——).
ments fixed, represents half of the ferritin molecules of the conjugate. Purification of the IgG/ferritin conjugates by column chromatography on Biogel A5m separates the conjugates, completely, from free IgG and multi-ferritin aggregates and, partially, from free ferritin (42). In conclusion, it is best to interpret the former results as indicating that a large part (at least 50%) of the ferritin molecules do not fix any of the F(ab) fragments either because they are free molecules or because of the nonreactivity of the anti-F(ab) conjugate; on the other hand, some of the reactive antibody/ferritin conjugates contain several anti-F(ab) sites and can be detected in the precipitation curve.

Biochemical Data

Table II summarizes some of the biochemical properties of the subcellular preparations. The absolute values of the enzyme activities for the homogenate agree with the data already published (1) and are not shown. The composition of the microsomal fraction is very similar to the one described by Amar-Costesec et al. (1): the main components were the ER-derived vesicles (~80%); plasma membrane, Golgi elements, and mitochondria were also present; they could account, respectively, for 10, 5, and 5% of the proteins. (See Leighton et al. [33] for the calculation procedure).

As shown by the relative specific activity (RSA) of the monoamine oxidase, the outer mitochondrial membranes were purified 33 times compared to the homogenate. The activity of the NADH cytochrome c reductase, which is also present in

![Figure 3 Immunoprecipitation curves between anti-F(ab) ferritin conjugates and F(ab) fragments. Anti-F(ab) ferritin conjugates (0.42 mg) were incubated in 0.3 ml of PBS solution with increasing amounts of F(ab) labeled with [3H]acetic anhydride. The antigen and antibody preparations were the same as in Fig. 2. After incubation for 1 h at 37°C and for 2 days at 4°C, the precipitates were washed twice with 10 ml of PBS and sedimented by centrifugation at 2,000 rpm for 20 min in the PR-J Centrifuge (International Equipment Co., Boston, Mass.). The pellets were dissolved in 2.5 ml of 0.1 N NaOH. The solutions were neutralized for radioactivity counting. Assays for iron, protein, and radioactivity countings were conducted as in Fig. 2. Controls, using anti-F(ab)/ferritin conjugates alone or [3H]F(ab), were very low and were subtracted from the test. Protein (●), iron (▲), 3H (■).]
these membranes, was very high. The main contaminants were the ER-derived vesicles which could represent ~5% of the proteins. In the peroxisomal preparation, we tried to minimize contamination by the ER-derived vesicles as much as possible. Data showed that these membranes account for only 1.3% of the membrane proteins. Very few mitochondria were present and, with respect to the protein content, they represented only 1% of the material; however, as a consequence of our drastic purification conditions, the amount of peroxisomes was very low. The NADH cytochrome c reductase showed a much higher RSA than the glucose 6-phosphatase and the NADPH cytochrome c reductase. This high NADH cytochrome c reductase activity in the density of peroxisome equilibration can be interpreted by the presence of the enzyme in peroxisomal membranes. We must, however, consider the presence of contaminating outer mitochondrial membranes which also contain the NADH cytochrome c reductase and account for ~20% of the activity of the preparation.

The inner mitochondrial membrane preparation contained mainly mitochondrial enzymes; however, we underestimated the cytochrome oxidase values in the mitochondrial preparation. Special treatment at high-speed centrifugation and in the presence of phosphate was responsible for its inactivation: compared to the homogenate, only 75% of the enzyme activity was recovered in the different fractions; this underestimation mostly affected the purified preparation in which the inactivation was probably >50%. As the soluble fraction did not contain any mitochondria (no cytochrome oxidase activity) but still showed 53% of the malate dehydrogenase activity, we could estimate an RSA of 4.6 for the bound mitochondrial enzyme. The RSA of 0.44 for the monoamine oxidase indicates that 90% of the outer membrane was released from the mitochondria. A few peroxisomes were present (~7% of the protein) but almost no ER-derived vesicles (~0.2%).

The lysosomal preparation was very pure with only slight contamination by some ER and Golgi elements (around 5 and 2%). Cytochrome b₅ could not be detected in the preparation.

Compared to the homogenate, the Golgi apparatus was purified 64 times; however, ER-derived vesicles still accounted for ~19% of the protein, and plasma membranes for 3.8%.

### Binding of Cytochrome b₅ to the Membranes

For the amounts of cytochrome b₅ bound to the membranes, see Tables III and IV. The nonspecific adsorption of T-cytochrome b₅ in the different preparations is very low. The values for the incorporation of D-cytochrome b₅ in the microsomes are similar to those of Strittmatter et al. (65); they are very high when expressed with respect to the phospholipid content. Outer mito-

