Distribution and Induction of Cytochrome P-450 in Rat Liver Nuclear Envelope

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ABSTRACT Induction of cytochrome P-450s by 3-methylcholanthrene (MC) and phenobarbital (PB) and distribution of P-450s in the rat liver nuclear envelope were investigated by biochemical analyses and ferritin immunoelectron microscopy using specific antibodies against the major molecular species of MC- and PB-induced cytochrome P-450. It was found, in agreement with Kasper (*J. Biol. Chem.*, 1971, 246: 577–581), that the total amount of cytochrome P-450s determined by biochemical analysis was markedly increased by MC, but not by PB, treatment. Immunoelectron microscopic analysis, however, showed marked and slight increases in ferritin labeling by MC and PB treatment, respectively. The latter finding was interpreted as resulting from the induction of a particular molecular species of PB-induced cytochrome P-450s. Ferritin immunoelectron microscopic analysis of intact isolated nuclei, naked nuclei from which the outer membrane of the nuclear envelope was partially detached (mechanically), and isolated nuclear envelopes have shown that the ferritin particles are found exclusively on the cytoplasmic face of the outer nuclear envelopes. Neither the nucleoplasmic face of the inner membrane of the nuclear envelope nor the cisternal face of both membranes of the nuclear envelope showed any labeling with ferritin. This indicates that cytochrome P-450 is located only on the outer membrane of the nuclear envelope and does not diffuse laterally into the domain of the inner membrane of the nuclear envelope across the nuclear pores. Our results suggest that a marked heterogeneity exists in the enzyme distribution between the outer and inner membrane of the nuclear envelope and that microsomal marker enzymes such as cytochrome P-450 exist exclusively in the outer membrane. In addition, it appears that cytochrome P-450 is probably not a transmembrane protein but an intrinsic protein located on the cytoplasmic face of the outer membrane of the nuclear envelope.

The nuclear envelope, which is composed of an inner and an outer membrane, forms the limiting barrier between the nucleoplasm and cytoplasm in all eucaryotic cells and occupies a strategic position in the control and regulation of cellular metabolism. The cytoplasmic surface of the outer leaflet of the nuclear envelope is studded with ribosomes and is sometimes continuous with the endoplasmic reticulum (29, 30). In addition to these structural similarities, the presence of a number of enzymes common in the nuclear envelope and endoplasmic reticulum has been reported (5, 6, 9, 31). For example, hepatic microsomal marker enzymes such as NADPH-cytochrome *c* reductase and cytochrome P-450 are also present in the nuclear envelope (4, 13–15, 25). Thus it has been claimed that these two membrane structures cannot be distinguished from one another and that they are practically identical (5).

It is possible, however, to point out several differences between the two membrane structures. In general, the specific enzymatic activities of the nuclear envelope are lower than those of the endoplasmic reticulum (9, 13, 25, 31). This is true for cytochrome P-450; the specific content of hepatic nuclear envelope was about one-half or one-third that of microsomes (25). Moreover, it has been reported by Kasper and co-workers (4, 14) that the induction of cytochrome P-450 by phenobarbital (PB) and by 3-methylcholanthrene (MC) are different in the two membranes: MC is an effective inducer for both the microsomal and the nuclear envelope P-450, whereas PB is

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effective only for the former. As to the induction experiment, however, contradictory results have been reported by several authors (24, 25), i.e., that PB treatment increased NADPH-cytochrome *c* reductase and cytochrome P-450 content of nuclei or the nuclear envelope.

Because the mass of nuclear envelope in the cell is only ~1% of the total mass of microsomal membrane protein (4), the possibility for serious contamination should always be considered. Recently, we have developed a quantitative immunoelectron microscopic technique and applied it successfully to the analysis of the distribution of cytochrome P-450 molecules on rat liver microsomes (19, 20). In addition to a biochemical approach, therefore, we used this technique in the hope of obtaining more direct information on the distribution and induction of cytochrome P-450 molecules in the nuclear envelope.

Biochemical determination indicated that nuclear cytochrome P-450 increases markedly in rat liver by treatment with MC but not with PB, whereas ferritin immunoelectron microscopic analysis showed marked and slight increase in ferritin labeling of the nuclear envelope by MC and PB treatment, respectively.

The ferritin immunoelectron microscopic analysis showed clearly that only the cytoplasmic surface of the outer membrane of the nuclear envelope is labeled with ferritin. Ferritin particles were hardly detected either on the cisternal surface of the outer membrane or on either surface of the inner membrane. Our findings are discussed in relation to the characteristic distribution of cytochrome P-450 on the two leaflets of the nuclear envelope and to the disposition of the enzyme within the outer membrane.

MATERIALS AND METHODS

Treatment of Animals

Male Sprague-Dawley rats, weighing ~150 g, were used in all experiments and were fed ad lib. with laboratory chow. They received an intraperitoneal injection of PB (100 mg/kg body wt, 100 mg/ml saline), MC (25 mg/kg body wt, 5 mg/ml corn oil), or β-naphthoflavone (β-NF; 80 mg/kg body wt, 8 mg/ml corn oil) daily for 4 d, or polychlorinated biphenyls (PCB; 25 mg/ml once), and were fasted overnight before sacrifice.

