MOLECULAR PHARMACOLOGY, 18, 304-312



# Effects of Phenobarbital, Pregnenolone-16α-Carbonitrile, and 3-Methylcholanthrene Pretreatments on the Distribution of NADPH-Cytochrome *c* (*P*-450) Reductase within the Liver Lobule

YOSHITSUGI TAIRA,[1] PHILLIP GREENSPAN,[2] GORDON F. KAPKE, JAN A. REDICK AND JEFFREY BARON[3]

*The Toxicology Center, Department of Pharmacology, College of Medicine, The University of Iowa, Iowa City, Iowa 52242*

Received February 11, 1980; Accepted May 6, 1980

SUMMARY

TAIRA, Y., P. GREENSPAN, G. F. KAPKE, J. A. REDICK AND J. BARON. Effects of phenobarbital, pregnenolone-16α-carbonitrile, and 3-methylcholanthrene pretreatments on the distribution of NADPH-cytochrome *c* (*P*-450) reductase within the liver lobule. *Mol. Pharmacol.* **18:** 304-312 (1980).

The effects of phenobarbital, pregnenolone-16α-carbonitrile, and 3-methylcholanthrene pretreatments on the distribution of NADPH-cytochrome *c* (*P*-450) reductase (NADPH: ferricytochrome oxidoreductase, EC 1.6.2.4) within the livers of male rats were investigated employing sheep antiserum produced against rat hepatic microsomal NADPH-cytochrome *c* reductase in indirect fluorescent antibody and unlabeled antibody peroxidase–antiperoxidase immunohistochemical staining techniques. In the livers of untreated and vehicle-pretreated rats, the extent of binding of the antireductase to hepatocytes was found to be similar within the centrilobular and midzonal regions of the lobule but significantly lower within the periportal regions of the lobule. Pretreatment of rats for 4 days with 3-methylcholanthrene did not result in alterations in either the pattern or the intensity of immunohistochemical staining for the reductase within the liver lobule. In the livers of rats which had been pretreated for 4 days with phenobarbital, microfluorometric analyses revealed a twofold increase in the extent of antireductase binding to hepatocytes within all regions of the lobule. In contrast to the effects of phenobarbital, pregnenolone-16α-carbonitrile markedly altered the pattern as well as the intensity of immunohistochemical staining for NADPH-cytochrome *c* (*P*-450) reductase within the liver lobule. After 4 days of pregnenolone-16α-carbonitrile pretreatment, there was a threefold increase in the extent of antireductase binding to hepatocytes within the periportal regions of the lobule, while the extent of antireductase binding to centrilobular and midzonal hepatocytes was increased by only 73 and 45%, respectively. Thus, in the livers of pregnenolone-16α-carbonitrile-pretreated rats, the antireductase bound to a similar extent to periportal and midzonal hepatocytes and to a significantly greater extent to centrilobular hepatocytes. These results demonstrate that, although both phenobarbital and pregnenolone-16α-carbonitrile induce NADPH-cytochrome *c* (*P*-450) reductase in hepatocytes throughout the liver lobule, phenobarbital appears to produce a uniform induction of the reductase within the lobule, while pregnenolone-16α-carbonitrile induces the reductase by significantly different extents within the three lobular regions.

## INTRODUCTION

The stimulatory effects of a multitude of drugs, carcinogens, steroids, and other chemicals on hepatic microsomal xenobiotic monooxygenations have been the subject of numerous investigations. It is now well recognized (1) that stimulation of hepatic microsomal monooxygenase activities produced by the prior administration of these substances results from an induction of the cytochromes *P*-450 which catalyze the monooxygenation reactions and, in most cases, of NADPH-cytochrome *c* (*P*-450) reductase, the enzyme which mediates the NADPH-dependent reduction of hepatic microsomal cytochromes

This investigation was supported by USPHS Grant 12675 from the National Institute of General Medical Sciences.

[1] Present address: Department of Pathology, Saga Medical School, Saga 840-01, Japan.

[2] Present address: Department of Pathology, Wake Forest University, Bowman Gray School of Medicine, Winston-Salem, N.C. 27109.

[3] Recipient of Research Career Development Award 00091 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

0026-895X/80/050304-09$02.00/0

*P*-450 (2). However, while PB[4] and PCN induce hepatic microsomal NADPH-cytochrome *c* (*P*-450) reductase (3–6), and while PB, PCN, and MC induce various forms of hepatic microsomal cytochrome *P*-450 (7–9) and stimulate the proliferation of smooth endoplasmic reticulum in hepatocytes (3, 6, 10–15), the effects of these and other xenobiotics on components of the microsomal monooxygenase enzyme systems within the different regions of the liver lobule have not been fully elucidated. Since highly reactive and toxic electrophilic metabolites are often formed during the cytochromes *P*-450-catalyzed monooxygenations of xenobiotics (16, 17), quantitative and qualitative differences in the inductions of cytochromes *P*-450 and NADPH-cytochrome *c* (*P*-450) reductase within the three regions of the liver lobule would profoundly influence the formation of these toxic metabolites within the different lobular regions and, thus, the location and severity of many types of xenobiotic-induced hepatotoxicities.

Employing fluorescent histochemical techniques, Wattenberg and Leong (18) demonstrated that MC produced the greatest enhancement of polycyclic aromatic hydrocarbon hydroxylation within the centrilobular regions of the liver lobule. Subsequently, Koudstaal and Hardonk (19) reported that histochemical staining for NADPH-tetrazolium reductase activity was increased most greatly within the centrilobular regions following the administration of PB to rats. More recently, Gooding *et al.* (20) employed a microspectrophotometric method to demonstrate that PB induces cytochrome *P*-450 to the greatest extent within centrilobular cells in rat liver. These findings are consistent with the observation that PB stimulates the proliferation of endoplasmic reticulum to the greatest extent within centrilobular hepatocytes (13, 15).

In an earlier study (21), we employed qualitative and quantitative immunohistochemical techniques to demonstrate that NADPH-cytochrome *c* (*P*-450) reductase exhibits a nonuniform pattern of distribution within the lobule in the livers of untreated rats. In the present study, these techniques have been used to investigate the responses of the reductase within the centrilobular, midzonal, and periportal regions of the liver lobule following the administration of PB and PCN, two chemicals which induce the hepatic microsomal enzymes (3–6), and MC, a xenobiotic which induces cytochrome *P*-450 but not NADPH-cytochrome *c* (*P*-450) reductase (5, 22).

