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Intranuclear Localization of Hepatic Cytochrome P_{448} by an Immunochemical Method

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SUMMARY

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By means of a combined histochemical-immunological technique, at least a part of the intracellular hepatic cytochrome P_{448} has been localized within nuclei isolated from 3-methylcholanthrene pretreated rats. In this technique, sections of nuclei are incubated initially with rabbit anti-cytochrome P_{448}, then with goat anti-rabbit immunoglobulin to which β-galactosidase had been chemically coupled. The preparation is then histochemically assayed for β-galactosidase using the histochemical substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in the presence of an oxidizing agent, nitroblue tetrazolium. A blue pigment is generated at the site of localization of β-galactosidase, and accordingly, of cytochrome P_{448}. When conjugate was employed in which the immunoglobulin was monospecific antibody to cytochrome P_{450} obtained from the livers of phenobarbital-treated rats, no demonstrable activity was observed within the nuclei obtained from 3-methylcholanthrene pretreated rats. This control adds considerable weight to our argument for the intranuclear localization of at least a portion of the cytochrome P_{448}.

INTRODUCTION

Evidence has been presented that favors the hypothesis that cytochrome P_{448} activity is present in isolated rat liver nuclei, prepared under conditions which preclude significant cytoplasmic contamination (1–7). However, the possible retention of fragments of endoplasmic reticulum in these nuclear preparations or the presence of hemoprotein *exclusively* in the outer nuclear membrane has not allowed for the definitive localization of cytochrome P_{448} within nuclei.

In our previous work, we had shown that hepatic nuclear and microsomal cytochrome P_{448} are immunologically identical (8). In order to definitively establish that rat liver cells express cytochrome P_{448} activity in their nuclei as well as within endoplasmic reticulum, we have developed an immunohistochemical staining technique for its direct visualization. The staining procedure depends upon the incubation of sections of rat liver nuclei with rabbit anti-rat

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liver microsomal cytochrome P_{448} antibody, followed by goat anti-rabbit immunoglobulin which was conjugated to β-galactosidase (Gz).[2] After incubation of the preparations with a histochemical substrate for β-galactosidase, the localization of the hemoprotein may be visualized by deposition of an insoluble blue dye. The results presented in this manuscript demonstrate that intranuclear localization of cytochrome P_{448}.

MATERIALS AND METHODS

β-D-galactosidase (Type IV, E.C. 3.2.1.23) was obtained from Sigma Chemical, St. Louis, Mo. as a crystalline suspension of 2.2 M $(NH_4)_2SO_4$. Five mg of the enzyme suspension was centrifuged at 6000 × *g* for 30 min at room temperature; the pellet was dissolved in 0.9 ml phosphate buffered saline, pH 7.4 (0.14 M NaCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 3.2 mM KCl, and 3.0 mM NaN_3) herein referred to as PBS. The dissolved enzyme solution was placed in Visking dialysis tubing and dialysed at 4° overnight against 4 liters of PBS.

Goat anti-rabbit immunoglobulin antiserum was obtained from Cappel Laboratories, Cochranville, PA. An immunoglobulin (Ig) fraction of this antiserum was prepared by Na_2SO_4 fractionation by the method of Voller *et al.* (9). The Ig concentration was estimated at 280 nm using $E^{1\%}_{1\,cm} = 13.0$. Two mg of Ig were dialyzed at 4° against 4 l PBS, then added to the dialysed β-galactosidase preparation. EM grade glutaraldehyde (Polysciences Inc., Warrington, Pa.) was added to the enzyme-antibody solution to a final concentration of 0.2%. After mixing, the conjugate preparation was allowed to stand at room temperature without agitation for 2 hr. The conjugate was dialysed overnight against 4 liters PBS containing 5 mM $MgSO_4$, 1 mM $MnSO_4$ and 1 mM $CaCl_2$. The conjugate was diluted with an equal volume of glycerine and stored at −20°.