Purification of Cytochrome P-450s and Preparation of Anticytochrome P-450 Antibodies

Cytochrome P-450s were purified as described by Masuda-Mikawa et al. (18) from liver microsomes of PB- or MC-treated rats, respectively. They are the major components of the hepatic microsomal cytochrome P-450s from PB- and MC-treated rats, and their molecular weights, estimated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), were 50,000 and 56,500, respectively (18). The latter is conventionally called cytochrome P-448. These molecular species of cytochrome P-450 will be indicated by PB-P-450 and MC-P-450 in this paper. Antisera against the purified PB-P-450 and MC-P-450 were prepared in male white rabbits as follows. Each animal received two subcutaneous injections of 1 mg each of purified enzymes mixed with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) at an interval of 1 wk. 1 wk after the second injection, 1 mg of purified enzymes was injected intraveously. 7 d later, the serum was obtained and the antibodies were prepared by ammonium sulfate fractionation followed by DEAE-cellulose column chromatography. The specificity of the antibodies has been described previously (18). Control IgG was also prepared from nonimmunized rabbit serum. Ouchterlony double diffusion analyses were carried out in 1% agarose containing 100 mM potassium phosphate buffer (pH 7.5).

Preparation of Nuclei and Nuclear Envelopes

Nuclei were prepared from rat liver by the procedure of Blobel and Potter (2), and nuclear envelopes were isolated by the procedure of Dwyer and Blobel (3).

Chemical and Enzyme Assays

Cytochrome P-450 was determined by the method of Omura and Sato (23), and phospholipid was extracted by the method of Schneider (26) and determined by the method of Lees (17). Drug-metabolizing activities of nuclear envelopes, such as 7-ethoxycoumarin *O*-deethylation, benzo(a)pyrene hydroxylation, and benzphetamine *N*-demethylation, were assayed as described by Ullrich and Weber (28), Nebert and Gelboin (22), Imai et al. (12), and Nash (21), respectively.

Preparation of Ferritin-Antibody Conjugates

Ferritin and anticytochrome P-450 antibodies were coupled according to the procedure of Kishida et al. (16) using glutaraldehyde as a coupling agent. The ferritin anti-PB-P-450 and anti-MC-P-450 antibody conjugates, with the molar ratio of immunoglobulin G (IgG) to ferritin of ~1:1, were isolated as described previously (19).

Labeling of Nuclei and Nuclear Envelopes and Electron Microscopy

Isolated nuclei and nuclear envelopes were suspended in 0.25 M sucrose in TKM (0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, 0.005 M $MgCl_2$) separately, and incubated for 2–3 h at 0–4°C with either the antibody or the control conjugates. Excess antibody conjugates were always added so as to label nuclei or nuclear envelopes at the saturation level (19, 20). The final sucrose concentration of the incubation mixture was always adjusted to 0.25 M. The samples were spun down in an Eppendorf 5412 centrifuge (Brinkmann Instruments, Inc., Westbruy, N.Y.) for 5 min, and the pellets were washed in TKM three times by centrifugation. The final pellets were fixed successively with glutaraldehyde and osmium tetroxide, dehydrated, and sectioned. The thin sections were observed under a Hitachi HU-12 electron microscope.

The structure of the pellets was well preserved throughout the procedures for electron microscopy, and a systematic electron microscopic survey from the top to the bottom of the pellets was performed.

Morphometry

Morphometry of electron micrographs was carried out with a Mutoh digitizer model G-2 (Mutoh Industrial Inc., Tokyo, Japan) connected to a Sord microcomputer model M 223 Mark III (Sord Inc., Tokyo, Japan). By using this modular system, the perimeters of the nuclear envelopes were measured, and the average number of ferritin particles per micrometer of the envelope was calculated.

RESULTS

Biochemical Studies

Table I shows the cytochrome P-450 content and the metabolic activities of the liver nuclear envelopes that were isolated from control, MC-, PB-, and PCB-treated rats, respectively. Treatment with MC markedly increased the cytochrome content and the metabolic activities of benzo(α)pyrene hydroxylase and 7-ethoxycoumarine *O*-deethylase, while benzphetamine *N*-demethylase activity remained almost at a control level. PB treatment failed to increase either the cytochrome P-450 content or the enzyme activities as shown in the same table, whereas PCB treatment slightly increased the P-450 content.

Ouchterlony Double-Diffusion Analysis of Nuclear and Microsomal Cytochrome P-450s

Immunological similarity of the nuclear and microsomal cytochrome P-450 has been tested by Ouchterlony double-diffusion analysis. In Fig. 1, the center well contained the antibody against microsomal MC-P-450; wells *1*, *3*, and *5* and wells *2*, *4*, and *6* contained partially purified MC-P-450 from MC-treated microsomes and nuclear envelopes, respectively. This result indicates that the nuclear MC-P-450 is immunologically indistinguishable from the microsomal counterpart. Immunological similarity between the nuclear PB-P-450 and its cytoplasmic counterpart was also demonstrated by the same

TABLE I

Cytochrome P-450s Content and Metabolic Activities in Control and Treated Rats‡*

Treatment	P-450 content	Turnover number		
	nmol/mg P§	Benzo(α)pyrene	Benzphetamine	7-Ethoxycoumarin
		nmol product formed/nmol P-450/min		
Control	1.75	2.63	3.2	1.02
3-Methylcholanthrene	4.25	8.46	4.0	8.18
Phenobarbital	1.47	1.90	3.8	1.04
Polychlorinated biphenyls	2.06	—	3.7	—

* Turnover numbers.
‡ Mean of two preparations of nuclear envelopes.
§ Phospholipid-P.

