IMMUNOELECTRON MICROSCOPE LOCALIZATION OF CYTOCHROME P-450 ON MICROSOMES AND OTHER MEMBRANE STRUCTURES OF RAT HEPATOCYTES

SHIRO MATSUURA, YOSHIAKI FUJII-KURIYAMA, and YUTAKA TASHIRO

From the Department of Physiology, Kansai Medical University, Moriguchi-shi, Osaka 570, Japan. Dr. Fujii-Kuriyama's present address is the Department of Biochemistry, Cancer Institute, Japanese Foundation for Cancer Research, Toshima-ku, Tokyo 170, Japan

ABSTRACT

Localization of cytochrome P-450 on various membrane fractions of rat liver cells was studied by direct immunoelectron microscopy using ferritin-conjugated antibody to the cytochrome. The outer surfaces of almost all the microsomal vesicles were labeled with ferritin particles. The distribution of the particles on each microsomal vesicle was usually heterogeneous, indicating clustering of the cytochrome, and phenobarbital treatment markedly increased the labeled regions of the microsomal membranes. The outer nuclear envelopes were also labeled with ferritin particles, while on the surface of other membrane structures such as Golgi complexes, outer mitochondrial membranes and plasma membranes the labeling was scanty and at the control level. The present observation indicates that cytochrome P-450 molecules are localized exclusively on endoplasmic reticulum membranes and outer nuclear envelopes where they are probably distributed not uniformly but heterogeneously, forming clusters or patches. The physiological significance of such microheterogeneity in the distribution of the cytochrome on endoplasmic reticulum membranes is discussed.

KEY WORDS ferritin immunoelectron microscopy · cytochrome P-450 · intracellular localization of cytochrome P-450 · membrane flow · microheterogeneity of microsomes

Cytochrome P-450 is a general term for microsomal hemoproteins which function as a terminal oxygenase in the drug-metabolizing enzyme system. These hemoproteins comprise ~5% and 15% of the protein of noninduced and phenobarbital-induced liver microsomes, respectively.

Recently, we reported that, in rat liver cells, phenobarbital inducible cytochrome P-450 is exclusively synthesized on membrane-bound ribosomes (30). We also found that the nascent P-450 molecules released from the ribosomes slide laterally to be inserted directly into the outer surface of rough endoplasmic reticulum (ER) membranes and then are transferred to smooth ER (13, footnote 1), the processes being essentially similar to the intracellular transport of nascent NADPH-cytochrome *c* reductase on rat liver microsomes (31, 35).

We attempted further to determine the distribution of cytochrome P-450 molecules on the membrane systems of liver cells; for example, are these molecules localized exclusively on ER mem-

[1] Fujii-Kuriyama, Y., M. Negishi, and Y. Tashiro. Manuscript in preparation.

branes or do they exist also on the other membrane structures such as nuclear envelopes, Golgi complexes and outer mitochondrial membranes, and also are these molecules distributed in a random manner or in clusters on the membrane structures? Biochemical data on the distribution of cytochrome P-450 in cytoplasmic membrane structures other than microsomes are controversial, as is taken up in the Discussion section. Instead of a biochemical approach, we utilized ferritin immunoelectron microscopy in the hope of obtaining more direct information on the distribution of cytochrome P-450 molecules.

We found that the cytoplasmic surface of the microsomal vesicles and of the outer nuclear envelopes was heavily labeled with ferritin, while labeling of other membrane structures such as Golgi complexes, outer mitochondrial membranes and plasma membranes was scanty and at the control level. We also found that the distribution of ferritin on the surface of each microsomal vesicle was heterogeneous, suggesting clustering of the cytochrome. Our findings are discussed in relation to our recent report on the distribution of NADPH-cytochrome *c* reductase on rat liver microsomes (29). A semiquantitative analysis of the distribution of cytochrome P-450 molecules on rat liver microsomes will be reported in a subsequent publication.[2]

MATERIALS AND METHODS

Preparation of Cytochrome P-450 and Antiserum

Male Sprague-Dawley rats (~200 g) were given a single daily intraperitoneal injection of phenobarbital (10 mg/100 g body weight) for 7 days and were fasted for 24 h before sacrifice. Cytochrome P-450 was purified from liver microsomes of phenobarbital-treated rats according to the procedures of Imai and Sato (18) with slight modification.[1]

Antiserum against the purified cytochrome P-450 was prepared in rabbits, and the antibody was partially purified by fractionation with ammonium sulfate as described recently (29). The double agar immunodiffusion test showed that the antibody gave a single precipitation line with purified cytochrome P-450 as well as with the microsomes solubilized with 0.6% sodium cholate. The properties of anti-cytochrome P-450 antibody will be reported in detail elsewhere.[1]

[2] Matsuura, S., Y. Fujii-Kuriyama, and Y. Tashiro. Manuscript in preparation.

Preparation of Ferritin-Antibody Conjugates

Ferritin and the antibody were coupled as described by Kishida et al. (23) using glutaraldehyde as a coupling agent. The ferritin anti-P-450 antibody conjugates (antibody conjugates) with the molar ratio of immunoglobulin G (IgG) to ferritin of approximately one were isolated by gel filtration on Bio-Gel A 1.5 M (Bio-Rad Laboratories, Richmond, Calif.), concentrated 20–30-fold and then treated with 0.1 M glycine to inactivate residual aldehyde groups (26, 47). The antibody conjugates were used for immunoelectron microscope studies without further purification.

Normal rabbit IgG and sheep IgG were also conjugated with ferritin by the same procedures, and these conjugates (control conjugates) were used for the control experiments.

Properties of the antibody conjugates were examined by double agar immunodiffusion and immunoelectrophoresis. To determine the specific antibody content in the conjugates and to calculate the molar ratio of IgG to ferritin, IgG was labeled with ^{125}I by the use of chloramine T according to Hunter's procedure (16), and the ^{125}I-labeled conjugates were prepared and isolated as described above. The conjugates were then adsorbed to an immunoadsorbent gel containing cytochrome P-450. After washing with 0.15 M and then 1.0 M potassium chloride, both in 0.01 M potassium phosphate buffer, pH 7.3, the specific conjugates were eluted with 0.2% formic acid. Their radioactivity was measured with a Packard 5330 autogumma spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.), and ferritin content was determined by spectrophotometry (25). These data were used for the calculation of the specific antibody content and the molar ratio of IgG to ferritin of the conjugates.

Preparation of Individual Subcellular Fractions

Smooth and rough microsomes were prepared by discontinuous sucrose density gradient centrifugation as described by McLaughlin and Pitot (28). Briefly, male rats weighing ~200 g were decapitated, and the livers were rapidly removed and homogenized in 2 vol of 0.44 M sucrose in TKM (0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, 0.005 M $MgCl_2$) in a Potter-Elvehjem homogenizer. A postmitochondrial supernate was prepared from the homogenate by centrifugation for 10 min at 14,000 *g*. An 8-ml portion of the postmitochondrial fraction adjusted to 1.35 M sucrose in TKM was layered over 3 ml of 2.0 M sucrose in TKM and overlaid with 2 ml of 0.44 M sucrose in TKM. This discontinuous gradient was centrifuged at 35,000 rpm for 15 h at 4°C in a RPS-40T Hitachi rotor (Hitachi Koki Co., Ltd. Katsuta, Japan). The smooth and the rough microsomes which floated at the boundary between the 0.44 M and

1.35 M, 1.35 M and 2.0 M sucrose layers, respectively, were collected. The solutions containing the smooth and rough microsomes were diluted with 2 vol of TKM and were centrifuged in a RP 40 rotor for 30 min, and both pellets were resuspended in 0.25 M sucrose in TKM.

Plasma membrane and nuclei were isolated by the method of Stein et al. (44) and Blobel and Potter (4), respectively.