## MATERIALS AND METHODS

*Chemicals.* Horse heart cytochrome *c* (Type VI), NADPH (Type III), nitroblue tetrazolium (Grade III), trisodium isocitrate (Type I), isocitrate dehydrogenase (Type IV), and MC were obtained from Sigmal Chemical Company. Ethylmorphine hydrochloride and sodium PB were purchased from Merck and Company, Inc. PCN was generously provided by The Upjohn Company. 7-Ethoxyresorufin was obtained from Pierce Chemical Company, and resorufin was purchased from Pfaltz and Bauer, Inc. All other chemicals and biochemicals employed were of the highest purity available.

*Animals and pretreatments.* Male albino Holtzman rats weighing 180–230 g were used in this study. Groups of rats were treated for 4 days with either PB (40 mg/kg/day, i.p., in saline), MC (20 mg/kg/day, i.p., in corn oil), or PCN (40 mg/kg every 12 h, p.o., in a solution containing 1 drop of Tween 80/10 ml water). Control rats were administered appropriate volumes of vehicle. Rats were fasted for 24 h prior to sacrifice by decapitation on the fifth day after initiation of treatment, and a portion of the median lobe was removed from each liver for immunohistochemical and histochemical analyses. After the remainder of each liver had been perfused with ice-cold saline, microsomes were prepared employing previously described methods (23).

*Immunohistochemical and histochemical procedures.* Details of the procedures for the immunohistochemical localization of NADPH-cytochrome *c* (*P*-450) reductase in liver sections using the indirect fluorescent antibody and the unlabeled antibody peroxidase–antiperoxidase staining techniques and for the histochemical demonstration of NADPH-tetrazolium reductase activity have been presented elsewhere (21). Following indirect fluorescent antibody staining, measurements of the intensity of fluorescence emitted at 525 nm from 2.5 × 2.5-μm areas on serial tissue sections which had been exposed to sheep antiserum to rat hepatic microsomal NADPH-cytochrome *c* reductase, normal (nonimmune) sheep serum, and adsorbed sheep antireductase serum were obtained and analyzed as described previously (21, 23, 24).

*Analytical procedures.* The NADPH-cytochrome *c* reductase activity catalyzed by hepatic microsomes was determined at 25°C as described previously (2, 23). The contents of cytochrome *P*-450 in the microsomal preparations were determined from carbon monoxide-difference spectra employing an extinction coefficient of 91 $mM^{-1}$ $cm^{-1}$ (25). Protein was determined by the microbiuret method (26) using bovine serum albumin as the standard.

The *N*-demethylation of ethylmorphine catalyzed by hepatic microsomes was determined by measuring the rate of formation of formaldehyde according to the method of Nash (27) as modified by Cochin and Axelrod (28). Incubations were performed in 25-ml Erlenmeyer flasks in a shaking bath at 25°C. Each 7-ml reaction mixture contained 14 mg of microsomal protein, 8 mM isocitrate, 8 mM ethylmorphine, 1 unit (0.28 mg) of isocitrate dehydrogenase, 150 mM KCl, 10 mM $MgCl_2$, and 50 mM Tris–HCl buffer, pH 7.4. After a 3-min preincubation, ethylmorphine *N*-demethylase activity was initiated by the addition of 200 μM NADPH, and 1-ml aliquots were removed every minute for the determination of formaldehyde.

The *O*-deethylation of 7-ethoxyresorufin catalyzed by hepatic microsomes prepared from corn oil- and MC-pretreated rats was determined fluorometrically at 25°C as described by Burke and Mayer (29). Each 2-ml reaction mixture contained 50–500 μg of microsomal protein, 100 mM sodium–potassium phosphate buffer, pH 7.8, and 0.25 μM 7-ethoxyresorufin. Changes in fluorescence were

[4] Abbreviations used: PB, phenobarbital; MC, 3-methylcholanthrene; PCN, pregnenolone-16α-carbonitrile.