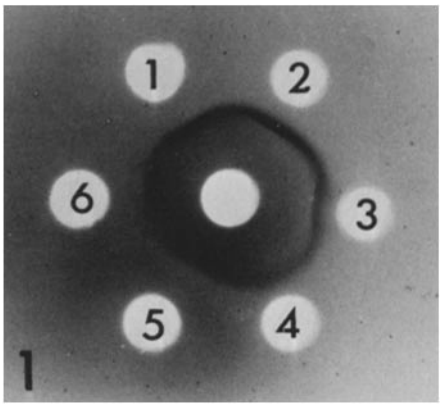

FIGURE 1 An Ouchterlony immunodiffusion analysis, comparing rat liver cytochrome P-448 (MC-P-450) of nuclear origin with that of microsomal origin. The center well was filled with 0.2 of mg antibody against microsomal MC-P-450. Wells *1*, *3*, and *5* contained 0.4, 0.2, and 0.1 nmol of partially purified MC-P-450 prepared from MC-treated microsomes (after amino octyl-Sepharose column chromatography) and wells *2*, *4*, and *6* contained 0.4, 0.2, and 0.1 nmol of partially purified MC-P-450 prepared from MC-treated nuclei, respectively.

analysis (data not shown). Our results are in agreement with the results reported by Thomas et al. (27) that hepatic MC-P-450 of nuclear origin is immunochemically indistinguishable from that of microsomal origin. In the present immunoelectron microscopic analysis of cytochrome P-450 in the nuclear envelope, we used the antibodies against microsomal cytochrome PB- and MC-P-450.

Immunoelectron Microscopic Observations of the Nuclear Envelopes

Fig. 2 shows a series of immunoelectron micrographs of hepatic nuclei isolated from control (*A*, *D*), PB- (*B*, *E*), and MC-treated rats (*C*, *F*), incubated with anti-PB-P-450 antibody conjugates (*A*, *B*, *C*) and anti-MC-P-450 antibody conjugates (*D*, *E*, *F*). Fig. 3 shows those nuclei from MC-treated rats incubated with control conjugates (*A*) and anti-MC-P-450 antibody conjugates (*B*).

In agreement with previous results (19), the outer membranes of the nuclear envelopes were heavily labeled with ferritin particles, except for those regions occupied by ribosomes, when incubated with anti-PB-P-450 antibody conjugates (Fig. 2 *A*, *B*, *C*). The degree of labeling in Fig. 2 *A*, *B*, and *C* is apparently different. Many more ferritin particles bound to the nuclear envelopes from PB-treated animals (Fig. 2 *B*), whereas the nuclear envelopes from MC-treated rats (Fig. 2 *C*) showed less labeling than those from control rats (Fig. 2 *A*).

When nuclear envelopes from control (Fig. 2 *D*) and PB-treated rats (Fig. 2 *E*) were incubated with anti-MC-P-450 antibody conjugates, the nuclear envelopes were only slightly labeled with ferritin particles. However, when envelopes from MC-treated rats were incubated with the corresponding conjugates, the envelopes were heavily labeled with ferritin (Fig. 2 *F*).

As a control experiment, hepatic nuclei prepared from MC-treated rats were incubated with the control conjugates. Hardly any ferritin particles were found on the nuclear envelopes (Fig. 3 *A*), but when the same nuclei were incubated with anti-MC-P-450 antibody conjugates they were heavily labeled with ferritin (Fig. 3 *B*, upper nucleus). Occasionally, the nuclear fraction contains nuclei from cells other than hepatocytes. The lower nucleus in Fig. 3 *B* is probably endothelial in origin, judging from the marked condensation of the large chromatin blocks. The nuclear envelopes of these cells were hardly labeled with ferritin, in marked contrast to the heavy labeling of hepatocyte nuclear envelopes. The nonhepatocyte nuclei thus serve as an internal marker for the specificity of the present ferritin-immunoelectron microscopic technique.

When nuclei from β-NF-treated rats were incubated with anti-MC-P-450 antibody conjugates, the nuclear envelopes were as heavily labeled with ferritin as those shown in Fig. 2 *F* (data not shown).

Morphometric Analysis of the Ferritin Immunoelectron Micrographs

To analyze the degree of labeling more quantitatively, the number of ferritin particles bound per micrometer of the outer membrane of the nuclear envelope was counted and is shown in Table II. It is apparent that hepatocyte nuclear envelopes from control rats bind more antibody conjugates against PB-P-450 than those against MC-P-450. If the antigenic determinants of each PB-P-450 molecule exposed to the cytoplasmic surface of the outer nuclear envelope, and thus accessible to the antibody conjugates, are equal in number to those of each MC-P-450 molecule, we could estimate that the molar ratio of the two molecular species of cytochrome P-450s is ~ 2:1 in the control rats. At present, however, we cannot compare these two numbers directly.

When rats were treated with PB, the number of ferritin particles increased slightly (150%), but treatment with MC markedly increased the number (381%). Treatment with β-NF also increased ferritin particles in a manner similar to the treatment with MC as shown in Table II. This result is to be