The Golgi fraction was prepared according to the procedures of Hino et al. (15) as follows: Rat livers were perfused with ice-cold 0.25 M sucrose, finely minced with scissors, and stained through a 100-mesh stainless sieve using a bamboo spatula. This homogenate was diluted with 2 vol of Hi-medium (0.5 M sucrose, 50 mM Tris-maleate buffer, pH 6.5, 1% dextran), centrifuged for 5 min at 3,000 rpm (Hitachi RP 20 rotor), and the supernate was further centrifuged for 10 min at 7,500 rpm. The pellets obtained were loaded directly onto ~3 ml of 1.2 M sucrose and centrifuged for 60 min at 100,000 *g* (Hitachi RPS 40 rotor). The materials which accumulated on the 1.2 M sucrose layer were collected, diluted approximately five times with Hi-medium, and then centrifuged for 10 min at 6,500 rpm. The resulting pellets were resuspended gently in Hi-medium and used as the Golgi fraction. The materials which were pelleted down through the 1.2 M sucrose layer were rich in mitochondria and used as the mitochondrial fraction.

Simultaneous Preparation of Various Subcellular Fractions from the Liver Homogenate from One Rat (Simultaneous Method)

For the immunoelectron microscope comparison of the distribution of cytochrome P-450 in the various cell organelles, it is preferable to prepare all the subcellular fractions from the liver homogenate from one animal so as to avoid differences due to individual variations among animals. For such a purpose, Golgi and mitochondrial fractions were first prepared from the rat liver homogenate according to the above described procedures, and all the other fractions were prepared from the rest of the same sources: From the pellets derived from the first centrifugation for 5 min at 3,000 rpm, plasma membrane and nuclei were prepared as described above, and the supernate derived from the second centrifugation for 10 min at 7,500 rpm was diluted three times with Hi-medium, and the total microsomes were pelleted down by centrifuging for 30 min at 100,000 *g* (Hitachi RPS 40 rotor) and were resuspended in 0.25 M sucrose containing TKM buffer. The subcellular fractions used for Figs. 3, 5, 7, 8, and 9 were prepared according to this simultaneous method.

Determination of Cytochrome P-450 and Protein

The cytochrome was determined by the method of Omura and Sato (36) from the CO difference spectra of the reduced samples, and protein was determined by the method of Lowry et al. (27) using bovine serum albumin as the standard.

Labeling of Subcellular Membrane Fractions and Electron Microscopy

In the present experiment, direct ferritin antibody method was used exclusively because this method gives a better resolution and a more quantitative result than the indirect method. Each subcellular fraction was incubated for 30 min, 5 h, or longer at 4°C with either the antibody or the control conjugates. The concentration of the specific antibodies in the antibody conjugates was adjusted to about three- to fivefold molar excess over the biochemically calculated cytochrome P-450 molecules in each fraction.

Three different control experiments were carried out: (*a*) Antibody conjugates were replaced by the same amount of rabbit or sheep control conjugates; (*b*) as a blocking test, the subcellular fraction was preincubated with 10–30-fold excess of anti-P-450 antibody, after which the antibody conjugates were applied; (*c*) antibody conjugates were preincubated with 10–30-fold excess of purified cytochrome P-450, and then unbound conjugates were incubated with the cell fractions.

In order to wash out unbound conjugates, the incubated solutions were layered onto a linear 0.45–1.25 M sucrose density gradient containing TKM buffer over a 1.65 M sucrose cushion containing the same buffer and were centrifuged at 100,000 *g* for 60 min (Hitachi RPS 40 rotor). Unbound conjugates banded at around the upper one-fourth of the linear sucrose gradient, while the membrane fractions were recovered either from the lower part of the sucrose density gradient or from the bottom of the tubes, depending on the kind and degree of labeling of the membranes. The membrane fractions were resuspended in TKM buffer and centrifuged at 10,000 *g* for 10 min. The thin pellets were fixed successively with glutaraldehyde and osmium tetroxide, dehydrated, and sectioned as described previously (29). The thin sections were observed under a Hitachi electron microscope, HU-12. The membranous structure of the pellets, even if such were fragmented, was well preserved throughout the procedures for electron microscopy, so that a systematic electron microscope survey from the top to the bottom of the pellets was easily performed.

RESULTS

Properties of Ferritin Antibody Conjugates

As shown in Fig. 1, the double agar diffusion test showed that a single precipitation line was formed between antibody conjugates and cytochrome P-450 as well as between the conjugates and sheep antiserum against rabbit Fab fragments of IgG.

To determine whether or not the antibody conjugate fraction through Bio-Gel A 1.5 M contained a polymer of IgG which might co-chromatograph with the conjugates, the fraction was examined by immunoelectrophoresis under the condition in which IgG had no electrophoretic mobility. The result (Fig. 2) demonstrated that the antibody conjugate fraction contained hardly any free IgG or polymers of IgG.

Table I shows that 19% of the IgG in the antibody conjugate fraction and 5% of the IgG in the control conjugate are adsorbed to the immunoadsorbent gel containing cytochrome P-450, respectively. This means that ~14% of IgG molecules in the conjugate fraction are monospecific to cytochrome P-450. A similar value was obtained by analysis of the unconjugated IgG fraction with the immunoadsorbent gel containing cytochrome P-450. These results show that antibody activity of the IgG as expressed by binding capacity to the immunoadsorbent gel was little affected by the conjugation. Chemical analyses of the monospecific conjugates eluted from the immunoadsorbent gel showed the molar ratio of IgG to ferritin to be 1.4.

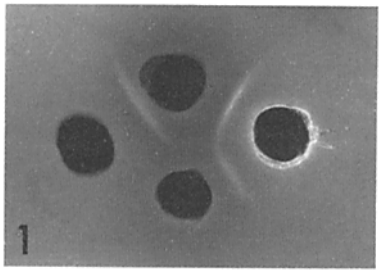


FIGURE 1 Ouchterlony double diffusion pattern of ferritin anti-P-450 antibody conjugates (upper well, 1.4 mg IgG/ml) against purified cytochrome P-450 (left well) and sheep antiserum against Fab fragment of rabbit IgG (right well). The lower well contains ferritin normal rabbit IgG conjugates (control conjugates, 1.4 mg/ml).

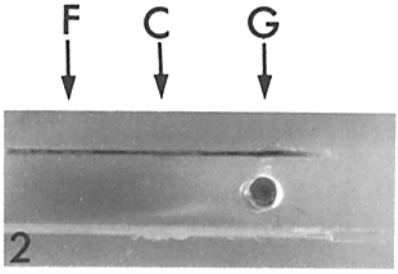


FIGURE 2 Immunoelectrophoresis of ferritin anti-P-450 antibody conjugates (center well, 1.4 mg IgG/ml). Upper and lower trough contains cytochrome P-450 and sheep antiserum against Fab fragment of rabbit IgG molecules, respectively. The location of free ferritin (*F*), ferritin antibody conjugates (*C*), and free IgG (*G*) are indicated by arrows. Immunoelectrophoresis was carried out as described by Kishida et al. (23).

TABLE I

Characterization of Ferritin Antibody Conjugates by Adsorption with an Immunoadsorbent Gel Containing Cytochrome P-450

	Ferritin antibody conjugates	Control Conjugates
	(%)	(%)
IgG in sample (*cpm*)*		
Initial	31,020	27,721
Washing	25,100 (81)	26,200 (95)
Adsorbed and eluted	5,920 (19)	1,521 (5)
Molar ratio IgG: ferritin adsorbed	1.4	

Ferritin antibody conjugates were adsorbed to an immunoadsorbent gel containing cytochrome P-450. After washing with 0.15 M and then with 1.0 M potassium chloride both in 0.01 M potassium phosphate buffer, pH 7.3, the specific antibody conjugates were eluted with 0.2% formic acid. The control conjugates were treated in the same manner. The amount of IgG in each sample was determined by radioactivity measurements, and the ferritin content was determined by spectrophotometry (25).

