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IMMUNOCYTOCHEMICAL LOCALIZATION OF CYTOCHROME P-450 IN HEPATIC AND EXTRA-HEPATIC TISSUES OF THE RAT WITH A MONOCLONAL ANTIBODY AGAINST CYTOCHROME P-450 *c*

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Abstract—The cellular distribution of cytochrome P-450 has been studied in the liver and a number of extrahepatic tissues in the rat by immunocytochemistry, using an antibody raised against cytochrome P-450 form *c*. Immunoreactive cytochrome P-450, most probably form *c*, was found in the proximal tubules of the kidney, in the Clara cells of the lung, and in the olfactory epithelium and Bowman's glands of the olfactory tissue, in addition to its location in the liver. Immunoreactive cytochrome P-450 was not found in the small intestine, the testes or the adrenal gland, although these organs are known to contain isoenzymes of cytochrome P-450. The use of antibody titration enabled the effects of phenobarbitone, β-naphthoflavone and clofibrate on the content and distribution of immunoreactive cytochrome P-450 to be studied in both the liver and in the other organs discussed. Phenobarbitone induces epitope-specific cytochrome P-450 in the centrilobular cells of the liver but has no effect in any of the other tissues studied. Clofibrate is without effect on the levels of immunoreactive cytochrome P-450 in any of the tissues studied. In contrast, β-naphthoflavone induces immunoreactive cytochrome P-450 in the periportal region of the liver, and also in the Clara cells of the lung, in the enterocytes of the small intestine and in the proximal tubules of the kidney. Of all of the tissues studied, in which immunoreactive cytochrome P-450 could be detected, only the olfactory epithelium failed to undergo enzyme induction following treatment with β-naphthoflavone.

The cytochrome P-450 dependent mixed function oxidase system plays an essential role in the metabolism of a broad range of both endogenous and exogenous substrates. There are multiple forms of cytochrome P-450 with unique, but overlapping substrate specificities [1]. The different isozymes function in both the activation and detoxication of foreign compounds and some forms of cytochrome P-450 have been implicated in the activation of a variety of chemical carcinogens [2–4].

Although the liver is the major site of xenobiotic biotransformation, other tissues such as the lung [5], kidney [6], and small intestine [7] also exhibit monooxygenase activity albeit often at low levels. However, this does mean that such tissues are able to catalyse the formation of highly reactive metabolites, leading to the tissue specific toxicity which occurs in some of these organs [8]. In addition, the cytochrome P-450 isozymes in many of these tissues are subject to induction following treatment with chemicals such as polycyclic aromatic hydrocarbons, phenobarbitone and pregnanalone-16α-carbonitrile [9–11].

Although there have been numerous studies on tissue differences in monooxygenase activity these have been confounded by the overlapping specificity of the isozymes of cytochrome P-450. Monoclonal antibodies, reacting with unique epitopes, are ideally suited to such studies [12]. The purpose of the present study was to investigate the distribution of the major hydrocarbon-inducible form of cytochrome P-450, for *c*, in rat tissue using a monoclonal antibody raised against this isozyme [13]. The antibody used in the present study, 3/4/2, has been shown to react with an epitope unique to cytochrome P-450 *c* and a structurally related phenobarbitone-inducible isozyme in the rat, but common to the analogous hydrocarbon-inducible isozyme in other species [14].

The identification of intra- and inter-organ distribution of cytochrome P-450 should enable possible target tissues to be pin-pointed where cytotoxicity due to metabolic activation might occur. Secondly, it should be possible to investigate the specificity of induction in tissues where levels of the enzyme are either too small or too focally distributed to be easily characterized by other techniques.

MATERIALS AND METHODS

Monoclonal antibodies. The production and purification of the monoclonal antibody, 3/4/2, used in these studies and its immunological specificity for

