MOLECULAR PHARMACOLOGY, 17, 374–381

An Immunohistochemical Study on the Localization and Distribution of NADPH–Cytochrome *c* (*P*-450) Reductase in Rat Liver

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Received October 14, 1979; Accepted December 21, 1979

SUMMARY

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).

The localization and distribution of NADPH–cytochrome *c* (*P*-450) reductase (NADPH: ferricytochrome oxidoreductase; EC 1.6.2.4) were investigated immunohistochemically in the livers of untreated rats employing both an unlabeled antibody peroxidase–antiperoxidase technique and an indirect fluorescent antibody method. The immunohistochemical localization of the reductase was accomplished through the use of sheep antiserum produced against NADPH–cytochrome *c* reductase which had been purified from hepatic microsomes of phenobarbital-pretreated rats. In both immunohistochemical procedures, staining for the reductase was detected in hepatocytes throughout the liver, although differences were noted in the intensity of immunostaining of hepatocytes within the liver lobule. Employing the indirect fluorescent antibody immunohistochemical staining method, the extents of binding of the antireductase to hepatocytes within the three regions of the liver lobule were determined. While hepatocytes within the centrilobular and midzonal regions bound the antireductase to similar extents, hepatocytes within the periportal regions of the lobule bound significantly less antireductase. Similar patterns of intralobular distribution for the antireductase were observed in the livers of male and female rats. Further, similar patterns of immunohistochemical staining for the reductase within the liver lobule were observed in the right, median, left, and caudate lobes. The results of this study thus demonstrate that NADPH–cytochrome *c* (*P*-450) reductase is not distributed uniformly throughout the liver lobule.

INTRODUCTION

In the endoplasmic reticulum of mammalian hepatocytes, the flavoprotein, NADPH–cytochrome *c* reductase, mediates the reduction of cytochromes *P*-450 (1) and is required for the cytochromes *P*-450-catalyzed monooxygenations of a wide variety of endogenous and exogenous substances including drugs, steroids, carcinogens, and fatty acids (1–4). While a considerable amount of evidence has been presented for the heterogeneous distributions of cytochromes *P*-450 and NADPH–cytochrome *c* reductase, as well as of certain other enzymes, in isolated hepatic microsomal membranes (5–11), the distributions of the components of the microsomal monooxygenase enzyme systems within the liver lobule have not been investigated as extensively.

Employing fluorescent histochemical techniques, Wattenberg and Leong (12) demonstrated that several polycyclic aromatic hydrocarbons were hydroxylated most extensively within the centrilobular regions in the livers of untreated rats. Subsequently, morphometric analyses showed that, while rough endoplasmic reticulum is distributed fairly uniformly throughout the liver lobule, hepatocytes within the centrilobular regions in the livers of untreated rats contain significantly more smooth endoplasmic reticulum than do parenchymal cells within the midzonal and periportal regions of the lobule (13). Consistent with these observations, histochemical staining for NADPH–tetrazolium reductase activity, an enzymatic activity which can be catalyzed by hepatic microsomal NADPH–cytochrome *c* reductase (14), has been reported (15) to be localized predominantly within the centrilobular regions in livers of untreated rats. More recently, Gooding *et al.* (16) employed a microspectrophotometric method to demonstrate that hepatocytes within the centrilobular regions in livers of untreated rats contain up to twice as much cytochrome *P*-450 as do those within the periportal regions of the lobule.

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

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0026-895X/80/030374-08$02.00/0

While these findings suggest that the components of the microsomal monooxygenase systems are present at the greatest concentrations within centrilobular hepatocytes, the exact distributions of these enzymes within the liver lobule remain unknown. In order to determine the distributions of these enzymes within the liver and other tissues, this laboratory has developed immunohistochemical techniques with which cytochromes *P*-450 and their associated enzymes can be localized (17–20). In the present study, antiserum produced against purified rat hepatic microsomal NADPH–cytochrome *c* reductase was employed in unlabeled antibody peroxidase–antiperoxidase and indirect fluorescent antibody immunohistochemical staining procedures to investigate the localization and distribution of NADPH–cytochrome *c* (*P*-450) reductase in the livers of untreated rats.

MATERIALS AND METHODS

Materials. Nitro blue tetrazolium (Grade III) and NADPH (Type III) were obtained from Sigma Chemical Company. Parabenzoquinone was purchased from Polysciences, Inc., and 3,3′-diaminobenzidine tetrahydrochloride was obtained from Hach Chemical Company. Normal (nonimmune) sheep serum, the soluble sheep peroxidase–antiperoxidase complex, rabbit antiserum to sheep immunoglobulin G (IgG), and fluorescein isothiocyanate conjugates of IgG prepared from rabbit antiserum to sheep IgG were obtained from Miles Laboratories, Inc. All other chemicals and biochemicals used were of the highest purity available.