### Table III

**Amount of Cytochrome b₅ Incorporated in the Preparations**

| Hemoprotein in the preparations | NADH reduced | Na₂S₂O₄ reduced | Cytochrome b₅ incorporated (T-C) |
|---------------------------------|-------------|----------------|---------------------------------|
|                                 | Control     | Test           | Control                         | Test       | NADH reduced | Na₂S₂O₄ reduced | Na₂S₂O₄/NADH reduced | NADH reduced % total |
| Preparations                    | (C)         | (T)            | (C)                            | (T)        |              |                |                  |                    |
| Microsomes                      | 0.46        | 0.51           | 9.31                           | 0.65       | 10.75        | 8.80            | 10.10             | 1.30               | 87                   |
| Outer mitochondrial membrane    | 0.47        | 0.63           | 9.03                           | 0.86       | 9.53         | 8.40            | 8.67              | 0.27               | 97                   |
| Peroxisomes                     | 0.01        | 0.03           | 1.97                           | 0.19       | 2.43         | 1.94            | 2.24              | 0.30               | 87                   |
| Inner mitochondrial membrane    | 0.04        | 0.04           | 0.44                           | 0.30       | 2.52         | 0.40            | 2.22              | 1.82               | 18                   |
| membrane and matrix             |             |                |                                |            |              |                  |                   |                    |
| Lysosomes                        | N.D.*       | 0.03           | 0.03                           | 0.05       | 1.18         | 0                | 1.13              | 1.13               | ~                    |
| Lysosomal membrane              | N.D.        | N.D.           | 0.04                           | 0.10       | 1.46         | 0.04            | 1.36              | 1.32               | 3                    |
| Golgi I                         | 0.10        | 0.17           | 1.16                           | 0.14       | 2.01         | 0.99            | 1.87              | 0.88               | 53                   |
| Golgi II                        | 0.10        | 0.16           | 2.22                           | 0.23       | 5.63         | 2.06            | 5.40              | 3.34               | 38                   |

Values are expressed in nanomoles of cytochrome b₅ (or equivalent) bound per milligram protein. The preparations were incubated with T-cytochrome b₅ in the control (C) and with D-cytochrome b₅ in the test (T).

* Not detected.
TABLE IV  
*Amount of Incorporated Cytochrome b₅ Compared to the Phospholipids*

| Preparations                  | Phospholipid composition | Incorporated cytochrome b₅ reducible by |
|-------------------------------|--------------------------|--------------------------------------|
|                               | µmol P₅L/mg protein      | NADH | Na₂S₂O₄ |
| Microsomes                    | 0.45                     | 19.56 | 22.44   |
| Outer mitochondrial membrane  | 0.79                     | 10.63 | 11.01   |
| Peroxisomes                   | 0.12                     | 16.67 | 18.33   |
| Inner mitochondrial membrane  | 0.14                     | 2.86  | 15.86   |
| Lysosomes                     | 0.27                     | 0     | 4.21    |
| Lysosomal membrane            | 0.65                     | 0.06  | 2.10    |
| Golgi I                       | 0.45                     | 2.20  | 4.20    |
| Golgi II                      | 0.52                     | 3.94  | 10.33   |

The values for cytochrome b₅ are expressed in millimoles of D-cytochrome b₅ bound per mole of phosphate from phospholipids.

Chondrial membranes fixed large amounts of cytochrome per mg protein; nearly all the hemoprotein (97%) could be reduced by the NADH cytochrome b₅ reductase. The amount of cytochrome b₅ incorporated by the peroxisomal preparation is low when expressed per milligram protein but it is very high when compared to the phospholipid content. In addition, 97% of the bound hemoprotein could be reduced by an endogenous reductase. Inner mitochondrial membranes fixed some cytochrome b₅, but only a small part was reducible when NADH was present. Low amounts of cytochrome were detected in the lysosomal preparation but were not in contact with the reductase. The incorporation of cytochrome b₅ into Golgi membranes was achieved under two different conditions (Table I). To maintain the Golgi structure, the incubation of the Golgi I preparation was carried out at 4°C for one night in the Morré medium. On the other hand, the Golgi II experiment was conducted at 37°C for 30 min on dissociated Golgi elements. Much more cytochrome was bound in the latter experiment, 38% of which was reduced by the reductase. All the binding experiments were performed at least twice and gave reproducible results except when lysosomes were concerned.

Several controls were carried out on the fixation conditions of cytochrome b₅ in the membranes. Firstly, we performed saturation curves of cytochrome binding on Golgi and microsomal membranes and showed that saturation was obtained in the experimental conditions used in this paper. Secondly, we tested the possibility of cytochrome b₅ destruction by lysosomal cathepsin because lysosomal extract is known to hydrolyse cytochrome b₅ from ER membranes (68). Total cathepsin activity of the lysosomal membrane preparation was measured at different pH but no cathepsin activity could be detected above pH 6.5. D-cytochrome b₅ was then incubated with a lysosomal preparation at different pH, and, after elimination of the membranes, we tested the ability of cytochrome to be incorporated into the microsomal membranes. The results are shown in Fig. 4. Cytochrome b₅ is destroyed below pH 7, exactly when the cathepsin activity begins to be detectable. The experiment was repeated at 0°C and 37°C with lysosomal membranes and with a soluble lysosomal extract. If the pH was maintained at 7.5, no alteration of cytochrome b₅ can be detected. The possible effect of Triton WR 1339 on the lysosomal membrane was also tested: microsomes were preincubated for 30 min at 37°C with different concentrations of Triton and Methods. Triton made no effect on the cytochrome b₅ binding in the treated microsomes.