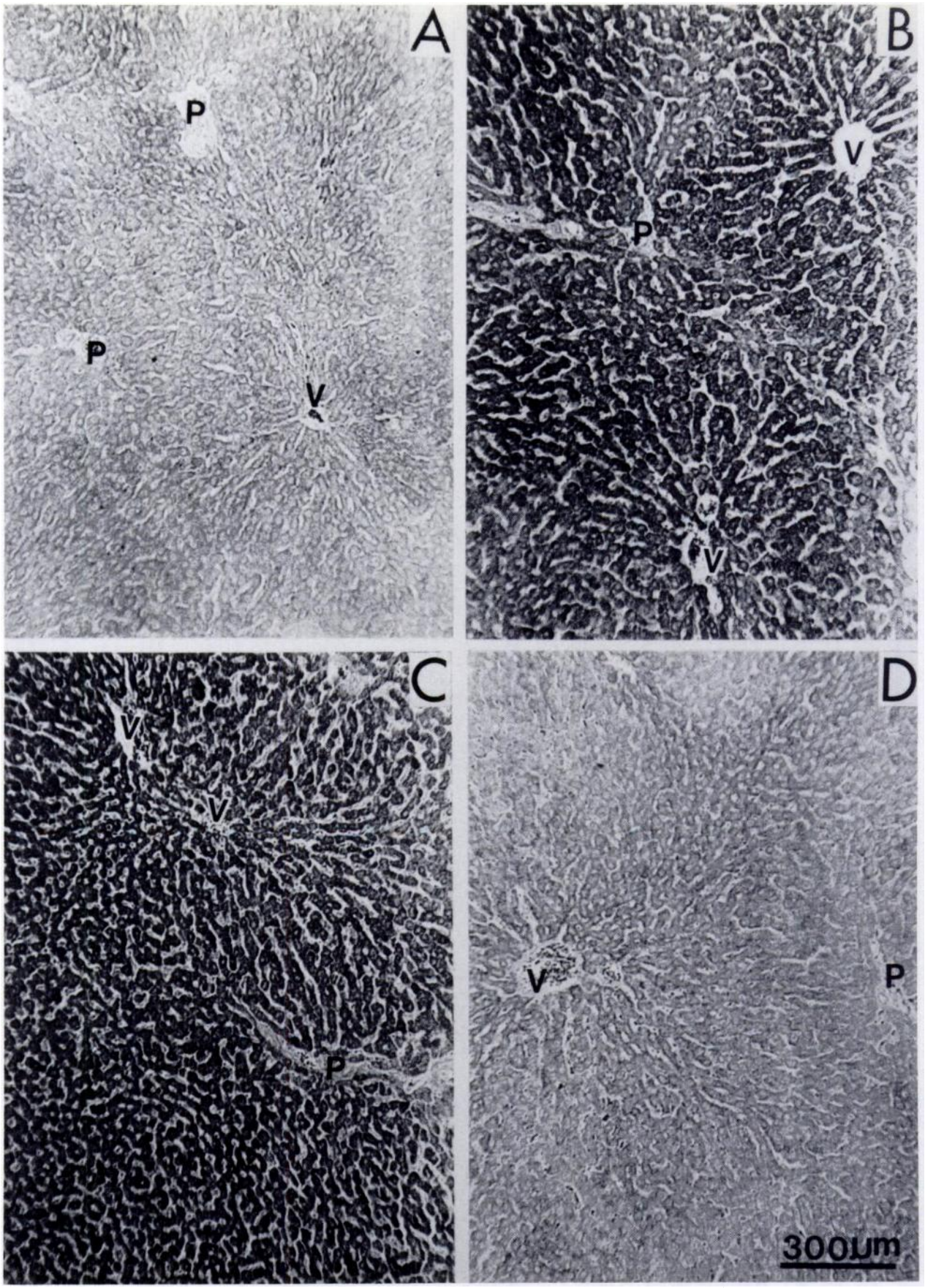


FIG. 1. *Immunohistochemical localization of NADPH-cytochrome c (P-450) reductase in livers of untreated and PB-, PCN-, and MC-treated rats*

The photomicrographs show areas in 5-µm-thick sections of livers from an untreated rat (A) and from rats pretreated with PB (B), PCN (C), and MC (D) which had been exposed to sheep antireductase serum in the unlabeled antibody peroxidase–antiperoxidase immunohistochemical staining procedure. Central veins (V) and portal triads (P) are indicated in each photomicrograph. 93×.

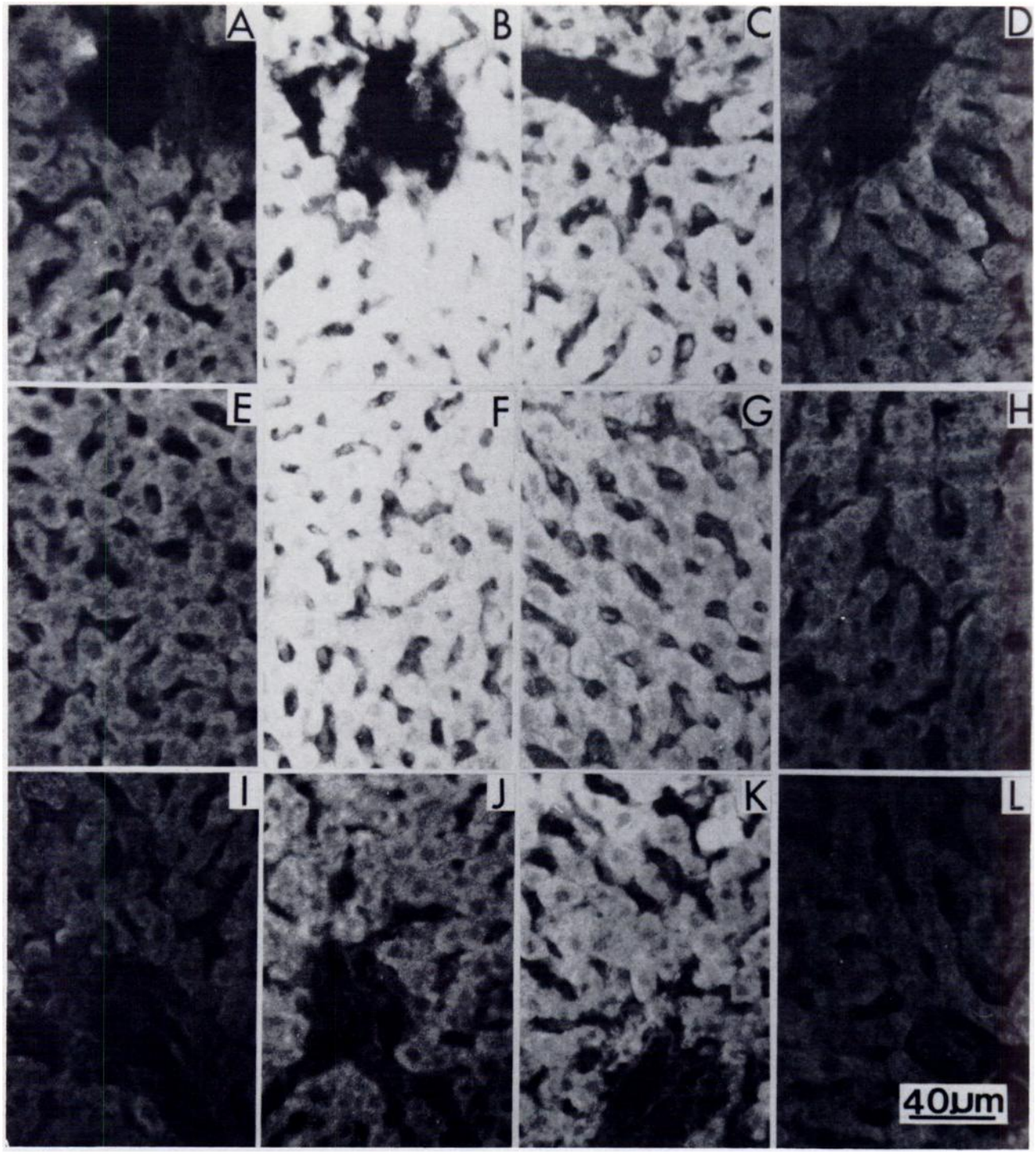


FIG. 2. *Effects of PB, PCN, and MC pretreatments on fluorescent immunohistochemical staining for NADPH-cytochrome c (P-450) reductase in the centrilobular (A–D), midzonal (E–H), and periportal (I–L) regions of rat liver*

