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EXPRESSION AND DISTRIBUTION OF CHOLESTEROL 7α-HYDROXYLASE IN RAT LIVER

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Abstract—The hydroxylation of cholesterol by cholesterol 7α-hydroxylase (CYP7) to 7α-hydroxycholesterol is the rate-limiting step in the production of bile acids. An anti-peptide antibody targeted to the C-terminus of CYP7 was produced by immunising rabbits with the synthetic peptide Tyr-Lys-Leu-Lys-His. The antibody bound to a single band of 54 kDa from rat hepatic microsomal fractions. The intensity of the band was subject to a diurnal variation and showed a significant increase ($P < 0.01$) in apoprotein at night. Treatment of rats with cholestyramine increased CYP7 apoprotein in the morning ($P < 0.005$) and at night ($P < 0.005$), but diurnal variation was maintained. CYP7 catalytic activity, measured using a specific gas chromatography/mass spectrometry assay, showed similar changes in the pattern of diurnal variation and induction. The distribution of CYP7 in rat liver tissue sections was investigated by immunocytochemistry. In sections from rats treated with cholestyramine, there was an even distribution of immunoreactivity, except in the proximal perivenous hepatocytes where immunoreactivity was slightly more intense. A similar distribution was found in sections from untreated rat liver, except immunoreactivity was overall slightly less intense. This study shows that the C-terminus of CYP7 is a useful epitope for the targeting of anti-peptide antibodies.

Key words: cholesterol 7α-hydroxylase; cholestyramine; antipeptide antibody; diurnal variation; immunocytochemistry; mass spectrometry

The hydroxylation of cholesterol by the hepatic microsomal enzyme cytochrome P450 CYP7† is the initial and rate limiting step in the conversion of cholesterol to bile acids [1]. Immunochemical and biochemical evidence exists to show that CYP7 is distinct from other hepatic P450 enzymes [2].

The regulation of CYP7 is known to be affected by the enterohepatic circulation of bile acids which act to repress enzyme levels. Biliary drainage or administration of anionic exchange resins, such as cholestyramine prevents feedback of bile acids to the liver which leads to a derepressive effect on CYP7 expression and an increase in the level of the enzyme [3]. It has recently been shown that the more hydrophobic bile acids (i.e. deoxycholate and taurodeoxycholate) have a stronger ability to repress CYP7 at the mRNA level [4, 5]. This indicates that down-regulation of the enzyme occurs as a result of decreased transcriptional activity of the CYP7 gene, but the exact mechanism of bile acid repression of the CYP7 gene has yet to be clearly defined. Expression of CYP7 is also known to be regulated by thyroid and steroid hormones [1]. The 5′ promoter region of both the human and rat CYP7 genes contains many liver specific transcription factor binding sites and putative hormone response elements which may be involved in the control of gene expression [6, 7].

Several groups have purified CYP7 from liver and have produced polyclonal antibodies to both rat and human forms [2, 8–11]. These antibodies have been used in immunoblotting studies and bind to CYP7 which migrates as a ~53 kDa protein [10]. However, in general these antibodies also bind to other proteins.

Here, a specific anti-peptide antibody was targeted towards CYP7. The antibody was used to study the expression and distribution of CYP7 in the rat liver.

MATERIALS AND METHODS

Materials. Cholestyramine and dithiothreitol were purchased from Sigma (Poole, Dorset, U.K.). Heptadeuterated 7α-hydroxycholesterol was obtained from CDN Isotopes (distributed by K&K-Greeff Ltd, Croydon, U.K.). All SDS-PAGE reagents were from National Diagnostics (Aylesbury, Bucks, U.K.). KLH was obtained from Calbiochem (Nottingham, U.K.). Polystyrene 96-well microtitre plates were from Dynatech Laboratories (Billingshurst, West Sussex, U.K.). Conjugates of immunoglobulins with HRP was from Sigma, and Strepavidin–biotin complex linked to HRP and swine anti-rabbit serum were obtained from Dako (High Wycombe, Bucks, U.K.). Gel filtration resins were

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† Abbreviations: CYP7, cholesterol 7α-hydroxylase; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; KLH, keyhole limpet haemocyanin; HRP, horseradish peroxidase; TLC, thin-layer chromatography; ELISA, enzyme-linked immunosorbent assay; ECL, enhanced chemiluminescence; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; HMG CoA reductase, 3-hydroxy 3-methylglutaryl-CoA reductase; DTT, dithiothreitol

from Pharmacia (Milton Keynes, U.K.). DB5 microbore gas chromatography columns were obtained from Jones Chromatography (Hengoed, Mid-Glamorgan, U.K.). LK5D strip linear K preadsorbent TLC plates were from Whatman (Maidstone, Kent, U.K.). *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide was obtained from Aldrich (Dorset, U.K.). All other chemicals were purchased from Sigma or from Merck-BDH (Lutterworth, Leicestershire, U.K.) and were of analytical grade or the best equivalent.

