

# 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

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### ABSTRACT

The localization of cytochrome  $b_5$  on the membranes of various subcellular organelles of rat liver was studied by a cytoimmunological procedure using anti-cytochrome  $b_5$ /anti-ferritin hybrid antibodies and ferritin as label. For this study, highly purified and biochemically characterized membrane preparations were employed. Outer mitochondrial membranes were found to be heavily labeled by the hybrid antibodies whereas Golgi and plasma membranes were not marked by the reagent. Peroxisome membranes were moderately labeled by the hybrid antibodies, suggesting that they may contain some cytochrome  $b_5$ . The preparation and purification of hybrid antibodies without peptic digestion is described and an analysis made of the composition of the final reagent product.

Cytochrome  $b_5$  was first found in the microsomal fraction of rat liver (10, 45) and later shown to be associated with outer membranes of mitochondria (17, 33, 43). This protein has also been reported to be present in the Golgi membranes (8, 16, 21) and NADH: cytochrome  $b_5$  reductase activity is said to be present in peroxisomes (9, 13). On the basis of these data, cytochrome  $b_5$  would appear to be present in membranes of several of the organelles of the liver cell. However, in these biochemical studies it is not always certain that the cytochrome  $b_5$  detected is truly associated with the organelle under study rather than being merely present as a subcellular contaminant of the preparations analyzed. In this paper, we report a sensitive ferritin-hybrid antibody technique that does

permit, by morphological analysis, the specific localization of cytochrome  $b_5$  to individual membrane elements. In the course of developing this assay, we studied in detail the various steps in the preparation of our morphologic label, an antigen-antibody complex between ferritin and the anti-cytochrome  $b_5$ /anti-ferritin hybrid antibody ( $ab_5/aF$ ).<sup>1</sup> We describe here our experience concerning

<sup>1</sup> *Abbreviations used in this paper:*  $ab_5/aF$ -ferritin, antigen-antibody complex between ferritin and the anti-cytochrome  $b_5$ /anti-ferritin hybrid antibody; anti-X, an immunoglobulin or a fragment of an immunoglobulin, the specificity of which is undefined;  $\rho$ , density; ER, endoplasmic reticulum; PBS, 0.01 M Na phosphate, pH 7.4, in 0.15 M NaCl; SDS, sodium dodecyl sulfate.

the preparation, purification, and final composition of this reagent. The validity of the technique is checked by its application on several purified subcellular fractions (outer membranes of mitochondria, plasma membranes, Golgi membranes, and peroxisomes), whose content in cytochrome  $b_5$  was assessed by biochemical methods. In the accompanying article (36), we describe the distribution of cytochrome  $b_5$  in microsomal subfractions using this technique.

A brief description of some of the immunological methods reported here has been presented before (35).

## MATERIALS AND METHODS

### Biochemical Procedures

#### PREPARATION OF ANTIGENS

Cytochrome  $b_5$  was obtained from tryptic digests of rat liver microsomes by the procedure of Omura et al. (31). The purity of the preparations ranged from 96 to 108% when assessed by comparing the amount of protein measured by the Lowry assay (25), using bovine serum albumin as standard, with that calculated by measuring the difference spectrum of the hemoprotein, and taking  $\Delta\epsilon_{424-405\text{ nm}} = 160\text{ cm}^{-1}\text{ mM}^{-1}$  and a mol wt of 12,000 for the cytochrome  $b_5$  fragment obtained after trypsinization (22). Results of sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis of cytochrome  $c$ , the cytochrome  $b_5$  preparation, and purified heme are shown in Fig. 1. The cytochrome  $b_5$ -containing gel shows two bands: one corresponds to a polypeptide chain that is similar in size to cytochrome  $c$ ; the second band did not stain black with amido black and displayed a green-yellow color. The latter band is probably the heme moiety of cytochrome  $b_5$  since it migrates to the same position as heme alone. Immunological purity was demonstrated by reacting anti-cytochrome  $b_5$  antiserum with cytochrome  $b_5$  purified after extraction with detergents (44), and with solubilized liver homogenate (Fig. 2). A line of identity was observed between the purified tryptic fragment of cytochrome  $b_5$ , the detergent-extracted cytochrome  $b_5$ , and solubilized liver homogenate. Two precipitin lines were seen with the cytochrome  $b_5$  derived from tryptic digests, as has been observed by others (34, 39). Commercial horse spleen ferritin was recrystallized six times with cadmium sulfate (4) before use.

#### PREPARATION OF SUBCELLULAR MEMBRANE FRACTIONS

External mitochondrial membranes were prepared from rat liver according to Parsons et al. (32), except for the flotation step which was carried out as follows. The crude membrane preparation obtained by differential centrifugation of the swollen mitochondrial fraction was

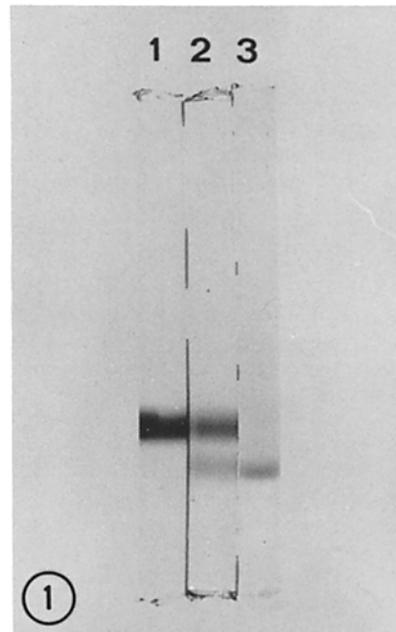


FIGURE 1 SDS-polyacrylamide gel electrophoresis of cytochrome  $c$  (1), cytochrome  $b_5$  (2), and purified heme (3). The preparations (2 mg/ml) were dissolved in 1% SDS, and 0.15 ml samples were applied to 5.6% polyacrylamide gels (7-mm diameter). Electrophoresis was carried out in 1% SDS/0.5 M Tris-acetate buffer, pH 7.4, at 8 ma/tube for 4 h. The gels were stained with 1% amido black. The faster moving band of the cytochrome  $b_5$  and heme preparations showed a green-yellow color, and did not bind the dye.

resuspended in a 43.1% sucrose solution.<sup>2</sup> To increase the equilibrium density of contamination fragments of plasma membranes, digitonin was added in an amount equivalent to 0.135 mg/g fresh weight of liver originally homogenized (3). This and all subsequent sucrose solutions contained 3 mM imidazole-HCl buffer, pH 7.4. The outer membranes were then separated from contaminants by flotation through a discontinuous sucrose gradient. This was done in a Ti-14 zonal rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) running for 90 min at 45,000 rpm and loaded with: 100 ml of 25.3% sucrose ( $\rho = 1.11$ ), 200 ml of 29.4% sucrose ( $\rho = 1.13$ ), 200 ml of 33.5% sucrose ( $\rho = 1.15$ ), 50 ml of the above mixture, and 100 ml of 52.2% sucrose ( $\rho = 1.25$ ) as a cushion. Purified outer membranes equilibrated at the 1.13/1.11 density interface.