**FIGURE 4**  
Effect of pH on cathepsin activity and on destruction of cytochrome b₅ by lysosomal enzymes. Total cathepsin activity (●) was measured on the washed lysosomal membranes using Gianetto and de Duve's method (24). With 1.7 mg of lysosomal membrane protein (0.17 U of cathepsin activity tested at pH 2.75), 0.5 mg of cytochrome b₅ was incubated for 30 min at 37°C in 0.6 ml of a 50 mM phosphate buffer at different pH. After incubation, the pH was adjusted to 7.5 and the solution was centrifuged at 39,000 rpm for 30 min in rotor n°40. 0.4 ml of the supernate was removed and incubated at 37°C for 30 min with 0.33 mg of microsomes. The amount of cytochrome b₅ incorporated into these membranes was measured as described in Materials and Methods (▲).
Thirdly, we looked for the effect of the incubation medium on the binding. The incorporation of D-cytochrome b₅ into microsomes was identical if the incubation occurred in phosphate-buffered saline (PBS), 0.25 M sucrose, or 0.5 NaCl medium. Strittmatter et al. (65) have already shown that, with an excess of cytochrome b₅, the binding was not dependent on the ionic strength of the buffer but on the temperature and incubation time.

**Morphological Observation**

From the four samples obtained in the labeling experiment of each subcellular fraction, only three are shown in the figures; indeed, both controls using normal F(ab) on membranes treated with either T- or D-cytochrome b₅ were identical, and therefore only the latter is presented.

The appearance of the microsomal fraction, after immunological labeling, is shown in Fig. 5. The difference between the control (Fig. 5 a) and the test (Fig. 5 b) is clear. The number of ferritin molecules per profile is 2.2 for the control and 6.5 for the test. On the other hand, the ferritin labeling after extra bound cytochrome b₅ (Fig. 5 c) is much heavier; the average ferritin number per profile is about 20. In one experiment, the labeling microsomes was realized as described in Materials and Methods, using an excess of D-cytochrome b₅, a ten-fold excess of F(ab) anti-cytochrome b₅, and a fivefold excess of anti-F(ab)/ferritin conjugate. The micrographs showed an average of 30 ferritin grains per profile in the test and six in the control.

It is evident by the ferritin labeling in Fig. 6 b that the outer mitochondrial membranes contain endogenous cytochrome b₅. Even after the incorporation of the hemoprotein (Fig. 6 c), the labeling is still much heavier, and some parts of the membranes must be close to saturation. Very few invaginations are labeled, probably because of some steric hindrances, but it is interesting to note that nonvesicular membranes are labeled on both sides. This indicates that cytochrome b₅ can be incorporated in vitro on both faces of the membrane whereas its natural location is only on the outer face (32).

The control of the peroxisomal preparation (Fig. 7 a) contains almost no ferritin. This is not so in the case of the endogenous cytochrome b₅ labeling (Fig. 7 b); indeed, several ferritin molecules are attached on well-defined peroxisomal membranes. This strengthens the observations of Fowler et al. (23) who used hybrid antibodies. After D-cytochrome b₅ incorporation (Fig. 8), the labeling is then very heavy and the ferritin is nicely distributed around all the vesicles.

Fig. 9 b presents the labeling of the endogenous cytochrome b₅ of the inner mitochondrial membrane preparation; only some small vesicles are labeled with ferritin. These are most probably outer mitochondrial membrane fragments which are present in this preparation and which contain the hemoprotein. Some of these vesicles are loose in the solution but a few others are attached to the inner membrane. Because endogenous cytochrome b₅ is fixed on the outer face of the outer membrane (32), these vesicles are right side out. We must assume that they first separated from the inner membrane, closed, and that then some of them became reattached. However, most of the vesicles fixed on the inner membrane are not labeled and could be considered as inside-out vesicles still attached by some contact sites on the inner membrane (25). When the membranes are incubated with D-cytochrome b₅ and treated with the immunochromical reagent (Fig. 10), all the well-recognized inner mitochondrial membranes are labeled with ferritin.

The labeling of the lysosomal membranes for endogenous cytochrome b₅ (Fig. 11 b) is very low and is no more pronounced than in the control (Fig. 11 a). A few, small, heavily labeled vesicles could be attributed to the ER-derived membranes. After cytochrome b₅ incorporation (Fig. 11 c), most of the ferritin appears on formless material usually with a low electron density. We consider that this material is derived from the lysosomes themselves. In fact, injection of the rats with Triton WR 1339, before the lysosome preparation, activates the autophagic process (49), and therefore this amorphous material could represent the autophagic residues. The large lysosomal membranes themselves carry very little or no ferritin and can be considered as not labeled. The experiment was repeated once under the same conditions and then again with solution buffered at pH 8. The ferritin pattern was identical.