Immunohistochemical procedures. NADPH–cytochrome *c* reductase, solubilized by tryptic digestion of hepatic microsomes prepared from phenobarbital-pretreated rats, was purified to homogeneity employing minor modifications (20) of the method described by Omura and Takesue (21). Sheep antiserum to the reductase was obtained as described previously (20). Whole sheep antireductase serum was employed in the immunohistochemical studies except for certain control experiments in which the antibody to the reductase was adsorbed with purified rat hepatic microsomal NADPH–cytochrome *c* reductase (20).

Male and female albino Holtzman rats weighing 180–230 g were used in these studies and were allowed food and water *ad libitum*. Rats were killed by decapitation, the livers were immediately excised, and the right, median, left, and caudate lobes were cut into blocks approximately 2 mm in thickness. The liver blocks were fixed at 4°C for a total period of 4 h by immersion in several changes of a solution containing 0.5% (w/v) parabenzoquinone and 0.02 M $CaCl_2$ in 0.2 M sodium cacodylate buffer, pH 7.4. After fixation, the blocks were embedded in paraffin, and serial sections 7 μm in thickness were prepared.

The immunohistochemical localization of NADPH–cytochrome *c* (*P*-450) reductase in rat liver was accomplished employing the following minor modifications of the unlabeled antibody peroxidase–antiperoxidase technique (17–20, 22) and the indirect fluorescent antibody method (17, 20). In the unlabeled antibody peroxidase–antiperoxidase immunohistochemical staining technique, after the endogenous peroxidase activity of the tissue had been blocked (17–20), the sections were exposed for 1 h to 10% (v/v) dimethylsulfoxide prior to the performance of the staining protocol. In the indirect fluorescent antibody immunohistochemical staining procedure, the sections were exposed similarly to dimethylsulfoxide, and after exposure to either the sheep antiserum to rat hepatic microsomal NADPH–cytochrome *c* reductase, normal sheep serum, or the adsorbed antireductase serum (each of which had been diluted 1:500 with 0.05 M Tris–HCl buffer, pH 7.75, containing 0.154 M NaCl), the sections were exposed for 2 h at 37°C to fluorescein isothiocyanate-conjugated IgG of rabbit antiserum to sheep IgG which had been diluted 1:100 with Tris-buffered saline. The liver sections were examined under a modified Leitz Orthoplan microscope (17, 20), and measurements of the intensity of fluorescence emitted at 525 nm from 2.5 × 2.5-μm areas on the tissue sections were obtained as described previously (17, 20). The relative extents of binding of the antireductase to hepatocytes within the three regions of the liver lobule 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 antiserum to the reductase. The microfluorometric determinations were analyzed statistically using the group Student's *t* test.

Histochemical staining for NADPH–tetrazolium reductase activity. NADPH–tetrazolium reductase activity was demonstrated histochemically employing the method of Farber and Louviere (23) as modified by Burstone (24). Fresh, frozen sections of liver, 20 μm in thickness, were incubated for 40 min at 37°C in 3 ml of a solution containing 0.88 mM NADPH, 0.28 mM nitro blue tetrazolium, and 0.03 M sodium barbital buffer, pH 7.4. In some instances NADPH was omitted from the incubation mixture, while in other instances the tissue sections were exposed to the sheep antireductase serum prior to performance of the histochemical staining procedure.

RESULTS AND DISCUSSION

Recently, we reported (18, 20) that an inhibitory antibody produced against trypsin-solubilized, rat hepatic microsomal NADPH–cytochrome *c* reductase could be used to localize the flavoprotein immunohistochemically in rat liver as well as in the adrenal cortex. The localization of the reductase at the light microscopic level was accomplished employing both a modification of the unlabeled antibody peroxidase–antiperoxidase technique described by Sternberger *et al.* (22) and an indirect fluorescent antibody method. In the peroxidase–antiperoxidase immunohistochemical staining technique, after the antireductase has interacted with and bound to NADPH–cytochrome *c* (*P*-450) reductase present on the tissue section, it is coupled to a peroxidase–antiperoxidase complex. The subsequent exposure of 3,3′-diaminobenzidine and H_2O_2 to this complex results in the oxidative polymerization of 3,3′-diaminobenzidine to form an insoluble product which, when chelated with OsO_4, can