Plasma membranes from rat liver were obtained according to the procedure of Song et al. (42), introducing

<sup>2</sup> All concentrations of sucrose expressed in percent are grams sucrose per 100 g solution.

the following modifications which take advantage of the density-shift of plasma membranes caused by digitonin, to increase the final purification. The crude preparation, suspended in a sucrose solution of density 1.22 (46.8%), was centrifuged for 3 h at 45,000 rpm in the Ti-14 zonal rotor loaded with: 80 ml of 29.4% sucrose ( $\rho = 1.13$ ), 150 ml of 39.3% sucrose ( $\rho = 1.18$ ), 30 ml of 43.1% sucrose ( $\rho = 1.20$ ), 325 ml of sample, and 65 ml of 52.2% sucrose ( $\rho = 1.25$ ). The fractions lighter than 1.18 were diluted in 1 vol of 1 mM NaHCO<sub>3</sub> and centrifuged for 40 min at 30,000 rpm (rotor n° 30, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pellet was suspended in 100 ml of 8.3% sucrose which contained 0.3 mg of digitonin per mg protein, and spun for another 3 h at 45,000 rpm in the Ti-14 zonal rotor loaded with the following sucrose solutions: 200 ml of 6.8% ( $\rho = 1.03$ ), 100 ml of sample, 20 ml of 35.5% ( $\rho = 1.16$ ), 100 ml of 39.3% ( $\rho = 1.18$ ), 150 ml of 41.2% ( $\rho = 1.19$ ), and 80 ml of 48.6% ( $\rho = 1.23$ ). Plasma membranes equilibrating above 1.19 density were collected.

A Golgi-rich fraction was obtained from rat liver following a modification of the method of Morr  et al. (28) introduced by Wibo et al.<sup>3</sup>

Peroxisomes were prepared as described by Leighton et al. (24).

#### CHEMICAL AND ENZYMIC DETERMINATIONS

Enzymic and chemical constituents of subcellular fractions were assayed as presented in detail by Beaufay et al. (7).

#### Immunological Methods

##### ANTISERA

Antisera were produced in rabbits by multiple site injections of antigen emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). After 5 wk, the animals were challenged with antigen dissolved in saline and bled 7 days later. Rabbits injected with ferritin produced high titers of antisera. In contrast, 11 out of 13 animals inoculated with cytochrome *b*<sub>5</sub> gave no significant response. However, rabbits injected with cytochrome *b*<sub>5</sub> polymerized with glutaraldehyde (27) responded very well. An IgG fraction was prepared from these sera by chromatography on DEAE Sephadex in 0.05 M Tris-HCl buffer, pH 8.0 (0°C) (41) and subsequent precipitation in 50% saturated ammonium sulfate.

##### IMMUNOLOGICAL ASSAYS

Quantitative immunological assays were done in two ways. Cytochrome *b*<sub>5</sub> binding activity was assessed by the method of Farr (14), employing as antigen cytochrome

<sup>3</sup> Wibo, M. D. Godelaine, A. Amar-Costesec, and H. Beaufay. 1976. Manuscript in preparation.

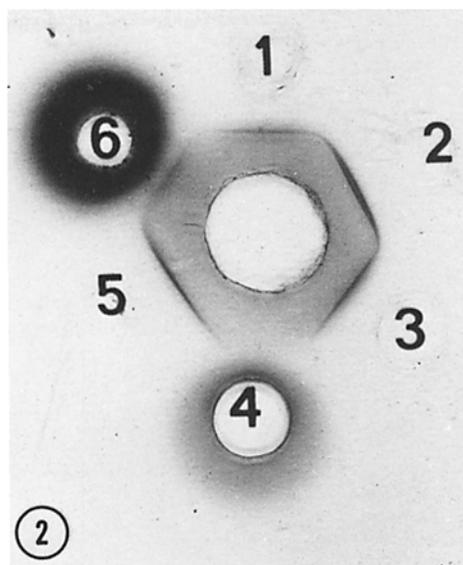


FIGURE 2 Ouchterlony pattern of anti-cytochrome *b*<sub>5</sub> antiserum (center) reacting with various antigen solutions: purified detergent-extracted cytochrome *b*<sub>5</sub> at 0.3 mg/ml (1 and 3), purified tryptic fragment of cytochrome *b*<sub>5</sub> at 0.06 mg/ml (2 and 5), crude liver homogenate at 100 mg/ml (4), and crude liver homogenate (90 mg/ml) dissolved in 1.5% Triton X-100 (6). Note the reaction of identity between the purified tryptic fragment of cytochrome *b*<sub>5</sub>, the detergent-extracted cytochrome *b*<sub>5</sub>, and solubilized liver homogenate.

*b*<sub>5</sub> labeled with [<sup>14</sup>C]acetic anhydride (23). Acetylation of the cytochrome *b*<sub>5</sub> did not affect its antigenic properties, and the assay was found to be linear with increasing amounts of antibody. One unit of antibody activity was expressed as the amount of IgG which binds 1 µg of cytochrome *b*<sub>5</sub>. Anti-ferritin antibodies were measured by a precipitation method using fluorescein-labeled antigen (46).

##### PURIFICATION OF ANTIBODIES

Antibodies were purified by immunoadsorption. Immunoadsorbent columns were made by coupling protein antigen (1 mg/ml packed agarose) in 0.1 M bicarbonate buffer, pH 9.0, to Sepharose 4B previously activated with cyanogen bromide (50 mg/ml packed agarose) at pH 10.5–11.0 (5). Before use, the columns were washed with 2.0% formic acid, then neutralized with 1 M phosphate buffer, pH 7.4, and equilibrated finally with 0.01 M Na phosphate, pH 7.4, in 0.15 M NaCl (PBS). Immunoadsorption steps were as follows: (a) IgG in PBS was slowly passed through the column three times; (b) non-adsorbed protein was thoroughly washed from the column with PBS; (c) antibodies were eluted from the column at 4°C over a period of 60 min with 2.0% formic

acid, pH 2.0. The eluted proteins were immediately neutralized with 1 M Tris-HCl buffer, pH 7.4. Quantitative immunologic studies show that 80% of the initial antiferritin activity was recovered in our antibody preparations. However, the yield for anti-cytochrome  $b_5$  activity ranged between only 50 and 65%.

#### PREPARATION OF THE $AB_5/AF$ HYBRIDS

Preparation and purification of  $ab_5/aF$  hybrids were done as illustrated schematically in Fig. 3. Prepurified anti-ferritin and anti-cytochrome  $b_5$  IgG in a molecular ratio of 9:1 were used for starting materials. After preparing the hybrids by selective reduction and dissociation as outlined below, side products were separated from  $ab_5/aF$  hybrid molecules. This was done by a two-step procedure involving successive immunoadsorption on Sepharose-linked cytochrome  $b_5$ , and on Sepharose-linked ferritin as described above. The final product was concentrated by filtration over an Amicon PM-30 membrane (Amicon Corp., Lexington, Mass.). The yield of cytochrome  $b_5$  binding activity after hybridization and purification was estimated to be on an average 18%.

Two methods for the preparation of hybrid antibodies were examined. Firstly, peptic fragments were prepared by the procedure of Hammerling et al. (19). In our hands, considerable loss of antigen-binding activity occurred by this method. We found, however, that good yields of hybrid could be obtained without using a preliminary digestion of gamma-globulin by pepsin. We adopted the following procedure based on early studies of rabbit gamma-globulin structure by Nisonoff and his associates (26, 29, 30). A mixture of purified rabbit anti-ferritin and anti-cytochrome  $b_5$  IgG (20 mg/ml, 0.04 M Tris-HCl, pH 8.0) was incubated under nitrogen for 90 min at 37°C with 30 mM mercaptoethanol, pH 8.0.<sup>4</sup> The reduced IgG was chilled and then adjusted to pH 2.3 with 1 M glycine-HCl buffer. The proteins were then passed through a Sephadex G-25 column pre-equilibrated with 25 mM NaCl adjusted to pH 2.3 with HCl. The eluted proteins were maintained at 4°C for 1 h in this low ionic strength medium. Monomers of gamma-globulin molecules obtained in this manner were randomly recombined to form hybrid molecules. This was achieved by adjusting the pH to 8.0 with 1 M Tris-HCl buffer, concentrating the proteins by ultrafiltration to at least 15 mg/ml, and finally incubating the mixture under an oxygen atmosphere, with gentle stirring, for no less than 8 h. The procedure described here was shown by Sephadex G-100 chromatographic analyses of the products to efficiently effect cleavage and recombination of rabbit gamma-globulin molecules. Immunoprecipitation studies also demonstrated that the hybrid antibodies prepared by this procedure were nonprecipitating as would

<sup>4</sup> The reduction conditions used maximize reduction of heavy chain-heavy chain linkages, while minimizing the cleavage of light chain-heavy chain disulfide bonds (20).