Selection and synthesis of peptide. It was decided to target an anti-peptide antibody to the C-terminus of CYP7, i.e. Tyr-Lys-Leu-Lys-His. This epitope was found to be unique among the cytochromes P450 and indeed does not occur in any other protein sequence listed on the Swiss-Prot database. Conjugation of the peptide to a carrier protein was achieved by synthesising a peptide with a cysteine residue at the N-terminus (Cys-Tyr-Lys-Leu-Lys-His). This decision was based on the success of targeting the C-terminus of other cytochromes P450 [12]. The peptide was synthesized on a solid-phase, continuous flow, semi-automatic peptide synthesizer (NovaSyn, Nottingham, U.K.) using a polyamide support and Fmoc amino acids as described previously [13]. The peptide was purified by gel filtration on a Sephadex G-15 column (15 mm × 320 mm) in 0.5 M acetic acid to give a product of >90% purity, as determined by high performance liquid chromatography using a C_{18} μBondapak column (Millipore, Middlesex, U.K.), with a gradient of 0–25% (v/v) acetonitrile in the presence 0.1% (v/v) trifluoroacetic acid over 20 min at a flow rate of 1 mL/min and with constant monitoring of eluent at 210 nm. The purified peptide was conjugated to carrier protein. KLH was derivatized with MBS and then added to the peptide at a ratio of 2.3 mg/μmol of cysteinyl peptide, as determined by the free thiol content of the preparation [14]. The pH of the mixture was adjusted to 7.3, mixed for 2 hr and dialysed three times against 500 mL of PBS (1.5 mM-KH_2PO_4, 8.1 mM-Na_2HPO_4, 2.7 mM-KCl, 137 mM-NaCl, pH 7.5). KLH-MBS-Cys was synthesized in a similar way by substituting cysteine for the thiolated peptide. A conjugate of KLH coupled to a peptide corresponding to the C-terminus of human CYP7, Cys-Lys-Phe-Lys-His-Leu was also synthesized.

Antibody production and assessment. Male New Zealand White rabbits (~3 kg body weight from Froxfield Farms Ltd, Hampshire, U.K.) were immunized by repeated injections of 200 μg of peptide conjugate in Freund's adjuvant in a total volume of 1 mL. The rabbits were initially immunised subcutaneously with complete adjuvant (day 1) then with incomplete adjuvant on day 14 and finally by intraperitoneal injection with incomplete adjuvant on day 21. Blood was taken on day 28 from a marginal ear vein. Whole serum was stored at −20°. Antibodies directed against the carrier protein and the linking group were depleted by affinity adsorption, as described previously [14]. The binding of the antibodies to the target peptide was assessed by ELISA as previously described [14].

Treatment of animals, preparation of hepatic microsomal fractions. Groups of six female Wistar rats (~250 g) from Harlan Olac Ltd (Bicester, U.K.) were fed with rat pellets (Special Diet Services, Essex, U.K.) or the same diet supplemented with 5% (w/w) cholestyramine. The supplemented diet was prepared by mixing powdered feed with cholestyramine and sufficient water to achieve a dough-like mixture. This was divided into pieces (1.5 cm × 10 cm × 2.5 cm), dried at 40° overnight, then broken into smaller pieces, ready for feeding. Animals were maintained under a normal light cycle (light from 5 a.m. to 5 p.m.). After 8 days three animals from each group were humanely killed in accordance with Home Office procedures at 10 a.m. and three were killed at 10 p.m. The livers were removed and microsomal fractions prepared as was described previously [15], except 5 mM dithiothreitol was added to all buffers. A sample of liver tissue was also removed for immunocytochemistry.