* IgG was previously labeled with [^{125}I]iodine as described in Materials and Methods.

P-450 Content of the Microsomes

Table II summarizes the content of cytochrome P-450 in the total microsomes and in the rough and smooth microsomes. On the protein basis, smooth microsomes contain more P-450 than do the rough microsomes, and phenobarbital treatment induced a marked increase in the P-450 content, as has also been reported by other workers (7, 10, 17, 37, 42).

Microsome Fraction

Fig. 3 shows representative electron micrographs of the total microsomal fraction after incubation for 30 min at 4°C with antibody conjugates (*A*) and with control conjugates (*B*), respectively. In Fig. 3*A*, the outer surfaces of most of the microsomal vesicles are studded with a number of ferritin particles, while in Fig. 3*B* only a few ferritin particles can be seen, thus clearly indicating the specificity of the present immunoelectron

TABLE II

Cytochrome P-450 Content of Liver Microsomes from a Control and Phenobarbital-Treated Rat

Total microsomes prepared by simultaneous method of preparation of various subcellular fractions	
	nmol/mg microsomal protein
Control	0.62 ± 0.05 (n = 6)
Phenobarbital treated*	1.81 ± 0.09 (n = 3)
Rough and smooth microsomes prepared by discontinuous sucrose density gradient centrifugation	
	nmol/mg microsomal protein
Control	
Smooth microsomes	1.02 ± 0.16 (n = 4)
Rough microsomes	0.45 ± 0.07 (n = 4)
Phenobarbital treated*	
Smooth microsomes	2.55 ± 0.38 (n = 3)
Rough microsomes	0.57 ± 0.05 (n = 3)

* Phenobarbital treatment was for 4 days.

microscope observation. Not only smooth microsomes but also rough ones are heavily labeled, except for those regions occupied by ribosomes. The particles were found exclusively on the outer or cytoplasmic surface, and no inner or luminal surface of the microsomes was labeled.

Labeled microsomes sometimes contained electron-opaque bodies ~50 nm in diameter within the vesicles as shown Fig. 3*A*, and some of these microsomes were studded with ribosomes (black arrows). This means that the microsomes are derived from rough ER and that the electron-opaque bodies are intracisternal particles, probably lipoprotein as reported by several authors (6, 14, 20). Sometimes, ferritinlike particles were found in these lipoprotein particles (white arrows). They probably do not represent the ferritin antibody conjugates, because the particles existed not on the surface but within the lipoprotein particles and were slightly smaller in size and more irregular in shape than the usual ferritin particles. Similar ferritinlike particles are also found in the control microsomal vesicles which were not incubated with the antibody conjugates.

Fig. 3*A* also shows that the number of the ferritin particles on each microsome is variable. Thus, some vesicles were labeled with a number of particles, while in others only a few particles were involved. Another interesting finding is that, on each microsomal vesicle, ferritin particles were usually distributed not uniformly on the microsomal surface but heterogeneously, forming clusters or patches. An *inset* to Fig. 3*A* indicates a typical patchy distribution of the ferritin particles (arrows). Occasionally, the clusters are so large that the vesicles are divided into two regions with and without ferritin particles. Heterogeneous distribution of the particles was also observed on grazing sections of the microsomes (Fig. 3*A*, arrowheads), and was independent of the time of incubation at 4°C. A similar heterogeneous distribution was observed after incubation for either 30 min, 5 h, or longer.

Phenobarbital treatment for 4 days resulted in a marked increase in the number of the particles in each microsomal vesicle as shown in Fig. 4. Thus, the region of the microsomal membrane labeled with ferritin particles increased while the particle-free region decreased, and some microsomal vesicles appear to be saturated with the particles. Heterogeneous distribution of the particles is not apparent on these microsomes. Detailed analyses of the distribution of cytochrome P-450 in microsomes from phenobarbital-treated rats will be published elsewhere.[2]

In order to exclude the possibility that incubation of microsomes with the antibody conjugates induced the heterogeneous distribution of the cytochrome P-450 molecules, liver microsomes were first fixed with 0.25% glutaraldehyde for 5–10 min at 4°C, and centrifuged for 2 min at 3,000 rpm at 4°C. The pellets were resuspended in TKM buffer containing 0.1 M glycine and then incubated with the antibody conjugates as before. Although the number of ferritin particles bound to the microsomal membranes decreased considerably after fixation, heterogeneous distribution of the ferritin particles was clearly observed as shown in Fig. 5 (see Fig. 3*A*).

Since loss of the antigenicity of cytochrome P-450 molecules by glutaraldehyde fixation is probably a random process, this result may indicate heterogeneous distribution of P-450 molecules on the microsomes.

Rough and Smooth Microsome Fractions

To compare more in detail the labeling of rough and smooth microsomes, both microsomes were prepared by discontinuous sucrose density gradient centrifugation as described in Materials and Methods, and incubated with ferritin antibody conjugates. As shown in Fig. 6*A*, almost all rough microsomes are labeled with ferritin particles, and the microsomes without ferritin particles were only 1–2% of the total. Heterogeneous distribu-