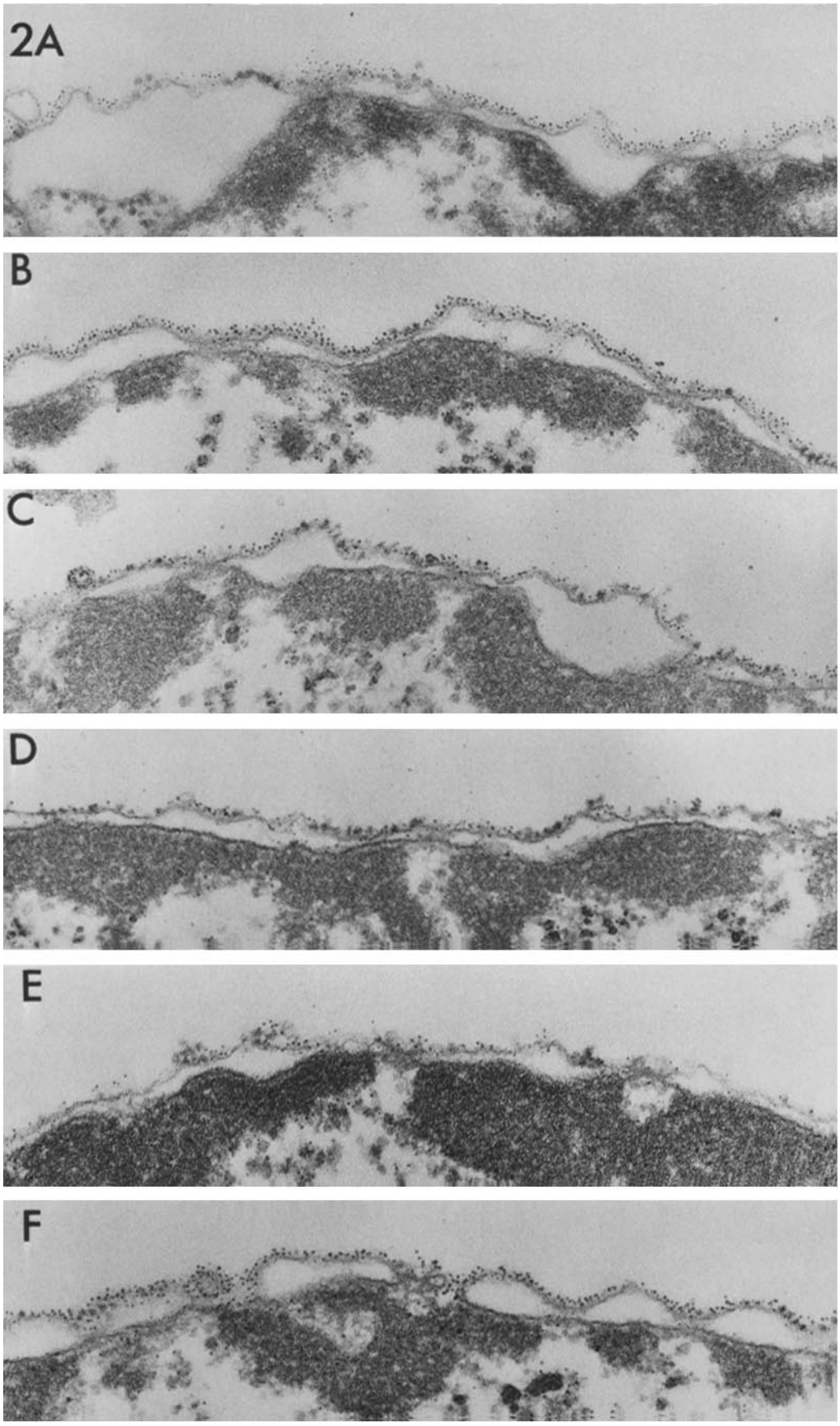

FIGURE 2 Rat liver nuclei incubated with the anti-phenobarbital-induced cytochrome P-450 (anti-PB-P-450), anti-3-methylcholanthrene-induced cytochrome P-450 (anti-MC-P-450) antibody conjugates and the control conjugates, respectively. Nuclei from control (*A*, *D*), PB- (*B*, *E*), and MC- (*C*, *F*) treated rats were incubated with the anti-PB-P-450 (*A*, *B*, *C*) and anti-MC-P-450 (*D*, *E*, *F*) antibody conjugates, respectively. × 67,500.

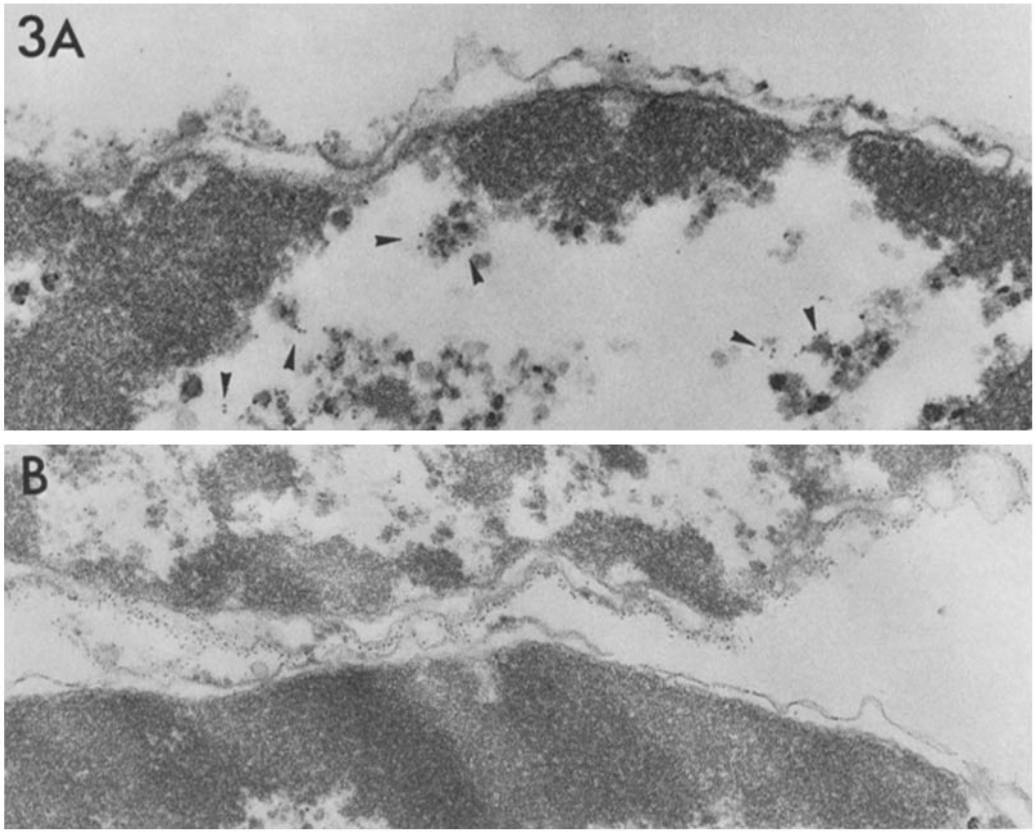

FIGURE 3 Rat liver nuclei incubated with the control (*A*) and anti-MC-P-450 antibody conjugates (*B*), respectively. Nuclei were prepared from MC-treated rats. Note the marked difference in labeling of the two nuclei in Fig. 3 *B*. The nuclear envelope of the lower nucleus, probably endothelial in origin, is not labeled with ferritin. It is to be noted in Fig. 3 *A* that a number of ferritin particles are observed within the nuclear interior, probably attached to chromatin blocks (arrowheads). × 67,500.

expected because it has been confirmed by SDS-PAGE that the molecular species of cytochrome P-450 induced by β-NF are not discriminated from those induced by MC (Masaki, R., manuscript in preparation).