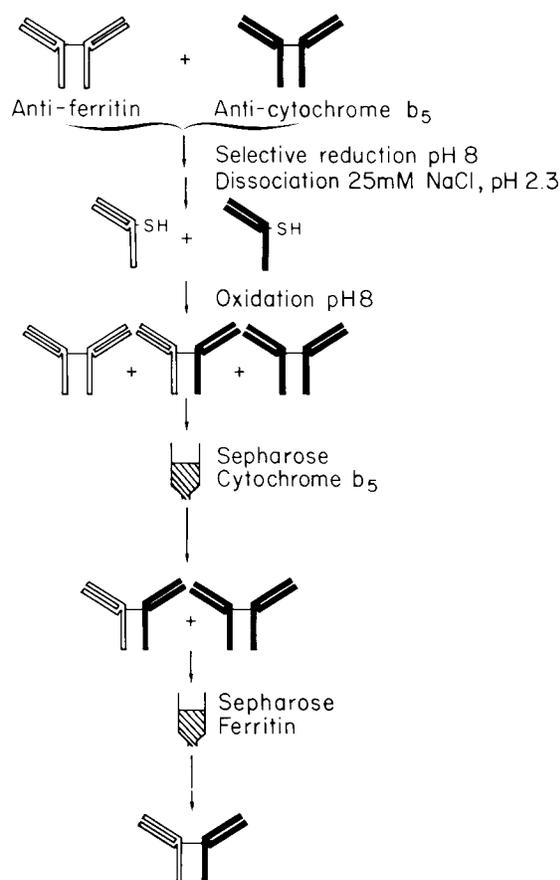


FIGURE 3 Schematic diagram of the sequential steps involved in the preparation and purification of hybrid  $ab_5/aF$  antibodies.

be expected for IgG molecules having a single reacting site. However, the hybrids do retain their capacity to bind antigen since antigen was recovered quantitatively in the precipitate formed when goat antirabbit serum was added to a mixture of hybrid plus antigen.

#### LABELING OF SUBCELLULAR MEMBRANE FRACTIONS WITH $AB_5/AF$ FERRITIN COMPLEX

**FORMATION OF HYBRID-FERRITIN COMPLEX:** Before incubation with membrane preparations, the  $ab_5/aF$  hybrid was first combined with ferritin. This was done with ferritin treated in the following manner: 150 mg of ferritin in 0.05 M acetate, pH 4.75, was first passed through a column (2.5 × 50 cm) of SP-Sephadex (G-25) equilibrated with the same buffer. The ferritin not retained by the resin (about 80%) was neutralized and used for subsequent steps. A complex of ferritin and

hybrid antibody was formed by careful addition of dilute antibody solution to a rapidly mixing solution of ferritin at neutral pH. The ferritin was in excess (molar ratio of 3-4:1) over the amount of hybrid antibody added. The  $ab_5/aF$ -ferritin complex (containing 10 mg IgG protein) was separated from the excess ferritin (about 65 mg) by chromatography on a small column (2.5 × 8.5 cm) of SP-Sephadex (G-25) equilibrated with 0.05 M acetate, pH 4.75. After washing away unreacted ferritin with the acetate buffer, the  $ab_5/aF$ -ferritin complex was eluted from the column using 0.1 M Tris-HCl, pH 7.4. Protein aggregates were removed by centrifugation of the preparation at 40,000 rpm for 15 min in a SW65 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

#### INCUBATION AND ISOLATION OF LABELED MEMBRANES

Subcellular preparations were incubated with the  $ab_5/aF$ -ferritin complex as described in the appropriate legend for each study. The  $ab_5/aF$ -ferritin complex was added in a three- to fivefold molar excess over the biochemically measured cytochrome  $b_5$  content of the subcellular fraction studied. Controls were done by preincubating the  $ab_5/aF$ -ferritin complex in 10-fold excess of purified cytochrome  $b_5$ .

After incubation, the reacted membranes were separated from the excess hybrid-ferritin complex by chromatography of 4 ml of the incubation mixture on a column (2.5 × 10 cm) of Biogel A-150 m agarose pre-equilibrated with the sucrose medium used for incubation. However, because the Golgi vesicles were damaged by passage through the agarose column, reacted Golgi membranes brought to a density of 1.16 with sucrose were separated from excess  $ab_5/aF$ -ferritin complexes by flotation through a three-layer sucrose gradient containing the Morré medium (37.5 mM Tris-maleate, pH 6.5, 5 mM  $MgCl_2$  and 1% Dextran 500). The membranes were recovered at the density 1.07-1.14 interface.

#### Morphological Procedures

The labeled membrane fractions to be examined by electron microscopy were collected over Millipore filters as described by Baudhuin et al. (6) and modified by Wibo et al. (47). Sections were stained with uranyl acetate for 2 min, then with lead citrate for 5 min (37).

#### Materials

Ferritin was a product from Fluka, A.G., Buchs, Switzerland. Digitonin was purchased from Merck A.G., Darmstadt, W. Germany. Sephadex G-25, DEAE-Sephadex, SP-Sephadex (G-25), and Sepharose 4B were obtained from Pharmacia, Uppsala, Sweden. Biogel A-150 m Agarose was a product of Bio-Rad Laboratories, Richmond, Calif.

## RESULTS

### *Analysis of the Hybrid Antibody Label*

#### COMPOSITION OF THE $ab_5/aF$ HYBRID PREPARATION

The specific activity of the  $ab_5/aF$  hybrid, based on its capacity to bind cytochrome  $b_5$ , was found to vary from 19.2 to 25.8 U per mg of IgG protein. The theoretical specific activity value, however, is approximately 80. Several possible explanations of this difference are: (a) the amount of anti-cytochrome  $b_5$  activity is underestimated because of a lower affinity of monovalent antibodies (38), (b) a large portion of the recovered antibody is denatured, or (c) the immunoadsorbent columns are not producing pure antibodies, and therefore a significant proportion of the hybrid preparation may not be of the  $ab_5/aF$  species. The first explanation was tested by diluting an anti-cytochrome  $b_5$  preparation four times with normal IgG to obtain a sp act of 6.1. After processing this mixture to obtain hybrid antibody, the sp act was found to be 6.5. Apparently, hybridization of the anti-cytochrome  $b_5$  does not produce a lower affinity to the antigen.

The precise composition of our hybrid preparation, therefore, was investigated. Hybrid  $ab_5/aF$  antibodies were acetylated with [ $^3H$ ]acetic anhydride and normal rabbit IgG with [ $^{14}C$ ]acetic anhydride, with each antibody molecule containing on an average only 1.5 acetyl groups. We assume that this level of acetylation does not impair antigen-antibody reaction. Two experiments were performed using a mixture of 60  $\mu g$  of  $^3H$ -labeled hybrid and 60  $\mu g$  of  $^{14}C$ -labeled IgG; the latter served as an internal control. In the first experiment, the mixture was incubated for 1 h at 4°C with an antigenic excess of Sepharose-linked cytochrome  $b_5$  to absorb all antibodies containing a cytochrome  $b_5$  binding site. After centrifugation, the supernate was incubated with an excess of Sepharose-linked ferritin to bind all anti-ferritin molecules not removed by the cytochrome  $b_5$  gel. After thorough washing with PBS, both gels were dissolved in a liquid scintillant, and the amount of bound radioactivity was determined. The counts were corrected for quenching and for the  $^{14}C$ -overlap in the tritium channel. In the second experiment, the order of gel absorption was reversed; hybrid antibodies were first adsorbed out

by treatment with Sepharose-linked ferritin, and the remaining anti-cytochrome  $b_5$  bearing molecules in the supernate were reacted with Sepharose-linked cytochrome  $b_5$ . Results of these two experiments are summarized in Table I. Approximately 40% of the hybrid preparation consists of  $ab_5/aF$  hybrids, 8.5% of the molecules contain a cytochrome  $b_5$  binding site but no binding site for ferritin ( $ab_5/aX$  or  $ab_5/ab_5$ ), and 27.5% of the preparation possesses antiferritin activity but no activity against cytochrome  $b_5$  ( $aF/aX$  or  $aF/aF$ ). The remaining 24% have no activity toward either ferritin or cytochrome  $b_5$ .