As already shown (23), when the Golgi apparatus is treated for the labeling of the endogenous cytochrome b₅, ferritin molecules are attached only to some vesicles and not to the characteristic Golgi elements (Fig. 12 b). However, after the in vitro incorporation of the hemoprotein, almost all the small vesicles are heavily labeled, and some parts of the distinctive Golgi elements carry ferri-
FIGURE 5 Electron micrographs of microsomes treated with anti-F(ab)/ferritin conjugate after incubation with (a) D-cytochrome $b_5$ and normal F(ab), (b) T-cytochrome $b_5$ and F(ab) anti-cytochrome $b_5$, (c) D-cytochrome $b_5$ and F(ab) anti-cytochrome $b_5$. See Materials and Methods for the experimental data. Note (a) the low ferritin density in the control; (b) much higher for the labeling of the endogenous cytochrome; (c) very heavy for the in vitro bound cytochrome. A few large profiles with very little or no ferritin grains (O). The rectangle indicates a microsome vesicle enlarged in the inset ($\times 80,000$) to show nonhomogeneous distribution of the ferritin. Bar, 0.5 $\mu$m. $\times 50,000$. 
FIGURE 6 Cytochrome $b_{5}$ labeling of an outer mitochondrial membrane preparation with F(ab) anti-cytochrome $b_{5}$ and anti-F(ab)/ferritin conjugate. Membranes were first incubated with T-cytochrome $b_{5}$ (b) or D-cytochrome $b_{5}$ (c). The control (a) was performed on membranes incubated with D-cytochrome $b_{5}$ but using normal F(ab) instead of the anti-cytochrome $b_{5}$ F(ab). Nonlabeled membrane invaginations (O). Membrane clearly labeled on both sizes (double arrows). The rectangle indicates a part of two vesicles enlarged in the inset (80,000) to show nonhomogeneous distribution of the ferritin. Bar, 0.5 μm.  x 50,000.
FIGURE 7 Peroxisomal preparation labeled for cytochrome b₅ by anti-F(ab)/ferritin conjugate (a) control obtained after incubation with D-cytochrome b₅ and normal F(ab); (b) endogenous cytochrome b₅ revealed after incubation with T-cytochrome b₅ and F(ab) anti-cytochrome b₅. The number of ferritin points attached to the membranes of well-recognized peroxisomes is significantly higher than in the control (arrows). Bar, 0.5 μm. × 50,000.
Figure 8  Peroxisomes labeled with anti-F(ab)/ferritin conjugate after incubation with D-cytochrome b₅ and F(ab) anti-cytochrome b₅. All the membranes are heavily labeled. PN, peroxisomes recognized by the nucleoid. PL, peroxisomes with the characteristic looplike projection described by Novikoff and Shin (45). MP, particles which could correspond to the microperoxisomes described by Novikoff and Novikoff (43). Bar, 0.5 μm. × 50,000.
Figure 9  Inner mitochondrial membranes labeled for cytochrome $b_5$ by anti-F(ab)/ferritin conjugate after incubation (a) with D-cytochrome $b_5$ and normal F(ab) and (b) with T-cytochrome $b_5$ and F(ab) anti-cytochrome $b_5$. Some rare ferritin molecules in the control (arrows). In the test, small labeled vesicles free or attached to the inner mitochondrial membranes (arrows); others without ferritin ($O$). The inner membrane itself is devoid of ferritin. Bar, 0.5 $\mu$m. $\times$ 50,000.
Figure 10 Inner mitochondrial membrane preparation labeled with goat anti-rabbit F(ab) bound to ferritin, after incubation with D-cytochrome b$_5$ and F(ab) anti-cytochrome b$_5$. Characteristic labeled inner mitochondrial membranes (M). Some small labeled vesicles attached to the outer mitochondrial membranes (arrows). Bar, 0.5 μm. × 50,000.
Figure 11 Electron micrographs of the lysosomal preparation. Membranes treated with (a) D-cytochrome b₅ and normal F(ab); (b) T-cytochrome b₅ and F(ab) anti-cytochrome b₅; (c) D-cytochrome b₅ and F(ab) anti-cytochrome b₅. All the membranes were then incubated with anti-F(ab)/ferritin conjugate. (a) Some ferritin grains (arrows). (b) A few labeled vesicular membranes (arrows). (c) Some labeled (arrows) and nonlabeled (O) membranes. Heavily labeled amorphous material (M). Inset: nonlabeled membrane containing ferritin attached to material inside the vesicle. Bar, 0.5 μm. × 50,000.
Figure 12  Golgi preparation I treated with anti-F(ab)/ferritin conjugate after incubation with (a) D-cytochrome bs and normal F(ab) and (b) T-cytochrome bs and F(ab) anti-cytochrome bs. All the manipulations were carried out at 4°C in the Morré medium (see Table I for details). (a) Very few ferritin grains (arrows). (b) Some small vesicles heavily labeled (arrows). Characteristic Golgi elements not labeled: saccules (S) and lipoprotein vesicles (LP). Bar, 0.5 μm. × 50,000.
tin grains (Fig. 13). These labeled spots are situated mostly on the edges of the Golgi aggregates facing the external medium. When the Golgi apparatus is disaggregated, incubated with D-cytochrome bs, and immunochemically treated (Fig. 14), most of the vesicles are labeled anyway.

DISCUSSION

Analysis of the Immunochemical Labeling

Because a quantitative analysis of the cytochrome bs labeling of the microsomes was already performed using hybrid antibodies (52), we wanted to compare the results we obtained on the same fraction (Fig. 5 b) in order to estimate the efficiency of our immunochemical material.

The labeling observed in this paper is similar to the one obtained with hybrid antibodies: we observed an average of 2.2 ferritin grains per profile for the control instead of 2.4, and 6.5 for the test instead of 9.5. The number of ferritin molecules observed with hybrid antibodies represented only 5% of the cytochrome bs molecules. The deficiency of our labeling compared to the amount of antigen is also very important. Several reasons can be responsible for this discrepancy. First, the low labeling could be the result of a deficiency of the immunochemical material. The amount of anti-cytochrome bs F(ab) incubated with the membranes was enough to bind twice the amount of cytochrome bs present in the membranes when tested for their activity. Moreover, from the inhibition curve (Fig. 1), we deduced that at least 61% of the cytochrome bs of the microsomes reacted with the F(ab) when using the labeling conditions. The activity of the anti-F(ab)/ferritin conjugate was also tested (Fig. 2 and 3), and we found that the molar ratio between the ferritin of the conjugate and the fixed F(ab) fragments was 0.5.