[2] The abbreviations used are: Gz, β-galactosidase; PBS; phosphate buffered saline; Ig, immunoglobulin; PBS-T, phosphate buffered Tween-20; BIG, β-galactosidase, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; NBT, nitro-blue tetrazolium; $E^{1\%}_{1\,cm}$, extinction coefficient of 1 mg/ml solution.

The highest concentration of conjugate which would not result in nonspecific staining of nuclei was determined by serial dilution. This amount was usually 1:500 (v/v).

Rat liver nuclei (isolated from rats that had received 20 mg/kg 3-methylcholanthrene 24 hr previously), 5–10 μ thick, were embedded in agar as described previously (5) and dried on 22 × 22 mm #2 cover slips. As discussed previously (5) this technique was designed so that the bulk, if not the entire nuclear population, would be sliced through and exposed areas on the slide would represent sections through the nucleus and not simply the outer nuclear membrane. These sections were hydrated for 5 min in PBS containing 5 mM $MgSO_4$, 1 mM $MnSO_4$, 1 mM $CaCl_2$, and 0.05% Tween 20 (hereafter referred to as PBS-T). Tween 20 was used to prevent nonspecific adsorption of antisera and conjugate to the surface of the cover slip (9). The moistened nuclear preparations were placed in a humidified chamber and flooded with monospecific rabbit Ig prepared against rat liver microsomal cytochrome P_{448} (3 mg/ml) (10) or nonimmune rabbit Ig (3 mg/ml), each diluted 1:500 in PBS-T, or buffer alone. The antibody was made monospecific by immunoabsorption with partially purified solid phase cytochrome P_{450} from rats treated with phenobarbital (10). This antibody recognized only one form of cytochrome in a highly specific manner. After overnight incubation at room temperature, the cover slips were drained, placed in Columbia staining jars (Fisher) and incubated in three changes of PBS-T at 5 min intervals to remove unbound protein. The preparations were then incubated with goat anti-rabbit-galactosidase conjugate diluted 1:100 in PBS-T, or buffer alone. For some experiments, antibody-treated preparations were incubated with goat anti-rabbit Ig (3 mg/ml) diluted 1:50 in PBS-T prior to the conjugate step as a control for specificity of conjugate adsorption.

The histochemical substrate for β-galactosidase, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BIG, Sigma) was dissolved in dimethylformamide, 10 mg/ml, and stored as a stock solution at −20°. A

stock solution of nitroblue tetrazolium (NBT), 1 mg/ml in PBS, was made immediately before used. Fresh substrate solution was constructed as a 1:20 dilution of BIG in dimethylformamide (500 mg/ml) containing a 1:200 dilution of NBT in PBS (50 mg/ml).

Nuclear preparations were flooded with fresh substrate and incubated at room temperature in a humid chamber. The substrate solution was frequently inspected for the appearance of a faint tinge of blue dye; visual appearance of pigment usually correlated well with adequate microscopic staining of the nuclei. Incubation times varied from 3–6 hours, depending on the intensity of the desired staining reaction.

After incubation with substrate, the cover slips were placed in Columbia jars containing PBS for 5 min, then drained and washed with several changes of distilled water. The preparations were dehydrated in methanol followed by xylene, then mounted on glass slides using Permount (Fisher).

In several experiments, a cytochrome P_{450} conjugate was employed as a control. The cytochrome P_{450} was purified from the livers of phenobarbital-treated rats and monospecific antibody produced as described (10). Monospecific antibody was obtained after negative immunoabsorption on cytochrome P_{448} Sepharose columns. The resultant antibody only recognized one form of cytochrome (10).