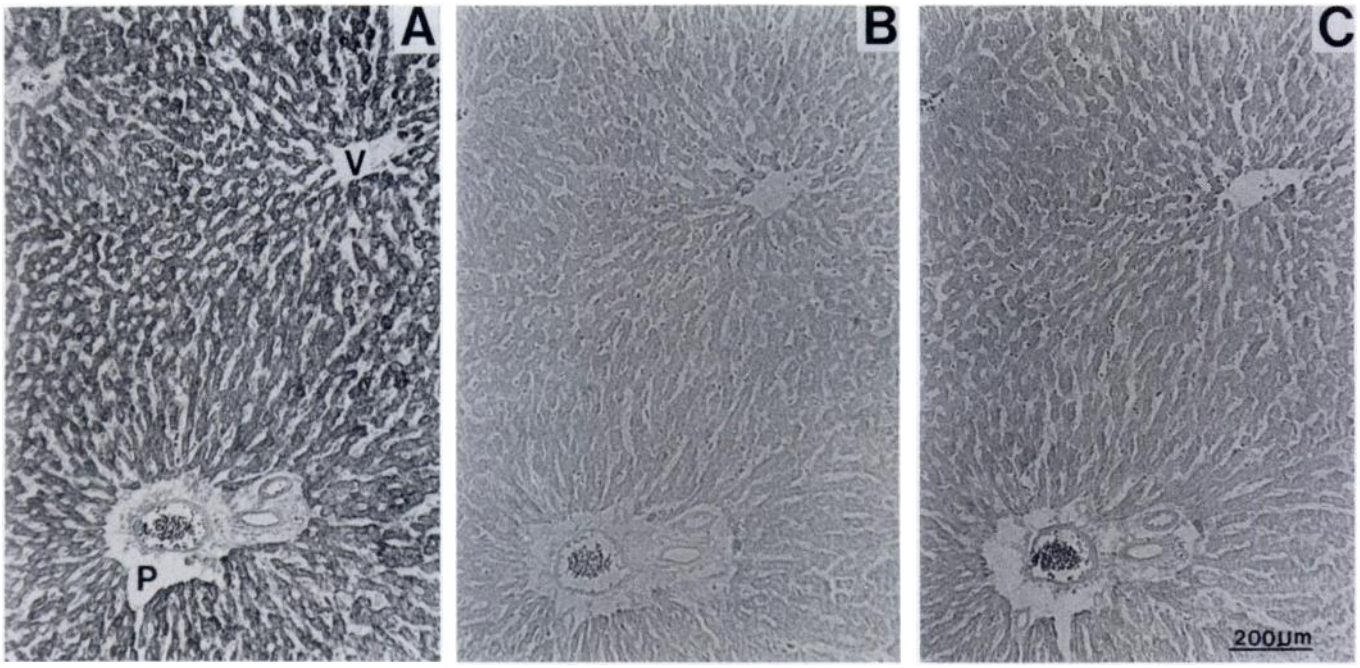

FIG. 1. *Immunohistochemical localization of NADPH–cytochrome c (P-450) reductase in the median lobe of rat liver*

The photomicrographs in the three panels show the same areas in serial sections of a liver of an untreated male rat. (A) A section which had been exposed to sheep antiserum to rat hepatic microsomal NADPH–cytochrome *c* reductase. (B) The corresponding area in a section which had been exposed to normal sheep serum. (C) A section which had been exposed to the adsorbed sheep antireductase serum. The antiserum, normal serum, and adsorbed antiserum had each been diluted 1:500 with 0.05 M Tris–HCl buffer, pH 7.75, containing 0.154 M NaCl. A central vein (V) and a portal triad (P) are seen in the upper right-hand and lower left-hand areas, respectively, in each photomicrograph. The photomicrographs in B and C have been overdeveloped slightly to accentuate the tissue morphology. 80×.

be seen easily under the light microscope as a brownish-black, particulate deposit at the site of the antibody complex (25). Representative findings on the localization of NADPH–cytochrome *c* (*P*-450) reductase in rat liver obtained using this immunohistochemical staining technique are presented in Figs. 1 and 2. When sections of livers of untreated male rats which had been exposed to the sheep antireductase serum were examined by transmitted light microscopy, immunohistochemical staining for NADPH–cytochrome *c* (*P*-450) reductase was evident in hepatocytes throughout the liver (Figs. 1A and 2A, D, and G). Although hepatocytes were stained intensely for NADPH–cytochrome *c* (*P*-450) reductase, immunostaining for the flavoprotein was not apparent in the connective tissue lying within portal areas (Figs. 1A and 2G). Since antibodies produced against the trypsin-solubilized, hepatic microsomal enzyme have been shown to be capable of interacting with NADPH–cytochrome *c* (*P*-450) reductase present in the nuclear envelope (26) and the Golgi fraction (27, 28) of rat liver, immunohistochemical staining for the reductase observed within hepatocytes (Figs. 2A, D, and G) is undoubtedly associated with these organelles in addition to the endoplasmic reticulum. Although the data are not presented, results which are essentially identical to those seen in Figs. 1 and 2 were obtained using livers of untreated female rats and of rats which had been fasted for 24 h prior to sacrifice. Further, comparable observations were made when the liver tissue had been fixed by perfusion *in situ* rather than by immersion.