Ferritin Immunoelectron Microscopic Observation of Isolated Nuclear Envelopes

In the series of immunoelectron micrographs shown in Fig. 2, the inner membrane of the nuclear envelope was never labeled with ferritin. It is not certain whether this is due to the inability of the antibody conjugates to reach the inner membrane of the nuclear envelope as a result of steric hindrance caused by the presence of the nuclear lamina and condensed chromatin. The nuclear lamina is always in close association with the inner membrane of the nuclear envelope in resting nuclei (3, 8). To avoid such steric hindrance, ferritin immunoelectron microscopic analysis had to be carried out on isolated nuclear envelopes.

First, hepatic nuclear envelopes were isolated by the procedures of Dwyer and Blobel (3) from MC-treated rats and incubated with the ferritin antibody conjugates against MC-P-450. The polarity of the nuclear envelope was decided by the exclusive attachment of ribosomes on the outer membrane and of residual chromatin on the inner membrane (Fig. 4). It is apparent from this figure that ferritin particles are exclusively found on the outer membrane of the nuclear envelope.

As shown in Fig. 4, the nuclear envelopes isolated by the procedures of Dwyer and Blobel are usually covered by lamina structures, even after washing with high KCl solution. The washing procedure was avoided in our experiment because of the deteriorative effects of the high salt washing on the ultrastructure of the nuclear envelope. It is not certain, therefore, whether or not this negative result of labeling of the inner membrane of the nuclear envelope is simply due to the steric hindrance by the presence of lamina structures.

Accidentally, we found that the hepatic Golgi fraction prepared according to the procedure of Hino et al. (11) contains

TABLE II

Binding of Ferritin-Antibody Conjugates to the Outer Nuclear Membranes from Control and Treated Rats

Source of hepatic nuclei	Incubated antibody conjugates	Number of ferritin particles bound per micrometer of nuclear envelopes*
Control	anti-PB-P-450 antibody	38.7 (100%)
PB-treated	anti-PB-P-450 antibody	56.7 (150%)
MC-treated	anti-PB-P-450 antibody	27.0 (68%)
Control	Anti-MC-P-450 antibody	17.1 (100%)
PB-treated	Anti-MC-P-450 antibody	17.2 (101%)
MC-treated	Anti-MC-P-450 antibody	57.6 (381%)
β-NF-treated	Anti-MC-P-450 antibody	52.3 (344%)
MC-treated	Control IgG	2.7 —

* Average of two morphometrical analyses. Ferritin particles counted were 500–1,000 for each measurement, and the total length of outer nuclear membranes surveyed was ~20 μm for each source of nuclei.

large sheets of nuclear envelopes that are easily identified by their characteristic double membrane profile and by the presence of nuclear pore complexes. The polarity of the nuclear envelopes was again easily decided by the presence of membrane-bound ribosomes. The nucleoplasmic surface of the inner membrane of the nuclear envelopes prepared by this procedure is usually smooth and probably not associated with the lamina structures as shown in Fig. 5 *A* and *B*. It appears that these structures have been detached from the nucleoplasmic surface of the nuclear envelopes during straining of liver tissues through a 100-mesh stainless sieve. It is evident that no ferritin particles are observed on the surface of the inner membrane of

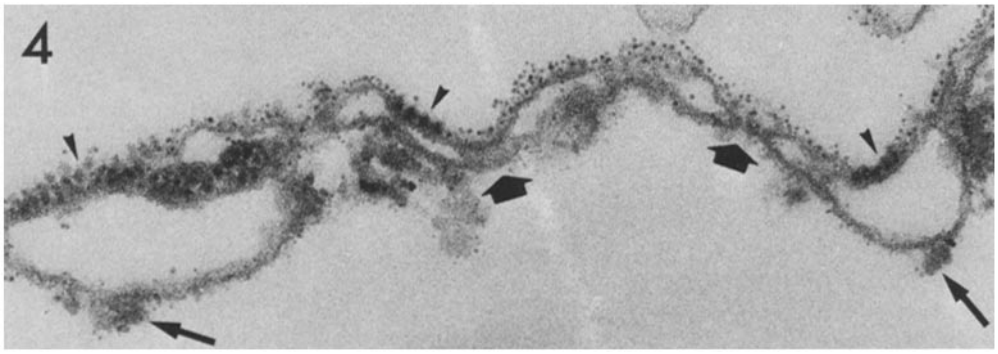

FIGURE 4 Hepatocyte nuclear envelopes isolated from MC-treated rats by the procedures of Dwyer and Blobel (3) and incubated with anti-MC-P-450 antibody conjugates. Long arrows, chromatin; short arrows, nuclear pores; arrowheads, ribosomes. × 80,000.