#### ASSESSMENT OF THE FERRITIN-HYBRID MOLECULAR RATIO

To estimate the number of hybrid molecules bound to each molecule of ferritin in the  $ab_5/aF$ -ferritin complex, the following experiment was done. Hybrid  $ab_5/aF$  antibodies (66  $\mu\text{g}$ ) were labeled by acetylation with [ $^{14}\text{C}$ ]acetic anhydride. A ferritin-hybrid complex was prepared as described in Materials and Methods, using 3.26 mg ferritin labeled with [ $^3\text{H}$ ]acetic anhydride. For a control,  $^{14}\text{C}$ -labeled normal rabbit IgG was substituted for the hybrid antibody and treated in an analogous

manner as the hybrid. To each preparation,  $ab_5/aF$ -ferritin complex or IgG-ferritin mixture was added 320  $\mu\text{g}$  of nonlabeled carrier rabbit IgG and, after mixing, 1 ml of goat antirabbit IgG antisera. After incubation for 12 h at  $4^\circ\text{C}$ , the resultant immunoprecipitate of hybrid and rabbit IgG was washed with PBS and dissolved in 0.1 N NaOH. The  $^{14}\text{C}$ - and  $^3\text{H}$ -radioactivities were then determined. The results are given in Table II. The molar ratio of IgG to ferritin was estimated to be 2.1. When the ratio is corrected, however, for the fact that only 40% of the hybrid preparation is true  $ab_5/aF$  and that ferritin also reacts with  $aF/aX$  and  $aF/aF$  as well as hybrid, the molar ratio becomes 1.4–2.0. The same experiment was then repeated, except that the hybrid-ferritin complex was adsorbed onto a Sepharose-linked cytochrome  $b_5$  gel. The data from this experiment are presented in Table II. Correcting the experimental value of 40.8  $\mu\text{g}$  IgG bound to the gel for the fact that  $ab_5/aF$  represents 82% of the total amount of cytochrome  $b_5$  binding IgG ( $ab_5/aF$ ,  $ab_5/aX$ ,  $ab_5/ab_5$ ), the molecular ratio of hybrid antibody to ferritin is found to be 2.5. On the basis of these two experiments, the molecular ratio of hybrid to ferritin in the complex appears to be near 2.

TABLE I  
Analysis of the Composition of the Hybrid Preparation

Binding of the hybrid preparation on Sepharose-cytochrome $b_5$ and Sepharose-ferritin immunoadsorbents*				
Protocol for adsorption of antibody	$\mu\text{g}$ of proteins bound to the adsorbent			Distribution of antibody (in %)
	IgG ( $^{14}\text{C}$ )	Hybrids ( $^3\text{H}$ )	Specific binding	
<b>Exp 1</b>				
(A) Sepharose-cytochrome $b_5$ (SB <sub>1</sub> )	1.0	22.2	21.2	46.6
(B) Sepharose-ferritin (SF <sub>1</sub> )	0.8	13.3	12.5	27.5
(C) Nonbound (N <sub>1</sub> )	62.1	11.8	(11.8)	25.9
<b>Exp 2</b>				
(A) Sepharose-ferritin (SF <sub>2</sub> )	1.2	34.8	33.6	68.8
(B) Sepharose-cytochrome $b_5$ (SB <sub>2</sub> )	0.6	4.8	4.2	8.5
(C) Nonbound (N <sub>2</sub> )	62.5	11.1	(11.1)	22.7
Calculation of the composition of the hybrid preparation				
Antibody specificity	$ab_5/aF$	$ab_5/aX$ $ab_5/ab_5$	$aF/aX$ $aF/aF$	$aX/aX$
Experimental values (in %)	SB <sub>1</sub> -SB <sub>2</sub> = 38.1 SF <sub>2</sub> -SF <sub>1</sub> = 41.3	SB <sub>2</sub> = 8.5	SF <sub>1</sub> = 27.5	N <sub>1</sub> = 25.9 N <sub>2</sub> = 22.7
Average content (%)	39.7	8.5	27.5	24.3

\* Further details of these experiments are given in the text.

*Ultrastructural Localization of  
Cytochrome b<sub>5</sub>*

BIOCHEMICAL DATA

In Table III are listed some biochemical properties of purified subcellular organelle preparations.

The composition of the preparations was calculated from the relative specific activities of marker enzymes as explained by Leighton et al. (24). Subcellular components derived from ER were the major contaminant in all cases. Outer mitochondrial membranes had a relative sp act of 35

TABLE II  
*Analysis of Molar Ratio of Hybrid-Ferritin Complex*

Experimental procedure*	Amount precipitated				Molar ratio (antibody/ ferritin)
	Experimental values		Corrected values		
	IgG ( <sup>14</sup> C)	Ferritin ( <sup>3</sup> H)	IgG ( <sup>14</sup> C)	Ferritin ( <sup>3</sup> H)	
	μg	μg	μg	μg	
Precipitation by goat anti-rabbit IgG					
Test (hybrid)	58.5	69.7	57.8 (88%)	69.1	2.1
Blank (normal IgG)	0.7	0.6			
Adsorption by Sepharose-cytochrome b <sub>5</sub>					
Test (hybrid)	41.0	36.7	40.8 (60%)	32.3	3.2
Blank (normal IgG)	0.2	4.4			

\* Further details are given in the text.

‡ A mol wt of 400,000 is taken for the ferritin molecule.

TABLE III  
*Biochemical Properties of the Purified Membrane Preparations*

Constituent	Subcellular preparations							
	Outer mitochondrial membrane		Plasma membrane		Golgi		Peroxisomes	
	Percent of H*	RSA‡	Percent of H	RSA	Percent of H	RSA	Percent of H	RSA
Protein	0.11	1	0.48	1	0.29	1	0.07	1
Glucose 6-phosphatase	0.01	0.09	—	—	0.18	0.62	0.04	0.57
NADH cytochrome c reductase	—	—	0.05	0.11	—	—	0.04	0.57
Cytochrome oxidase	ND§	—	0.02	0.04	0.02	0.06	0.02	0.28
Monoamine oxidase	3.8	<b>34.6</b>	0.04	0.08	0.03	0.09	—	—
Acid phosphatase	—	—	—	—	1.8	6.55	0.09	1.28
Alkaline phosphodiesterase-I	ND	—	18.3	<b>38.2</b>	0.83	2.85	0.03	0.43
5'-Nucleotidase	—	—	11.4	<b>23.8</b>	—	—	0.04	0.57
Galactosyltransferase	ND	—	—	—	18.3	<b>62.9</b>	—	—
Catalase	—	—	—	—	—	—	2.69	<b>38.43</b>
Urate oxidase	—	—	—	—	—	—	4.70	<b>67.14</b>
Cytochrome b <sub>5</sub> content (μg/mg protein)	9.6		ND		0.9		(—) <sup>  </sup>	

\* Percent of homogenate.

‡ Relative specific activity.