In the labeling experiment, the amount of F(ab) fragments fixed on the membranes was not directly measured, and the amount of anti-F(ab)/ferritin conjugate added was thus calculated in order to obtain two ferritin molecules for each cytochrome bs. Under these conditions, the number of anti-F(ab) active sites introduced was equal to the number of cytochrome bs molecules. The insufficiency of the immunological material can only partially explain the large deficiency observed in the labeling of cytochrome bs. This conclusion is strengthened if we observe the labeling of the microsomes after incorporation of D-cytochrome bs (Fig. 5c). The immunological reagent introduced for the labeling together with the amount of cytochrome bs in the membranes were both 22 times greater than in the natural microsomes. Under these conditions, the average number of ferritin molecules per profile was only 20 and represented <1% of the cytochrome bs molecules.

Another explanation for the low labeling efficiency is the redistribution of the antigens on the membranes. This rearrangement can be induced by some anti-cytochrome bs dimers which account for 6% of the F(ab) preparation and by some of the anti-F-(ab)/ferritin conjugates which contain several antibody active sites. Finally, cytochrome bs molecules can be either nonhomogeneously distributed on the membranes or associated in groups of molecules. The analysis of some of the pictures, particularly those of the microsomes (Fig. 5c) as well as of the inner (Fig. 10) and outer mitochondrial membranes (Fig. 6c), sustains the hypothesis of a heterogeneous distribution of the antigens (natural or induced); on the same profile, parts of the membrane can be heavily labeled while others are devoid of ferritin. On the bases of biochemical assays, Ito (28) proposed that about 50 cytochrome bs molecules were associated with about five NADH cytochrome bs reductase molecules in the microsomal membrane; this functional association would result from a spatial clustering of the cytochrome bs molecules on the membrane. The results of Rogers and Strittmatter (55) are contradictory, indicating that the molecules are randomly distributed on the membrane. On the other hand, NADPH cytochrome c reductase was localized on the microsomal membrane by Morimoto et al. (39) using an immunochemical method. The number of ferritin molecules was lower than the number of antigens, and the explanation proposed by the authors was that three to five enzyme molecules were associated on the membrane. Because the significance of the absolute amount of ferritin is not definite, we will use the comparisons of the ferritin labeling in the other preparations on a qualitative basis and also as a tool to solve the problems raised by the presence of some contaminant materials.

Incorporation of Cytochrome bs into the Intracellular Membranes

As stated earlier and described in Table I,
FIGURE 13  Golgi preparation I incubated successively with D-cytochrome b₅, F(ab) anti-cytochrome b₅, and anti-F(ab)/ferritin conjugate. All the manipulations were carried out at 4°C in the Morré medium (see Table-I for experimental details). Most of the small vesicles are heavily labeled. Parts of the distinctive Golgi elements showing ferritin grains (arrows). Saccules (S), tubules (T), lipoprotein vesicles (LP). Bar, 0.5 µm. × 50,000.
different incubation and centrifugation conditions were used in the cytochrome b₅ incorporation experiments for the different subcellular preparations; moreover, each organelle was isolated under particular conditions in order to purify and preserve its structure. The comparison of the absolute amount of cytochrome b₅ incorporated by the different membranes is thus possible only with the assumption that differences in preparatory techniques do not affect the binding, and hence the comparative data in this discussion should be considered tentative, subject to validation of the above-mentioned assumption in future experiments.

The labeling experiments with the ER, outer mitochondrial, and peroxisomal membranes indicate that cytochrome b₅ is naturally present in these three membranes. The low cytochrome b₅ content of the peroxisomal preparation and the low labeling of the membranes indicate that the amount of cytochrome b₅ in these membranes is very low. These observations confirm published results (17, 23, 52).

When incubated in vitro with D-cytochrome b₅, ER, outer mitochondrial, and peroxisomal membranes incorporated large amounts of the hemoprotein, as indicated by the spectrophotometric data (Tables III and IV) and by the heavy labeling of the membranes (Figs. 5c, 6c, and 8). Moreover, most of the cytochrome was reduced in the presence of NADH. Because the main contaminant membranes of the peroxisomal preparation represented no more than 5% of the membrane protein and because all the vesicles of the preparation were heavily labeled, we conclude that the peroxisomal membrane contained an NADH cytochrome b₅ reductase. This result confirms the biochemical analysis of the peroxisomal preparation in which the activity of the NADH cytochrome c reductase was higher than that of the other ER markers. The proportion of membrane proteins in the peroxisomal preparation is difficult
to estimate, because as shown in the electron micrographs (Figs. 7 and 8), most of the peroxisomes are broken or altered. Cytochrome b₅ incorporation is thus best expressed with respect to the phospholipid content of the preparation; the values are then particularly high compared to those for the other membranes.

Although the inner mitochondrial membrane does not naturally contain any cytochrome b₅, it binds it very well in vitro and shows a very nice ferritin pattern in the EM (Fig. 10). The reduction of a fraction (18%) of the bound cytochrome b₅ in the presence of NADH (Table III) is not surprising because 10% of the outer membranes of the mitochondria are still present in the preparation. We conclude, then, that the major part of D-cytochrome b₅, not reducible by an NADH-dependent reductase, is bound to the outer face of the inner mitochondrial membrane. In fact, there is one NADH cytochrome c reductase on the inner face of the inner membrane, and it would be interesting to know whether the enzyme could reduce the cytochrome b₅ fixed on the same side. If the results are expressed with respect to phospholipids (Table IV), the values become really high and similar to those of the first three membranes. This effect results from the low phospholipid content of the inner mitochondrial membrane and matrix compared to the protein.

