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An Immunohistochemical Study on the Localization and Distribution of Epoxide Hydrolase within Livers of Untreated Rats

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SUMMARY

Antiserum produced against epoxide hydrolase (EC 3.32.3) which had been purified to apparent homogeneity from rat hepatic microsomes was employed in unlabeled antibody peroxidase-antiperoxidase and indirect fluorescent antibody staining techniques to investigate the localization and distribution of the enzyme within livers of untreated rats. Using both immunohistochemical procedures, staining for epoxide hydrolase was detected within hepatocytes throughout the liver. However, differences were noted in the intensity of immunohistochemical staining of hepatocytes within the liver lobule. Microfluorometric determinations of the relative extents of binding of the anti-epoxide hydrolase to hepatocytes within the three regions of the liver lobule revealed that, although midzonal and periportal hepatocytes bound the antibody to similar extents, centrilobular hepatocytes bound significantly more anti-epoxide hydrolase. Similar patterns of intralobular distribution for the anti-epoxide hydrolase were determined within livers of untreated male and female rats. Furthermore, similar intralobular patterns of immunohistochemical staining were observed within the right, median, left, and caudate lobes of the liver.

INTRODUCTION

In liver, cytochromes P-450-containing monooxygenase enzyme systems associated with microsomal and nuclear membranes mediate the oxidative metabolism of a multitude of drugs, mutagens, carcinogens, and other xenobiotics (1, 2). During the oxidative metabolism of olefinic and aromatic substances, highly reactive and toxic electrophilic epoxides are frequently generated (3–5). These reactive metabolites can either attack nucleophilic sites on cellular macromolecules or be inactivated by conjugation with reduced glutathione, by rearrangement to yield phenols and analogous aliphatic compounds, or by hydration to their corresponding *trans*-dihydrodiols (3–6). The latter reaction is catalyzed by epoxide hydrolase (formerly referred to as epoxide hydrase or epoxide hydratase), an enzyme which is also associated with hepatic microsomal and nuclear membranes (6–9). Although dihydrodiols are not electrophilically reactive (4, 10–12), in certain instances they can serve as precursors for highly mutagenic and carcinogenic dihydrodiol epoxides (4, 8, 11, 12). Since the hydration of epoxides by epoxide hydrolase is important for both the inactivation and activation of xenobiotics, the distribution of epoxide hydrolase, as well as of the monooxygenases which generate epoxides from xenobiotics, within the liver lobule may be of critical importance in determining the locations and severities of xenobiotic-induced hepatotoxicities which result from the formation of epoxide metabolites.

One means by which the distributions of enzymes within tissues can be investigated is through the application of immunohistochemical techniques. Employing these techniques, this laboratory and other investigators have provided evidence for the heterogeneous distributions of cytochromes P-450 (13–15), NADPH-cytochrome *c* (P-450) reductase (14, 16–18), and, more recently, of epoxide hydrolase (14, 19, 20) within the lobule in livers of untreated rats. In the present study, antiserum produced against epoxide hydrolase which had been purified to apparent homogeneity from rat hepatic microsomes was employed in qualitative and semiquantitative immunohistochemical procedures to examine in greater detail the localization and distribution of epoxide hydrolase within livers of untreated male and female rats.

MATERIALS AND METHODS

Materials. Parabenzoquinone was obtained from Polysciences, Inc. (Warrington, Pa.), and 3,3′-diaminobenzidine tetrahydrochloride was purchased from Hach Chemical Company (Loveland, Colo.). Normal (nonimmune) rabbit serum was obtained from Cappel Labora-

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tories, Inc. (Cochranville, Pa.), and the soluble rabbit peroxidase-antiperoxidase complex, sheep antiserum to rabbit IgG,[2] and fluorescein isothiocyanate conjugates of IgG prepared from sheep antiserum to rabbit IgG were purchased from Miles Laboratories, Inc. (Elkhard, Ind.). All other chemicals used were of the highest purity available.