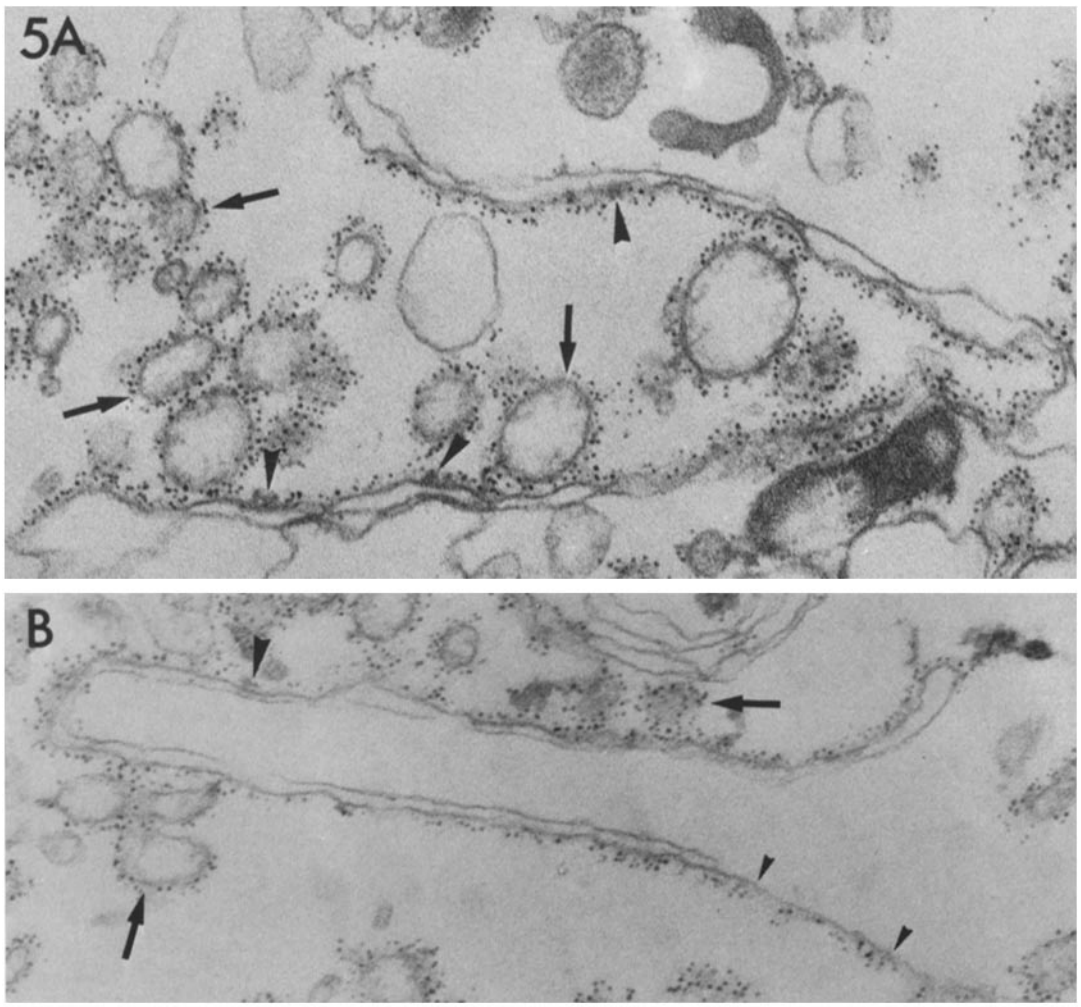

FIGURE 5 Rat liver Golgi fraction prepared by the procedures of Hino et al. (11) from 3-MC-treated rat was incubated with anti-MC-P-450 antibody conjugates. This fraction occasionally contains smooth nuclear envelopes that are probably not associated with lamina structures. Note heavy labeling of the outer membranes of the nuclear envelope, which is in marked contrast to the complete lack of labeling of their inner counterparts. Large arrowheads indicate the ribosomes attached to the outer nuclear membranes, while small arrowheads indicate complete absence of ferritin particles on the cisternal surface of the outer membranes. It is to be noted here that the Golgi fraction, when prepared from PB- or MC-treated rats, is contaminated markedly with smooth microsomal vesicles (arrows) that are heavily labeled with ferritin particles. (*A*) × 82,000; (*B*) × 90,000.

the nuclear envelope. This result is in marked contrast to the heavy labeling of the outer membrane of the nuclear envelope.

Occasionally, we observed nuclear envelopes that had partially lost their inner membrane as shown in Fig. 5 *B* (small arrowheads). Ferritin particles were not found on the cisternal surface of the outer membrane.

Ferritin Immunoelectron Microscopic Observation of the Naked Hepatic Nuclei

Because of the importance of the lack of labeling of the cisternal surfaces of both membranes of the nuclear envelope, we tried to prepare naked nuclei. When isolated nuclei were vigorously homogenized with a tight Teflon-glass homogenizer, the outer nuclear membranes were sometimes partially detached from the nuclear surface, and nuclei, covered simply by the inner nuclear membrane, were prepared as shown in Fig. 6. When these nuclei were incubated with the antibody conjugates, it was clearly shown that the cytoplasmic surface of the outer nuclear envelope membrane was heavily labeled with ferritin, but that the cisternal surfaces of both the outer and inner nuclear envelope membranes were hardly labeled (Fig. 6 *A* and *B*).

Occasionally, we observed blister formation from the outer nuclear envelope membrane as shown in Fig. 7. This blister formation was also observed in the conventional nuclei that were not treated with a tight homogenizer. These blisters were easily recognized, because they were smooth and not labeled with ferritin particles. In some cases, these regions were flattened and occupied a considerably vast area of the outer nuclear envelope. These blisters were probably formed artificially by glutaraldehyde fixation, as in the case of plasma membranes (10). The specificity of the present immunoelectron microscopy is also supported by the complete lack of labeling of the blister membranes.