The absence of cytochrome b₅ spectra in the lysosomal preparation and the low ferritin labeling of the membranes indicate that cytochrome b₅ is not naturally present in the lysosomal membranes. After the incorporation of cytochrome b₅, especially if the values refer to the phospholipids, the amount of incorporated cytochrome b₅ is very low. Also, after the immunochromical reaction, the number of ferritin molecules observed on the lysosomal membranes is very low. When the experiment was performed on intact lysosomes, the incorporation of cytochrome b₅ into the preparation was also low (Tables III and IV) and, after the labeling, ferritin was not detectable on the membranes but on the amorphous material (not shown). However, the lysosomes broke during manipulation. From all the negative results, we conclude that the lysosomal membranes do not incorporate the hemoprotein. One explanation of the negative results could be the presence of Triton WR 1339 which accumulates in the lysosomes. We have shown that the contact of this molecule with the microsomes did not change their ability to fix the protein. We cannot, however, exclude the fact that, in vivo, the prolonged contact of the membrane with Triton produces an irreversible change in the membrane. The possibility of cytochrome b₅ digestion by cathepsin was also investigated (Fig. 4). No digestion occurred at pH 7.5 or above, where negative results of the presence of cytochrome b₅ molecules on the membranes were observed. Lysosomal membranes appear to be different from the others because neither the presence of residual hydrolase activity nor prior exposure to Triton WR 1339 can explain the lack of binding. The presence of glycoproteins and negatively charged sialic acid in the membrane could be responsible for these negative results, because they can prevent the hemoprotein from reaching the hydrophobic part of the membrane. Such a situation was already found in the plasma membrane (52). This assumption is also likely for the lysosomal membrane because the latter contains twice as many carbohydrates as, and 1.7 times more sialic acid than, the plasma membrane (26). Another explanation for the lack of binding which can also be applied to the lysosomal membrane has been proposed by Enomoto and Sato (20). They suggest that the high cholesterol content of the membrane inhibits the binding of cytochrome b₅.

The Golgi preparation is contaminated by ~20% of ER-derived vesicles. It was shown by Fowler et al. (23) that Golgi membranes do not normally possess cytochrome b₅, and the presence of some labeled vesicles in Fig. 12b is explained by the presence of contaminant membranes. The electron micrographs are completely different after the incorporation of D-cytochrome b₅; only the membranes deeply inserted inside the Golgi apparatus are not labeled (Fig. 13). The Golgi membrane labeling is confirmed by the heavy ferritin pattern obtained when the Golgi complex is dissociated before incorporation of cytochrome b₅ (Fig. 14). The presence of some lysosomal and plasma membranes in this preparation can explain the few nonlabeled membranes. The fact that the hemoprotein is incorporated into the Golgi membrane is also sustained by the presence of a large amount of cytochrome b₅ which could not be reduced in the presence of NADH (Tables III and IV). We conclude that a large part, if not all, of the Golgi membranes, can incorporate D-cytochrome b₅ and that it is not reduced by the reductase.

The fact that inner mitochondrial, Golgi, and plasma membranes, not possessing endogenous
cytochrome \( b_5 \), are able to incorporate the hemoprotein in vitro gives rise to the idea that the anchorage of integral protein in membrane is a nonspecific phenomenon. This conclusion supports the idea, with respect to the fluid mosaic membrane model (61), that the only reason for an integral protein to be inserted in a membrane is its hydrophobic association with the hydrophobic portion of the lipids.

Rogers and Strittmatter (56) have shown that the maximum amount of cytochrome \( b_5 \) bound to egg lecithin liposomes corresponds to one protein molecule per 11 phospholipid molecules. In agreement with the results of Strittmatter et al. (65), the data in Table IV show that in the microsomes one cytochrome \( b_5 \) molecule corresponds to 45 phospholipid molecules. Compared to artificial membranes, the lower incorporation of cytochrome \( b_5 \) into natural membranes can be explained not only by differences in the physical and chemical properties but also by the presence of other integral proteins which limits the incorporation of new integral proteins. In fact, Rogers and Strittmatter (57) have shown that two integral proteins restrict each other in their in vitro binding on a membrane. In this context, and taking into account the cautions already mentioned with respect to the value of the data, the amount of cytochrome \( b_5 \) incorporated into the different subcellular membranes could be a way of measuring their saturation in integral proteins.

Even in the membranes where cytochrome \( b_5 \) is naturally present, there is a large gap between the amount of cytochrome \( b_5 \) present and the ability of the membrane to bind the protein. Thus, the low cytochrome \( b_5 \) content of the membranes is not limited by the anchorage of the protein but is the result of a steady state between its synthesis and its degradation (60).

**Origin of the Specificity of the Subcellular Membranes**

If we try to ascribe physiological implications to the results obtained on the in vitro incorporation of cytochrome \( b_5 \) in the subcellular membranes, we have to postulate that, for this particular problem, the membranes preserved their natural properties during the purification procedure. Two arguments sustain this supposition: firstly, extrabound cytochrome \( b_5 \) is enzymatically active and is indistinguishable from natural cytochrome \( b_5 \) (65); secondly, the fact that some membranes (lysosomal membrane and the external face of the plasma membrane) do not incorporate the hemoprotein indicates that the homogenization process does not induce in all the membranes alterations that would be nonspecific for the incorporation of cytochrome \( b_5 \).