To demonstrate the specificity of immunohistochemical staining for NADPH–cytochrome *c* (*P*-450) reductase using the unlabeled antibody peroxidase–antiperoxidase technique, two approaches were taken. In the first approach, the sheep antireductase serum was replaced with normal sheep serum during the staining procedure. As can be seen in the photomicrographs in Figs. 1B and 2B, E, and H, immunostaining for the reductase was not apparent in hepatocytes in sections which had been exposed to normal sheep serum. The second approach used to demonstrate the specificity of immunohistochemical staining was to substitute the adsorbed sheep antiserum for the antireductase serum in the staining protocol. The photomicrographs in Figs. 1C and 2C, F, and I show that this substitution resulted in a very marked decrease in the intensity of staining for NADPH–cytochrome *c* (*P*-450) reductase throughout the liver.

Visually, the intensity of immunohistochemical stain-

FIG. 2. *Immunohistochemical localization of NADPH–cytochrome c (P-450) reductase in the centrilobular, midzonal, and periportal regions in the median lobe of rat liver*

The photomicrographs in A, D, and G show regions in a section of a liver of an untreated male rat which had been exposed to sheep antiserum to the reductase. The photomicrographs in B, E, and H show the corresponding regions in a serial section which had been exposed to normal sheep serum, while those in E, F, and I show these regions in a serial section which had been exposed to the adsorbed sheep antireductase serum. The centrilobular region is seen in A, B, and C, the midzonal region is seen in D, E, and F, and the periportal region is seen in G, H, and I. 450×.