RESULTS

Prior to presenting the results, it is important to discuss possible artifacts in the technique. Artifactual staining reactions using BIG as a histochemical indicator of Gz activity have been noted by several investigators (11, 12, and Boraker, unpublished observations). Such artifactual staining occurs as a result of the diffusion of a colorless, soluble indoxyl cleavage product of BIG, which must then be further oxidized to the insoluble indoxan form (13). Lojda *et al.* (14) were able to eliminate this objection to the use of BIG by incorporating nitro blue tetraozlium (NBT) in the substrate incubation step. NBT acts as an oxidizing agent for the indoxyl intermediate of BIG cleavage, and is itself reduced to a blue insoluble diformazan dye (14). Thus, our experiments were performed using a substrate admixture of BIG and NBT; the stain which deposits at the sites of cytochrome P_{448} localization is, therefore, a mixture of insoluble indigo from BIG and diformazan from NBT.

In order to test the possibility that the deposits of stain (seen in Figs. 1 and 2) were artifactual, the following experiment was performed. Substrate solution (BIG, 1 mg/ml; NBT, 100 μg/ml) in PBS-T was cooled in an ice bath. An equal volume of cold Gz was added to aliquots of substrate solution to a final concentration of 2.5 μg/ml, and 0.5 μg/ml. Coverslip preparations of sectioned rat liver nuclei were immediately flooded with the enzyme-substrate mixtures, and allowed to incubate in a humid chamber at room temperature. After 18 min, all three dilutions of Gz had produced extensive substrate hydrolysis, as judged by the intense blue color reaction in the fluid covering the nuclei. Nuclear preparations flooded with each of the three enzyme dilutions were washed in distilled water, dehydrated in methanol, and mounted for microscopic examination. The remaining nuclear preparations were allowed to incubate with enzyme and substrate for 1 hr 40 min, after which the intensely blue incubation solution was drained, and the coverslips mounted. None of the preparations incubated for 18 min showed even faint staining, in spite of the extensive enzyme-substrate reaction which occurred in the solution in which they were incubated. Faint uniform staining occurred in nuclei incubated for 1 hr 40 min in substrate containing 2.5 μg/ml Gz; however, the staining reaction was quite unlike that of nuclei treated as in Figs. 1 and 2 in that no discrete precipitates corresponding to the macro- and micro-deposits were seen. This preparation also showed considerable deposits of crystalline indigo-diformazan dye complex, indicating that the enzyme-substrate conditions were in enormous excess of the conditions under which nuclei were treated in the experiments outlined in Table 1. Thus, we were unable to demonstrate staining

reactions comparable in morphology to that seen in antibody- and conjugate-treated nuclei. We conclude that the substrate incubation conditions used in these experiments exclude artifactual staining due to diffusion and spurious oxidation of soluble indoxyl from BIG.

The various combinations of interactions are presented in Table 1. The preparations were examined by both conventional bright field and phase-contrast illumination. The only nuclear preparations which demonstrated significant staining were those of Series I, VIII and XI, Table 1. Intense blue deposits of indoxan-formazan complex were seen in virtually all nuclei packets of Series I, VIII and XI (Fig. 1a and b). The major deposits of stain were isolated into intranuclear packets resembling nucleoli in size and distribution (macro-deposits). This is not to imply that these areas were indeed nucleoli. In addition, micro-deposits of stain were distributed throughout the nuclear contents, producing a "stippled" appearance, which is very apparent in the phase contrast photomicrograph of Figure 1b.

The specificity of the staining reaction was demonstrated by the complete absence of indoxan dye in all preparations outlined in Table I except Series I, VIII and XI. In addition, Series VII demonstrated that goat anti-rabbit Ig could completely inhibit the adsorption of the conjugate, for such preparations were completely free of indoxan dye. Series IV yielded a faint but visible indoxan precipitate, due either to non-specific absorption of nonimmune rabbit Ig, or low-titer cross-reacting antibody present in this Ig preparation. That this reaction was due to adsorption of rabbit Ig was verified in Series IX, since goat (anti-rabbit Ig) Ig completely inhibited the faint indoxan precipitate seen in Series IV.