In keeping with the assumption mentioned above, we would like to compare the specificity of the subcellular organelles with respect to the in vitro binding and the in vivo location of cytochrome \( b_5 \) and ask whether the absence of cytochrome \( b_5 \) in some membranes is the result of a structural incompatibility between the membranes and the protein. This does not seem to be the case because cytochrome \( b_5 \) can be incorporated into all the membranes, except for the lysosomes. So mechanisms must exist in vivo to prevent the incorporation of newly synthesized cytochrome \( b_5 \) into a broad range of different intracellular membranes. We will discuss them in view of what is known about the biogenesis of cytochrome \( b_5 \) and about the relationships between the subcellular organelles in the cell. The turnover of cytochrome \( b_5 \) was extensively studied (8) but the site of its synthesis is still a matter of discussion (58). Cytochrome \( b_5 \) is an endoprotein, and some authors believe that these proteins are made on free ribosomes and diffuse to the membranes where they insert spontaneously (6, 34, 36). This hypothesis is compatible with our results only if we postulate that the endoproteins are synthesized as precursors which are incapable of being inserted into membranes but which are activated by proteolytic enzymes specific to the target membrane (35, 58). A simpler alternative is that cytochrome \( b_5 \) is made on membrane-bound ribosomes (47, 59); its insertion into the ER membrane is then easy to imagine because the hydrophobic part of the protein, situated at the carboxyl terminus of the peptide chain, is immediately in contact with the membrane. Such a mechanism has already been proposed for the biosynthesis of the NADPH-cytochrome c reductase (41). In view of the results of Blobel and Dobberstein (7), the ribosomes on which the apocytochrome is synthesized would be directed to the ER membrane with a special signal sequence (58).

The presence of cytochrome \( b_5 \) in the ER can explain the presence of the same molecule in the peroxisomal membranes. In fact, when one looks at the amount of cytochrome \( b_5 \) bound with respect to the phospholipid content, in the ER and peroxisomal membrane, it is tempting to
correlate the similarity of behavior of the two membranes with Novikoff's theory on the biogenesis of peroxisomes (44). Novikoff proposes that peroxisomes arise by budding from the smooth ER and differentiate into microperoxisomes before acquiring their characteristic nucleoid. In this hypothesis, the membranes of the peroxisomes originate from the ER, and it is not surprising that both membranes behave in the same way. Several biochemical results are, however, contradictory, for example, the low amount of natural cytochrome b₅ and the absence of some specific ER enzymes in the peroxisomal membrane. Outer mitochondrial and ER membranes are similar in some ways, for example, for their cytochrome b₅ content and incorporation, and are different in other respects. The significance of their similarity and the mechanism of their possible relationship are still unclear.

The Golgi apparatus does not contain the cytochrome in its membrane but is able to incorporate it; how, then, is this compatible with the continual and dynamic relationship between the ER and the Golgi apparatus (48)? In fact, cytochrome b₅ represents only one of the differences between the two membranes which have different protein, enzyme, and lipid compositions (2, 5, 9, 21, 22, 37, 38). Several hypotheses explain the spatial relationship between the organelles: Morré et al. (40) and Claude (11) proposed a permanent continuity between the ER and the Golgi apparatus. Other authors (5, 29, 45, 48) prefer the idea of an intermittent relationship between these two subcellular compartments through the intermediary of small vesicles. Considering this latest hypothesis and the fact that the Golgi membrane is not in contact with cytochrome b₅ and that a restriction for the latter occurs in the formation of the small vesicles which carry secretory proteins from the ER to the Golgi apparatus.

As a general rule, we proposed that fusion and shuttling processes are specific for some integral proteins. Such a phenomenon is well known in the formation of some virus membranes. In this case, special peripheral proteins are responsible for the specific assembling of the integral virus proteins (10). A similar mechanism would explain the maintenance of the specific membrane composition in the cell and the discrepancy observed between the in vitro binding of cytochrome b₅ and the specificity of the natural subcellular membranes.