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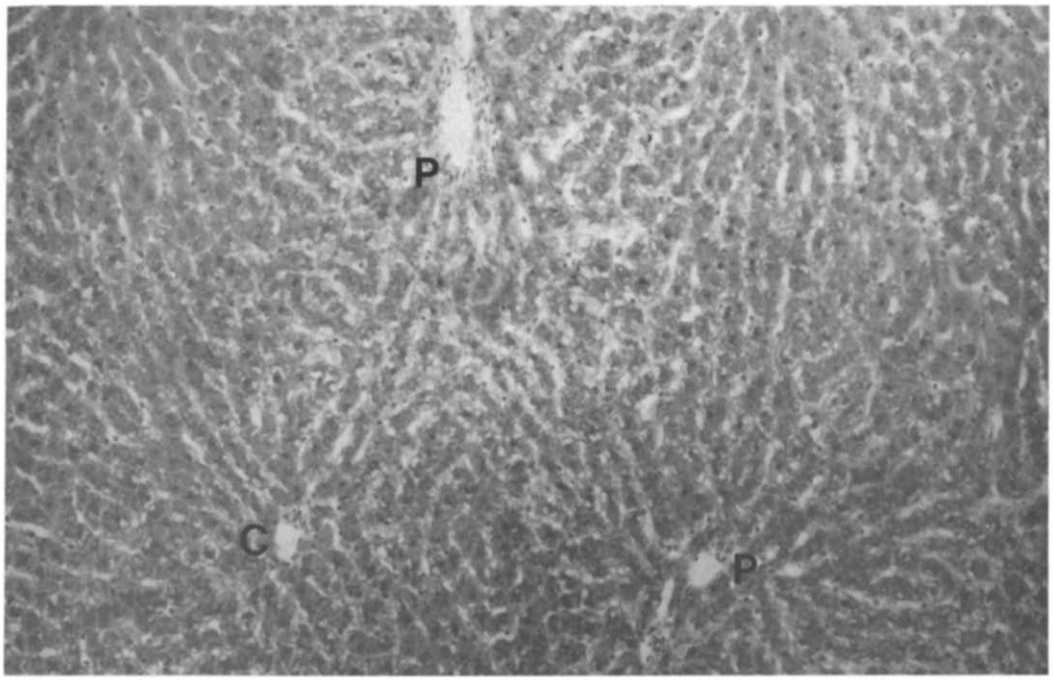
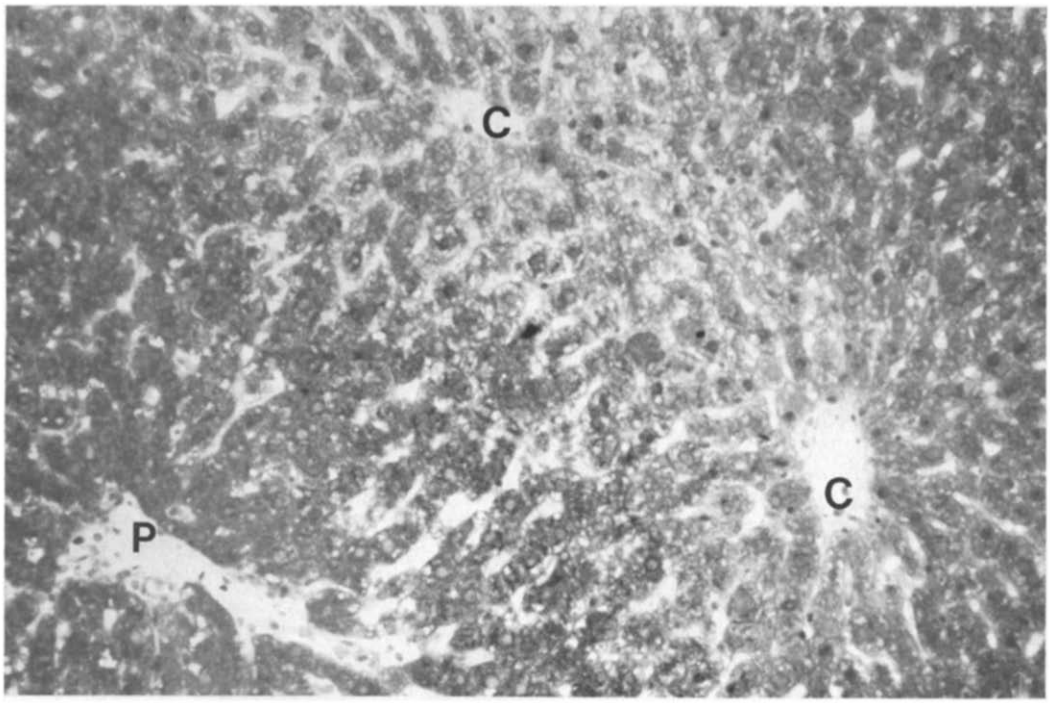
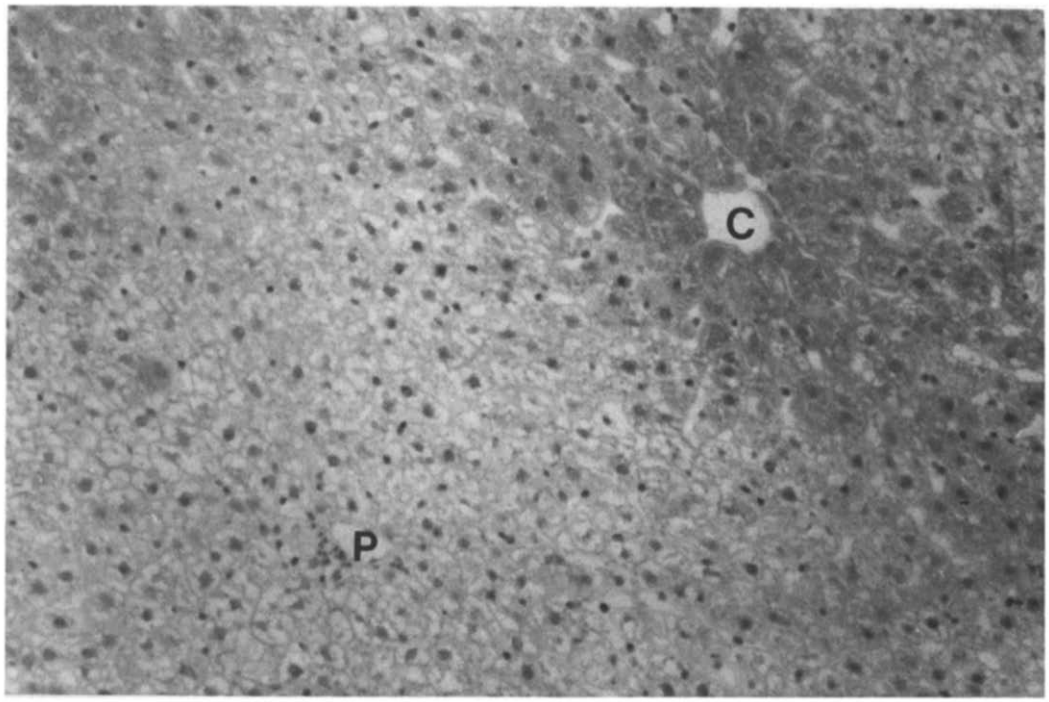


(a)

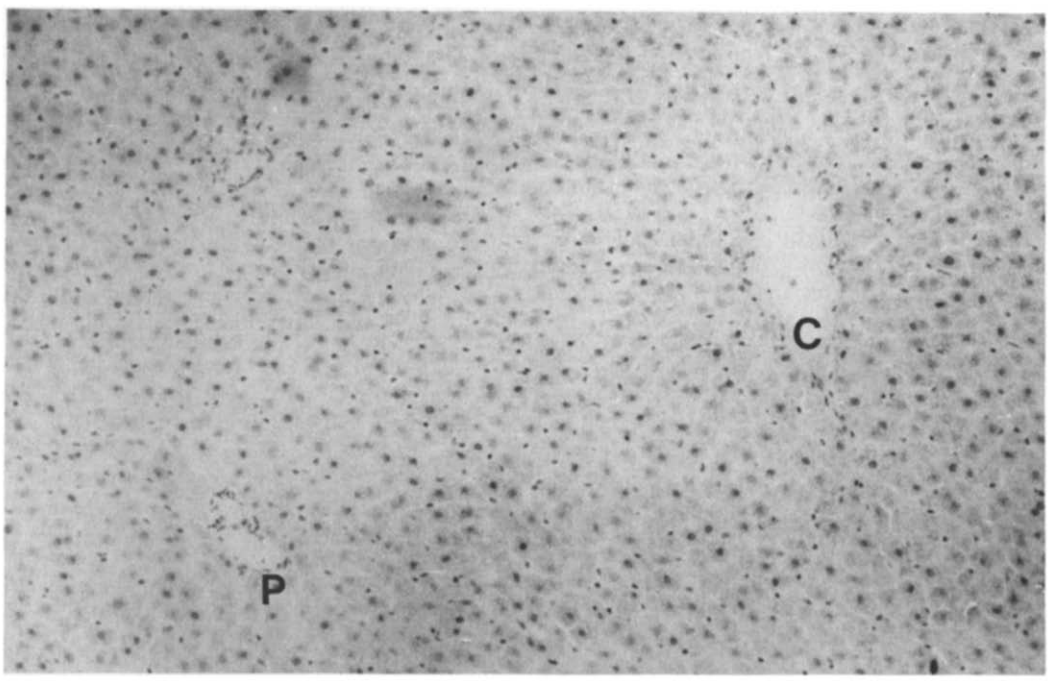


(b)