§ Not detected.

|| Interfering material prevented direct assay of this preparation.

for monoamine oxidase and were very pure. ER components accounted for about 2% protein of the outer mitochondrial membrane preparation which contained 9.6  $\mu\text{g}$  of cytochrome  $b_5$  per mg protein. Plasma membranes were also very pure, containing some 2–3% ER contaminants, based on NADH cytochrome  $c$  reductase content. The average relative sp act of plasma membrane marker enzymes (alkaline phosphodiesterase I and 5'-nucleotidase) in the preparation was 31. The relative specific activity of galactosyltransferase was 63 in the Golgi fraction, which was 70–75% pure and contained 10–15% protein belonging to ER components. The cytochrome  $b_5$  content of the Golgi fraction was found to be 0.9  $\mu\text{g}$ /mg protein, which is about the amount expected to be present from contaminating ER components (2). Contamination of the peroxisome preparation was about 10% by ER and 6% by mitochondria. Interfering material prevented direct assay of this preparation for cytochrome  $b_5$ .

#### ELECTRON MICROSCOPE EXAMINATION OF LABELED MEMBRANES

**OUTER MITOCHONDRIAL MEMBRANES:** The appearance of outer mitochondrial membranes labeled with the  $ab_5/a\text{F}$ -ferritin complex is shown in Fig. 4. Most of the vesicles have characteristic invaginations of their membranes which appear in cross-sections as small circular profiles in the interior of the vesicle. In a few of the membrane profiles there are areas which suggest a continuity between the interior vesicle and the external one. The control preparation (Fig. 4a) contains very little ferritin. Most of that which is seen is in the form of aggregates. Very few ferritin molecules are associated with the membrane surface. Some aggregates of ferritin are seen within the internal vesicles. They apparently have been

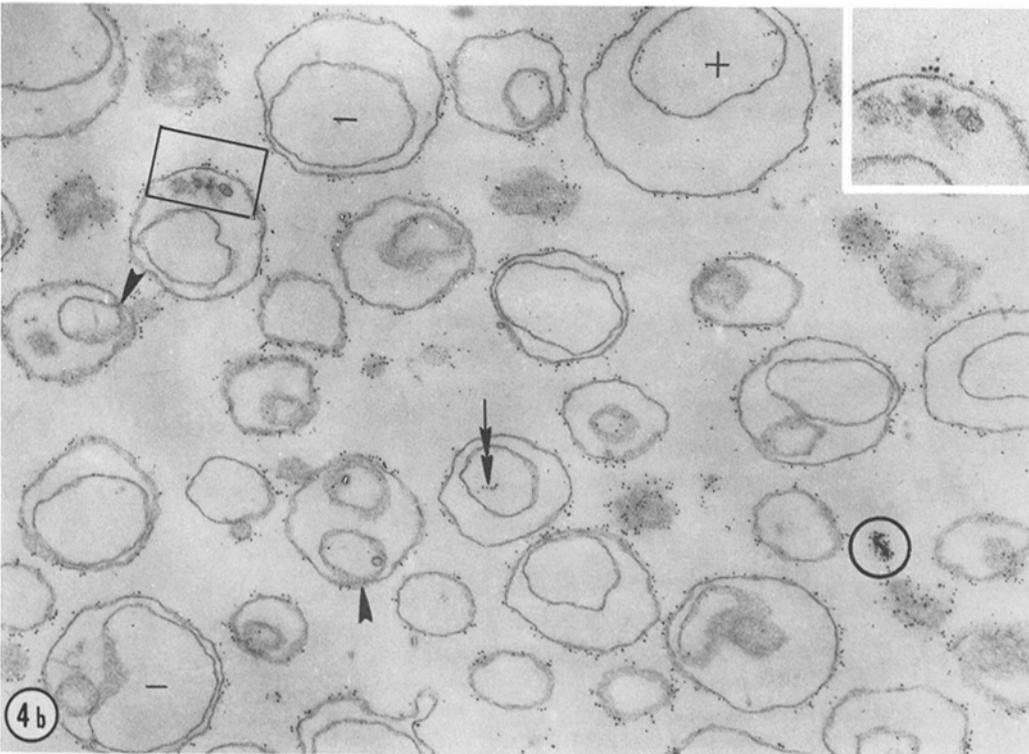
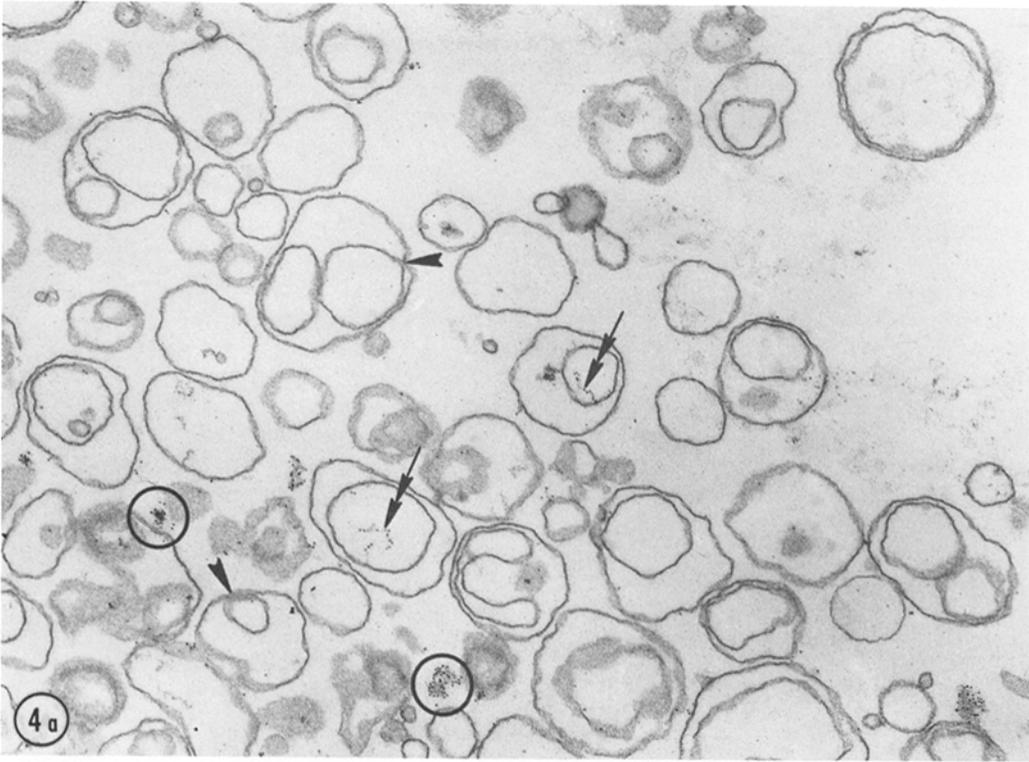
trapped within the invaginations and were not removed during the washing procedure.

In the preparation treated with active hybrid-ferritin complex (Fig. 4b), the outer surfaces of the mitochondrial membranes are heavily labeled with ferritin molecules. A few ferritin aggregates are also seen as in the control. Much of the ferritin label stands away from the membrane surface by a short space of about 12 nm. This distance is compatible with the molecular dimensions of ferritin (radius of the protein shell = 5 nm [15]) and gamma-globulin (maximum distance between reactive sites is 13 nm [11]), assuming that the hybrid molecule is sandwiched between the ferritin and the membrane surface. Most of the dense images of ferritin are associated with the external face of the membranes. The fact that the internal face of the external vesicle is not labeled does not preclude the occurrence of anti-cytochrome  $b_5$  binding sites on the internal surface, because the latter was not exposed to the  $ab_5/a\text{F}$ -ferritin complex under the conditions of incubation. Note that in a few instances one side of the interior vesicle membrane is labeled with ferritin, which suggests that this face of the invaginated membrane is in contact with the external medium.