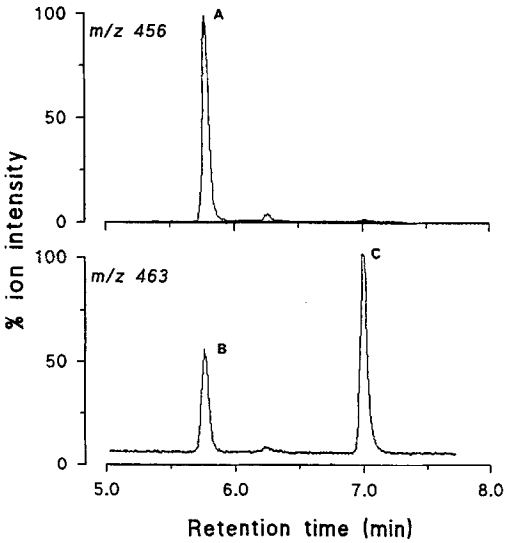

Fig. 1. Gas chromatography/mass spectrometry selected monitoring of ions at *m/z* 456 and *m/z* 463 of the trimethylsilyl esters of 25 ng of 7α-hydroxycholesterol and 25 ng of heptadeuterated 7α/7β-hydroxycholesterol. The elution of (A) authentic 7α-hydroxycholesterol, (B) deuterated 7α-hydroxycholesterol, and (C) deuterated 7β-hydroxycholesterol is shown.

Immunoblotting. Microsomal samples were separated on 9% polyacrylamide gels and then transferred onto nitrocellulose as described previously [13]. The polyclonal anti-peptide antibody directed against CYP7 (1:4000 dilution) and a goat anti-rabbit peroxidase coupled antibody (1:30,000 dilution) were used with an enhanced chemiluminescence method (ECL-Amersham International, Bucks, U.K.) to detect apoprotein.

Immunoreactivity was quantified by scanning immunoblots using an LKB Ultrascan XL laser densitometer.

Assay for 7α-hydroxylase activity. The method used was based on those described previously [16, 17]. The incubation mixture consisted of 0.2 mg of microsomal protein, 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, 5 mM DTT,

0.015% (w/v) CHAPS, 50 mM NaF, 1 mg/mL BSA in a total volume of 0.9 mL. The reaction was started by addition of 1 mM of NADPH (final concentration) in 0.1 mL of water. For each microsomal sample the amount of endogenous 7α-hydroxycholesterol was determined under similar assay conditions except that NADPH was omitted (reaction blank). The enzyme reaction was carried out at 37° for 30 min. The reaction was stopped by the addition of 0.5 mL of ice cold methanol. Then 3 mL of petroleum ether and an internal standard of 500 ng deuterium labelled 7α/7β-hydroxycholesterol dissolved in 50 μL of ethyl acetate was added. After mixing for 5 min, the upper ether layer containing the sterol fraction was removed and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μL of ethyl acetate and loaded onto a Whatman LK5D linear K preadsorbent TLC plate as were standards of cholesterol and 7α-hydroxycholesterol. Cholesterol was separated from 7α-hydroxycholesterol using a mobile phase consisting of ethyl acetate/toluene 1:1. The migration positions of cholesterol and 7α-hydroxycholesterol standards were visualized as red and blue bands, respectively, following development by spraying with 5 M chlorosulphonic acid/1 M acetic acid (1:1) and heating to about 120°. Silica in the region equivalent to the migration position of 7α-hydroxycholesterol in the unknown samples was removed from the TLC plate and extracted with 0.5 mL of ethyl acetate/toluene. The solvent was evaporated to dryness and the residue derivatised overnight with *N*-methyl-(trimethylsilyl)trifluoroacetamide at 50°. This was evaporated to dryness and the residue dissolved in 20 μL of dodecane. Measurement of 7α-hydroxycholesterol was carried out by gas chromatography/mass spectrometry using electron impact ionization. Samples of 1–2 μL were injected onto a DB5 column, with a helium carrier gas (pressure of 10 psi (1 psi ≈ 6.9 kPa)) and over a temperature gradient of 200° to 320° in 7 min. The elution time of 7α-hydroxycholesterol was ~6 min. The ratio of standard ion (456) to the deuterated ion (463) was assessed for each sample (Fig.1). The amount of 7α-hydroxycholesterol formed during the incubation was calculated by subtracting the amount present in the reaction blank and was expressed as pmol/min/mg protein. The amount of 7α-hydroxycholesterol in the reaction blank was always less than 10% of the total amount measured.