The photomicrographs in A, E, and I show regions in a 7-μm-thick section of a liver from an untreated rat which had been exposed to sheep antireductase serum in the indirect fluorescent antibody immunohistochemical staining procedure. The photomicrographs in B, F, and J show the corresponding regions in a section of a liver from a PB-pretreated rat, those in C, G, and K show these regions in a section of a liver from a PCN-pretreated rat, and those in D, H, and L are from a section of a liver from a MC-pretreated rat. The photomicrographs are of typical areas from which the microfluorometric determinations presented in Table 1 and Fig. 4 were taken. 342×.

recorded at an excitation wavelength of 510 nm and an emission wavelength of 586 nm using an Aminco Bowman spectrophotofluorometer. 7-Ethoxyresorufin *O*-deethylase activity was initiated by the addition of 200 μM NADPH, and the progressive increase in fluorescence was monitored. For calibration of the fluorometer, after

the reaction had proceeded for approximately 2 min, 0.1 nmol of resorufin was added to the cuvette and the resulting increase in fluorescence was recorded.

## RESULTS

*Distribution of NADPH-cytochrome c (P-450) reductase in the livers of untreated and vehicle-pretreated rats.* When sections of livers from untreated male rats are exposed to sheep antiserum to rat hepatic microsomal NADPH-cytochrome *c* reductase in both the unlabeled antibody peroxidase–antiperoxidase and the indirect fluorescent antibody immunohistochemical staining procedures, hepatocytes throughout the liver are stained for the enzyme (Fig. 1A and Figs. 2A, E, and I). Measurements of the intensity of fluorescence emitted from 2.5 × 2.5-μm areas within the three lobular regions in sections after indirect fluorescent antibody staining reveal that, while the antireductase binds to the same extent to hepatocytes within the centrilobular and midzonal regions of the lobule, significantly less ($P < 0.05$) antibody binds to periportal hepatocytes (Table 1). The data presented in Table 1 are consistent with our earlier findings (21) and demonstrate further that antireductase binding to all hepatocytes is abolished by adsorption of the antibody with the purified rat hepatic microsomal enzyme.

Differences in the intensity of immunohistochemical staining for NADPH-cytochrome *c* (*P*-450) reductase observed within the liver lobule are not as striking as those seen for the intralobular distribution of histochemical staining for NADPH-tetrazolium reductase activity (Fig. 3A), an enzymatic activity which can be catalyzed, in part, by microsomal NADPH-cytochrome *c* (*P*-450) reductase (30).

Pretreatment of rats with the three vehicles used in this study, saline, corn oil, and Tween 80/water, had no effect ($P > 0.05$) on either NADPH-cytochrome *c* reductase activity catalyzed by isolated hepatic microsomes (Table 2), immunohistochemical staining for hepatic NADPH-cytochrome *c* (*P*-450) reductase (Fig. 4), or histochemical staining for hepatic NADPH-tetrazolium reductase activity (data not presented).

*Effect of PB pretreatment on NADPH-cytochrome c (P-450) reductase within the liver lobule.* The data presented in Table 2 show that the pretreatment of rats with PB for 4 days resulted in the induction of hepatic microsomal NADPH-cytochrome *c* reductase activity, cytochrome *P*-450, and ethylmorphine *N*-demethylase activity. At the same time, immunohistochemical staining for NADPH-cytochrome *c* (*P*-450) reductase (Fig. 1B and Figs. 2B, F, and J) and histochemical staining for NADPH-tetrazolium reductase activity (Fig. 3B) were both enhanced markedly in all regions of the liver lobule. In comparison to the extent of antireductase binding to hepatocytes within the three lobular regions in the livers of saline-pretreated rats, 105% more antibody bound to centrilobular and midzonal hepatocytes and 126% more antibody bound to periportal hepatocytes in the livers of rats which had been treated for 4 days with PB (Fig. 5). Thus, the intralobular pattern of distribution of the reductase was not affected significantly ($P > 0.05$) by the administration of PB (Fig. 4).

TABLE 1

*Binding of antibody to NADPH-cytochrome c reductase within the centrilobular, midzonal, and periportal regions in the livers of untreated rats*

Refer to Fig. 2 for the location of regions from which the microfluorometric measurements were taken. The values of relative fluorescence units given represent the mean ± SE of at least 20 measurements taken within the specified region. The relative extents of antireductase binding to hepatocytes within the three lobular regions were determined by subtracting the mean emitted fluorescence intensity from regions in sections exposed to normal sheep serum from each individual microfluorometric measurement obtained from corresponding regions in serial sections exposed to the sheep antireductase serum. The microfluorometric determinations were analyzed statistically using the group Student's *t* test.

| Region | Serum[a] | Emitted fluorescence | Antibody binding |
|---|---|---|---|
| Centrilobular | NSS | 42.90 ± 2.39* | |
| | SARS | 69.12 ± 3.45** | 25.81 ± 3.45**** |
| | A-SARS | 44.48 ± 3.97* | |
| Midzonal | NSS | 43.27 ± 4.35* | |
| | SARS | 68.96 ± 3.57** | 25.38 ± 3.57**** |
| | A-SARS | 44.42 ± 4.13* | |
| Periportal | NSS | 43.53 ± 3.33* | |
| | SARS | 56.57 ± 2.91*** | 12.84 ± 2.91***** |
| | A-SARS | 43.09 ± 3.22* | |

[a] NSS = normal sheep serum; SARS = sheep antireductase serum; A-SARS = adsorbed sheep antireductase serum.

* Values do not differ significantly from each other, $P > 0.05$.

** Significantly greater than corresponding values obtained within the centrilobular and midzonal regions using normal sheep serum and adsorbed sheep antireductase serum, $P < 0.01$.

*** Significantly greater than corresponding values obtained within the periportal region using normal sheep serum and adsorbed sheep antireductase serum, $P < 0.05$.