**PLASMA MEMBRANES:** Fig. 5 shows pictures of purified plasma membranes incubated with the ferritin-hybrid label. The broken appearance of the membranes is characteristic of plasma membranes treated with digitonin. The membranes are not fragmented since the sedimentation coefficient of plasma membranes treated with digitonin is not decreased (3), and since by negative staining techniques the digitonin-treated membranes appear whole (12). In this experiment, as was the case in the control (not shown), very little labeling of the membranes is observed. Similar observations were made on plasma membrane

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FIGURE 4 Electron micrographs of purified outer mitochondrial membranes incubated with the  $ab_5/a\text{F}$ -ferritin complex. (a) Control: only occasional ferritin molecules are seen on the surfaces. (b) Test: all profiles are seen to be labeled with ferritin on their outer surfaces. In some profiles, ferritin label is observed on the inner surface of the internal vesicle membrane (+) while in others it is not (-). Arrowheads: regions suggesting a continuity between the internal and external vesicles. Double arrow: aggregates of ferritin molecules trapped within internal vesicles. Circles: aggregates of ferritin in the external medium. The rectangle indicates the membrane profile enlarged in the inset to show the ferritin label attached to the membrane surface. The membranes (0.5 mg protein) were incubated with  $ab_5/a\text{F}$ -ferritin (cytochrome  $b_5$  binding capacity = 14.5  $\mu\text{g}$ ) for 30 min at 25°C in a 2 ml volume of 0.25 M sucrose containing 25 mM sodium phosphate, pH 7.4.  $\times 50,000$ .



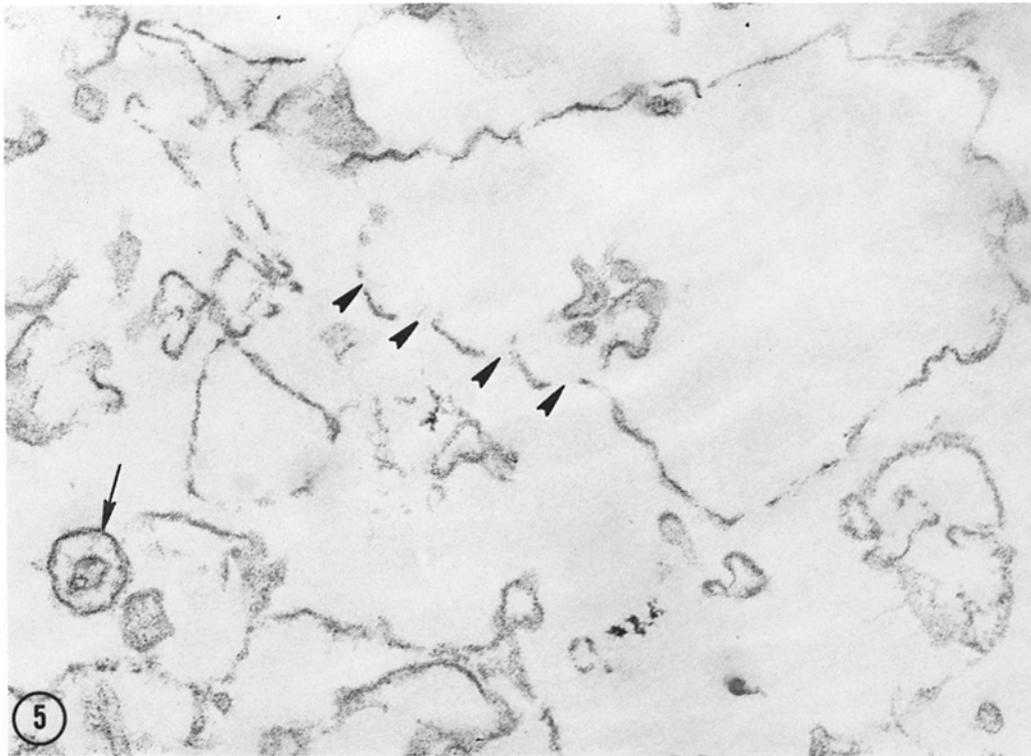


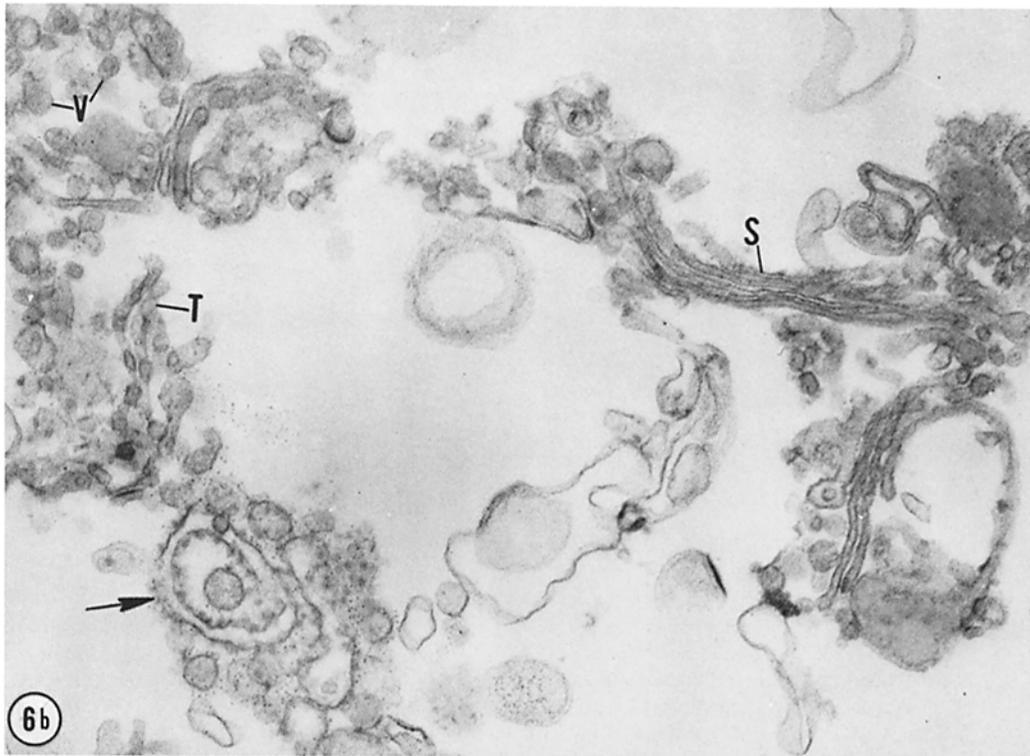
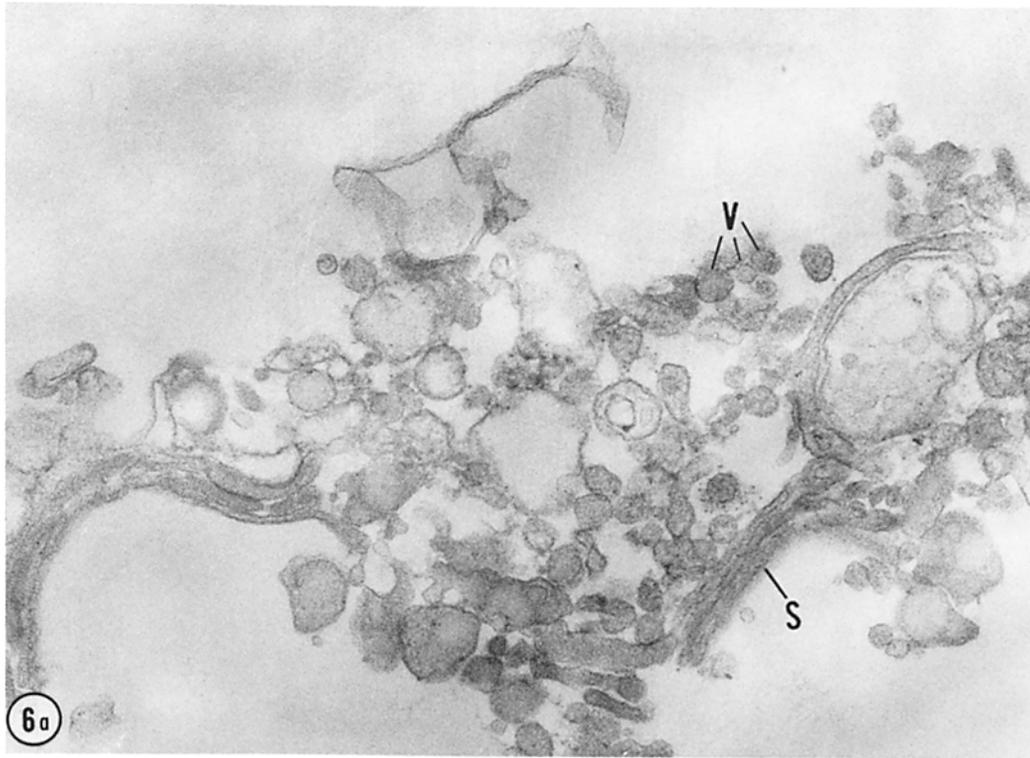
FIGURE 5 Electron micrograph of purified plasma membranes incubated with  $ab_5/aF$ -ferritin label. The broken appearance of the plasma membrane results from the treatment with digitonin (arrowheads). Only occasional ferritin molecules are seen associated with such membranes. Small vesicles (arrow), however, were labeled with ferritin. A similar picture was observed in the control for this experiment. The plasma membranes (0.5 mg protein) were incubated with  $ab_5/aF$ -ferritin (cytochrome  $b_5$  binding capacity = 12  $\mu$ g) for 12 h at 0°C in 2 ml of a medium consisting of 0.25 M sucrose, 25 mM phosphate, pH 7.4, 0.15 M NaCl, 0.02% Triton WR-1339, 0.5% bovine serum albumin, and 0.02%  $NaN_3$ . Triton WR-1339 was added to reduce the nonspecific adsorption of the ferritin label on the membranes.  $\times 50,000$ .