Methods. The epoxide hydrolase "A" fraction was isolated and purified to apparent homogeneity from hepatic microsomes prepared from phenobarbital-pretreated rats as described by Guengerich *et al.* (21). Using styrene-7,8-oxide as substrate, the specific activity of the purified enzyme at 37° was 1471 nmoles of diol formed per minute per milligram of protein (21). Rabbits were immunized with epoxide hydrolase, and antiserum was obtained as described previously (22). Whole rabbit antiserum to epoxide hydrolase was employed except for certain experiments in which the anti-epoxide hydrolase was removed from the antiserum by adsorption with the purified enzyme. To accomplish this, the antiserum was diluted 1:50 with 0.05 M Tris-HCl buffer (pH 7.75) containing 0.154 M NaCl and was incubated first for 45 min at 37° and then overnight at 4° with epoxide hydrolase, which was present at a concentration of 1.6 mg/ml. The antigen-antibody complex was removed from the antiserum by centrifugation at 10,000 × *g* for 30 min.

Male and female albino Holtzman rats weighing 200–230 g were used in these studies and were fasted for 24 hr prior to sacrifice. 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° for a total period of 4 hr by immersion in several changes of a freshly prepared solution containing 0.35% (w/v) parabenzoquinone and 0.02 M $CaCl_2$ in 0.2 M sodium cacodylate buffer (pH 7.4). After fixation, the blocks were dehydrated, cleared, and embedded in paraffin, and serial sections 7 μm in thickness were prepared.

The immunohistochemical localization of epoxide hydrolase in rat liver was accomplished by employing minor modifications of the unlabeled antibody peroxidase-antiperoxidase technique (13–17, 19, 20, 23) and the indirect fluorescent antibody method (14, 15, 17, 18, 23). In the indirect fluorescent antibody staining method, after the sections had been exposed to rabbit antiserum to epoxide hydrolase, normal rabbit serum, or adsorbed rabbit anti-epoxide hydrolase serum [each of which had been diluted 1:100 with 0.05 M Tris-HCl buffer (pH 7.75) containing 0.154 M NaCl], the sections were exposed for 1 hr at 37° to fluorescein isothiocyanate-conjugated IgG of sheep antiserum to rabbit IgG which had been diluted 1:100 with Tris-buffered saline. The liver sections were then examined by incident-light fluorescence microscopy using a modified Leitz Orthoplan microscope, and measurements of the intensities of fluorescence emitted from 28–μm^2 circular areas on the tissue sections were obtained as described previously (14, 15, 17, 23). The relative extents of binding of the antibody to epoxide hydrolase to hepatocytes within the three regions of the liver lobule were determined by subtracting the mean fluorescence intensity emitted from regions in sections exposed to normal rabbit serum from each microfluorometric measurement obtained from corresponding regions in serial sections exposed to rabbit anti-epoxide hydrolase serum. The microfluorometric determinations were analyzed statistically using the group Student's *t*-test.

[2] The abbreviation used is: IgG, immunoglobulin G.

RESULTS AND DISCUSSION

Recently, we reported in preliminary communications (14, 19) that antiserum produced against phenobarbital-induced rat hepatic microsomal epoxide hydrolase could be used in a modification of the unlabeled antibody peroxidase-antiperoxidase staining technique described by Sternberger *et al.* (24) to localize epoxide hydrolase in livers of untreated rats at the light microscopic level. Bentley *et al.* (20) have also employed this immunohistochemical method to localize epoxide hydrolase in rat liver, using a purified antibody produced against the hydrolase which had been purified from livers of untreated rats. In the unlabeled antibody peroxidase-antiperoxidase staining procedure, after the anti-epoxide hydrolase has interacted with and bound to the enzyme present in the tissue section, it is coupled to a peroxidase-antiperoxidase complex. The subsequent exposure of this complex to 3,3′-diaminobenzidine and H_2O_2 results in the oxidative polymerization of 3,3′-diaminobenzidine to form an insoluble reaction product which, when chelated with OsO_4, can be easily seen under the light microscope as a brownish-black deposit at the site of the antigen-antibody complex (25). Representative findings on the localization of epoxide hydrolase within the median lobe in livers of untreated male rats obtained using this immunohistochemical staining technique are presented in Fig. 1.