**** Values do not differ significantly from each other, $P > 0.05$.

***** Significantly lower than corresponding values from the centrilobular and midzonal regions, $P < 0.05$.

*Effect of PCN pretreatment on NADPH-cytochrome c (P-450) reductase within the liver lobule.* The administration of PCN to rats resulted in the induction of hepatic microsomal NADPH-cytochrome *c* reductase activity, cytochrome *P*-450, and ethylmorphine *N*-demethylase activity (Table 2). PCN also produced a marked enhancement of immunohistochemical staining for NADPH-cytochrome *c* (*P*-450) reductase (Fig. 1C and Figs. 2C, G, and K) and of histochemical staining for NADPH-tetrazolium reductase activity (Fig. 3C) within the liver. Visually, the effect of PCN appeared to be most pronounced within the periportal regions of the liver lobule. This observation was verified by microfluorometric analyses of liver sections from PCN- and Tween 80/water-pretreated rats after indirect fluorescent antibody staining (Figs. 4 and 5). The data presented in Fig. 5 show that while PCN pretreatment resulted in a 203% increase in the extent of antireductase binding to periportal hepatocytes, antibody binding to centrilobular and midzonal hepatocytes increased by only 73 and 45%, respectively. Thus, after pretreatment of rats with PCN for 4 days, the intralobular pattern of distribution of NADPH-cytochrome *c* (*P*-450) reductase is altered so that, while centrilobular hepatocytes bind antireductase

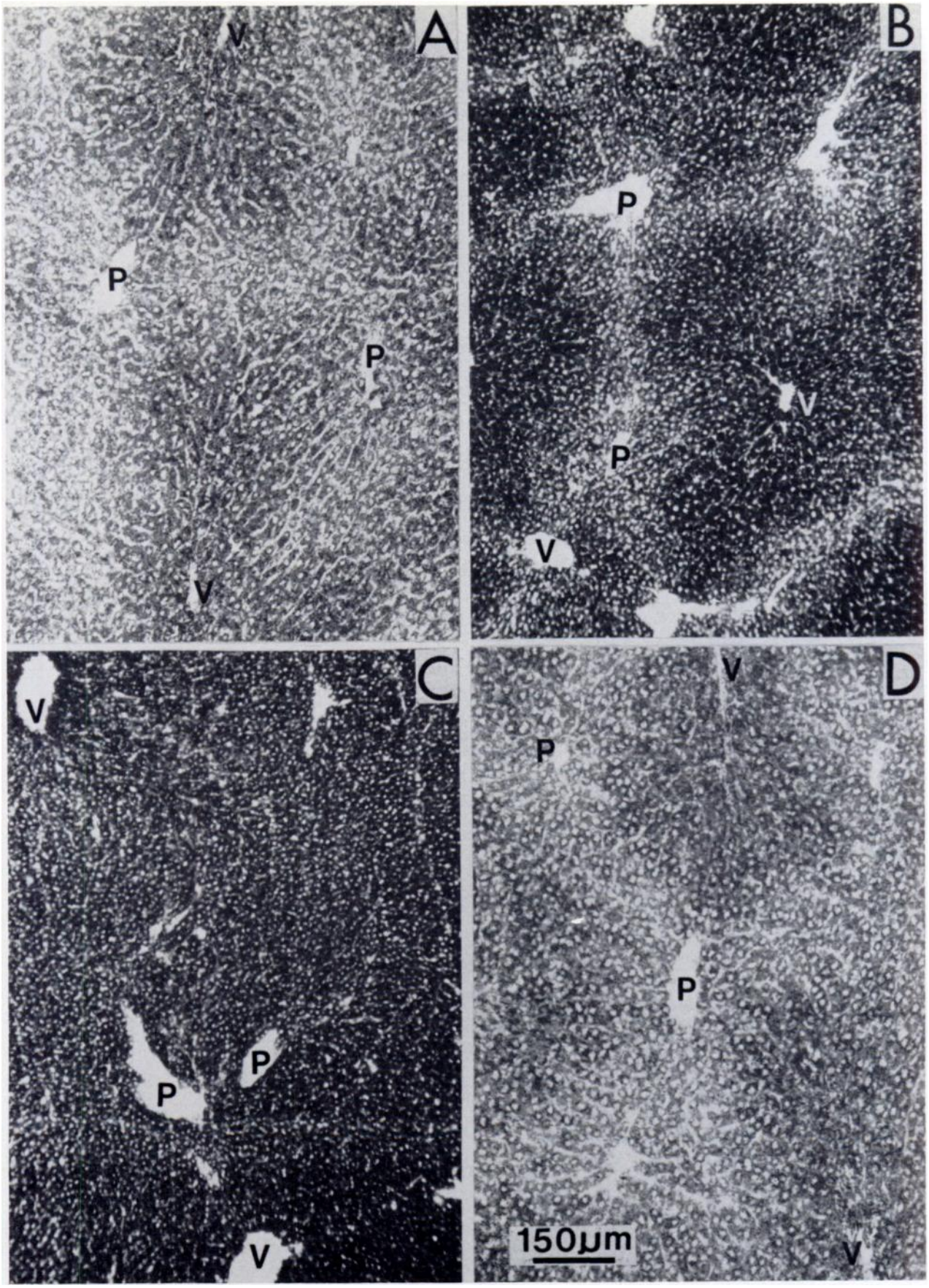


FIG. 3. *Effects of PB, PCN, and MC pretreatments on histochemical staining for NADPH-tetrazolium reductase activity in rat liver*

The photomicrographs show areas in 12-μm-thick sections of livers from untreated (A), PB-pretreated (B), PCN-pretreated (C), and MC-pretreated (D) rats after incubation with nitroblue tetrazolium and NADPH. Central veins (V) and portal triads (P) are indicated in each photomicrograph. 60×.