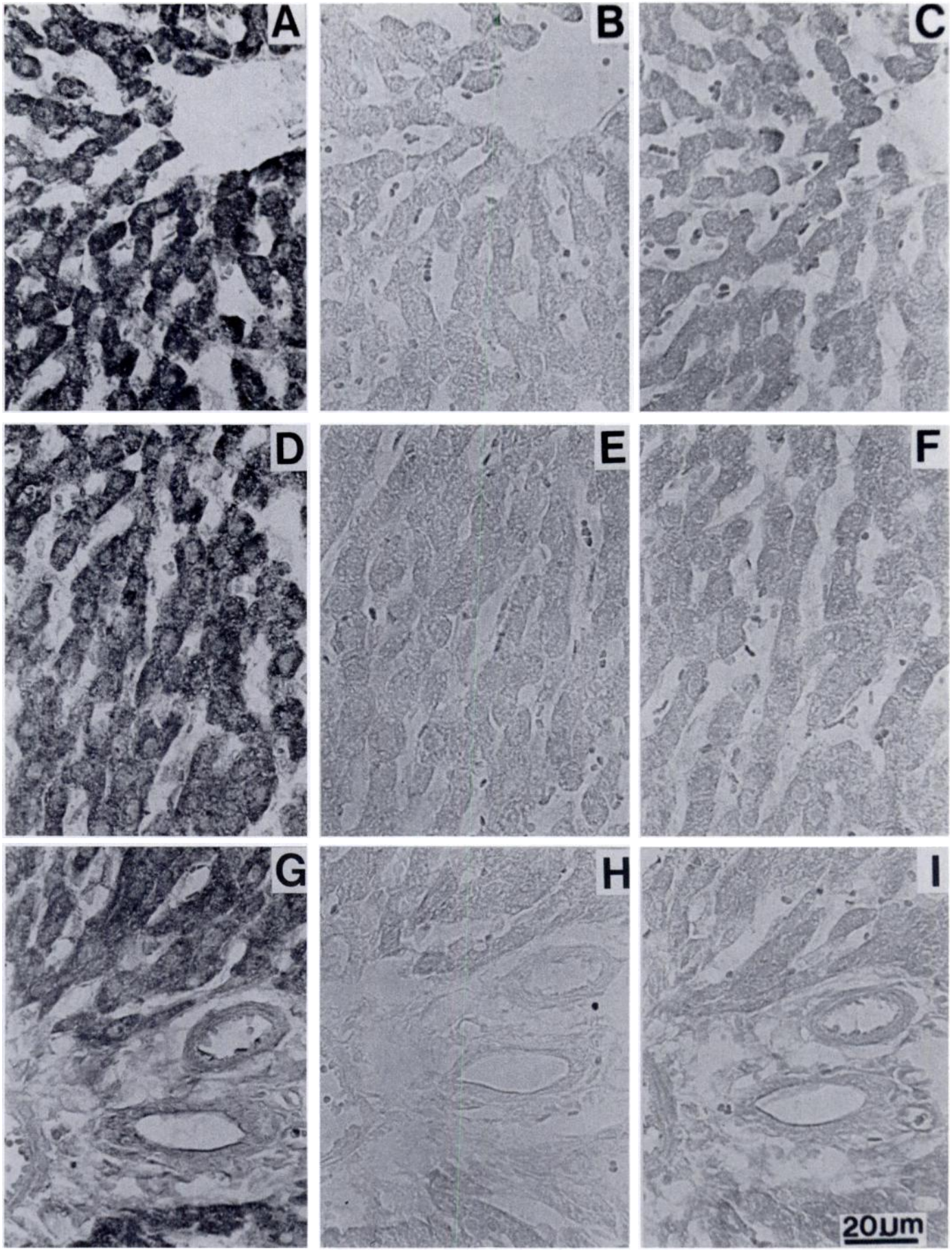

FIG. 2

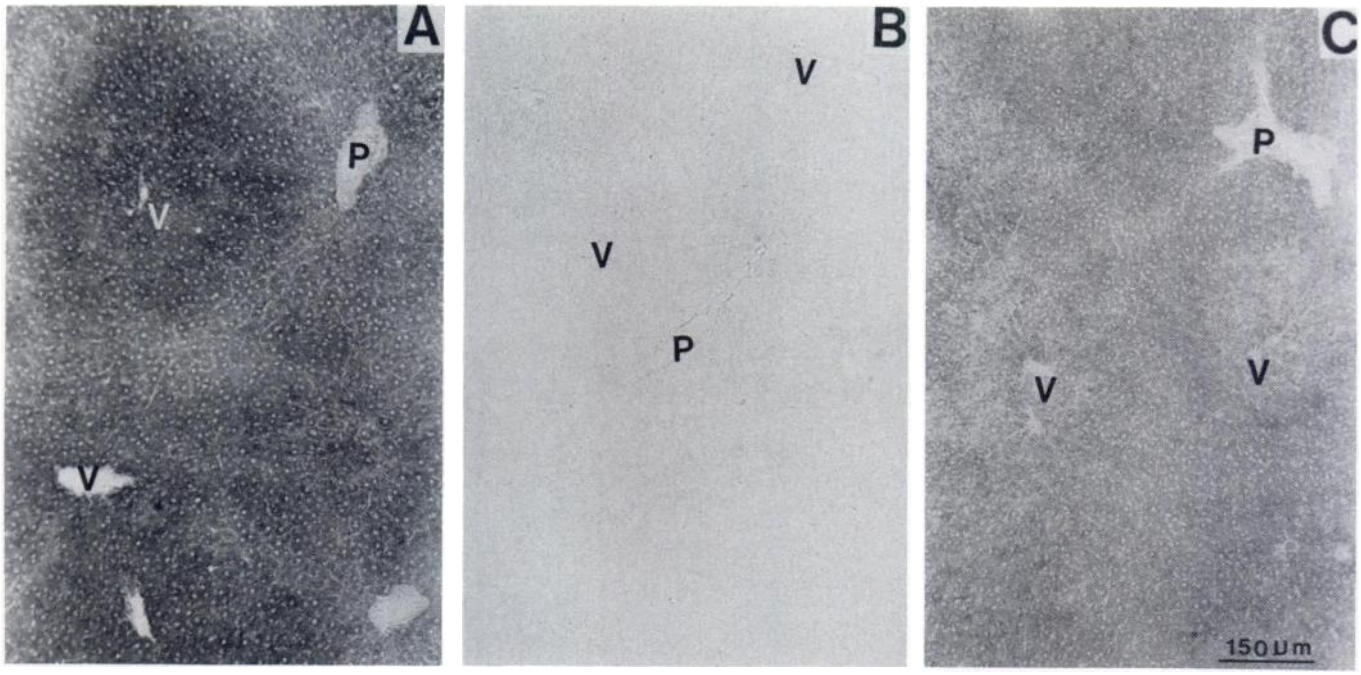

FIG. 3. *Histochemical demonstration of NADPH-tetrazolium reductase activity in the median lobe of rat liver*

The photomicrograph in A is of a section of a liver of an untreated male rat after incubation in the presence of nitro blue tetrazolium and NADPH. The section shown in B had been incubated with nitro blue tetrazolium in the absence of NADPH. The section shown in C had been exposed for 1 h at 37°C to sheep antiserum to NADPH-cytochrome *c* reductase prior to incubation with nitro blue tetrazolium and NADPH. The sheep antireductase serum had been diluted 1:20 with 0.05 M Tris-HCl buffer, pH 7.75, containing 0.154 M NaCl. This dilution was found to produce the greatest suppression of histochemical staining for NADPH-tetrazolium reductase activity. Although not shown, exposure of liver sections to normal sheep serum did not affect histochemical staining for NADPH-tetrazolium reductase activity. 50×.