The nuclear preparations were examined with both bright field and phase-contrast illumination (Fig. 1). The blue macro- and microdeposits were readily visible by both methods of illumination (Fig. 1a and b). When negative control preparations (e.g., Series V, Table 1) were examined by conventional illumination, the nuclei were only faintly visible (Fig. 1c) and intranuclear

TABLE 1

Experimental and control preparations for the immunohistochemical demonstration of intranuclear cytochrome P448

Series	Step 1	Step 2	Step 3	Step 4
I	R anti-rat cytochrome[a]	Conjugate[b]	Substrate[c]	
II	R anti-rat cytochrome	PBS-T[d]	Substrate	
III	PBS-T	Conjugate	Substrate	
IV	NRIg[e]	Conjugate	Substrate	
V	PBS-T	PBS-T	Substrate	
VI	PBS-T	PBS-T	PBS-T	
VII	R anti-rat cytochrome	Goat anti-rabbit Ig[f]	Conjugate	Substrate
VIII	R anti-rat cytochrome	NGIg[g]	Conjugate	Substrate
IX	NRIg	Goat anti-rabbit Ig	Conjugate	Substrate
X	NRIg	NGIg	Conjugate	Substrate
XI	NGIg[h]	R anti-rat cytochrome	Conjugate	Substrate
XII	NGIg	NRIg	Conjugate	Substrate

[a] Immunoglobulin (Ig) fraction of monospecific rabbit anti-rat cytochrome P_{448} (3 mg/ml) diluted 1:500 in PBS containing 0.5% Tween 20.

[b] Ig fraction of goat anti-rabbit immunoglobulin conjugated to β-D-galactosidase, diluted 1:100 in PBS-T.

[c] 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 500 mg/ml and nitro blue tetrazolium 50 mg/ml in PBS containing 5 mM Mg^{2+}, 1 mM Ca^{2+}, and 1 mM Mn^{2+}.

[d] Phosphate-buffered saline containing 0.5% Tween 20.

[e] Nonimmune rabbit Ig (3 mg/ml) diluted 1:500 in PBS-T.

[f] Immunoglobulin fraction of goat anti-rabbit immunoglobulin (Cappel Laboratories), 3 mg/ml, diluted 1:50 in PBS-T.

[g] Nonimmune goat Ig (3 mg/ml) diluted 1:50 in PBS-T.

[h] Nonimmune goat Ig (3 mg/ml) diluted 1:500 in PBS-T.