Fig. 1(a, b)



(c)



(d)

Fig. 1. Immunocytochemical localization of cytochrome P-450 in the livers of rats using monoclonal antibody 3/4/2. Sections were from (a) untreated rat, 3/4/2 used at a dilution of 1:10; (b) BNF-treated rat, 3/4/2 used at a dilution of 1:200; (c) PB-treated rat, 3/4/2 used at a dilution of 1:50; (d) untreated rat, control antibody B3A3 used at a dilution of 1:10. C, central vein; P, portal vein. Magnification ×204.8.

cytochrome P-450 have been reported in a previous publication [13]. Clone 3/4/2 was derived from the fusion of the mouse myeloma cell line P3.NS1.1-Ag4-1 (NS1 cells) with splenic lymphocytes obtained from a Balb/c mouse immunized with partially purified cytochrome P-450 form *c*. The cytochrome P-450 preparation was obtained from the livers of rats treated with β-naphthoflavone (BNF) (Sigma Chemical Company, Poole) by i.p. injection, at a dose of 100 mg/kg in corn oil daily for 4 days, as previously described [13].

Treatment of animals. Tissue was obtained from male rats of the Alp/Apk strain (Wistar derived) of body weight in the range 100–150 g. Animals were given food and water at *ad lib*. Groups of 4 animals were treated with BFN in corn oil at 100 mg/kg/day for 4 days by i.p. injection or by gastric intubation, with sodium phenobarbitone (McArthur's, London) at 80 mg/kg/day for 5 days by i.p. injection or with clofibrate (ICI Pharmaceuticals Division) at 200 mg/kg/day for 10 days by i.p. injection. Animals were killed 24 hr after the last dose of the inducer by an overdose of sodium pentobarbitone (Sagatal, May & Baker Limited, London) administered by i.p. injection. Various tissues were rapidly dissected from the animals, snap frozen in liquid nitrogen and stored at −70% until required for sectioning. Tissues were sectioned at 7–10 μm at −20° and mounted onto acetone-cleaned glass slides for immediate use or were stored at −20° until required for subsequent immunocytochemistry.

Immunocytochemistry. Tissue sections were washed in phosphate-buffered saline (PBS) prior to fixation in 0.5% p-benzoquinone (BDH, Poole) in 0.2 M sodium cacodylate/0.02 M calcium chloride buffer for 5 min. They were washed for a further 5 min in PBS. Sections were then incubated with monoclonal antibody 3/4/2 at various dilutions (1:10–1:1000) and treated according to the method of Wolf *et al.* [15], with the exception that the second antibody used was peroxidase-labelled anti-mouse IgG (Miles Scientific, Slough). Sections were counterstained in Harris' haematoxylin, dehydrated and mounted in DPX. Control incubations were performed by substitution of the specific monoclonal antibody with a monoclonal antibody that did not cross-react with cytochrome P-450, at the same immunoglobulin concentration. This control antibody was clone B3A3, which was derived against bovine growth hormone. In other control incubations, the peroxidase-labelled anti-mouse IgG was omitted. In all control sections staining was minimal (for example, see Figs. 1d, 3c, 4b and 5b).

The inducibility of cytochrome P-450 in each tissue following treatment with the various compounds was assessed qualitatively from the changes in the staining characteristics of the sections by the monoclonal antibody 3/4/2 when used at varying dilutions, i.e. induction of cytochrome P-450 in a tissue should result in binding of 3/4/2 at lower concentrations (higher dilutions) than in the uninduced tissues. The rat tissues examined for binding of monoclonal antibody 3/4/2 were liver, lung, kidney, adrenal, testis, duodenum and the olfactory tissue from the nose. In each case the results have been expressed for each tissue individually from both treated and untreated animals. In all cases the entire surface of at least five sections from at least five different animals was examined by light microscopy, and a subjective assessment of staining intensity and distribution was made blind to the treatment of the animals and the nature of the monoclonal antibody (i.e. test or control). The results for the animals within each group were qualitatively similar in all cases.

RESULTS

Liver

In liver from untreated animals, specific immunostaining was detected with antibody 3/4/2. Immunoreactive cytochrome P-450 was evenly distributed across the liver lobule (Fig. 1a) but could be visualized only when the antibody was used at a dilution factor of 1:10 (Table 1).

Liver from animals treated with BNF showed high levels of staining in the periportal region of the lobule with lower levels in the centrilobular region (Fig. 1b). In addition, visualization could be achieved at antibody dilutions as low as 1:1000 (Table 1) indicating that BNF-treatment had resulted in induction of immunoreactive cytochrome P-450.

Liver from animals treated with phenobarbitone showed high levels of staining for cytochrome P-450 in the centrilobular region, with very low levels of staining in the periportal region, below that observed in untreated animals (Fig. 1c). Visualization of cytochrome P-450 was achieved at an antibody dilution of 1:50, again indicating that induction of cytochrome P-450 had occurred in the centrilobular region following phenobarbitone treatment.

When antibody 3/4/2 was replaced with the control antibody B3A3, there was an almost total lack of staining of the liver sections (Fig. 1d).

Although induction of cytochrome P-450 was apparent after treatment with both BNF and phenobarbitone, the antibody could be used at a greater dilution with liver from BNF-treated rats than from phenobarbitone-treated animals (Table 1).

Treatment of rats with clofibrate had no effect on either the distribution or staining intensity of immunoreactive cytochrome P-450 in the liver.

Lung

Staining of lung sections from untreated animals with 3/4/2 localized cytochrome P-450 in the Clara cells of the bronchioles (Fig. 2). No specific staining was apparent when antibody 3/4/2 was replaced with the control antibody B3A3 (data not shown).

The distribution of cytochrome P-450 was not changed following treatment of animals with BNF, phenobarbitone or clofibrate. However, although the dilution factor at which visualization could be achieved in sections from untreated, phenobarbitone-treated and clofibrate-treated animals was the same (1:10), in sections from rats treated with BNF, the dilution factor was increased to 1:50 (Table 2). Immunoreactive cytochrome P-450 could be detected only in the bronchiolar Clara cells, all other cell types in the lung failing to show any preferential binding of the antibody.