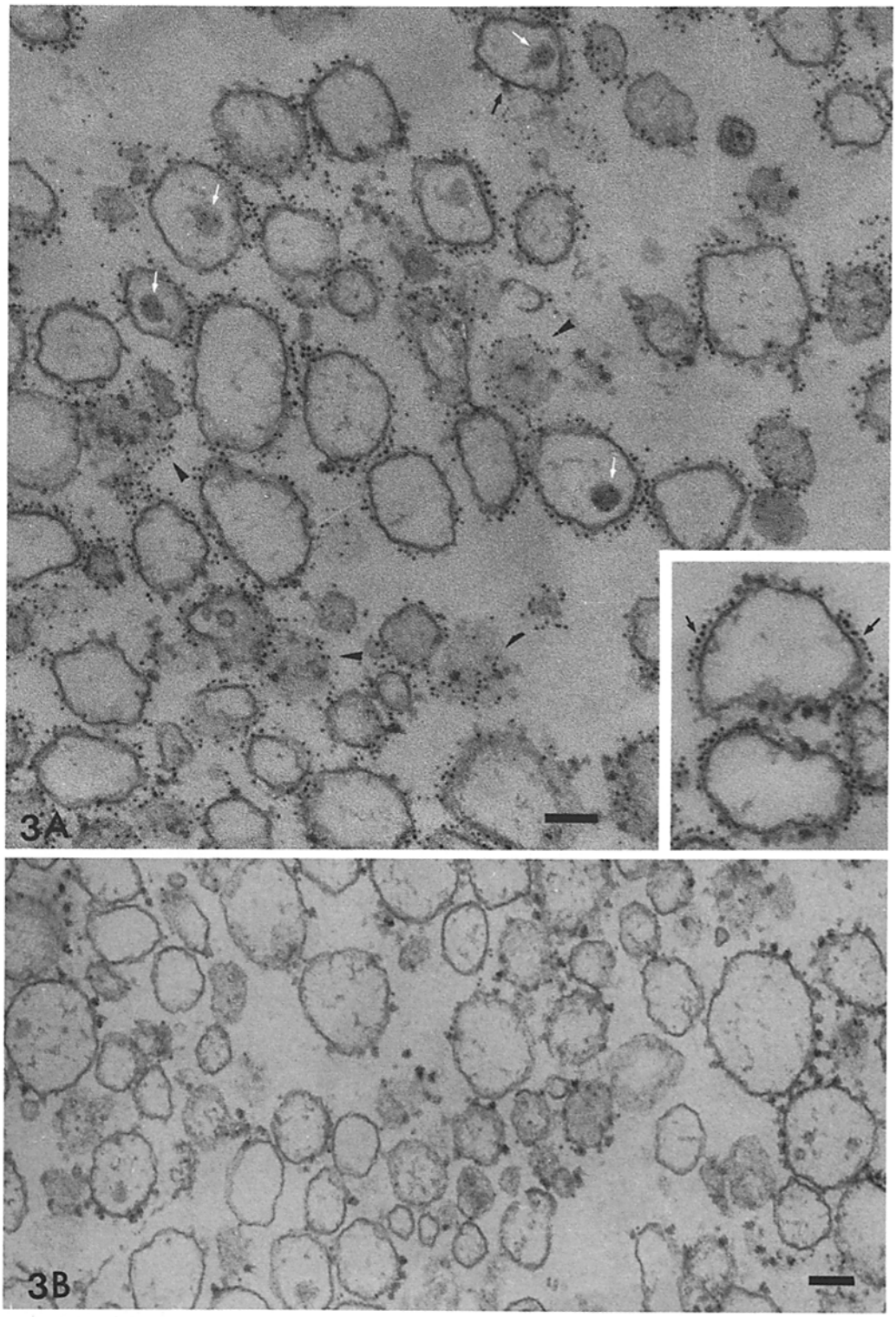


FIGURE 3 Rat liver microsomes incubated with ferritin anti-P-450 antibody conjugates (*A*) and with control conjugates (*B*) at 4°C, respectively. Black arrows in Fig. 3*A* indicate rough microsomes containing intracisternal particles, probably lipoprotein; white arrows indicate ferritinlike particles in the intracisternal particles, and arrowheads indicate grazing section of microsomes. *Inset* to Fig. 3*A* shows typical patchy distribution of ferritin particles on rough microsomes. (*A*) × 78,000; (*B*) × 65,000. Bar, 0.1 μm.

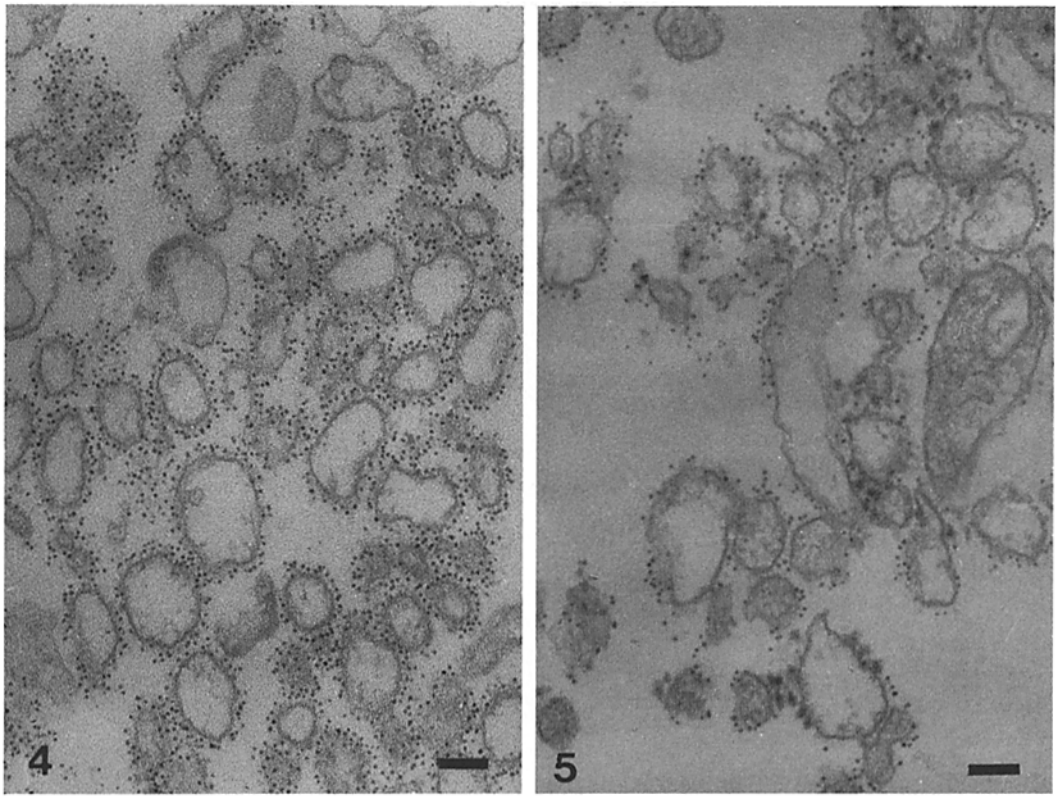


FIGURE 4 Marked increase in labeling of liver microsomes isolated from phenobarbital-treated rat. The rat was treated with the drug for 4 days as described in Materials and Methods. × 65,000.

FIGURE 5 Microsomes were fixed for 5–10 min at 4°C with 0.25% glutaraldehyde and then incubated with the antibody conjugates. Heterogeneous distribution of ferritin particles as shown in Fig. 3*A* is clearly observed. × 65,000.

tion of the particles on the outer surface of the microsomes is also apparent, and hardly any ferritin particles were found in regions of the microsomal vesicles loaded with ribosomes. This is clearly shown in the grazing sections of the rough microsomes (Fig. 6*A*, arrowheads).

Labeling of the outer surface of the smooth microsomes was even more impressive (Fig. 6*B*). Heterogeneous distribution of ferritin particles on the smooth microsomal vesicles is again evident. Some of the vesicles (arrows) contained lipoprotein particles as shown in Fig. 3*A*. The number of the smooth microsomal vesicles not labeled with ferritin particles (stars) was 5–7% of the total. The rough and smooth microsome fractions are contaminated with a small number of the other cytoplasmic organelles such as mitochondria and the membrane sheet structures which are presumably derived from plasma membrane. These structures which can be discriminated morphologically were excluded from the calculation.

Nuclear Membranes

As shown in Fig. 7 A, the outer envelopes of nuclear membranes were always heavily labeled with ferritin particles except for those regions occupied by ribosomes (arrowhead). This is in marked contrast with the control micrograph (Fig. 7*B*) where nuclei were incubated with the control conjugates. Some ferritin particles were found in the nuclear matrix (Fig. 7*A*, arrows). A comparable number of particles was also noted in the matrix of the control experiment (Fig. 7*B*, arrows), indicating that such particles were trapped within the nuclei during incubation and were not removed by the washing procedure. The inner nuclear envelopes were not labeled as shown in Fig. 7*A*. It is not certain whether this negative