Ferritin Immunoelectron Microscopic Observation of the Nuclear Chromatin Blocks

When the hepatic nuclei prepared from MC-treated rats were incubated with corresponding ferritin antibody conjugates, some ferritin particles were found in the nuclear interior,

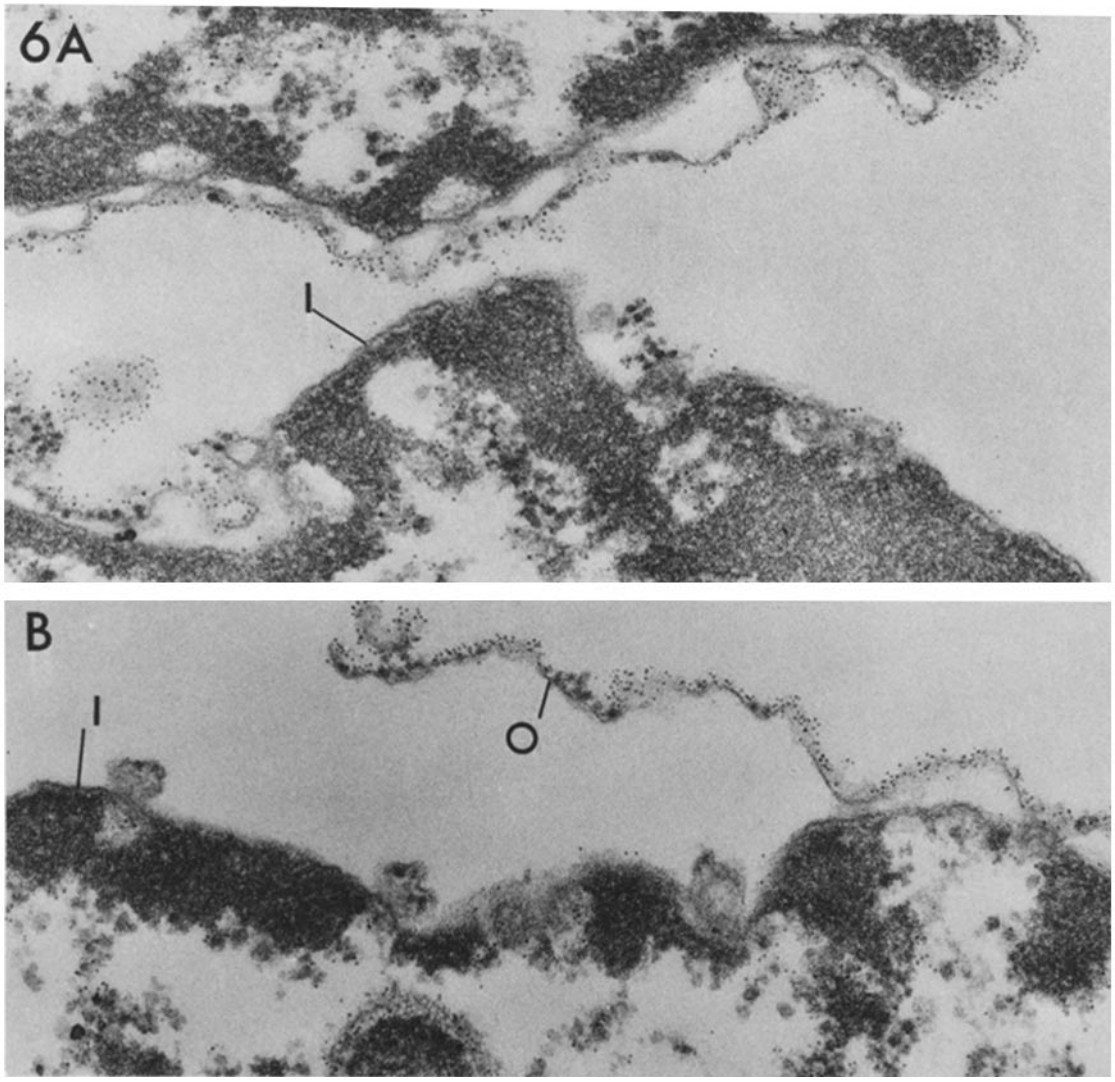

FIGURE 6 Rat liver nuclei prepared from 3-MC-treated rats were homogenized in a tightly Teflon-glass homogenizer and incubated with anti-MC-P-450 antibody conjugates. These preparations contain stripped nuclei covered only with inner nuclear membranes (*A*, lower nucleus) or having partially detached outer membranes (*B*). Ferritin particles were exclusively found on the cytoplasmic surface of the outer membranes. *O*, *I*, outer and inner nuclear membranes, respectively. (*A*) × 68,000; (*B*) × 65,000.

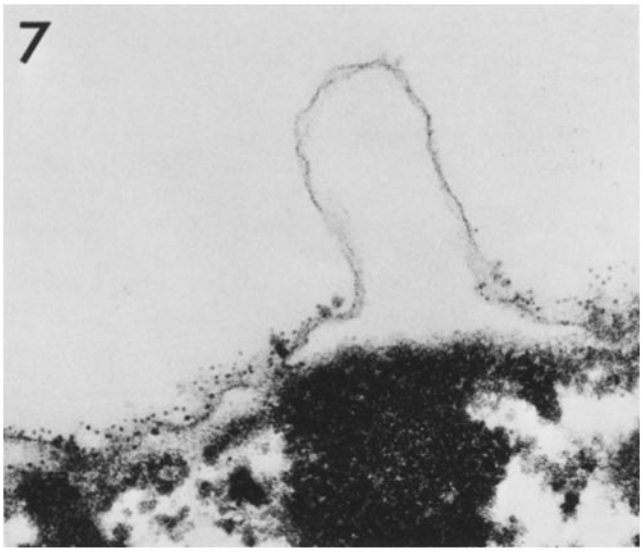

FIGURE 7 Blister formation from the outer nuclear membranes. Liver nuclei prepared from 3-MC-treated rats were incubated with anti-MC-P-450 antibodies. Note the blister that formed on the outer membrane which is not labeled with ferritin. These blisters were probably formed artificially by glutaraldehyde fixation. × 77,500.

sometimes associated with chromatin blocks as shown in Fig. 6. A similar degree of labeling with ferritin was observed when the nuclei from control animals were incubated with the same conjugates. Even when these nuclei were incubated with control conjugates, ferritin particles were found within the chromatin blocks and were not removed by repeated washings with various buffer solutions (Fig. 3*A*). We conclude, therefore, that the presence of ferritin particles within the nuclear interior is due to nonspecific adsorption of the antibody or control conjugates to nuclear chromatin blocks.