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REFERENCES
1. AMAR-COSTEIX, A., H. BEAU~AY, M. WIBO, D. THIBASSE~MOUX, E. FEYTMAN, M. ROBB, 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.
2. AMAR-COSTEIX, A., M. WIBO, D. THIBASSE~MOUX, H. BEAU~AY, 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 propanol-2. J. Cell Biol. 62:717-745.
3. ANRAMEA, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry. 6:43-52.
4. BEAU~AY, H., A. AMAR-COSTEIX, E. FEYTMAN, D. THIBASSE~MOUX, M. WIBO, M. ROBB, and J. BERTHET. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Biochemical methods. J. Cell Biol. 61:188-200.
5. BERGERON, J. M., H. H. EBERHARNE, P. SIEKEVITZ, and G. E. PALADE. 1973. Golgi fractions prepared from rat liver homogenates. II. Biochemical characterization. J. Cell Biol. 59:73-96.
6. BRETCHER, M. S. 1973. Membrane structure: some general principles. Science (Wash. D. C.), 181:622.
7. BLOOM, J., and J. DUBINSTEIN. 1973. Transfer of proteins across membranes. I. Presence of proteolytical processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. 60:855.
8. BLOCK, K. W., and P. SIEKEVITZ. 1970. Turnover of inner and protein moieties of rat liver microsomal cytochrome b₅. Biochem. Biophys. Res. Commun. 41:374-380.
9. CHEETRE, R. D., D. J. MOORE, and W. YOUNGSMITH, 1970. Isolation of a Golgi apparatus-rich fraction from rat liver. II. Enzymatic characterization and comparison with other cell fractions. J. Cell Biol. 44:492-500.
10. CHOPP, P. W., R. W. COMANS, A. SCHEID, J. H. MCMAHON, and S. G. LAXEROTTIE. 1972. Structure and assembly of viral membranes. In Membrane Research, C. F. Fox, editor, Academic Press, Inc., New York. 163-186.
11. CLAUDE, A. 1970. Growth and differentiation of cytoplasmic membranes in the course of lipoprotein granule synthesis in the hepatic cell. I. Elaboration of elements in the Golgi complex. J. Cell Biol. 47:745-766.
12. COLEBAU, A., J. NACHBAU, and P. M. VIGNARE. 1971. Enzymatic characterization and lip composition of rat liver subcellular membranes. Biochim. Biophys. Acta. 249:492-499.
13. DE DUN, C., B. R. PESSEMA, R. GIANETTO, R. WATTAUX, and F. APPELMAN. 1955. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. Biochem. J. 60:604-617.
14. DE DUN, C., R. WATTAUX, and P. BACHMANN. 1962. Distribution of enzymes between subcellular fractions in animal tissues. Adv. Enzymol. 24:291-358.
15. DE FIERE, J. W., and M. L. KARNOLFSKY. 1973. Plasma membranes of mammalian cells. A review of methods for their characterization and isolation. J. Cell Biol. 66:373-383.
16. DONALDSON, R. P., H. E. TOUBERT, and S. SCHWARZENBERGER. 1972. A comparison of microbody membranes with mitochondria from plant and animal tissue. Arch. Biochem. Biophys. 152:199-215.
17. DUDERKO, J., R. BIEDRON, and C. LUBIAN. 1976. Binding of bovine cytochrome b₅ to phosphotidylcholine liposomes. Characterization of the reconstituted lipid-protein vesicles. Biochim. Biophys. Acta. 438:228-236.
18. ENOMOTO, K., and R. SATO. 1973. Incorporation in vitro of purified cytochrome b₅ into liver microsomal membranes. Biochem. Biophys. Res. Commun. 51:1-7.
20. ENOMOTO, K., and R. Sato. 1970. Asymmetric binding of cytochrome b to the membrane of human erythrocyte ghosts. Biochim. Biophys. Acta 241:274-277.

21. FLEISCHER, B., and S. FLEISCHER. 1970. Preparation and characterization of Golgi membranes from rat liver. Biochim. Biophys. Acta 219:276-297.

22. FLEISCHER, B., and S. FLEISCHER. 1970. Preparation and characterization of Golgi membranes from bovine liver. J. Cell Biol. 46:119-130.

23. FOWLER, S. J., J. REMACLE, A. TROUTH, H. BEAUFAY, J. BERTHET, M. WAO, and P. HAUSER. 1970. Analytical study of microsomes and isolated subcellular membranes from rat liver. V. Immunological localization of cytochrome b by electron microscopy: methodology and application to various subcellular fractions. J. Cell. Biol. 74:421-430.

24. GIOVITO, R., and C. DE DUVE. 1967. Isolation and characterization of Golgi membranes from rat liver. J. Biochem. 54:421-430.

25. HACKEMANN, G. L., and V. T. MACKENZIE. 1971. The distribution of NADPH cytochrome c reductase on rat liver microsomes. J. Biol. Chem. 246:577-584.

26. HACKEMANN, G. L., and R. J. MILLER. 1975. The distribution of anionic sites on the surfaces of mitochondrial membranes. Visual probing with polycationic ferritin. J. Cell Biol. 66:15-60.

27. HENNIG, H., D. H. KALLEN, and W. STOFFEL. 1970. Isolation and chemical composition of the lysosomal and the plasma membrane of rat liver cell. Hoppe-Seyler’s Z. Physiol. Chem. 351:191-199.

28. HOLLOWAY, P. M., and J. T. KATZ. 1975. Effect of cytochrome b on the size, density and permeability of phosphatidylcholine vesicles. J. Biol. Chem. 250:902-907.

29. ITO, A. 1974. Evidence obtained by cathepsin digestion of microsomes for the assembly of cytochrome b and its reductase in the membrane. J. Biochem. 75:279-297.

30. JAMESON, J. D., and G. E. PALADE. 1968. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the perinuclear and Golgi complexes. J. Cell Biol. 34:777-793.

31. JAMESON, J. D., and G. E. PALADE. 1968. Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Lipids. J. Cell Biol. 34:777-793.

32. JAMESON, J. D., and G. E. PALADE. 1968. Intracellular transport of secretory proteins in the pancreatic exocrine cell. III. Localization of NADPH cytochrome b reductase in the membrane. J. Cell Biol. 34:777-793.

33. KLOPAGER, M. 1958. Pigments of rat liver microsomes. Hoppe-Seyler’s Z. Physiol. Chem. 235:1238.

34. KLOPAGER, M. 1958. Pigments of rat liver microsomes. Hoppe-Seyler’s Z. Physiol. Chem. 235:1238.

35. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

36. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

37. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

38. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

39. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

40. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

41. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

42. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

43. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

44. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.

45. LODISH, H. F., and B. F. SMALL. 1975. Membrane proteins synthesized by rabbit reticulocytes. J. Cell Biol. 64:387-405.