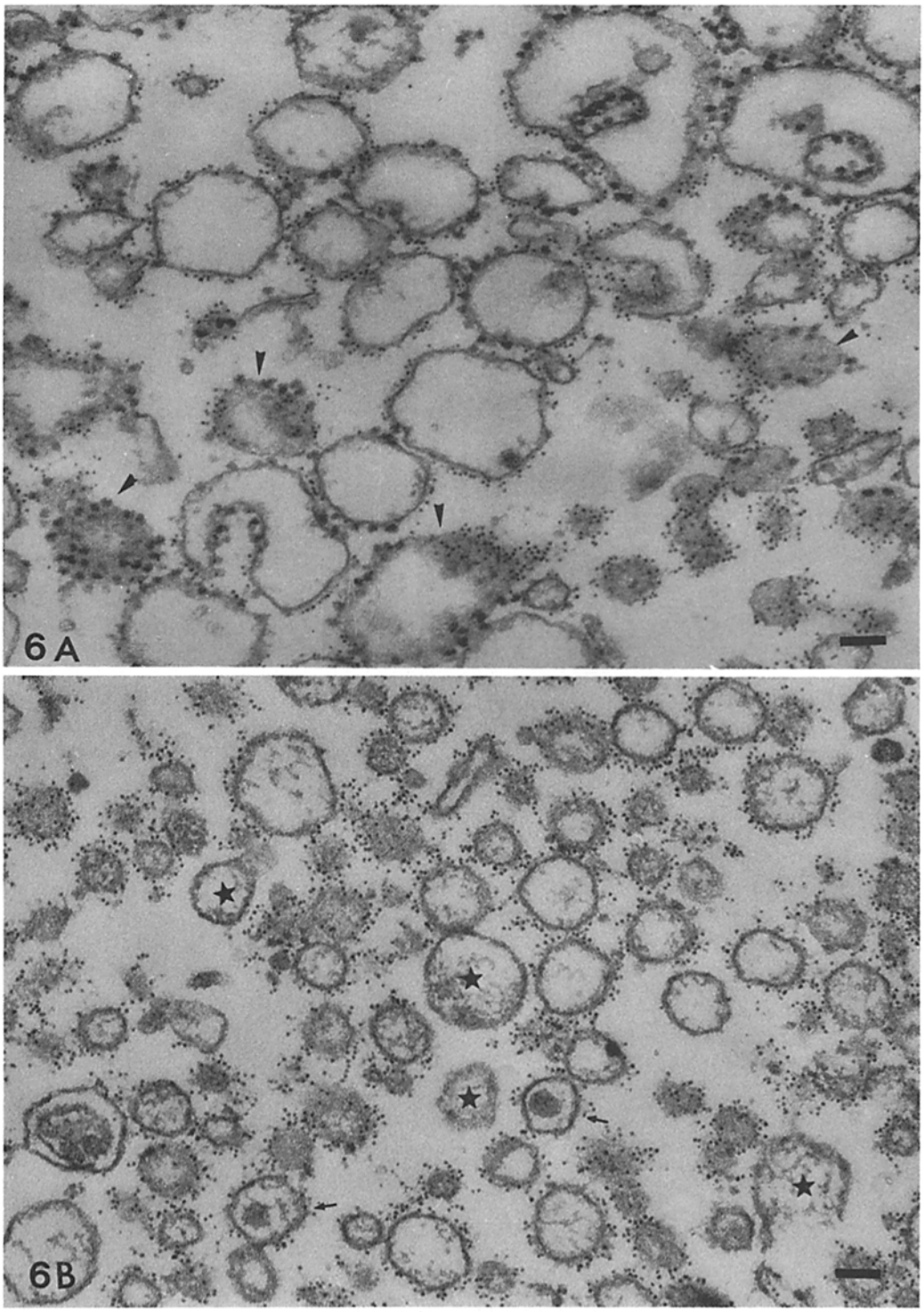


FIGURE 6 Fig. 6*A* and *B* are rat liver rough and smooth microsome fractions, respectively, which were incubated with the antibody conjugates. Arrowheads in Fig. 6*A* show grazing profiles of rough microsomes. Note that the regions studded with ribosomes are not labeled with ferritin. Arrows in Fig. 6*B* show microsomes containing intracisternal lipoprotein particles, while stars indicate microsomal vesicles which are not labeled with ferritin particles. × 65,000. Bar, 0.1 μm.

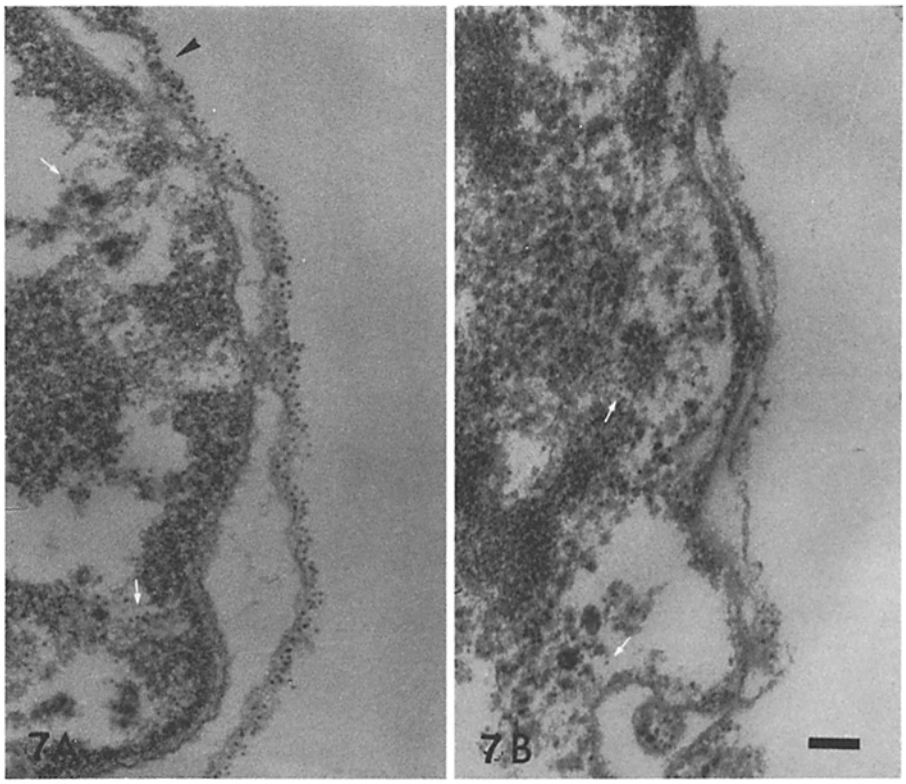


FIGURE 7 Fig. 7*A* and *B* show rat liver nuclear fractions incubated with the antibody conjugates and with control conjugates, respectively. Arrowhead shows ribosomes attached to the outer nuclear envelope, while white arrows indicate ferritin particles trapped within the nucleus. In Fig. 7*A*, the outer nuclear envelope was sectioned slightly tangentially so that numerous ferritin particles could be observed. × 65,000. Bar, 0.1 μm.

result is due to the difficulty of access of the conjugates to the inner nuclear envelope or to steric hindrance by the presence of condensed chromatins.[3]

Golgi Fraction

Fig. 8*A* and *B* show representative sections of the Golgi fraction isolated by the procedure of Hino et al. (15) and incubated with the antibody conjugates (*A*) and with control conjugates (*B*), respectively. As reported by these authors, the structural organization of Golgi complexes is well preserved, showing the forming (cis) and the maturing (trans) face of the complexes. In our experiment, effort was made to preserve the structural integrity of the complexes at the sacrifice of the purity of the fraction. The Golgi fraction was, therefore, contaminated with other organelles such as microsomal vesicles, and condensing vesicles or secretory granules (small arrows) loaded with very low density lipoproteins (VLDL).

Fig. 8*A* shows only a few ferritin particles on the two Golgi complexes, one sectioned vertically and the other tangentially (large arrow). The degree of labeling of the complexes, however, was comparable to that of the control (Fig. 8*B*), and no special labeling was observed on the membrane of the outermost saccules of the Golgi complexes. This finding was repeatedly confirmed by observing a number of Golgi complexes.

The vesicles heavily labeled with ferritin particles (Fig. 8*A*, arrowheads) are most probably microsomal vesicles, since some are studded with ribosomes. They serve as an internal marker for the specificity of the present immunocytochemical reaction. Only a few ferritin particles were noted on the surface of secretory granules loaded with VLDL's (small arrows), and the labeling was

[3] Recently, we found that the inner nuclear envelopes are not labeled with the anti-P-450 antibody conjugates.