Table 1. Immunotitration of the binding of antibody 3/4/2 to liver

Treatment	Antibody dilution					
	1:10	1:50	1:100	1:200	1:500	1:1000
Control	++	+/−	−	−	−	−
Phenobarbitone sodium	+++	++	+/−	−	−	−
β-Naphthoflavone	+++	+++	+++	+++	++	+
Clofibrate	++	+/−	−	−	−	−

+++ intense staining; ++ moderate staining; + slight staining; +/− some positive areas; − no specific staining.

Kidney

In kidney sections from untreated animals, 3/4/2 localized cytochrome P-450 in a small proportion of the cells in the S_3 portion of the proximal convoluted tubule (Fig 3a). There was considerable variation in the extent and percentage of cells staining positively amongst individual animals, and a similar pattern of staining was observed in rats treated with phenobarbitone or clofibrate. In addition, there was no difference in the dilution factor at which positive staining could be detected following treatment with these inducers (Table 3). In contrast, in kidneys from animals treated with BNF, cytochrome P-450 could be detected throughout the entire length of the proximal tubules of the cortex (Fig. 3b) although even in these sections, the intensity of staining was still stronger in the S_3 segment. The glomeruli and distal tubules of the cortex and the medulla failed to show positive staining. Sections in which 3/4/2 was replaced with B3A3 were negative (Fig. 3c). The dilution factor of 3/4/2 at which positive staining occurred in sections from untreated, phenobarbitone-treated and clofibrate-treated animals was 1:10, whereas in sections from animals treated with BNF, positive staining was obtained at dilution factors of 1:100 to 1:200 (Table 3).

Adrenal

Antibody 3/4/2 failed to show any specific staining in sections from the adrenal glands of untreated, phenobarbitone-, BNF- or clofibrate-treated animals, even at dilutions of 1:5.

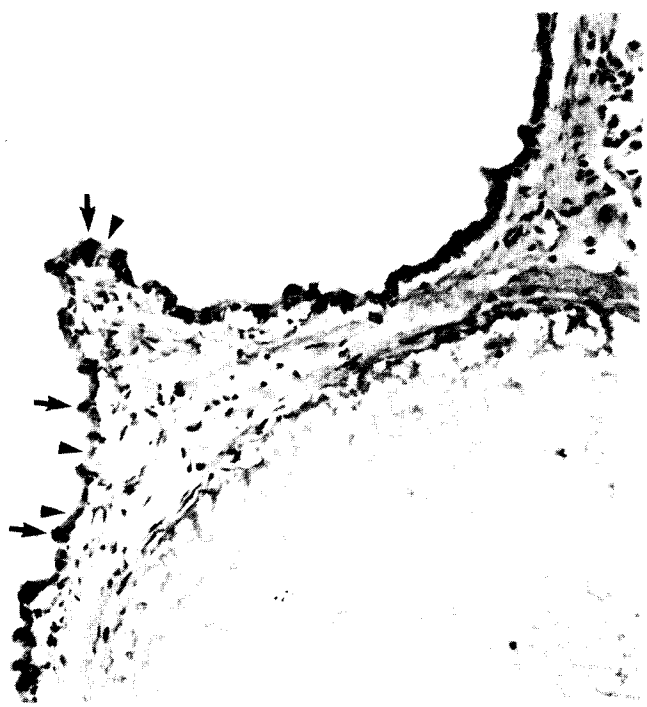


Fig. 2. Immunocytochemical localization of cytochrome P-450 *c* in the bronchiolar region of the lung of a rat treated with BNF using monoclonal antibody 3/4/2 at a dilution of 1:50. Staining of the isoenzyme is apparent in the domed Clara cells of the bronchiolar epithelium (↑), but is absent from the ciliated cells (▲). Magnification ×204.8.

Table 2. Immunotitration of the binding of antibody 3/4/2 to lung

Treatment	Antibody dilution			
	1:10	1:50	1:100	1:500
Control	++	–	–	–
Phenobarbitone sodium	++	–	–	–
β-Naphthoflavone	+++	++	–	–
Clofibrate	++	–	–	–

+++ intense staining; ++ moderate staining; – no specific staining.

Testis

Antibody 3/4/2 was also negative in sections from the testes of untreated animals and of those treated with phenobarbitone, BNF and clofibrate, even when the antibody was used at dilutions as low as 1:5.

Small intestine

No specific staining was obtained with antibody 3/4/2 with sections of small intestine from untreated, phenobarbitone-treated or clofibrate-treated animals (Table 4). However, with sections from BNF-treated animals, immunoreactive cytochrome P-450 could be detected in the enterocytes of the intestinal villi (Fig. 4a). Appropriate control sections showed no staining (Fig. 4b). In animals treated with BNF orally, positive staining of the enterocytes was evident throughout the length of the villi, but the cells of the crypts of Lieberkuhn were negative. In contrast, in those animals treated by i.p. injection, both the cells lining the villi and those in the crypts showed positive staining. Following both oral and i.p. dosing with BNF, visualization of cytochrome P-450 was achieved at an antibody dilution of 1:50 (Table 4).

Olfactory tissue

In sections from unrelated animals immunoreactive cytochrome P-450 was detected in the apical cytoplasm of the cells of the olfactory epithelium and the underlying Bowman's glands (Fig. 5a). However, it was not possible to determine the differential distribution of the isozyme amongst the cells of the olfactory epithelium due to the thickness of the section and the interdigitating nature of the cell to cell contacts. Treatment of animals with BNF, phenobarbitone or clofibrate failed to alter either the distribution of the enzyme or the dilution factor at which positive staining was obtained (Table 5). Appropriate control sections were negative (Fig. 5b).