TABLE 2

*Cytochrome P-450 concentrations and NADPH-cytochrome c reductase, ethylmorphine N-demethylase, and 7-ethoxyresorufin O-deethylase activities in hepatic microsomes prepared from untreated and PB-, PCN-, MC-, and vehicle-pretreated rats*

Animals were pretreated and analytical determinations were performed as described in Materials and Methods. 7-Ethoxyresorufin *O*-deethylase activity was determined using hepatic microsomes prepared from MC- and corn oil-pretreated rats only. Each value given represents the mean ± SE of three experiments.

| Pretreatment | Cytochrome *P*-450 | NADPH-cytochrome *c* reductase | Ethylmorphine *N*-demethylase | 7-Ethoxyresorufin *O*-deethylase |
|---|---|---|---|---|
| | *nmol/mg protein* | | *nmol/min/mg protein* | |
| None | 0.91 ± 0.09 | 109.9 ± 6.8 | 5.41 ± 0.41 | — |
| PB | 2.28 ± 0.17* | 199.7 ± 9.2* | 14.30 ± 0.83* | — |
| Saline | 0.89 ± 0.02 | 106.4 ± 6.9 | 5.54 ± 0.35 | — |
| PCN | 2.17 ± 0.14* | 200.7 ± 7.5* | 15.60 ± 1.46* | — |
| Tween 80/water | 0.86 ± 0.02 | 117.2 ± 3.6 | 5.05 ± 0.28 | — |
| MC | 1.56 ± 0.04* | 108.9 ± 6.3*** | 4.84 ± 0.46*** | 5.92 ± 0.96** |
| Corn oil | 0.89 ± 0.04 | 106.3 ± 5.2 | 3.87 ± 0.51 | 0.20 ± 0.09 |

* $P < 0.001$ when compared to values in untreated and vehicle-pretreated controls.
** $P < 0.01$ when compared to values in untreated and corn oil-pretreated controls.
*** $P > 0.05$ when compared to values in untreated and corn oil-pretreated controls.

to the greatest extent ($P < 0.05$), midzonal and periportal hepatocytes bind the antireductase to similar extents (Fig. 4).

*Effect of MC pretreatment on NADPH-cytochrome c (P-450) reductase within the liver lobule.* The administration of MC to rats for 4 days resulted in the induction of hepatic microsomal cytochrome *P*-450 and 7-ethoxyresorufin *O*-deethylase activity without affecting hepatic microsomal NADPH-cytochrome *c* reductase or ethylmorphine *N*-demethylase activities (Table 2). In addition, MC pretreatment did not appear to affect either immunohistochemical staining for NADPH-cytochrome *c* (*P*-450) reductase (Fig. 1D and Figs. 2D, H, and L) or histochemical staining for NADPH-tetrazolium reductase activity (Fig. 3D) within the liver. These visual findings were confirmed when the extents of binding of the antireductase to hepatocytes within the three regions of the lobule were determined in sections of livers from MC- and corn oil-pretreated rats (Figs. 4 and 5).

## DISCUSSION

In a previous communication (21), we presented immunohistochemical evidence for the nonuniform intralobular distribution of NADPH-cytochrome *c* (*P*-450) reductase within the livers of untreated rats. The results of the present study confirm this earlier finding and demonstrate further that different xenobiotics exert characteristic effects on the reductase within hepatocytes in the three regions of the liver lobule. It must be cautioned, however, that antibodies produced against trypsin-solubilized, hepatic microsomal NADPH-cytochrome *c* reductase are also capable of interacting with the enzyme

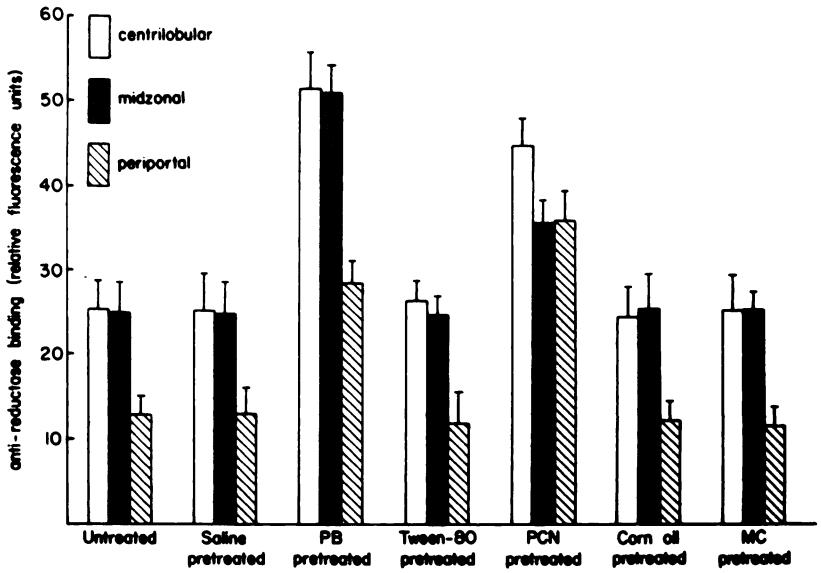


FIG. 4. *Binding of antibody to NADPH-cytochrome c reductase within the centrilobular, midzonal, and periportal regions in the livers of untreated and PB-, PCN-, MC-, and vehicle-pretreated rats*

Each bar represents the mean ± SE extent of antireductase binding within the specified lobular region. The data presented for the binding of the antireductase within the livers of untreated rats were taken from Table 1.

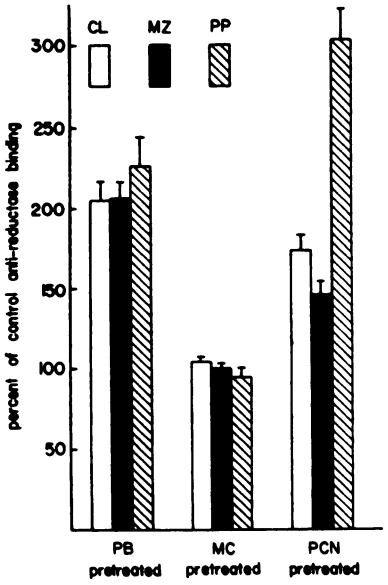


FIG. 5. *Effects of PB, PCN, and MC pretreatments on NADPH-cytochrome c (P-450) reductase within the centrilobular (CL), midzonal (MZ), and periportal (PP) regions of the liver lobule*

Each bar represents the mean ± SE percent of control antireductase binding within the specified lobular region. Percentage of control antibody binding was determined from the data presented in Fig. 4 for the binding of antireductase within regions in the livers of xenobiotic- and vehicle-pretreated rats.

present in the nuclear envelope and Golgi membranes in rat liver (31, 32). Thus, immunohistochemical staining for the reductase observed within hepatocytes is undoubtedly associated with these organelles as well as with the endoplasmic reticulum. Furthermore, some degree of immunohistochemical staining would also be associated with nascent reductase present in hepatocytes (33). While the presence of nascent enzyme could explain, in part, the discrepancies observed between immunohistochemical staining for NADPH-cytochrome *c* (*P*-450) reductase and histochemical staining for NADPH-tetrazolium reductase activity in the liver, our previous finding that antiserum to hepatic microsomal NADPH-cytochrome *c* reductase could not suppress completely histochemical staining for hepatic NADPH-tetrazolium reductase activity (21) indicates that the histochemical staining reaction is not totally specific for NADPH-cytochrome *c* (*P*-450) reductase.