preparations obtained without digitonin treatment. Then, however, the frequency of ferritin-labeled vesicles was slightly greater in the test, in agreement with the less complete purification achieved.

**GOLGI MEMBRANES:** The appearance of vesicles of the Golgi complex after reaction with the  $ab_5/aF$ -ferritin complex is shown in Fig. 6. In

the control, the principal characteristic components of the Golgi complex, the large flat saccules and tubules, contained very few ferritin images. In the test, the labeling of the same elements with ferritin is about the same order of magnitude. These observations suggest therefore that the larger elements at least of the Golgi complex are not endowed with cytochrome  $b_5$ . In contrast,

FIGURE 6 Appearance of Golgi membranes after incubation with  $ab_5/aF$ -ferritin complex. Saccules (S), tubules (T) and vesicles (V) similar in appearance to those seen in the Golgi apparatus *in situ* are observed. (a) Control: a few ferritin molecules are associated with these structures. (b) Test: characteristic Golgi elements contain little ferritin label. In contrast, vesicles clearly of ER origin (arrow) are strongly labeled. Incubation of Golgi preparation (0.5 mg protein) with  $ab_5/aF$ -ferritin (cytochrome  $b_5$  binding capacity = 12  $\mu$ g) was done for 12 h at 0°C in a 2 ml vol consisting of 0.25 M sucrose, 37.5 mM Tris-maleate, pH 6.5, 0.15 M NaCl, and 1% Dextran 500.  $\times 50,000$ .



many small vesicles of the preparation are labeled in the test. Some of these vesicles have ribosomes on their surfaces. This and the fact that biochemically the principal contaminants of this preparation of the Golgi membranes are ER marker enzymes indicate that most of the small labeled vesicles are contaminating membranes of ER origin.

**PEROXISOMES:** The labeling of peroxisomes with the  $ab_5/aF$ -ferritin complex is presented in Fig. 7. The peroxisomes are most easily recognized when they are seen with their dense, urate oxidase-containing core. In the control, these particles are observed to have associated with their membrane a few ferritin images. In the test, clumps of ferritin are observed in the sample, as are free cores and small membranous vesicles. The peroxisome profiles themselves are marked with more ferritin molecules than are the peroxisome profiles in the control. The degree of labeling, however, is not nearly so extensive as that observed for the contaminating microsomal vesicles in this preparation, which are also labeled, or for outer mitochondrial membranes (Fig. 4*b*). These photographs suggest the possibility that peroxisomes may possess some cytochrome  $b_5$  on their membranes.

Besides those shown here, other preparations of various membrane fractions were examined during the development of the experimental method. Labeling patterns similar to those shown here were observed. The controls appeared the same whether incubated with  $aF/aF$ -ferritin complexes or with complexes of  $ab_5/aF$ -ferritin preincubated with excess cytochrome  $b_5$ .

## DISCUSSION

### *Preparation and Analysis of the Hybrid Antibody Label*

The method of hybrid preparation we describe here eliminates the proteolytic digestion step of earlier published procedures (19) and thus avoids subjecting the antibody to possible denaturation.

Highly purified antigens are used to prepare the immunoabsorbent columns on which the antibodies are purified. Use of these columns ensures the antigenic specificity of the hybrid reagent produced. The cytochrome  $b_5$  antigen employed was obtained as a tryptic fragment from rat liver microsomes. Antisera prepared against this peptide cross-reacted with the whole molecule of cytochrome  $b_5$  obtained after detergent extraction (Fig. 2). We found that some molecules of ferritin spontaneously adsorbed to our membrane preparations. The reactive molecules could be removed, however, by adsorption on SP-Sephadex at pH 4.75, before the preparation of the ferritin-hybrid complex.