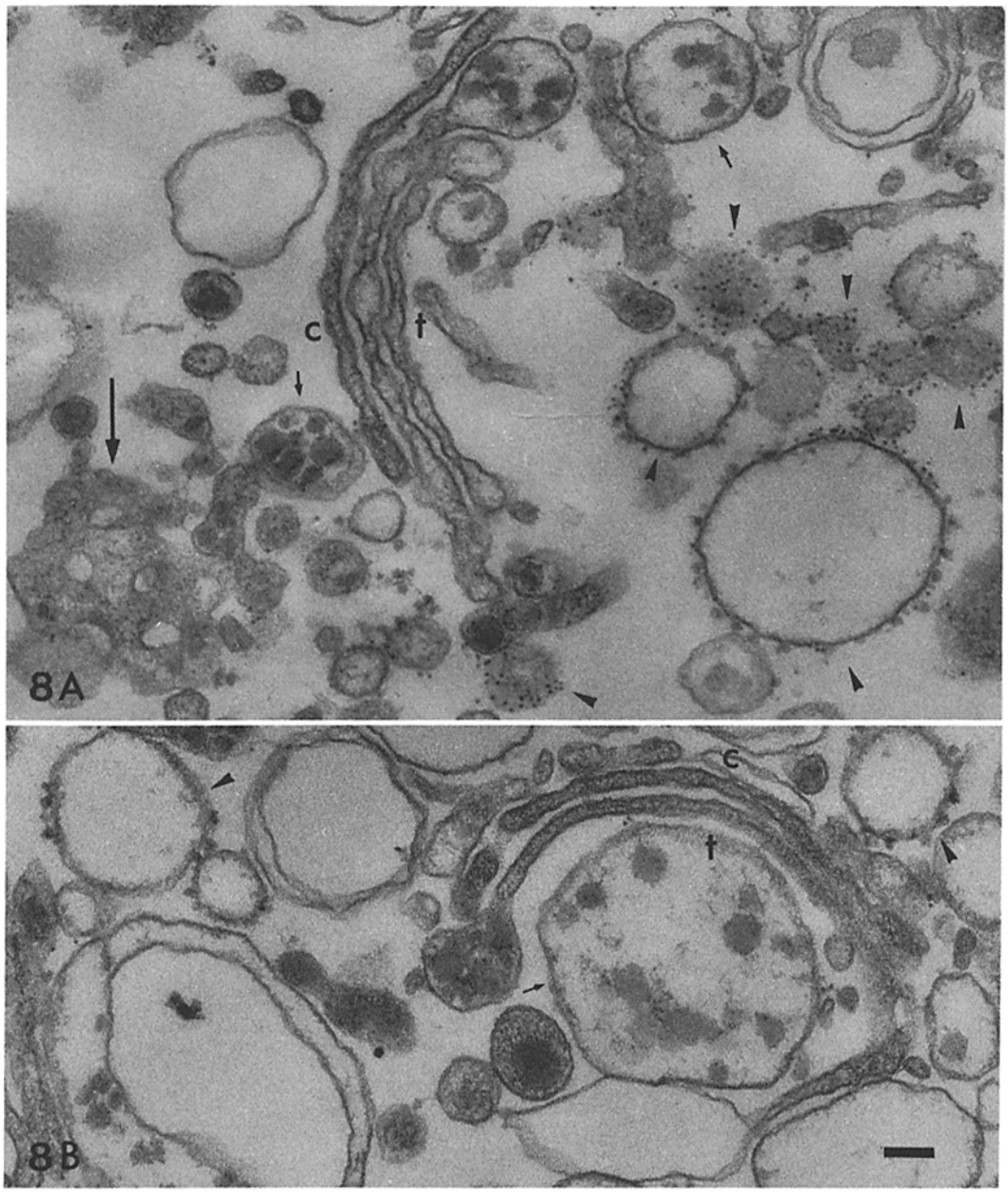
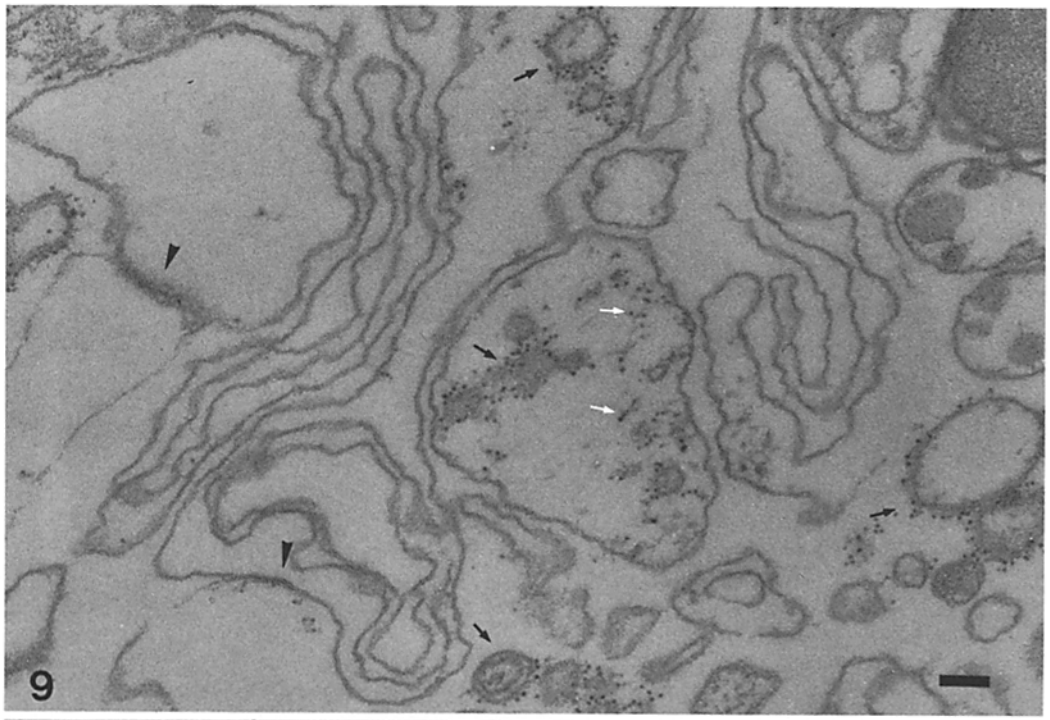


FIGURE 8 Fig. 8*A* and *B* are rat liver Golgi fractions incubated with the antibody conjugates and control conjugates, respectively. *C*: cis or forming face of the Golgi complex; *t*: trans or maturing face of the Golgi complex. Arrowheads show microsomal vesicles, and small arrows show the secretory granules loaded with VLDL particles. Large arrow in Fig. 8*A* shows grazing section of a Golgi complex. × 67,000. Bar, 0.1 μm.

comparable to that of the same structures in the control experiments.

Plasma Membrane Fraction

Fig. 9 shows the plasma membrane fraction incubated with the antibody conjugates. Some plasma membranes are easily identified by their characteristic sheet profile and by the presence of junctional elements and/or bile canaliculi (arrowheads). There were no ferritin particles on such