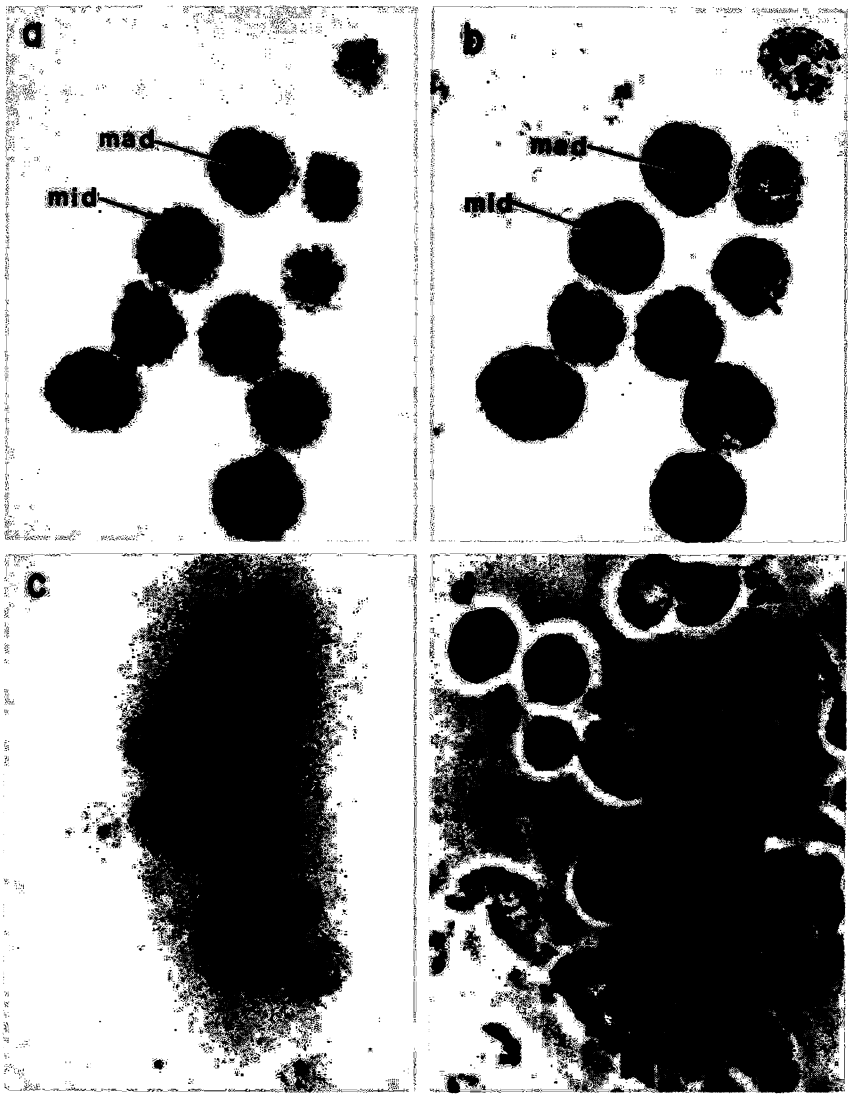

FIG. 1. *Bright field and phase contrast photomicrography of stained nuclei*

a) Bright field photomicrograph treated with an immunoglobulin fraction of rabbit anti-rat cytochrome P_{448}, followed by goat anti-rat immunoglobulin-β-galactosidase conjugate and the histochemical substrate, BIG in NBT (Series I, Table 1). Magnification 1250×. Macrodeposit, mad; microdeposit, mid. Both *mad* and *mid* were intensely blue. b) Photomicrograph of stained nuclei of 1a taken with phase contrast illumination. ×1250. The positive stain seen as a blue coloration of *mad* and *mid*. c) Bright field photomicrograph of nuclei treated with nonimmune rabbit immunoglobulin, followed by conjugate and substrate (Series IV, Table 1). d) Phase contrast photomicrography of nuclei treated as in 1c. The nucleoli can be easily seen in these preparations. These are *not* of the same coloration as in 1b.

organization was difficult to discern due to the hyaline nature of these preparations. However, when such preparations were examined with phase-contrast illumination, intranuclear "organelles" resembling nucleoli in size were readily visualized (Fig. 1d). The very visible nucleoli of Fig. 1d are *not* stained blue. Furthermore, no stippled blue appearance of the nuclei is seen. Careful adjustment of the focus of indoxan-stained nuclei at 2000 ×, e.g., Series 1 of Table 1, showed that the macrodeposits were distributed at different depths within the nuclei (Fig. 2). The microdeposits might