When sections prepared from the median lobes of livers from untreated male rats were exposed to rabbit antiserum to epoxide hydrolase in the unlabeled antibody peroxidase-antiperoxidase staining procedure and then examined by transmitted light microscopy, immunohistochemical staining for epoxide hydrolase was evident within hepatocytes throughout the liver (Fig. 1, *panels A*, *D*, *G*, and *J*). However, cells associated with the hepatic vasculature, Kupffer cells, and sinusoidal cells did not appear to be stained for this enzyme. When sections were exposed to normal rabbit serum rather than to the rabbit anti-epoxide hydrolase serum, immunohistochemical staining was not apparent within hepatocytes (Fig. 1, *panels B*, *E*, *H*, and *K*). Furthermore, the adsorption of the anti-epoxide hydrolase with the purified enzyme resulted in a very marked reduction in the intensity of immunohistochemical staining for the hydrolase throughout the liver (Fig. 1, *panels C*, *F*, *I*, and *L*). These observations demonstrate that immunohistochemical staining produced using the unlabeled antibody peroxidase-antiperoxidase technique was specific for epoxide hydrolase.

Upon inspection of the higher magnification photomicrographs in *panels D*, *G*, and *J* of Fig. 1, it can be seen that immunohistochemical staining for epoxide hydrolase produced using the unlabeled antibody peroxidase-antiperoxidase technique was of a fine, particulate nature and was uniformly dispersed throughout the cytoplasm