Immunocytochemistry. Tissues were fixed overnight in 10% (v/v) formalin or methacarn (methanol/chloroform/glacial acetic acid, 6:3:1) and then mounted in parafin wax. Sections 5 μm thick were cut and applied to poly-L-lysine coated glass slides. After blocking endogenous peroxidase by incubation in 0.3% (v/v) hydrogen peroxide, sections were incubated for 10 min with normal swine serum diluted 1:20 in PBS. This was followed by a 2 hr incubation with antiserum (dilution 1:200 in PBS containing 0.1% BSA) raised against the C-terminus of rat CYP7. After rinsing with PBS, biotinylated swine anti-rabbit immunoglobulin diluted 1:500 in PBS was applied for 45 min. Sections were then rinsed with PBS and incubated with HRP conjugated Streptavidin complex for 30 min. After rinsing in PBS, sections were developed for 2–7 min with 0.05% diaminobenzidine and 0.03% H_2O_2 in PBS, counterstained with Harris's haematoxylin, dehydrated, cleared and mounted in Pertex.

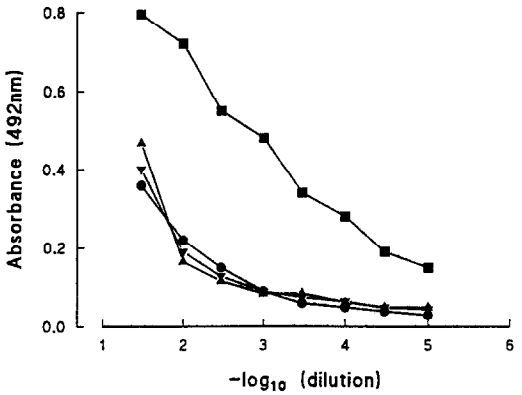

Fig. 2. The relative affinity of binding of the anti-peptide antibody to various peptide conjugates. Microtitre wells were coated with either KLH (▼), KLH-MBS-Cys (▲), KLH-MBS-Cys-Tyr-Lys-Leu-Lys-His (■) (target epitope) or KLH-MBS-Cys-Lys-Phe-Lys-His-Leu (●). To each group a series of dilutions of the anti-peptide antibody (depleted of antibodies against KLH-MBS-Cys) was added and the relative binding was determined as described in the text.

RESULTS

The binding of the antiserum depleted of antibodies against KLH-MBS-Cys to various antigens was determined by ELISA (Fig. 2). The immunodepleted antiserum showed little binding to the null conjugate, KLH-MBS-Cys, or carrier protein, KLH, but showed strong binding to the immunising conjugate KLH-MBS-Cys-Tyr-Lys-Leu-Lys-His indicating that antibodies to the peptide were successfully raised. Binding of antibodies to another conjugate KLH-MBS-Cys-Lys-Phe-Lys-His-Leu was similar to that found for binding to the negative control null conjugate, KLH-MBS-Cys. Therefore, no binding was detected to the peptide moiety of this conjugate, thus showing that the binding of the anti-peptide antibodies were sequence dependent.

In immunoblotting, the anti-peptide antibody bound to a single protein band with a molecular weight of 54,000 in hepatic microsomal fractions from untreated and cholestyramine treated female Wistar rats (Fig. 3). The intensity of the band was increased in rats killed at night ($P < 0.01$) and was also increased in rats treated with cholestyramine ($P < 0.005$) (Figs 3 and 4a).

Rats fed the control diet had a microsomal CYP7 activity of 29 ± 3.3 pmol/min/mg in the morning which was significantly elevated by 2.4-fold to 72.2 ± 2.7 at night ($P < 0.005$) (Fig. 4b). Immunoblotting showed that there was a similar 2.2-fold increase ($P < 0.01$) in apoprotein levels in samples taken at night compared with morning (Figs 3 and 4a). Treatment of rats with cholestyramine resulted in CYP7 activity of 110 ± 10.0 pmol/min/mg (morn-

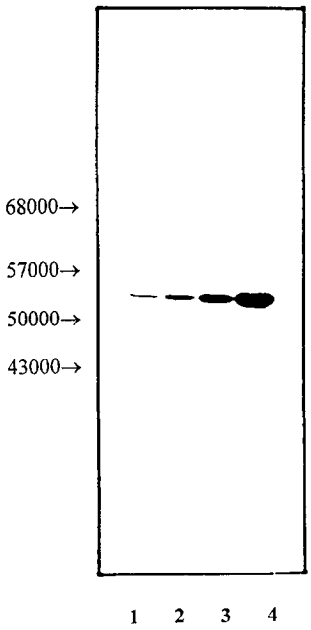

Fig. 3. Immunoblot of CYP7 apoprotein. A single band of M_r 54,000 following SDS-PAGE of hepatic microsomes from untreated and cholestyramine treated female Wistar rats. The immunoblot was developed with an anti-peptide antibody raised against the C-terminus of rat CYP7. Samples of 20 μg of hepatic microsomal fraction from untreated rats killed in the morning (lane 1), and at night (lane 2), cholestyramine-treated rats killed in the morning (lane 3) and at night (lane 4), were loaded onto the gel and developed as described in the text.