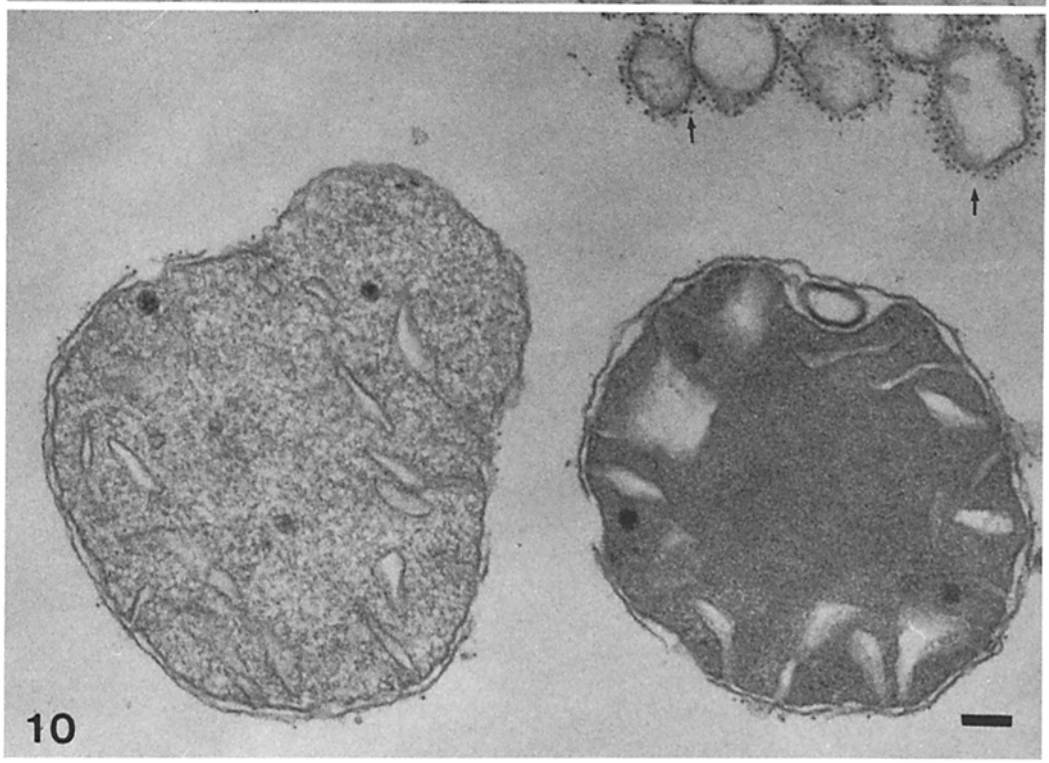


FIGURE 9 Rat liver plasma membrane fraction incubated with the antibody conjugates. Plasma membrane was identified by characteristic sheets or large vesicle profiles and by the presence of junctional elements (arrowheads) and/or bile canaliculi. Note presence of a number of ferritin particles on contaminating microsomal vesicles (black arrows). Sometimes ferritin particles were also noted on the actinlike filaments (white arrows) which existed enclosed within the large vesicle of the plasma membrane. The latter particles are probably due to nonspecific adsorption of the conjugates to the actinlike filament (see text). × 65,000. Bar, 0.1 μm.

FIGURE 10 Rat liver mitochondria incubated with the antibody conjugates. In this experiment, rats were treated with phenobarbital for 4 days, and the mitochondrial fraction was fixed previously with 0.25% glutaraldehyde. Note marked labeling on microsomal vesicles (arrows). × 65,000. Bar, 0.1 μm.

plasma membranes, while microsomal vesicles which existed as contaminants in the plasma membrane fraction were heavily labeled with the particles (black arrows).

It is noted here that some ferritin particles were adsorbed to the actinlike filaments occasionally found in the space surrounded or enclosed by the plasma membrane (white arrows). As similar labeling of the same filaments was also found in the control experiment where control conjugates were incubated with plasma membrane fraction, these ferritin particles are probably due to nonspecific adsorption of the conjugates to the actinlike filaments.

Mitochondrial Fraction

Fig. 10 shows a representative electron micrograph of the mitochondrial fraction which was incubated with the antibody conjugates. A few ferritin particles were noted on the outer mitochondrial membrane. A similar degree of labeling of the outer membrane was also observed when the same fraction was incubated with the control conjugates.

The mitochondrial fraction prepared by the simultaneous method was considerably contaminated with microsomal vesicles as is evident by the marked labeling with ferritin particles (Fig. 10, arrows). In this preparation, a rat had been treated with phenobarbital for 4 days.

DISCUSSION

In the present experiment, we were most interested in the distribution of cytochrome P-450 in the organelles and the mutual relationships *in situ*. For this purpose, the best way may be to localize antigens on the thin sections of the cells. So far, experimental approaches using frozen thin sections or thin sections of the cells embedded in plastic or protein have not been successful.

Then we applied the procedures used by Olsen and Prockop (34). Liver cells prefixed with 1% formaldehyde were homogenized with a Teflon glass homogenizer, and the cell fragments sedimented by centrifugation at 20,000 *g* for 10 min were resuspended and incubated with ferritin antibody conjugates. This approach has also not been successful.

We have, therefore, used cell fractionation techniques to obtain the cell organelles. As mentioned above, our primary interest was to investigate the distribution of P-450 molecules in hepatic cells *in situ*. Therefore we made efforts to preserve the original structural integrity of the cell organelles as much as possible. For the preparation of Golgi bodies, for example, we used Hino's procedure which does allow for preservation of the integrity of Golgi bodies at the sacrifice of purity.

Biochemical studies of liver cells have shown that cytochrome P-450 is exclusively localized in rough and smooth microsomes (10, 17). This conclusion is supported by the present immunoelectron microscope observation that most of the microsomal vesicles were labeled with the ferritin-antibody conjugates.

Administration of phenobarbital caused a marked increase in the number of ferritin particles on microsomes as is shown in Fig. 4. This observation is consistent with the previous reports that phenobarbital provokes not only an increase in liver weight and, at the subcellular level, a proliferation of smooth ER but also an increase in the amount of cytochrome P-450 per milligram of microsomal protein (37, 42).

The existence of cytochrome P-450 in other fractions such as nuclear membrane (11, 21), outer mitochondrial membrane (5, 46, 49), and Golgi complex (3, 10, 17) remains controversial. The amount of P-450 found in these fractions is usually small, and it is not clear whether P-450 activity actually exists in these fractions or is due to contamination of these fractions by microsomal membranes. The present immunoelectron microscope approach makes feasible visualization and comparison of the distribution of P-450 molecules in these organelles. Since tissue from a single rat was used to prepare all the subcellular fractions, differences due to individual variations among animals were avoided.

It was evident that the outer nuclear envelopes can be labeled as heavily as microsomal vesicles with the ferritin-antibody conjugates. This finding is in agreement with the recent biochemical data from T. Omura's laboratory[4] that the amount of cytochrome P-450 per microgram phospholipid P in nuclear envelopes in approx. one-third that found in microsomes and increases with phenobarbital treatment.

We also found that cytochrome P-450 can hardly be detected either on the Golgi complex or on the plasma membrane. This is consistent with the biochemical studies of Bergeron et al. (3) on the Golgi complex, and of Ichikawa et al. (17)

[4] Sagara, Y., T. Harano, and T. Omura. 1978. *J. Biochem.* **83**:807–812.

and Fleischer et al. (10) on the plasma membrane, respectively.

It has been reported that a cytochrome P-450 exists on the outer mitochondrial membrane (49). The labeling of the outer surface of the mitochondrial membrane with the ferritin-antibody conjugates was, however, very slight and at the control level. This means that at least the phenobarbital-inducible cytochrome P-450 molecules do not exist in any detectable amount on the outer mitochondrial membranes.

It is generally accepted that the main component of the microsomal fraction of rat liver is composed of pieces of the ER, which fragments upon homogenization into self-sealing small vesicles (39). It was observed, however, that 1–2% and 5–7% of the microsomal vesicles in the rough and the smooth microsomal fractions, respectively, are not labeled with ferritin. This result is to be expected. Since the enzyme was shown to be present in groups of molecules, some negative sections would exist. In addition, ER membrane is the major, but not the sole constituent of the microsomal vesicles thus prepared.

According to Wibo et al. (51) and Beaufay et al. (2), rat liver microsomes prepared by their procedures contain, on a protein basis, 7–8% of plasma membrane, 6% of mitochondria, 4–5% of Golgi complex, 3% of external mitochondrial membrane, 1% of lysosomes, and 1% of peroxisomes. Thus, microsomal elements derived from ER account for no more than 77% of all the microsomal protein.

In our calculation, cytoplasmic organelles such as mitochondria, Golgi complex, and plasma membrane in a long sheet or in large vesicle structures, all of which are easily identified by electron microscope observation of microsomal fraction, were excluded from the count. This would explain why in our experiment the microsomal vesicles not labeled with ferritin accounted for only 1–2% in the rough and 5–7% the smooth microsomal fractions, respectively. Slight differences in the degrees of labeling of the two fractions suggest that most of the smooth microsomes that are not labeled with ferritin particles are a fragmented and vesiculated form of plasma membrane, Golgi complex, and/or external mitochondrial membrane.