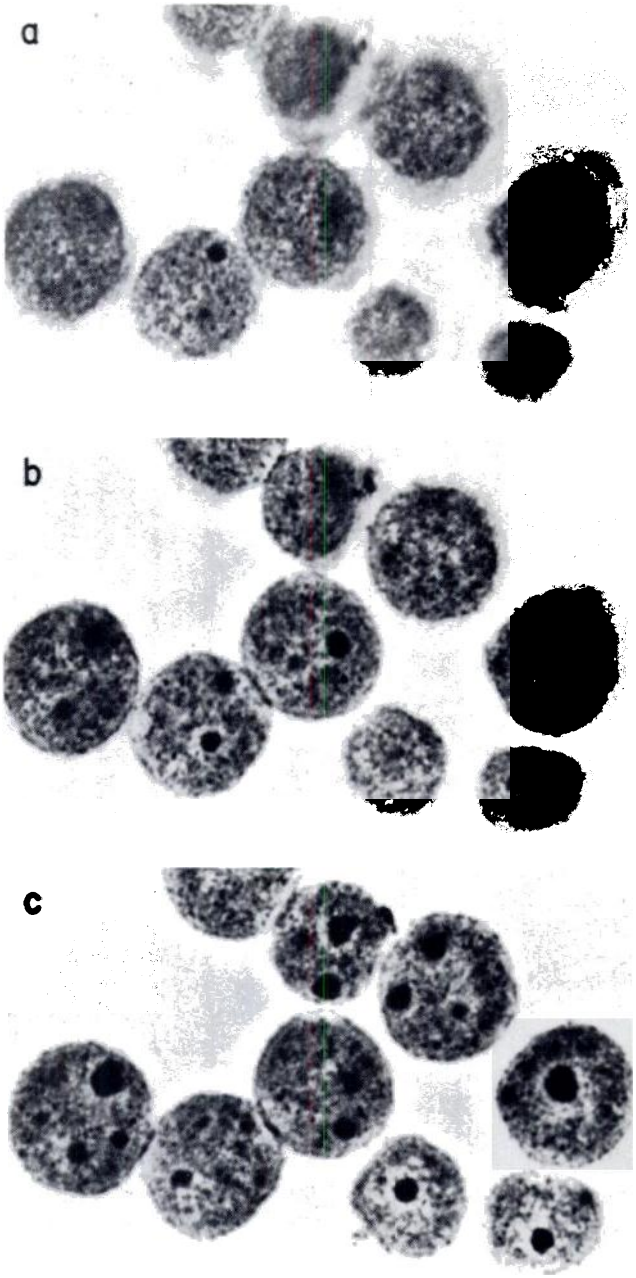

FIG. 2. *A series of phase contrast photomicrographs of nuclei stained for Cytochrome P_{448}*
The photomicrographs in a, b, c, were taken at different focusing distances from the plane of the coverslip on which the nuclear sections were "fixed." The sequences demonstrate that the *mad* of histo-chemical stain are distributed within the nuclear contents in a 3-dimensional array. ×2000.

represent elements of the cytonuclear skeleton.

To further test the specificity of the staining reaction, we prepared conjugate containing monospecific antibody to cytochrome P_{450} (from phenobarbital treated rats). The protocol for these experiments is outlined in Table 2. In the Series A to D, only Series C exhibited easily detectable micro- and macrodeposits. In Series A, no

TABLE 2

Protocol for demonstration for lack of reaction with cytochrome P_{450}

Series	Step 1	Step 2	Step 3
A	PBS-T	Conjugate[b]	Substrate[c]
B	NRIg[a]	Conjugate	
C	R-anti-rat cyto P_{448}[d]	Conjugate	
D	R-anti-rat cyto P_{450}[e]	Conjugate	

[a] Nonimmune rabbit Ig (3 mg/ml) diluted 1:500 in PBS-T.

[b] Ig fraction of goat anti-rabbit immunoglobulin conjugated to β-galactosidase; diluted 1:100 in PBS-T.

[c] 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 500 mg/ml, and nitro blue tetrazolium, 50 mg/ml, in PBS containing 5 mM Mg^{+2}, 1 mM Ca^{+2}, and 1 mM Mn^{+2}.

[d] Ig fraction of monospecific rabbit anti-rat cytochrome P_{448} (3 mg/ml) diluted 1:500 in PBS containing 0.5% Tween 20.