DISCUSSION

Immunohistochemical localization of cytochrome P-450 has been performed utilizing a monoclonal antibody raised against rat cytochrome P-450 form *c*. The only other protein in rat liver recognized by this antibody is a phenobarbitone-inducible form of the enzyme, apparently not yet purified by any other group [14]. Although raised against the hepatic isozyme, the antibody has been used successfully to localize the haemoprotein in liver, kidney, lung, duodenum and olfactory tissue. In a separate study (manuscript in preparation) it was shown by Western blotting that antibody 3/4/3 reacts with only a single protein in kidney, lung and small intestine from 3-

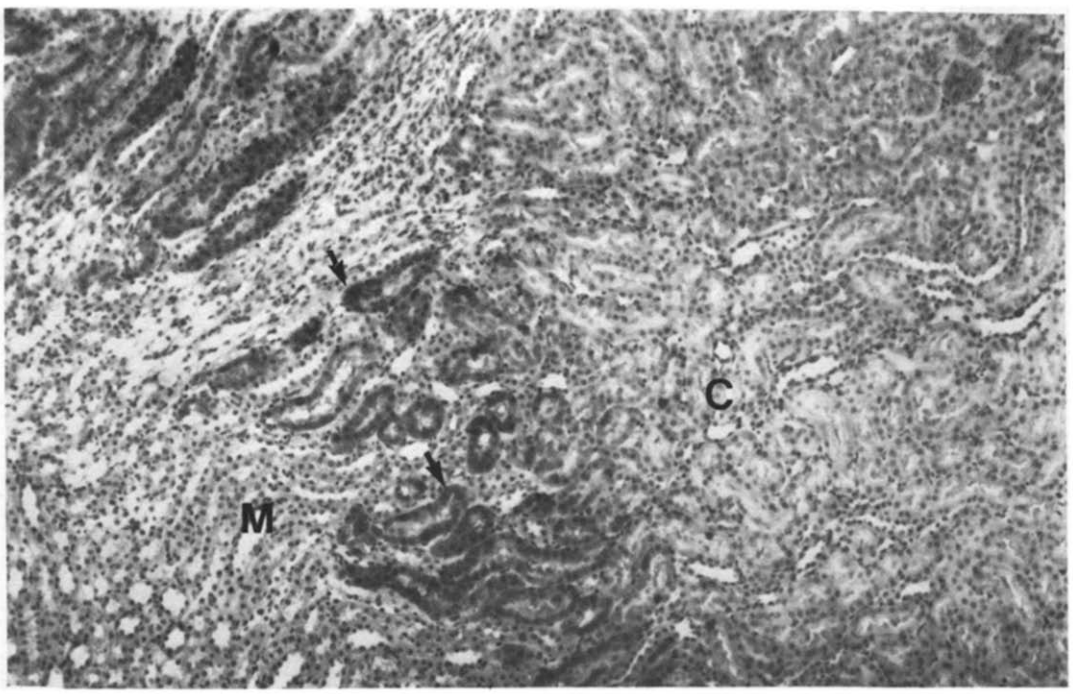
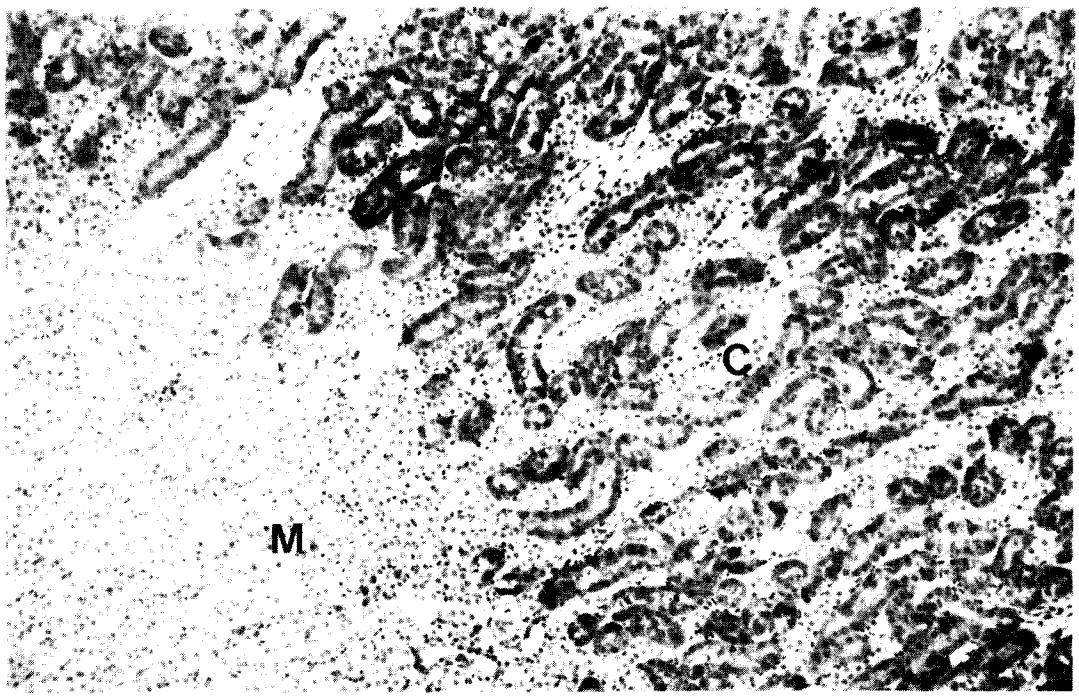


Fig. 3 (a)



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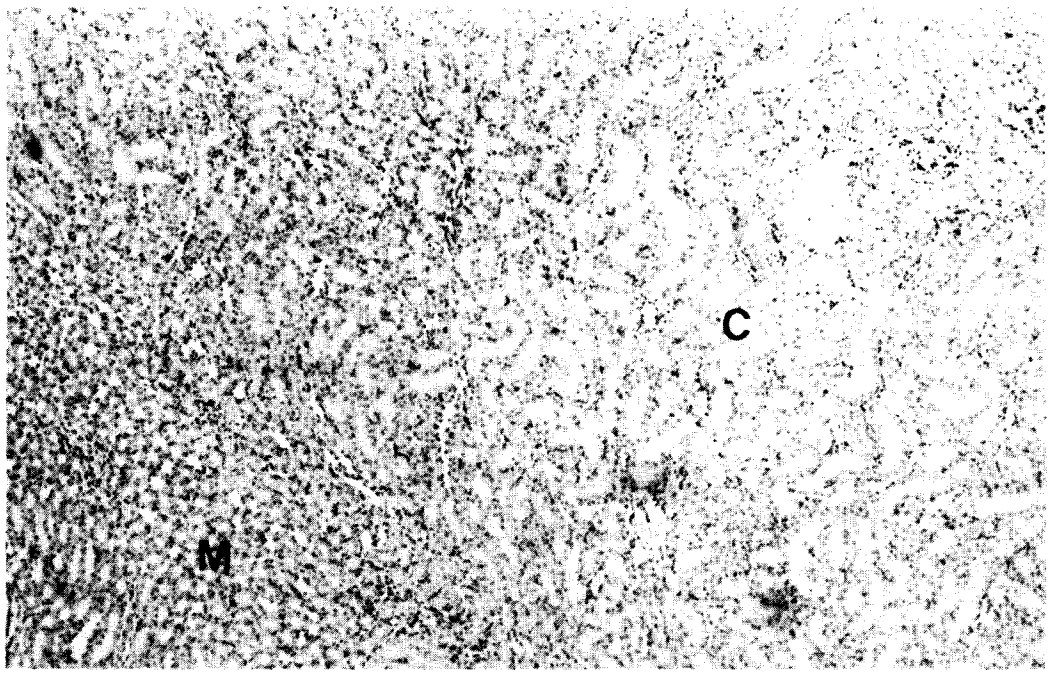