We conclude, therefore, that almost all of the rough and smooth microsomes derived from ER membrane contain cytochrome P-450 molecules and that the enzymes are widely distributed on ER membrane.

The present observation also suggests that at least one or some of the antigenic sites of the cytochrome are exposed to the outer or cytoplasmic surface of microsomal vesicles. Welton and Aust (50) reported that cytochrome P-450 molecules in microsomes are labeled with ^{125}I by lactoperoxidase procedures, and we found the demethylating activity of benzphetamine by rat liver microsomes to be markedly inhibited (more than 80%) by incubation with anti-P-450 antiserum.[1]

It is not clear, however, whether some of the antigenic sites of the cytochrome are also exposed to the inner or luminal surface of the microsome vesicles (8). Permeability of the liver microsomal membrane has been studied extensively by several investigators (8, 19, 32), and it is apparent that the ferritin antibody conjugates are too large to penetrate the microsomal membranes.

A heterogeneous distribution of cytochrome P-450 molecules was noted on the microsomal membrane. It could be argued that the clustering may be formed artificially by lateral diffusion and artificial aggregation of membrane proteins during the immunocytochemical procedures, because the immunoreaction was carried out with ferritin-antibody conjugates containing divalent antibodies. To avoid such artifacts, three methods appear to be applicable: (*a*) One is to use monovalent conjugates such as ferritin-Fab conjugates, (*b*) the second is to incubate cell organelles with antibody conjugates at low temperature, and (*c*) the third is to immobilize membrane proteins by mild prefixation.

(*a*) The first method may be ideal, if good monovalent conjugates are available. Preparation of ferritin-Fab conjugates has been described by several authors. Kraehenbuhl and Jamieson (24) reported solid phase conjugation of ferritin to Fab, and the molar ratio of Fab to ferritin in their conjugates was up to 3. Kishida et al. (23) reported conjugation of Fab with ferritin after purification by immunoadsorbent gel, and the molar ratio was 1.8. Abbas et al. (1) purified ferritin-Fab conjugates by preparative block electronphoresis using Pevikon (Mercer Consolidated Corp., Yonkers, N. Y.) as the supporting medium. The molar ratio was 2.0–1.0. All these data clearly indicate that the preparations contain polyvalent conjugates such as ferritin + 2 Fab or ferritin + 3, which could induce aggregation of protein molecules on membrane, even though Fab was used for the preparation of the conjugates. Therefore, we have to isolate monovalent ferritin Fab conjugates (ferritin + 1 Fab) by gel filtration or by

some other technique. We felt that the isolation of the monovalent conjugates is most difficult, if not impossible, because the difference in mol wt between monovalent ferritin Fab conjugates (mol wt, ~600,000 + 25,000), free ferritin (mol wt, ~600,000) and polyvalent ferritin Fab conjugates (mol wt, ~600,000 + 25,000 × n, n = 2, 3, 4, etc.) is so little that isolation of monovalent conjugates by gel filtration is not successful. Thus, the situation is quite different from fluorescence antibody techniques where preparation of monovalent conjugates can be readily facilitated.

In this paper, we have applied the direct method using ferritin IgG conjugates with a molar ratio of IgG ferritin of 1.4. In addition to a better resolution in immunoelectron microsocopy, the direct method is free from complications due to multiple binding of ferritin Fab conjugates in the second step of the labeling procedure. It is also to be pointed out that a considerable number of the ferritin IgG conjugates that we have used behave like monovalent antibodies, probably because of steric hindrance due to the large size of ferritin molecules as described in the previous paper from this laboratory (29).

(*b*) We employed incubation with the ferritin antibody conjugates at a low temperature (~4°C) for a short time (30 min). It has been reported that a thermotropic phase change in the microsomal membranes occurs within the 19°-22°C range (9, 41, 48) and that lateral diffusion of membrane-bound monoribosomes and intramembrane particles of microsomes does not occur at 4°C (33). In our experiment, heterogeneous distribution of ferritin particles was observed by the incubation of microsomes with the antibody conjugates at 4°C for as short as 30 min and remained unchanged at this temperature for several hours or longer.

(*c*) We have also used prefixation with 0.25% glutaraldehyde, expecting to immobilize membrane proteins, because aldehyde fixation has been reported to immobilize surface antigens on the cell membrane, thus preventing any subsequent movement or redistribution of the antigens (1). Even after the prefixation, we could demonstrate a similar patchy distribution of cytochrome P-450.

One interpretation may be that the heterogeneous distribution was induced by aldehyde fixation, because glutaraldehyde could aggregate membrane protein. Indeed, Steck (43) has reported preferential cross-linking of some of the membrane polypeptides by fixation with glutaraldehyde. His interpretation was, however, that some of the membrane polypeptides exist not in a random array but in a specific oligomeric association and that glutaraldehyde cross-linked these membrane polypeptides. We are of the opinion that glutaraldehyde can cross-link randomly neighboring molecules but cannot induce specific aggregation of certain molecules, for example, P-450 molecules, on membrane.

We favor the interpretation, therefore, that heterogeneous clustering of cytochrome P-450 molecules is not due to artificial aggregation of the immunoreaction products but rather to the existence of such microclusters in vivo on rough and smooth ER membranes.

The existence of such clusters has also been suggested from kinetic studies of the enzyme (12, 40) and from sedimentation (45) and immunochemical analyses (22) of sonicated microsomes. The possibility of artificial aggregation induced by multivalent ligands, however, should not be excluded, because microclustering of cytochrome P-450 on the microsomal membrane would require diffusion of less than 1 μm.

Since NADPH-cytochrome *c* reductase is suggested to be distributed heterogeneously within microsomes in groups of three–five molecules (29), and since the number of cytochrome P-450 molecules is 10–20-fold greater than the number of reductase molecules (12, 40), microsomal multi-enzyme oxygenase systems presumably exist in clusters or in patches, each of which is composed of three–five molecules of NADPH-cytochrome reductase and 30–100 molecules of cytochrome P-450. Such clustering may facilitate efficient electron transport for the hydroxylation reaction on ER membranes. A more quantitative analysis of such clusters will be published elsewhere.[2]

Another possible significance of such microheterogeneity on ER membrane is for membrane flow. It has been generally accepted that the pathway followed by the secretory proteins leads from rough ER to the transitional elements of this system, then to the small peripheral vesicles on the cis side of the Golgi complex and finally either to condensing vacuoles or to Golgi stacks. In order to make such a transport process possible, ER membrane should flow from the ER to the Golgi complex, simultaneously maintaining the specific enzymatic and polypeptide composition of the Golgi membrane by some mechanisms (38).

One possibility is that the ER membrane incorporated into Golgi complex is selectively degraded enzymatically or selectively eliminated from Golgi membrane by forming small vesicles to be transported back to ER membrane as suggested by Palade (38).

This type of mechanism suggests that at least the outermost saccules of the Golgi complex, which are assumed to be formed by fusion of the small vesicles, would be heavily labeled with ferritin particles. No such labeling was detected on the Golgi complex. This finding and the heterogeneous distribution of cytochrome P-450 and NADPH-cytochrome *c* reductase within the microsomal vesicle suggest the following alternative possibility. On the ER membrane facing the Golgi complexes, special regions appear which have a chemical composition similar to that of the Golgi membrane but are lacking in the microsomal mixed function oxygenase system. These regions can form small vesicles by a budding mechanism, and secretory proteins are, by some unknown mechanism, collected and enclosed therein for intracellular transport. This hypothesis of the preassembling of Golgi membrane on a special region of ER membrane could avoid the difficulties imposed on the conception of intracellular membrane flow.

We thank M. Ohara and K. Miki for assistance with the manuscript.

This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by a grant from the Naito Research Fund.

Received for publication 15 June 1977, and in revised form 28 March 1978.

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