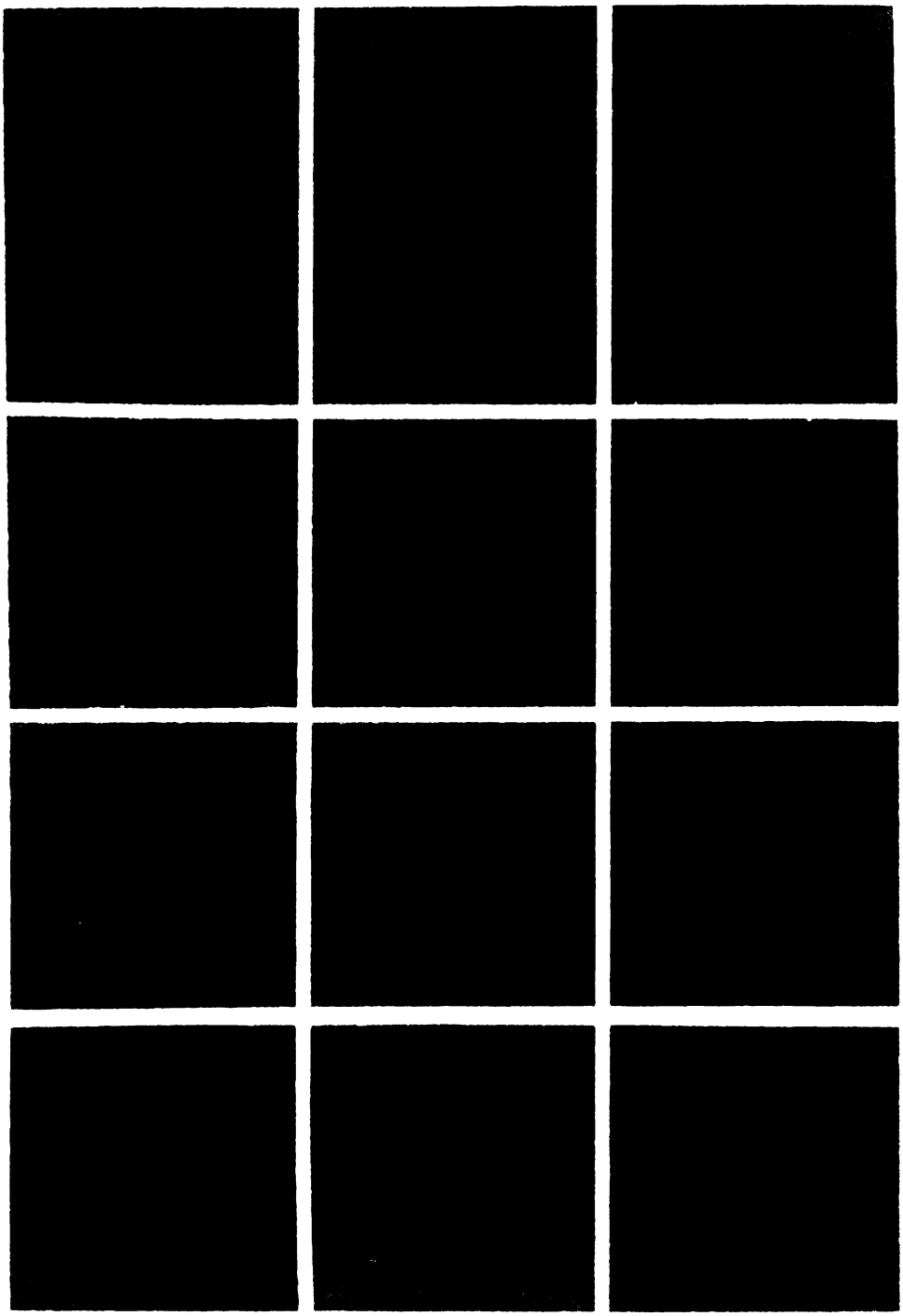

FIG. 1. *Immunohistochemical localization of epoxide hydrolase in the median lobe of rat liver*

Panels A, *B*, and *C* show the same areas in serial sections prepared from the liver of an untreated male rat. *A*, Section exposed to rabbit antiserum to epoxide hydrolase in the unlabeled antibody peroxidase-antiperoxidase staining protocol; *B*, section exposed to normal rabbit serum; *C*, section exposed to adsorbed rabbit anti-epoxide hydrolase serum. The *boxes* in *panel B* indicate areas from which the higher magnification photomicrographs in *panels D–L* were taken. The centrilobular region is seen in *D*, *E*, and *F*; the midzonal region is seen in *G*, *H*, and *I*; and the periportal region is seen in *J*, *K*, and *L*. *D*, *G*, and *J*, regions in the section exposed to rabbit anti-epoxide hydrolase serum; *E*, *H*, and *K*, regions in the serial section exposed to normal rabbit serum; *F*, *I*, and *L*, regions in the serial section exposed to the adsorbed antiserum. The antiserum, normal serum, and adsorbed antiserum had each been diluted 1:100 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 indicated in *panel C*.

of hepatocytes. This type of immunohistochemical staining is similar to that observed for both NADPH-cytochrome *c* (P-450) reductase (17) and the phenobarbital-inducible form of cytochrome P-450 (15) within hepatocytes in livers of untreated male rats. However, it is quite different from the type of staining observed for the 3-methylcholanthrene-inducible form of cytochrome P-450, which occurs as much larger, particulate deposits

concentrated within discrete areas in the cytoplasm of hepatocytes (15). Thus, these findings indicate that there are marked differences in the intracellular distributions of epoxide hydrolase and various forms of cytochrome P-450 within hepatocytes in livers of untreated male rats. These differences may be of great importance during the metabolic activation and inactivation of mutagens, carcinogens, and other xenobiotics in liver.

Although hepatocytes have been reported to contain a soluble form of epoxide hydrolase (26), only very low levels of the cytosolic enzyme have been detected in livers of male rats (26), and Guenthner *et al.* (27) recently reported that antibodies raised against homogeneous rat liver microsomal epoxide hydrolase do not cross-react with the cytosolic enzyme. Thus, the presence of cytosolic epoxide hydrolase would not be expected to contribute to the over-all staining observed within hepatocytes. Furthermore, although immunohistochemical staining for epoxide hydrolase was clearly visible within hepatocyte cytoplasm, hepatocyte nuclei did not appear to be stained appreciably (Fig. 1, *panels D*, *G*, and *J*). On the other hand, the perinuclear area in many hepatocytes was observed to be more intensely stained for the hydrolase than was the surrounding cytoplasm. This observation is consistent with the findings of Bentley *et al.* (20) and with the observation that the epoxide hydrolase activity of isolated hepatocyte nuclei is predominantly associated with the nuclear envelope (9).