(c)

Fig. 3. Immunocytochemical localization of cytochrome P-450 *c* in the kidney of rats using monoclonal antibody 3/4/2. Sections were from (a) untreated rat, 3/4/2 used at a dilution of 1:10; (b) BNF-treated rat, 3/4/2 used at a dilution of 1:100; (c) untreated rat, control antibody B3A3 used at a dilution of 1:10. The isoenzyme is apparent only in the proximal tubules of in the cortico-medullary area of control animals (↟), but is stained throughout the proximal tubules of the cortex following BNF treatment. M, medulla; C, cortex. Magnification ×128.

Table 3. Immunotitration of binding of antibody 3/4/2 to kidney

Treatment	Antibody dilution				
	1:10	1:50	1:100	1:200	1:500
Control	+/−	−	−	−	−
Phenobarbitone sodium	+/−	−	−	−	−
β-Naphthoflavone	+++	+++	++	+	−
Clofibrate	+/−	−	−	−	−

+++ intense staining; ++ moderate staining; + slight staining; +/− some positive areas; − no specific staining.

MC treated rats. This protein has the same molecular weight as cytochrome P-450 *c*. Another monoclonal antibody, 12/2/3/2, which reacts with form *c* at an epitope spatially distinct from that with which 3/4/2 reacts [14], also recognizes this protein. Thus, it seems likely that the protein recognized by 3/4/2 in extra-hepatic tissues is, indeed, cytochrome P-450 form *c*.

Cytochrome P-450 has been identified in all of the tissues examined in the present study [6, 7, 16–20] and certain isozymes have been localized immunohistochemically in liver [15, 20–25], kidney [22], lung [22, 26, 27] and adrenal [28, 29]. Monoclonal antibody 3/4/2 exhibited its greatest affinity in the liver and, in particular in liver from animals treated with BNF, as expected. However, considerable binding also occurred in liver from animals treated with phenobarbitone, albeit at a lower dilution factor, and in liver from control animals, but at relatively high antibody concentrations. Treatment of rats with clofibrate at a dose known to induce cytochrome P-452 [30] had no effect on either the distribution or intensity of staining of the liver, indicating that the specific isozyme induced by this compound does not contain the epitope recognized by 3/4/2.

The localization of cytochrome P-450 in uninduced liver by 3/4/2 was similar to that found by Baron *et al.* [21] with a polyclonal antibody against 3-methylcholanthrene inducible cytochrome P-450, although these authors found slightly more intense staining of the centrilobular region. Wolf *et al.* [15] reported very low intensity of staining with a polyclonal antibody against a 3-methylcholanthrene-inducible isozyme of cytochrome P-450, with slightly more intense staining of the periportal region.

The localization of hepatic cytochrome P-450 following treatment of rats with phenobarbitone was centrilobular, and similar to that shown by Wolf *et al.* [15], using polyclonal antibodies raised against phenobarbitone-inducible forms of hepatic cytochrome P-450. Thus, although different isozymes were localized in these two studies, their distribution following phenobarbitone-treatment was very similar. The localization of cytochrome P-450 in liver following BNF-treatment was very similar to that reported by Wolf *et al.* [15] using a polyclonal antibody derived against a 3-methylcholanthrene inducible form of hepatic cytochrome P-450 (MC_2), which was almost certainly the same isozyme against which 3/4/2 was raised, based on molecular weight, substrate specificity, absorption maximum and chromatographic properties.

No immunoreactive cytochrome P-450 could be detected with 3/4/2 in the testes or adrenal, from either untreated or induced animals. However, cytochrome P-450 has been identified in these organs [18, 28, 29]. Thus, presumably, the isozymes of cytochrome P-450 present in these tissues do not possess the epitope with which 3/4/2 reacts. Both adrenal and testicular cytochromes P-450 are primarily involved in steroid biosynthesis and as such they presumably exhibit such structural differences as to prevent binding of the antibody. In addition, hydrocarbon treatment of rats had little or no effect in these tissues on the amount of the isozyme of cytochrome P-450 against which 3/4/2 was raised [11]. This explanation may also apply to the form of cytochrome P-450 present in the small intestine from uninduced rats, a tissue in which cytochrome P-450 has previously been demonstrated [7, 31]. However,

Table 4. Immunotitration of the binding of antibody 3/4/2 to small intestine

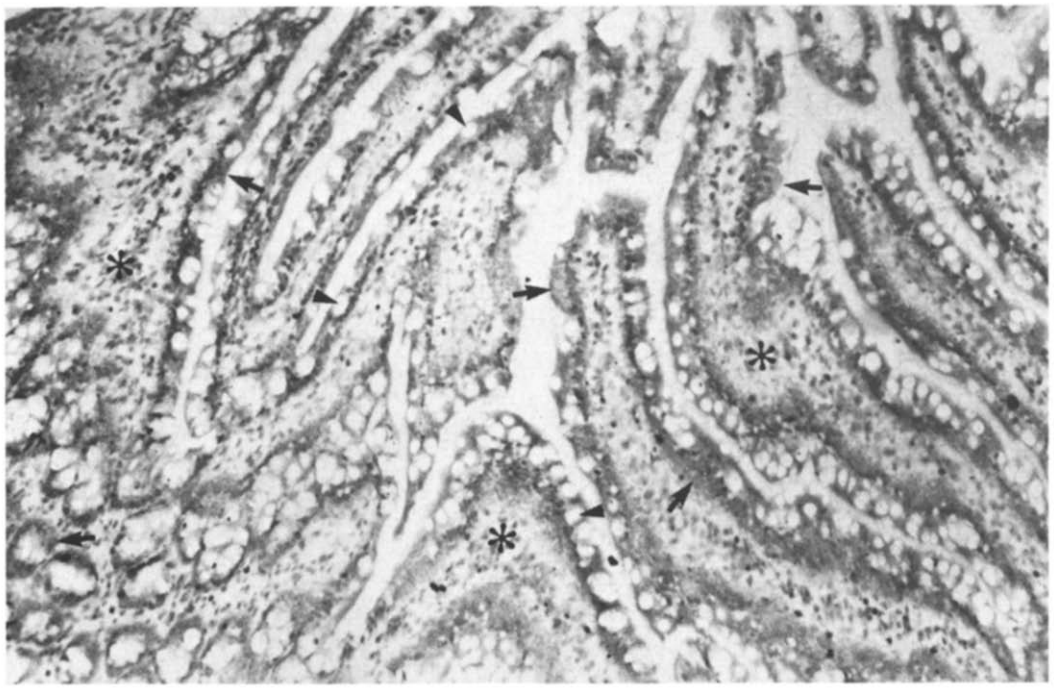
Treatment	Antibody dilution			
	1:10	1:50	1:100	1:500
Control	−	−	−	−
Phenobarbitone sodium	−	−	−	−
β-Naphthoflavone	+++	++	−	−
Clofibrate	−	−	−	−

+++ intense staining; ++ moderate staining; − no specific staining.