Of the three xenobiotics employed in this study, only MC has been shown to induce hepatic microsomal cytochrome *P*-450 and xenobiotic monooxygenations, as well as to stimulate the proliferation of hepatocyte endoplasmic reticulum, without causing the concomitant induction of NADPH-cytochrome *c* (*P*-450) reductase (3, 5, 7, 8, 11, 12). The findings that neither immunohistochemical staining for the reductase nor histochemical staining for NADPH-tetrazolium reductase activity was altered as a result of MC pretreatment are consistent with biochemical evidence for the refractoriness of hepatocyte NADPH-cytochrome *c* (*P*-450) reductase to MC.

In contrast to the effects of MC and other polycyclic aromatic hydrocarbons, both PB and PCN induce hepatic microsomal NADPH-cytochrome *c* (*P*-450) reductase in addition to inducing cytochrome *P*-450 and xenobiotic monooxygenations and stimulating the proliferation of hepatocyte endoplasmic reticulum (3–15). PB and PCN pretreatments also result in the enhancement of immunohistochemical staining for NADPH-cytochrome *c* (*P*-450) reductase and of histochemical staining for NADPH-tetrazolium reductase activity in hepatocytes throughout the liver lobule. However, the results of this study demonstrate that the barbiturate and the steroid exert different effects on NADPH-cytochrome *c* (*P*-450) reductase within the liver lobule.

The results of our immunohistochemical analyses reveal that the reductase is induced by similar extents within centrilobular, midzonal, and periportal hepatocytes after 4 days of PB pretreatment. It is interesting to note that a very good correlation exists between the stimulatory effects of PB on the specific activity of NADPH-cytochrome *c* reductase in isolated hepatic microsomal membranes (an increase of 88 ± 9% over that in microsomes prepared from saline-pretreated rats) and on the binding of the antireductase to hepatocytes (increases of 105 ± 12, 105 ± 11, and 126 ± 18% to centrilobular, midzonal, and periportal hepatocytes, respectively). Thus, while the results of microspectrophotometric (20) and morphometric (13, 15) analyses have shown that cytochrome *P*-450 content and endoplasmic reticulum proliferation are most greatly stimulated by PB within centrilobular hepatocytes, these immunohistochemical findings indicate that after the administration of PB, the degree of induction of the reductase in hepatocytes does not parallel that of cytochrome *P*-450 or the degree of stimulation of endoplasmic reticulum proliferation.

Unlike the effects of PB on the reductase, PCN does not produce a uniform induction of the enzyme throughout the liver lobule. Rather, after 4 days of pretreatment with PCN, the reductase is induced by a much greater extent within periportal hepatocytes than within either centrilobular or midzonal hepatocytes. PCN pretreatment similarly results in the greatest enhancement of histochemical staining for NADPH-tetrazolium reductase activity within the periportal regions of the liver lobule. The differential induction of the reductase within centrilobular, midzonal, and periportal hepatocytes following the administration of PCN could be the consequence of a concentration gradient of PCN in blood as it flows from the portal triads toward the central veins. This would result in the exposure of cells more distal to the afferent blood to lower concentrations of the steroid. Although this possibility remains to be tested, it does not appear to be a viable explanation since the degree of induction of the reductase following PCN pretreatment is significantly greater in centrilobular hepatocytes than in midzonal hepatocytes. Alternatively, the results of this study can be interpreted to indicate that periportal hepatocytes are considerably more sensitive to the stimulatory action of PCN than are either centrilobular or midzonal hepatocytes, at least with respect to induction of NADPH-cytochrome *c* (*P*-450) reductase. These results suggest further that after PCN pretreatment, peri-

portal hepatocytes possess a greatly enhanced capacity to oxidatively metabolize xenobiotics. This suggestion is consistent with the protection afforded by PCN against the centrilobular necrosis which results from the cytochrome *P*-450-mediated formation of highly reactive and toxic electrophilic metabolites from certain xenobiotics (6).