ing for NADPH-cytochrome *c* (*P*-450) reductase produced using the unlabeled antibody peroxidase-antiperoxidase technique appears to be fairly uniform throughout the liver lobule (Figs. 1A and 2A, D, and G). Similar results have been obtained using wide ranges of dilutions of the sheep antireductase serum in the immunohistochemical staining procedure. This finding is in marked contrast to the pattern of histochemical staining for NADPH-tetrazolium reductase activity observed within the lobule in the livers of untreated rats (Fig. 3A). Consistent with the findings of Koudstaal and Hardonk (15), the photomicrograph shown in Fig. 3A demonstrates that much less intense staining for NADPH-tetrazolium reductase activity is produced within the periportal regions of the lobule than within either the midzonal or the centrilobular regions. The photomicrograph in Fig. 3C shows that the prior exposure of the liver section to the sheep antiserum to hepatic microsomal NADPH-cytochrome *c* reductase resulted in the suppression of histochemical staining for NADPH-tetrazolium reductase activity. However, suppression of histochemical staining beyond that seen in this photomicrograph could not be produced, even in the presence of far greater concentrations of the antireductase. Although microsomal NADPH-cytochrome *c* (*P*-450) reductase can catalyze the NADPH-dependent reduction of tetrazolium dyes (14), other hepatic enzymes, such as the NADPH-dependent flavoprotein dehydrogenase (hepatoredoxin reductase) associated with mitochondrial cytochrome *P*-450 (29) and certain NADH-linked dehydrogenases which would be supplied with NADH via mitochondrial transhydrogenase activity, could also participate in this histochemical staining reaction. Moreover, these enzymatic activities may not be distributed uniformly throughout the liver lobule, a phenomenon which has been demonstrated histochemically for a number of enzymes (15, 30–32). The observed inability of the antibody to NADPH-cytochrome *c* reductase to suppress completely histochemical staining for NADPH-tetrazolium reductase activity is consistent with this explanation for the discrepancy between the immunohistochemical and histochemical observations.

Since the unlabeled antibody peroxidase-antiperoxidase immunohistochemical staining technique does not lend itself readily to quantitation, the localization and distribution of NADPH-cytochrome *c* (*P*-450) reductase

FIG. 4. *Fluorescent immunohistochemical localization of NADPH-cytochrome c (P-450) reductase in the centrilobular (A, B, and C), midzonal (D, E, and F), and periportal (G, H, and I) regions in the median lobe of rat liver*

The photomicrographs in A, D, and G show regions in a section of a liver of an untreated male rat which had been exposed to the sheep antiserum to rat hepatic microsomal NADPH-cytochrome *c* reductase. The photomicrographs in B, E, and H show the corresponding regions in a serial section which had been exposed to normal sheep serum, while those in C, F, and I show these regions in a serial section which had been exposed to the adsorbed sheep antireductase serum. The photomicrographs are of typical areas from which the microfluorometric measurements presented in Tables 1 and 2 were taken. 560×.