DISCUSSION

Biochemical evidence for the existence of cytochrome P-450s in hepatic nuclei, especially in the nuclear envelope, has been presented by a number of investigators (4, 6, 13–15, 24, 25). The exact localization of the cytochromes, however, has not yet been settled. Do they exist exclusively in the nuclear envelope or do they also exist within the nucleus? Because the mass of nuclear envelope in the cell is only 1% of the total mass of microsomal membrane protein (4), the possibility exists that the apparent enzyme activity was not associated with nuclei, but with the contaminating endoplasmic reticulum. In addition to a biochemical approach, therefore, we used ferritin immunoelectron microscopy methods that had been successfully applied to the analysis of the distribution of cytochrome P-450 on hepatic microsomes (19, 20).

Our observations clearly indicate that ferritin particles are found exclusively on the cytoplasmic surface of the outer membrane of the nuclear envelope. The other surfaces of the nuclear envelope, that is, the nucleoplasmic surface of the inner membrane and the cisternal surfaces of both the inner and outer membranes of the nuclear envelope were only minimally labeled with ferritin.

This result indicates that cytochrome P-450 molecules are exposed exclusively on the cytoplasmic surface of the outer nuclear membrane and that they cannot diffuse laterally to the domain of the inner nuclear membrane. It is suggested, therefore, that the nuclear pore complexes could be diffusion barriers for cytoplasmic-face-membrane proteins such as cytochrome P-450.

Lack of labeling of the cisternal surface of the outer membrane suggests that the polypeptide chain of cytochrome P-450 does not penetrate the outer nuclear membrane. Therefore, cytochrome P-450 is not a transmembrane protein but an intrinsic protein of the cytoplasmic surface of the outer nuclear membrane. This suggestion is supported by the fact that the nascent chains of cytochrome P-450, newly synthesized on the membrane-bound ribosomes, are directly inserted into the cytoplasmic surface of endoplasmic reticulum membranes (1, 7). A possibility still exists, however, that cytochrome P-450 is indeed a transmembrane protein but has no antigenic determinants on the cisternal side of the outer membrane of the nuclear envelope.

The next question is whether or not cytochrome P-450 exists within the nucleus. In spite of several trials, we have failed to get any positive evidence showing the existence of cytochrome P-450 within the nuclear interior. In the course of this study, however, we occasionally found that internalization of microsomal vesicles occurred when nuclear envelopes were broken during the incubation with the antibody or control conjugates (data not shown). This finding suggests that the localization of cytochrome P-450 within nuclei was produced artifically by the inclusion of microsomal vesicles in the nuclear interior.

We conclude, therefore, that nuclear cytochrome P-450 is exclusively localized on the outer membrane of the nuclear envelope. This conclusion is supported by the finding of Rogan et al. (24) that washing of nuclei with a detergent (Triton N-10) removed the nuclear envelope and, simultaneously, nuclear cytochrome P-450.

It has been generally accepted that the enzyme profile of the nuclear envelope is similar to that of the endoplasmic reticulum and that usually the specific enzyme activities of the nuclear envelope are approximately one-third or one-half of those of endoplasmic reticulum (9, 13, 31). In fact, Sagara et al. (25) reported that the cytochrome P-450 content of rat liver nuclei is approximately one-half that of microsomes on a phospholipid basis. If phospholipids are equally distributed in the outer and inner nuclear membranes, the content of cytochrome P-450 on a phospholipid basis is expected to be about one-half of that of microsomes.

Thus it is strongly suggested that the microsomal-type enzymes in nuclei are associated mostly with the outer nuclear membrane and that the properties of the inner nuclear membrane are considerably different from those of its outer counterpart. More ferritin immunoelectron microscopic evidence on the distribution of many other microsomal enzymes must be presented before this generalization can be accepted.

As to the inducibility of nuclear cytochrome P-450 by PB treatment, controversial results have been reported as described earlier in this paper. Our biochemical analysis has shown that the total nuclear envelope cytochrome P-450 remained the same in control and PB-treated animals. Ferritin immunoelectron microscopic analysis, however, clearly indicated a slight induction of the cytochrome by PB treatment (1.5 times). This apparently inconsistent result could be reconciled if one considers that, although the absolute amount of nuclear envelope cytochrome P-450 was not increased by PB induction, there could be an induction of one molecular species of cytochrome P-450 that reacts specifically with the antibody against PB-P-450, a major molecular species of PB-induced cytochrome P-450s. A biochemical study on the induction of lidocaine-bind-

ing form of cytochrome P-450 by PB induction without producing a net increase of nuclear cytochrome P-450 has also been reported by Fahl et al. (4).

In agreement with the biochemical results of a number of investigators (4, 14, 24, 25), our present biochemical and immunoelectron microscopic observations clearly indicate a marked induction of MC-P-450 in nuclear envelopes by MC or β-NF treatment. Thus MC treatment may induce biosynthesis of MC-P-450 in the outer membrane of the nuclear envelope that may be ultimately involved in the generation of carcinogens. The proximity of the site of generation of these metabolites to the site of DNA modification may be important for chemical carcinogenesis.

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