Although the data are not presented, results essentially identical with those presented in Fig. 1 were obtained when other lobes from the livers of untreated male rats and when livers from untreated female rats were examined. Furthermore, comparable observations were made when the rabbit antiserum to epoxide hydrolase was used in a wide range of dilutions in the staining protocol and when unfixed, cryostat sections of liver were examined.

Immunohistochemical staining for epoxide hydrolase produced within the lobule in livers of untreated rats using the unlabeled antibody peroxidase-antiperoxidase technique has previously been reported to be nonuniform, with centrilobular hepatocytes being more intensely stained for the hydrolase than are either midzonal or periportal hepatocytes (14, 19, 20). The photomicrographs shown in *panels A*, *D*, *G*, and *J* of Fig. 1 are consistent with these earlier observations. Moreover, similar observations were made when epoxide hydrolase was localized in livers of untreated rats by means of an indirect fluorescent antibody staining method which permits the semiquantitation of the extent of antibody binding to cells (14, 15, 17, 23). In this immunohistochemical technique, after the rabbit anti-epoxide hydrolase has interacted with and bound to the enzyme on the tissue section, it is coupled to fluorescein isothiocyanate conjugates of sheep IgG prepared against rabbit IgG. The tissue section is then examined by incident-light fluorescence microscopy. Representative observations on the localization of epoxide hydrolase within the median lobe in livers of untreated male rats are presented in Fig. 2. The liver section exposed to the rabbit antiserum to epoxide hydrolase (*panel A*) emits fluorescence which is considerably more intense than that emitted from the serial sections exposed to either normal rabbit serum (*panel B*) or the adsorbed rabbit anti-epoxide hydrolase serum (*panel C*). The fluorescence of weak intensity emitted from liver sections exposed to normal rabbit serum or the adsorbed antiserum is due to both nonspecific tissue autofluorescence and nonspecific binding of the fluorescein isothiocyanate-conjugated IgG.