[e] Ig fraction of monospecific rabbit anti-rat cytochrome P_{450} (3 mg/ml) diluted 1:500 in PBS containing 0.5% Tween 20.

stain was detectable while in B and D, a very faint stain of the nuclear envelope was apparent; no micro- or macrodeposits could be seen. The faint positive reaction seen in series D is in accordance with the low amount of cytochrome P_{450} (Phenobarbitol) reported in microsomes from 3-methylcholanthrene pretreated rats (10).

DISCUSSION

Benzo(a)pyrene is a known procarcinogen which requires metabolic activation to exert its mutagenic and carcinogenic activity (15–17). The enzymes which catalyze these reactions (the cytochrome P_{450}-dependent mixed function oxidase system and epoxide hydrase) are localized primarily in the endoplasmic reticulum of liver and other tissues. However, the resultant ultimate carcinogenic metabolite, a benzo(a)pyrene 7,8-diol-9,10-epoxide (18), would have to "survive" a veritable sea of nucleophilic substances, e.g., cytosolic protein, in order to interact with the cell's genetic material. The fundamental assumption is of course that such interaction is a prerequisite to the ensuing neoplastic transformation. The possibility of the highly reactive benzo(a)pyrene 7,8-diol-9,10-epoxide successfully gaining access to the nucleus under these circumstances seemed small. This laboratory as well as others therefore sought a mechanism which would circumvent this problem. The discovery of a mixed function oxidase system associated with nuclei (1–7) appeared to fulfill that objective. Unfortunately, the evidence still remained a bit equivocal. The possibility still existed that the apparent enzyme activity was *not* associated with nuclei but with contaminating endoplasmic reticulum and the product of the reaction diffused into the nuclei.

Using an immunochemical technique, we have presented strong evidence for the presence of a cytochrome P_{448} in the nucleus. Furthermore, the hemoprotein is *not* restricted to the nuclear membrane. The basis of our experimental design is a combined histochemical-immunological technique in which we have taken advantage of the immunological specificity of antibody for microsomal and nuclear cytochrome P_{448} (8). In addition, we have coupled anti-antibody with the easily-detectable enzyme, β-galactosidase, and have demonstrated the localization of the latter immunological complex by a histochemical assay in which insoluble blue indigo and diformazan dyes are deposited at the appropriate site. The assay has been well-controlled in terms of obviating non-specificity (see Table 1).

Recently, Matsuura et al. (19) demonstrated, by immunoelectron microscopy, the localization of cytochrome P_{450} on the outer surfaces of microsomes and nuclei derived from hepatocytes of rats treated with phenobarbital. The distribution of ferritin-labeled antibody in these experiments was heterogeneous, suggesting that cytochrome P_{450} was located on the envelopes of these subcellular constituents in clusters or patches. Elaborate control experiments mitigated against the possibility that the ferritin-antibody conjugate caused redistribution of cytochrome P_{450} through cross-linkage by bivalent antibody. However, as clearly suggested by Matsuura *et al.* (19), there is considerable doubt as to the capability of the ferritin conjugate to penetrate the nuclear membrane. The clustering of cytochrome P_{450} on the outer surface of the nuclear envelope may be analogous to the patchy distribution of immunohistochemi-

cal stain seen in our preparations, described as microdeposits. However, the definitive localization of cytochrome P_{448} within the nuclei (Fig. 2) suggests that sectioned nuclei are the most appropriate targets for immunocytochemical reagents designed to reveal the full extent of cytochrome enzyme distribution both on the surface of nuclei, and throughout the nuclear contents.

Although leakage of the cytochrome P_{448} from the nuclear envelope might be responsible for our observed results, all our evidence would not be in concert with this phenomenon. Thus, we *only* see P_{448} *within* the nucleus and not *outside* of the nucleus. When nuclei are incubated for as long as 1 hr in buffer, no cytochrome P_{448} is demonstrable in the buffer.

With this evidence, it is now clear that an activating system for conversion of at least one procarcinogen, benzo(a)pyrene, exists in the region of the cell in which the prime target (DNA) for mutagenesis and carcinogenesis is located.

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