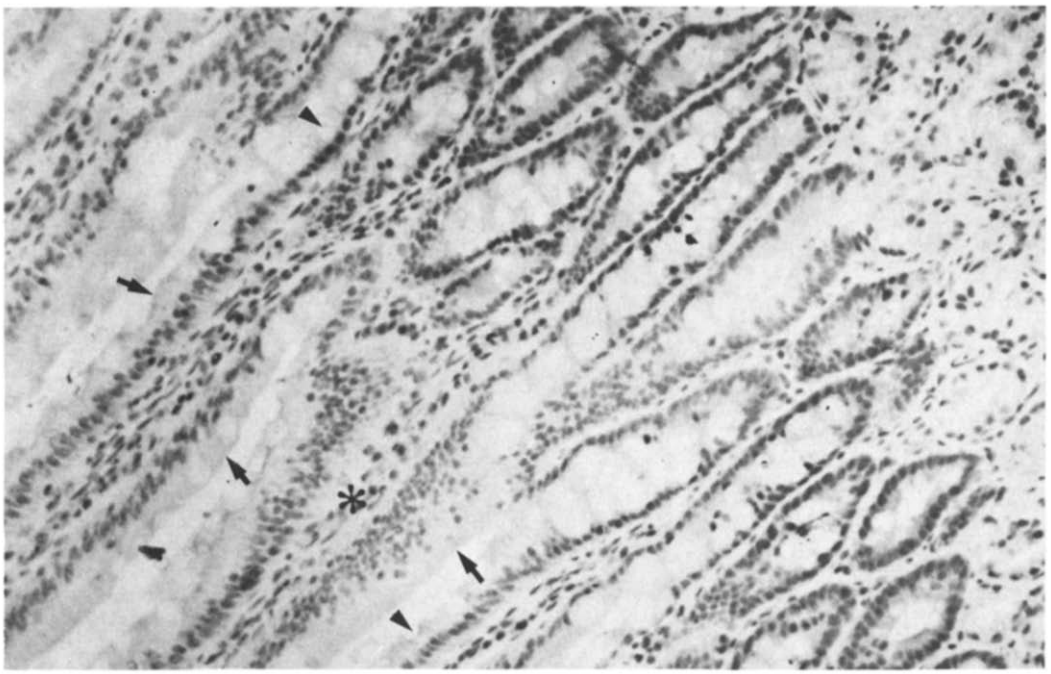
Table 5. Immunotitration of the binding of antibody 3/4/2 to olfactory epithelium

Treatment	Antibody dilution		
	1:10	1:50	1:100
Control	++	+/−	−
Phenobarbitone sodium	++	+/−	−
β-Naphthoflavone	++	+/−	−
Clofibrate	++	+/−	−

++ moderate staining; + slight staining; +/− some positive areas; − no specific staining.

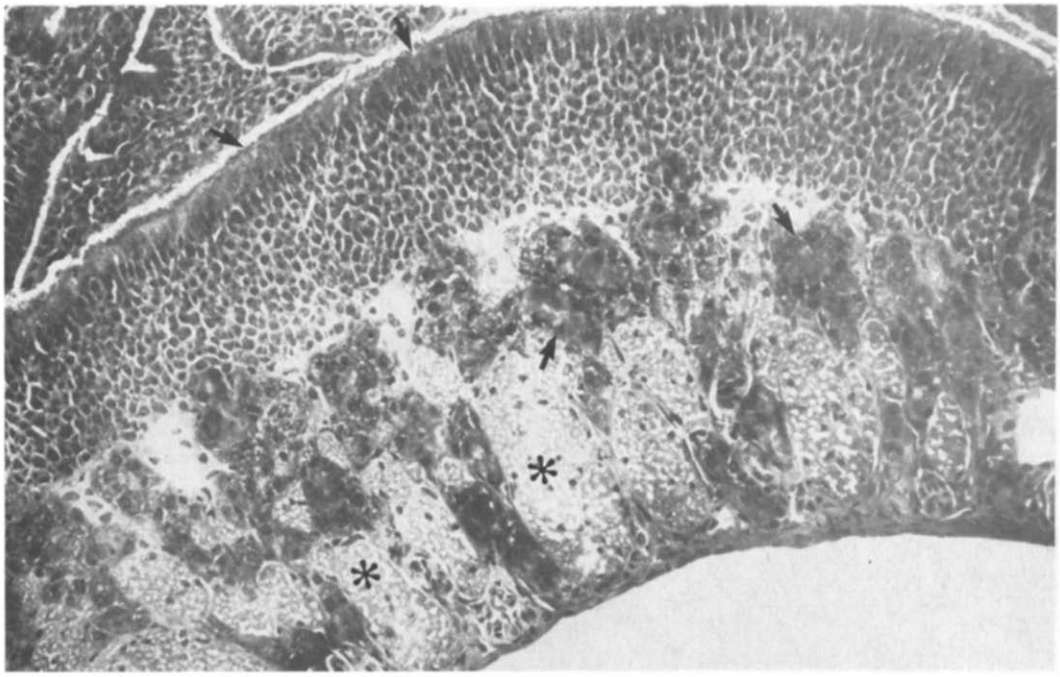


(a)



(b)

Fig. 4. Immunocytochemical localization of cytochrome P-450 *c* in the small intestine of rats using monoclonal antibody 3/4/2. Sections were from (a) rat treated with BNF orally. Positive staining is apparent in the enterocytes throughout the cilli and in the cells of the crypts of Lieberkuhn (↑), 3/4/2 used at a dilution of 1:50. The "vacuolated" goblet cells (▲) and the lamina propria (*) are negative; (b) BNF-treated rat, using control antibody B3A3 at a dilution of 1:50. The lack of differential staining between the lamina propria (*) and the enterocytes (↑) in this section are in marked contrast to that seen on exposure of sections to antibody 3/4/2 as in Fig. 4a.