## REFERENCES


1. Conney, A. H. Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* **19:** 317–366 (1967).
2. Masters, B. S. S., J. Baron, W. E. Taylor, E. L. Isaacson and J. LoSpalluto. Immunochemical studies on electron transport chains involving cytochrome P-450. I. Effects of antibodies to pig liver microsomal reduced triphosphopyridine nucleotide-cytochrome *c* reductase and the non-heme iron protein from bovine adrenocortical mitochondria. *J. Biol. Chem.* **246:** 4143–4150 (1971).
3. Ernster, L. and S. Orrenius. Substrate-induced synthesis of the hydroxylating enzyme system of liver microsomes. *Fed. Proc.* **24:** 1190–1199 (1965).
4. Kuriyama, Y., T. Omura, P. Siekevitz and G. E. Palade. Effects of phenobarbital on the synthesis and degradation of the protein components of rat liver microsomal membranes. *J. Biol. Chem.* **244:** 2017–2026 (1969).
5. Lu, A. Y. H., A. Somogyi, S. West, R. Kuntzman and A. H. Conney. Pregnenolone-16α-carbonitrile: A new type of inducer of drug-metabolizing enzymes. *Arch. Biochem. Biophys.* **152:** 457–462 (1972).
6. Tuchweber, G., J. Werringloer and P. Kourounakis. Effect of phenobarbital or pregnenolone-16α-carbonitrile (PCN) pretreatment on acute carbon tetrachloride hepatotoxicity in rats. *Biochem. Pharmacol.* **23:** 513–518 (1974).
7. Guengerich, F. P. Separation and purification of multiple forms of microsomal cytochrome P-450. Activities of different forms of cytochrome P-450 towards several compounds of environmental interest. *J. Biol. Chem.* **252:** 3970–3979 (1977).
8. Ryan, D. E., P. E. Thomas, D. Korzeniowski and W. Levin. Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. *J. Biol. Chem.* **254:** 1365–1374 (1979).
9. Birnbaum, L. S., M. B. Baird and H. R. Massie. Pregnenolone-16α-carbonitrile-inducible cytochrome P-450 in rat liver. *Res. Commun. Chem. Pathol. Pharmacol.* **15:** 553–562 (1976).
10. Remmer, H. and H. J. Merker. Effect of drugs on the formation of smooth endoplasmic reticulum and drug-metabolizing enzymes. *Ann. N.Y. Acad. Sci.* **123:** 79–96 (1965).
11. Fouts, J. R. and L. A. Rogers. Morphological changes in the liver accompanying stimulation of microsomal drug metabolizing enzyme activity by phenobarbital, chlordane, benzpyrene or methylcholanthrene in rats. *J. Pharmacol. Exp. Ther.* **147:** 112–119 (1965).
12. Garg, B. D., J. A. Blascheck and K. Kovacs. A comparative study on drug-induced proliferation of smooth-surfaced endoplasmic reticulum in hepatocytes. *Tohoku J. Exp. Med.* **104:** 205–214 (1971).
13. Wanson, J.-C., P. Drochmans, C. May, W. Penasse and A. Popowski. Isolation of centrolobular and perilobular hepatocytes after phenobarbital treatment. *J. Cell Biol.* **66:** 23–41 (1975).
14. Garg, B. D., P. Kourounakis, B. Tuchweber, S. Szabo and K. Kovacs. Studies on drug metabolism and liver ultrastructure after conjoint treatment with pregnenolone-16α-carbonitrile and dl-ethionine. *Arch. Int. Pharmacodyn. Ther.* **227:** 283–293 (1977).
15. Massay, E. D. and W. H. Butler. Zonal changes in the rat liver after chronic administration of phenobarbitone: An ultrastructural, morphometric and biochemical correlation. *Chem. Biol. Interact.* **24:** 329–344 (1979).
16. Jerina, D. M. and J. W. Daly. Arene oxides: A new aspect of drug metabolism. *Science* **185:** 573–582 (1974).
17. Heidelberger, C. Chemical carcinogenesis. *Annu. Rev. Biochem.* **44:** 79–121 (1975).
18. Wattenberg, L. W. and J. L. Leong. Histochemical demonstration of reduced pyridine nucleotide dependent polycyclic hydrocarbon metabolizing systems. *J. Histochem. Cytochem.* **10:** 412–420 (1962).
19. Koudstaal, J. and M. J. Hardonk. Histochemical demonstration of enzymes related to NADPH-dependent hydroxylating systems in rat liver after phenobarbital treatment. *Histochemie* **20:** 68–77 (1969).
20. Gooding, P. E., J. Chayen, B. Sawyer and T. F. Slater. Cytochrome P-450 distribution in rat liver and the effect of sodium phenobarbitone administration. *Chem. Biol. Interact.* **20:** 299–310 (1978).
21. Taira, Y., J. A. Redick and J. Baron. An immunohistochemical study on the localization and distribution of NADPH-cytochrome *c* (P-450) reductase in rat liver. *Mol. Pharmacol.* **17:** 374–381 (1980).
22. von der Decken, A. and T. Hultin. Inductive effects of 3-methylcholanthrene on enzyme activities and amino acid incorporation capacity of rat liver microsomes. *Arch. Biochem. Biophys.* **90:** 201–207 (1960).
23. Taira, Y., J. A. Redick, P. Greenspan and J. Baron. Immunohistochemical studies on electron transport proteins associated with cytochromes P-450 in steroidogenic tissues. II. Microsomal NADPH-cytochrome *c* reductase in the rat adrenal. *Biochim. Biophys. Acta* **583:** 148–158 (1979).
24. Baron, J., J. A. Redick, G. F. Kapke and L. S. Van Orden, III. Immunohistochemical localization of adrenal ferredoxin and distribution of adrenal ferredoxin and cytochrome P-450 in rat adrenal. *Biochim. Biophys. Acta* **540:** 443–454 (1978).
25. Omura, T. and R. Sato. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J. Biol. Chem.* **239:** 2379–2385 (1964).
26. Itzhaki, R. F. and D. M. Gill. A micro-biuret method for estimating proteins. *Anal. Biochem.* **9:** 401–410 (1964).
27. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55:** 416–421 (1953).
28. Cochin, J. and J. Axelrod. Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine. *J. Pharmacol. Exp. Ther.* **125:** 105–110 (1959).
29. Burke, M. D. and R. T. Mayer. Ethoxyresorufin: Direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab. Dispos.* **2:** 583–588 (1974).
30. Roerig, D. L., L. Mascaro and S. D. Aust. Microsomal electron transport: Tetrazolium reduction by rat liver microsomal NADPH-cytochrome *c* reductase. *Arch. Biochem. Biophys.* **153:** 475–479 (1972).
31. Sagara, Y., T. Harano and T. Omura. Characterization of electron transport enzymes in the envelope of rat liver nuclei. *J. Biochem. (Tokyo)* **83:** 807–812 (1978).
32. Hino, Y., A. Asano and R. Sato. Biochemical studies on rat liver Golgi apparatus. II. Further characterization of isolated Golgi fraction. *J. Biochem. (Tokyo)* **83:** 925–934 (1978).
33. Negishi, M., T. Sawamura, T. Morimoto and Y. Tashiro. Localization of nascent NADPH-cytochrome *c* reductase in rat liver microsomes. *Biochim. Biophys. Acta* **381:** 215–220 (1975).


**Send reprint requests to:** Jeffrey Baron, The Toxicology Center, Department of Pharmacology, College of Medicine, The University of Iowa, Iowa City, Iowa 52242.