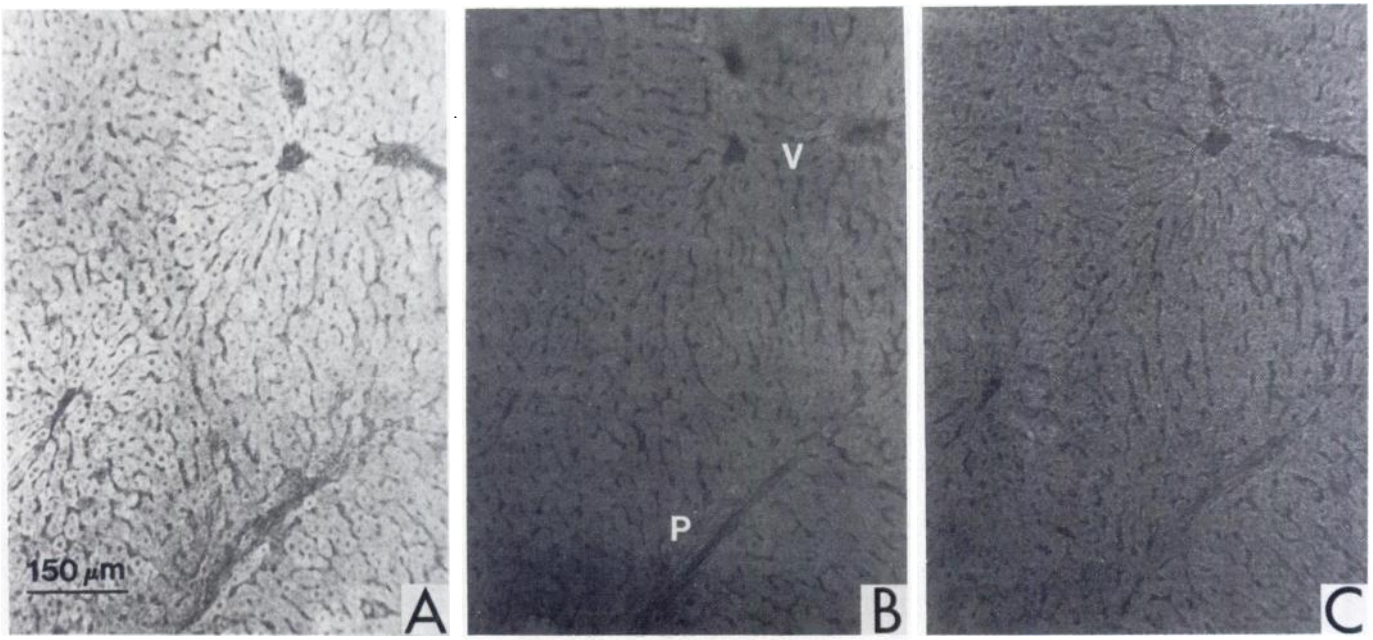

FIG. 2. *Fluorescent immunohistochemical localization of epoxide hydrolase in the median lobe of rat liver*

The three panels show the same areas in serial sections prepared from the liver of an untreated male rat. *A*, Section exposed to rabbit antiserum to epoxide hydrolase in the indirect fluorescent antibody staining protocol; *B*, section exposed to normal rabbit serum; *C*, section exposed to the adsorbed rabbit anti-epoxide hydrolase serum. A group of three central veins (*V*) and a portal triad (*P*) are indicated in *panel B*.

TABLE 1

Binding of antibody to epoxide hydrolase to regions within the median lobe in livers of untreated male and female rats

The values of relative fluorescence units given represent the mean ± standard error of at least 10 measurements taken within the specified region. The extents of anti-epoxide hydrolase binding to hepatocytes within the three lobular regions were determined as described under Materials and Methods. Values of relative fluorescence units emitted from regions in sections exposed to normal rabbit serum (NRS) were in all instances significantly less ($p < 0.001$) than those emitted from corresponding regions in serial sections exposed to rabbit antiserum to epoxide hydrolase (RAEH).

Region	Serum	Emitted fluorescence	Antibody binding
Male rats			
Centrilobular	NRS	17.71 ± 1.06	
	RAEH	81.21 ± 0.89	63.50 ± 0.89[a, b]
Midzonal	NRS	16.71 ± 1.39	
	RAEH	68.27 ± 2.07	51.56 ± 2.07[c]
Periportal	NRS	15.15 ± 1.21	
	RAEH	63.97 ± 2.28	48.82 ± 2.28[c]
Female rats			
Centrilobular	NRS	19.96 ± 1.32	
	RAEH	80.54 ± 2.00	60.58 ± 2.00[a, b]
Midzonal	NRS	17.56 ± 1.80	
	RAEH	70.18 ± 1.19	52.62 ± 1.19[c]
Periportal	NRS	20.25 ± 1.26	
	RAEH	69.33 ± 1.48	49.08 ± 1.48[c]

[a] Values are not significantly different from each other ($p > 0.05$).

[b] Significantly greater than corresponding values obtained from the midzonal and periportal regions ($p < 0.01$).

[c] Values are not significantly different from each other ($p > 0.05$).