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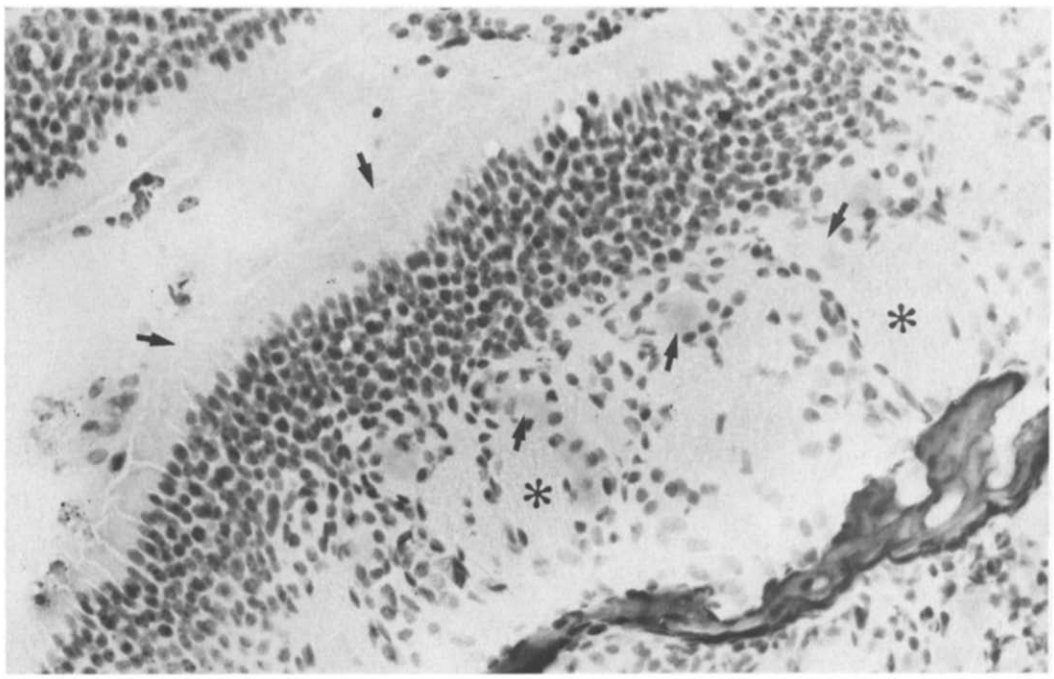

(b)

Fig. 5. Immunocytochemical localization of cytochrome P-450 *c* in the olfactory tissue of rats, using monoclonal antibody 3/4/2. Sections were from (a) untreated rat using antibody 3/4/2 at a dilution of 1:10. Positive staining for cytochrome P-450 *c* is apparent in the olfactory epithelium and in the cells of Bowman's glands (↑), but the nerve bundles (*) in the lamina propria are negative; (b) untreated rat using control antibody B3A3 at a dilution of 1:10. No staining was apparent in the olfactory epithelium and Bowman's glands (↑). Magnification ×204.8.

there is an alternative explanation for this observation. Immunohistochemistry, like other methods of analysis, has a lower limit of detection, below which there is insufficient binding of the antibody to enable visualization of reaction product. There is evidence [32, 33] based on the substrate specificities of cytochrome P-450 in liver and small intestine that the isozymes in the two tissues are similar, but that in the latter tissue levels are very low. This has recently been confirmed by Bokovsky *et al.* [34].

Following treatment of animals with BNF, immunoreactive cytochrome P-450 was readily detected in the small intestine with 3/4/2. No induction of intestinal cytochrome P-450 was apparent following treatment with phenobarbitone. Both BNF and phenobarbitone have been reported to induce cytochrome P-450 in this tissue, although BNF is three times more potent [11, 31, 34]. Thus, failure to detect induction of cytochrome P-450 by phenobarbitone in the present study is due, presumably, to the lack of the epitope for 3/4/2 on the induced isozyme, rather than levels of the isozyme below the limits of detection. One observation that awaits an explanation is the differential distribution of staining observed following oral and i.p. administration of BNF. This is most likely due to differences in exposure of the intestinal cells to the inducing compound when administered by the different routes. To the authors' knowledge this is the first description of the distribution of cytochrome P-450 in the small intestine.

In the kidney from untreated animals antibody 3/4/2 stained cytochrome P-450 in the S_3 portion of the proximal convoluted tubule, a finding similar to that previously reported [35]. Neither treatment with phenobarbitone nor clofibrate altered the distribution of staining with 3/4/2 in the kidney and the lack of effect with phenobarbitone is in agreement with results of other workers [10]. There is also biochemical evidence that phenobarbitone does not induce renal cytochrome P-450 in the rat [36]. In contrast, treatment with BNF was shown in the present study to markedly induce renal cytochrome P-450, and literature data support this finding [22, 35, 36].

In the lung, immunoreactive cytochrome P-450 was detected only in the Clara cells of the bronchiolar epithelium, and no alteration in staining was observed following treatment with either clofibrate or phenobarbitone. In contrast, treatment with BNF increased the intensity of the staining but did not change the distribution of positive cells. Localization of hydrocarbon inducible cytochrome P-450 within bronchiolar Clara cells is similar to that reported by Serabjit-Singh *et al.* in the rabbit [26], but differs from that in other studies [22, 27] where strong staining of alveolar type II cells was also observed. The absence of staining of type II cells in the present study is good evidence for the presence of different isozymes in the two cell types in the rat, as has been reported for the rabbit [18].

Comparatively little is known about cytochrome P-450 in olfactory tissue. Although it has been shown that the haemoprotein is present in this tissue [19] there have been no previous reports on its cellular distribution. Cytochrome P-450 was detected in the apical cytoplasm of the endothelial cells and the underlying Bowman's glands. Unlike the isozyme present in the other tissues, treatment with BNF had no effect on the distribution or content of this isoenzyme in olfactory tissue.

The monoclonal antibody used in these studies, 3/4/2, is particularly suitable for immunohistochemical studies of the distribution of cytochrome P-450. It is highly specific and does not cross react with other proteins. In the present study, the localization of cytochrome P-450 and its response to treatment of animals with inducers, has been determined in a number of tissues, including small intestine and olfactory epithelium, not previously studied. In addition, assessing inducibility of isozymes *in situ* in tissues where the distribution is focal and limited, making assessment by other techniques impractical, has been shown to be of considerable potential value.

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