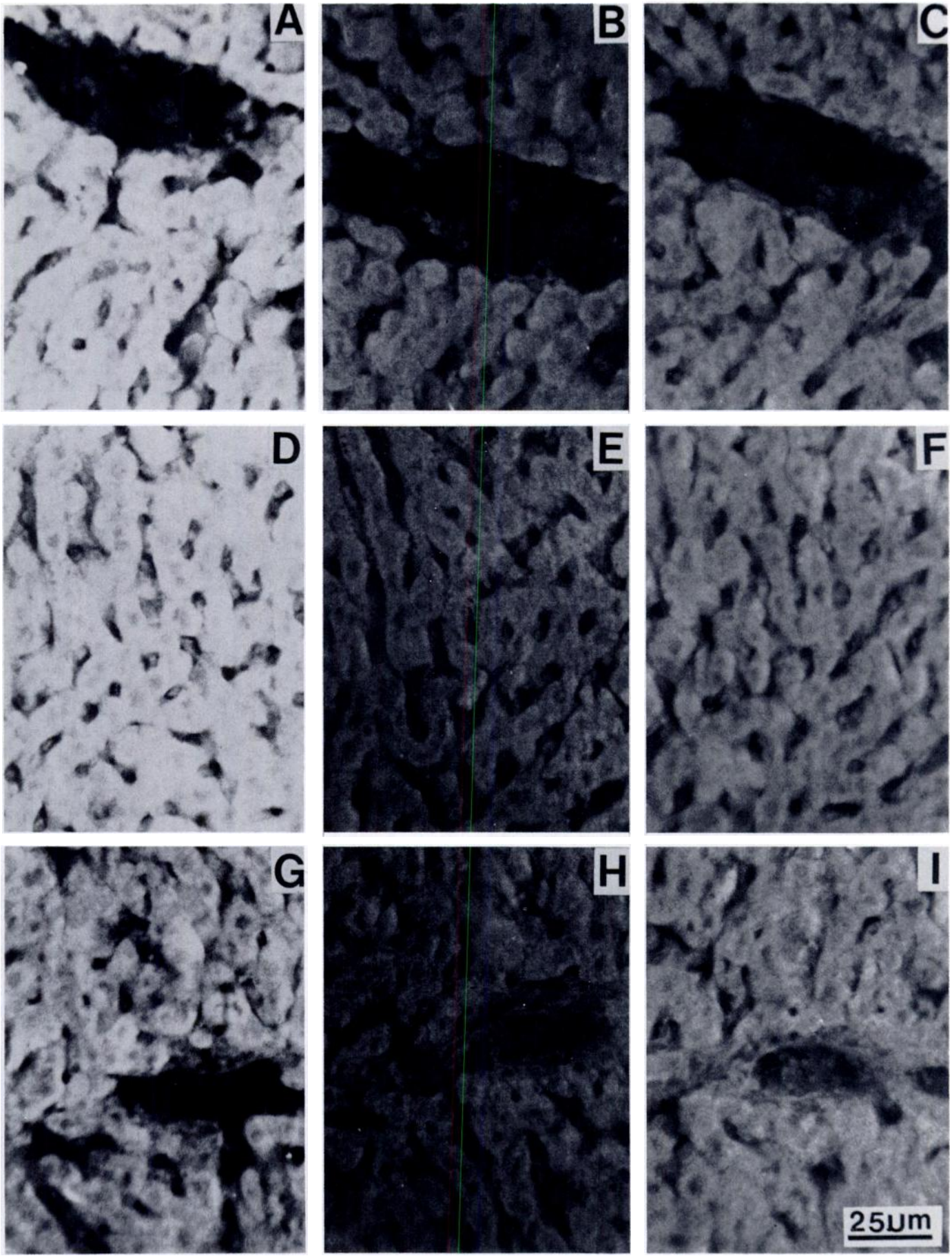

FIG. 4

within the heaptic lobule were investigated further by means of an indirect fluorescent antibody staining method (17, 20). In this immunohistochemical staining technique, after the sheep antireductase has bound to NADPH–cytochrome *c* (*P*-450) reductase present on the tissue section, it is coupled to fluorescein isothiocyanate conjugates of IgG prepared from rabbit antiserum to sheep IgG. Representative results obtained employing this method are presented in Fig. 4. As can be seen from the photomicrographs in this figure, liver sections which had been exposed to the sheep antiserum to rat hepatic microsomal NADPH–cytochrome *c* reductase (Figs. 4A, D, and G) emit fluorescence which is considerably more intense than that emitted from serial sections which had been exposed to either normal sheep serum (Figs. 4B, E, and H) or the adsorbed sheep antireductase serum (Figs. 4C, F, and I). In agreement with the observations made using the unlabeled antibody peroxidase–antiperoxidase staining technique (Figs. 1 and 2), fluorescent immunostaining for the reductase was not evident in connective tissue lying within the portal areas (Fig. 4G). The fluorescence of weak intensity emitted from liver sections exposed to normal sheep serum (Figs. 4B, E, and H) or to the adsorbed sheep antireductase serum (Figs. 4C, F, and I) is due to both nonspecific tissue autofluorescence and nonspecific binding of the fluorescein isothiocyanate-conjugated IgG.

A comparison of the photomicrographs shown in Figs. 4A, D, and G reveals that the intensity of fluorescence emitted from hepatocytes within the periportal regions of the liver lobule is significantly less than that emitted from cells within the centrilobular and midzonal regions. This visual observation was confirmed when measurements of the intensity of emitted fluorescence were taken from 2.5 × 2.5-μm areas within the three lobular regions. It can be seen from the data presented in Tables 1 and 2 that significant binding of the antireductase occurs within the centrilobular, midzonal, and periportal regions of the lobule in the livers of untreated rats. It is also apparent that the extent of binding of the antireductase is similar within the corresponding regions in the livers of both male and female rats (Table 1) and within the right, median, left, and caudate lobes (Table 2). However, while the antireductase binds to the same extent to hepatocytes within the centrilobular and midzonal regions of the liver lobule in untreated rats, significantly less (approximately 55% less) antibody binding occurs to cells lying within the periportal regions (Tables 1 and 2).

The results of this immunohistochemical study demonstrate that NADPH–cytochrome *c* (*P*-450) reductase is present in hepatocytes throughout the liver in untreated male and female rats. However, it is apparent that there is a significant regional difference in the distribution of the enzyme within the liver lobule. The data reported for the extent of binding of the antibody to rat hepatic microsomal NADPH–cytochrome *c* reductase indicate that cells lying within the centrilobular and midzonal regions of the lobule contain approximately twice as much NADPH–cytochrome *c* (*P*-450) reductase as do those cells lying within the periportal regions. This finding is consistent with the reported distributions of

TABLE 1

Binding of antibody to NADPH–cytochrome c reductase to regions within the medium lobe in the livers of untreated male and female rats

Refer to Fig. 4 for the localization of regions from which the microfluorometric measurements were taken. The values of relative fluorescence units given represent the mean ± SE of at least 20 determinations taken within the specified region. The extent of antibody binding within the indicated regions was determined as described in Materials and Methods. Although the data are not presented, values of relative fluorescence units of regions in sections exposed to the adsorbed antireductase serum were not significantly different ($P > 0.05$) from those of corresponding regions in sections exposed to normal sheep serum.