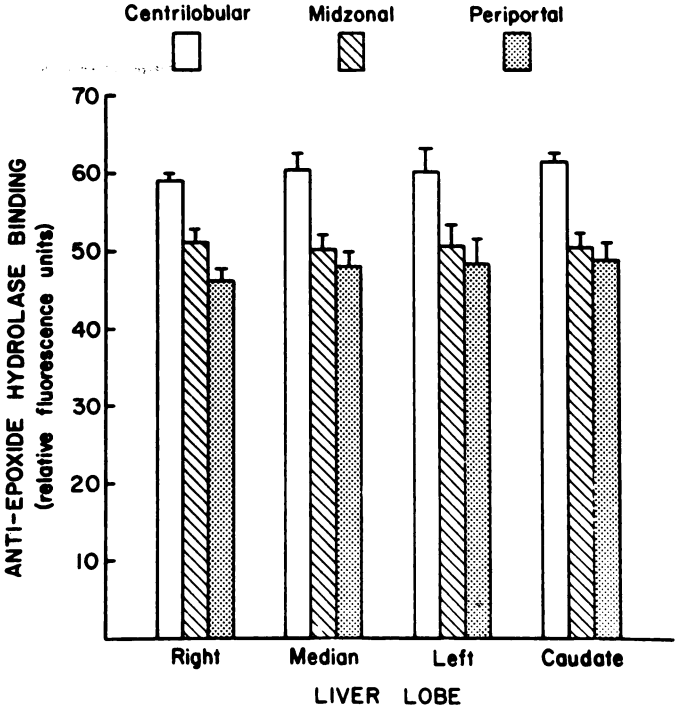

FIG. 3. *Binding of anti-epoxide hydrolase to centrilobular, midzonal, and periportal hepatocytes within livers of untreated male rats*

Each *bar* represents the mean (± standard error) extent of antibody binding within the specific lobular region.

Consistent with observations made using the unlabeled antibody peroxidase-antiperoxidase staining technique, hepatocyte nuclei did not appear to be stained for epoxide hydrolase using the indirect fluorescent antibody method (Fig. 2, *panel A*). Furthermore, fluorescence immunohistochemical staining for the hydrolase was not evident in cells associated with the hepatic vasculature or in either Kupffer or sinusoidal cells. *Panel A* of Fig. 2 also demonstrates that centrilobular hepatocytes are more intensely stained for epoxide hydrolase than are midzonal and periportal cells.

Differences in the intensity of staining of hepatocytes for epoxide hydrolase observed visually with both immunohistochemical staining techniques were confirmed when measurements of the intensities of emitted fluorescence were taken within the three lobular regions (Table 1; Fig. 3). Although the data are not presented, microfluorometric determinations revealed that, after the anti-epoxide hydrolase had been adsorbed with the purified antigen, antibody binding to hepatocytes throughout the liver lobule was reduced by 90–95%.

The data presented in Table 1 show that significant binding of the anti-epoxide hydrolase occurs within the centrilobular, midzonal, and periportal regions of the lobule in livers of untreated rats. It is also apparent that the anti-epoxide hydrolase binds to very similar extents within corresponding regions in livers of untreated male and female rats. The data presented in Fig. 3 further show that the anti-epoxide hydrolase binds to similar extents within corresponding regions in the right, median, left, and caudate lobes of rat liver. However, although the anti-epoxide hydrolase binds to the same extent ($p > 0.05$) to hepatocytes within the midzonal and periportal regions of the liver lobule in untreated rats, significantly more ($p < 0.05$) antibody binds to cells lying within the centrilobular regions (Table 1; Fig. 3).

The results of this immunohistochemical study demonstrate that epoxide hydrolase is present within hepatocytes throughout the livers of untreated male and female rats. However, there are significant regional differences in the concentration of the enzyme within the liver lobule. The data reported for the extent of binding of the anti-epoxide hydrolase indicate that hepatocytes lying within the midzonal and periportal regions of the lobule contain significantly less enzyme than do centrilobular hepatocytes. Thus, the pattern of intralobular distribution determined in the present study for epoxide hydrolase within livers of untreated rats differs from those reported previously for both NADPH-cytochrome *c* (P-450) reductase, which is present in equal concentrations within centrilobular and midzonal hepatocytes (14, 17), and the phenobarbital-inducible form of cytochrome P-450 whose content decreases from the central vein toward the portal triad (14, 15). On the other hand, the distribution of epoxide hydrolase within the lobule in livers of untreated rats is quite similar to that reported for the 3-methylcholanthrene-inducible form of cytochrome P-450 (14, 15). These findings suggest that, although centrilobular hepatocytes possess the greatest capacity to generate reactive and toxic electrophilic epoxide metabolites from xenobiotics, they also possess the greatest capacity to inactivate these epoxides by hydration to form *trans*-dihydrodiols.

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