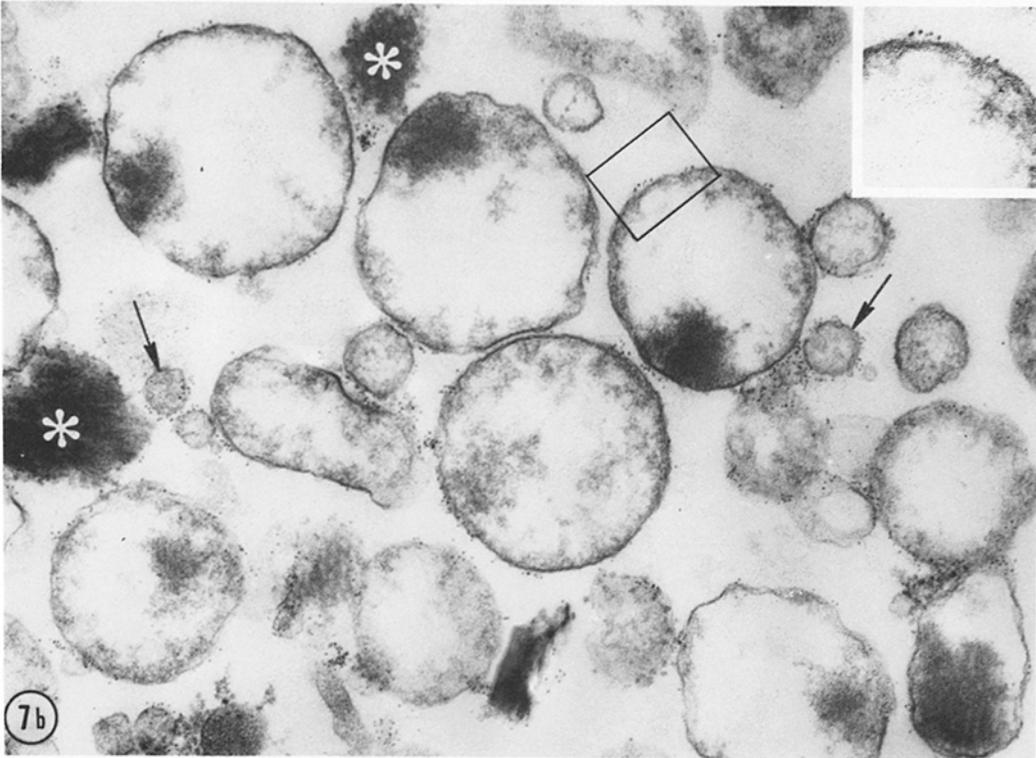
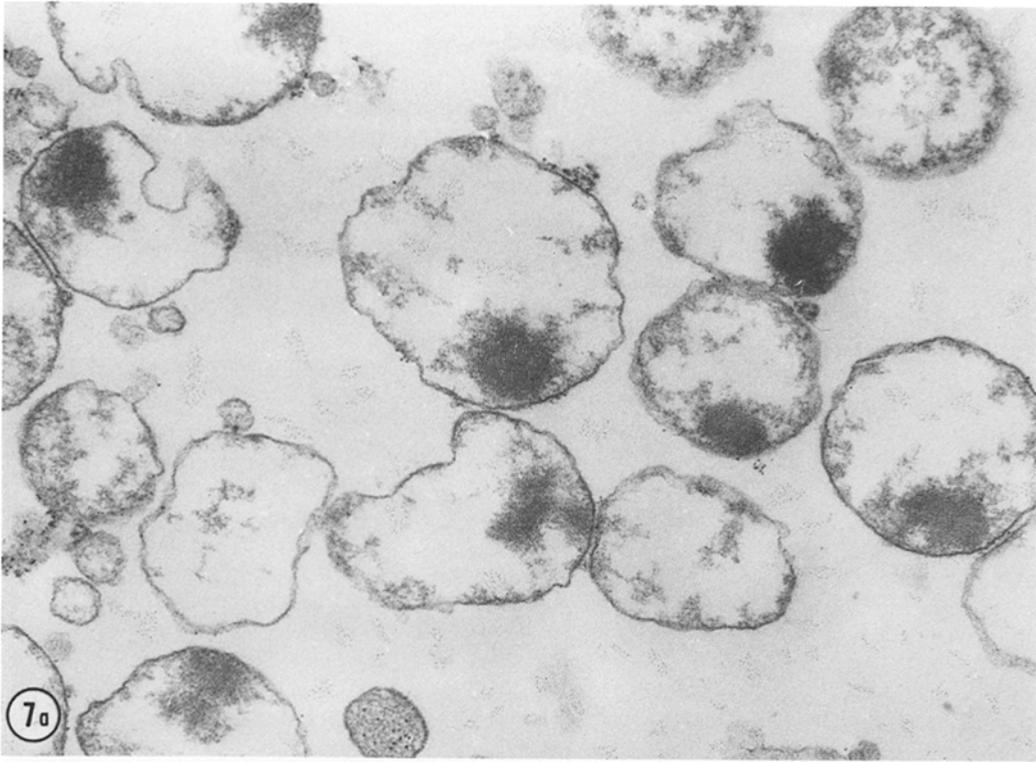
The results of experiments summarized in Table I indicate that nearly half of the final hybrid antibody product consists of gamma-globulins bearing anti-cytochrome  $b_5$  activity. However, about 18% of this activity is associated with molecules of the  $ab_5/aX$  or  $ab_5/ab_5$  type which in incubations could react with membrane-bound cytochrome  $b_5$  but would not be observed by electron microscopy. Such molecules should not significantly affect the results of labeling experiments, however, unless the experiments are intended to be quantitative in nature. The average molecular ratio of hybrid antibody to ferritin in the  $ab_5/aF$ -ferritin complex was found to be near 2 (Table II). Some ferritin-hybrid labeling therefore could involve reaction with more than one molecule of cytochrome  $b_5$ . Clumping of labeled membranes by means of ferritin links was very seldom observed, however.

### *Localization of Cytochrome $b_5$ on Intracellular Membranes*

The  $ab_5/aF$ -ferritin complex clearly labeled outer membranes of mitochondria confirming earlier biochemical findings on the presence of cytochrome  $b_5$  in this membrane (17, 33, 43). Since all the membranes in the preparation are marked, our results provide strong evidence against the contention of Allmann et al. (1) and Green et al.

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FIGURE 7 Electron micrographs of peroxisomes incubated with the  $ab_5/aF$ -ferritin complex. (a) Control: surfaces of the peroxisomes bear only occasional ferritin molecules. (b) Test: all peroxisome profiles contain ferritin label, but the degree of labeling is not great. Small vesicles, probably of ER origin, are strongly labeled (arrows). Asterisks: free peroxisome cores. The rectangle indicates a portion of a peroxisome enlarged in the inset to show the ferritin label to better advantage. Incubation of 0.5 mg peroxisomes with  $ab_5/aF$ -ferritin (cytochrome  $b_5$  binding capacity = 9.5  $\mu$ g) was done in 2 ml of a medium consisting of 0.25 M sucrose and 3 mM imidazole-HCl, pH 7.4, at 25°C for 10 min.  $\times$  50,000.



(18) that the rotenone-insensitive NADH:cytochrome *c* reductase system found in outer mitochondrial membranes originates from contaminating microsomes. The pattern of ferritin labeling on the profiles appears to be a random one; no large patches, at least of ferritin labeling, were evident. However, we cannot be certain that this impression about the labeling pattern is a true reflection of the distribution of cytochrome *b*<sub>5</sub> on the membrane surface, since with our method as little as 5–10% of the total antigen present may have been labeled with the hybrid-ferritin complex (36).

Incubation of purified preparations of plasma membranes with the *ab*<sub>5</sub>/aF ferritin label indicates the absence of accessible cytochrome *b*<sub>5</sub> in this subcellular component. None of the large nonvesicular fragments was significantly labeled, indicating that cytochrome *b*<sub>5</sub> was not present on either of the two faces of the plasma membrane. The few vesicles which did specifically label can often, on the basis of their size and appearance, be attributed to ER contaminants.

The findings of our studies on the Golgi membranes do not support the reports of others (8, 16, 21) that the membranes of this organelle contain cytochrome *b*<sub>5</sub>. Instead, our results suggest that most of the cytochrome *b*<sub>5</sub> measured in the Golgi fractions may be attributed to ER contamination of the preparations.

The data on the labeling of the peroxisomes indicate that the membranes of this organelle may contain a small amount of cytochrome *b*<sub>5</sub> and therefore give some support to the claim of Donaldson et al. (13) that liver and kidney peroxisomes contain NADH:cytochrome *b*<sub>5</sub> reductase activity. The degree of ferritin labeling of the peroxisome profiles, however, is low and conclusions concerning the presence of ER enzymes in peroxisome membranes must be considered tentative until further studies are done.

The virtue of the hybrid antibody labeling method is that it ensures a fine localization of antigen. This fact was used to great advantage in the present work where the subject material consisted of membranous vesicles closely spaced to one another. Attempts to use antibody to which ferritin was coupled chemically (40) were unsuccessful, because to each antibody several ferritin molecules were usually coupled. After incubation with subcellular membranes, it is difficult to be certain about which vesicle these large ferritin-antibody aggregates are really associated. This was

particularly true for studies on microsomes. With the hybrid antibody method, on the other hand, assignment of ferritin label to one vesicle or another was much less a problem. We anticipated that the uniqueness of the hybrid antibody would allow a more detailed quantitative analysis of the topographic distribution of cytochrome *b*<sub>5</sub> on the membranes. However, we were not able to achieve this goal with the present method, for reasons discussed in the following paper (36).

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