Sex	Region	Serum[a]	Emitted fluorescence	Antibody binding
Male	Centrilobular	NSS	43.70 ± 2.56	
		SARS	69.64 ± 3.43*	25.95 ± 3.43**
	Midzonal	NSS	42.49 ± 3.81	
		SARS	69.40 ± 4.40*	26.37 ± 4.41**
	Periportal	NSS	43.85 ± 4.61	
		SARS	54.01 ± 4.28***	11.24 ± 4.28****. *****
Female	Centrilobular	NSS	43.96 ± 3.15	
		SARS	68.90 ± 4.11*	26.06 ± 4.10**
	Midzonal	NSS	42.68 ± 4.12	
		SARS	69.93 ± 3.67*	26.17 ± 4.67**
	Periportal	NSS	42.72 ± 3.87	
		SARS	55.29 ± 4.26***	11.54 ± 4.24****. *****

[a] Sheep antireductase serum = SARS; normal sheep serum = NSS.

* Significantly greater than corresponding value obtained using normal sheep serum, $P < 0.01$.

** Values are not significantly different from each other, $P > 0.05$.

*** Significantly greater than corresponding value obtained using normal sheep serum, $P < 0.05$.

**** Significantly lower than corresponding values from other regions, $P < 0.05$.

***** Values are not significantly different from each other, $P > 0.05$.

TABLE 2

Binding of antibody to NADPH–cytochrome c reductase to regions within different liver lobes

Untreated male rats were employed. Conditions were the same as those described for Table 1.

Lobe	Region	Serum	Emitted fluorescence	Antibody binding
Right	Centrilobular	NSS	44.31 ± 3.27	
		SARS	69.39 ± 3.19*	26.22 ± 3.19**
	Midzonal	NSS	43.72 ± 2.84	
		SARS	67.89 ± 3.61*	25.19 ± 3.76**
	Periportal	NSS	43.90 ± 4.40	
		SARS	55.28 ± 3.22***	12.04 ± 3.21****. *****
Median	Centrilobular	NSS	43.77 ± 2.56	
		SARS	69.36 ± 3.93*	25.99 ± 3.93**
	Midzonal	NSS	44.32 ± 3.48	
		SARS	68.11 ± 4.27*	24.67 ± 4.27**
	Periportal	NSS	43.23 ± 4.69	
		SARS	56.17 ± 3.18***	11.95 ± 3.18****. *****
Left	Centrilobular	NSS	44.65 ± 4.20	
		SARS	69.45 ± 3.61*	26.31 ± 3.61**
	Midzonal	NSS	42.47 ± 4.42	
		SARS	68.35 ± 5.14*	25.10 ± 5.14**
	Periportal	NSS	43.24 ± 4.33	
		SARS	55.78 ± 3.59***	11.45 ± 3.59****. *****
Caudate	Centrilobular	NSS	43.97 ± 2.96	
		SARS	67.54 ± 3.87*	25.34 ± 3.94**
	Midzonal	NSS	44.09 ± 4.22	
		SARS	67.21 ± 4.15*	26.17 ± 4.15**
	Periportal	NSS	44.40 ± 3.47	
		SARS	56.63 ± 4.12***	12.16 ± 4.37****. *****

* Significantly greater than corresponding value obtained using normal sheep serum, $P < 0.01$.

** Values are not significantly different from each other, $P > 0.05$.

*** Significantly greater than corresponding value obtained using normal sheep serum, $P < 0.05$.

**** Significantly lower than corresponding values from other regions, $P < 0.05$.

***** Values are not significantly different from each other, $P > 0.05$.

cytochrome P-450 (16) and monooxygenase activity (12) within the lobule in the livers of untreated rats. The intralobular distribution observed for NADPH-cytochrome c (P-450) reductase is also consistent, in large measure, with the intralobular locations of a number of toxicities which result from the cytochrome P-450-mediated formation of reactive, toxic metabolites from certain xenobiotics such as acetaminophen, carbon tetrachloride, bromobenzene, and furosemide (33, 34).

ACKNOWLEDGMENTS

The authors would like to express their thanks to Drs. Gordon F. Kapke, Phillip Greenspan, and Thomas K. Shires for their helpful suggestions. The technical assistance of Mrs. Cheryl Standing Eckart is gratefully acknowledged.

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