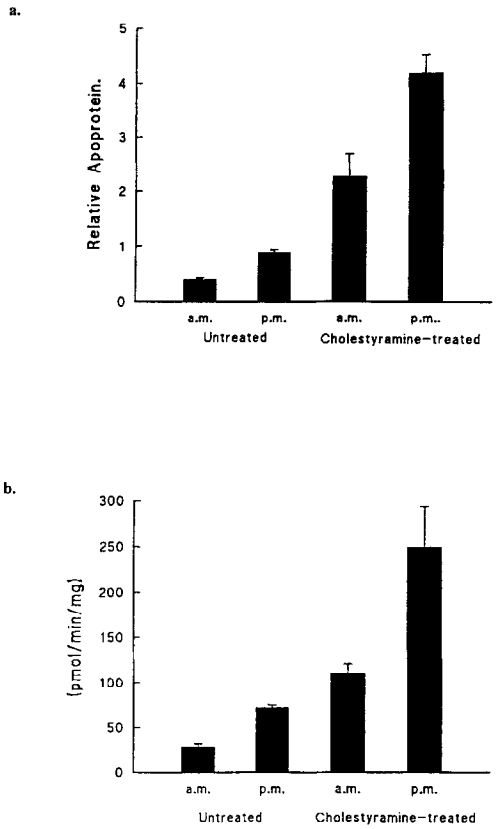

Fig. 4. (a) Relative CYP7 apoprotein levels of microsomes from untreated (a.m. and p.m. killed) and cholestyramine treated (a.m. and p.m. killed) female Wistar rats. For each group (N = 3), band densities were measured on an LKB laser densitometer. (b) Hepatic microsomal CYP7 activity in untreated (a.m. and p.m. killed) and cholestyramine treated (a.m. and p.m. killed) female Wistar rats. The activity was measured using a specific mass spectrometry/gas chromatography assay as described in the text. The results shown are mean values ± SEM (N = 3).

ing), and 249.4 ± 44.4 pmol/min/mg (night), i.e. 3.7-fold ($P < 0.01$) and 3.4-fold ($P < 0.025$) increases over the respective control groups (Fig. 4b). The apoprotein levels were increased by 5.7-fold ($P < 0.005$) in the morning and increased by 4.7-fold ($P < 0.005$) at night following cholestyramine treatment (Figs 3 and 4a).

The antipeptide antibody was tested in immunocytochemical studies. Whole tissue sections were fixed in either formalin or in methacarn. The antibody failed to bind to formalin fixed sections (results not shown), but bound avidly to methacarn fixed sections (Fig. 5). In sections from cholestyramine treated rats, immunoreactivity was evenly distributed in all hepatocytes except around proximal perivenous regions where immunoreactivity was slightly more intense. In sections from untreated rats, a similar pattern was observed except immunoreactivity was less intense.

DISCUSSION

An antipeptide antibody was produced by targeting the C-terminus of rat CYP7. In previous attempts to raise anti-peptide antibodies, the C-terminus of cytochromes P450 has been found to be a highly successful region for the production of specific antibodies [12].

In ELISA, the anti-peptide antibody bound to the target peptide and did not cross react with other antigens, especially the synthetic peptide corresponding to the human C-terminus, thus, indicating the sequence selectivity of the antibody. In immunoblotting, the antibody recognized a single protein band of 54 kDa in hepatic microsomal fractions from untreated female Wistar rats. The molecular weight is similar to that described by other groups [2, 10, 11]. A diurnal variation was observed in the levels of CYP7 apoprotein with significantly higher levels being found at night and this corresponded with CYP7 activity. Diurnal variation has also been shown by Sundseth and Waxman [2] and Chiang *et al.* [10]. Treatment of rats with cholestyramine caused a significant increase in the levels of CYP7 apoprotein in both samples taken in the morning and at night. Again, the levels corresponded to an increase in CYP7 activity. The effect of cholestyramine administration and the maintenance of diurnal variation has been shown previously and indicates that the factors controlling

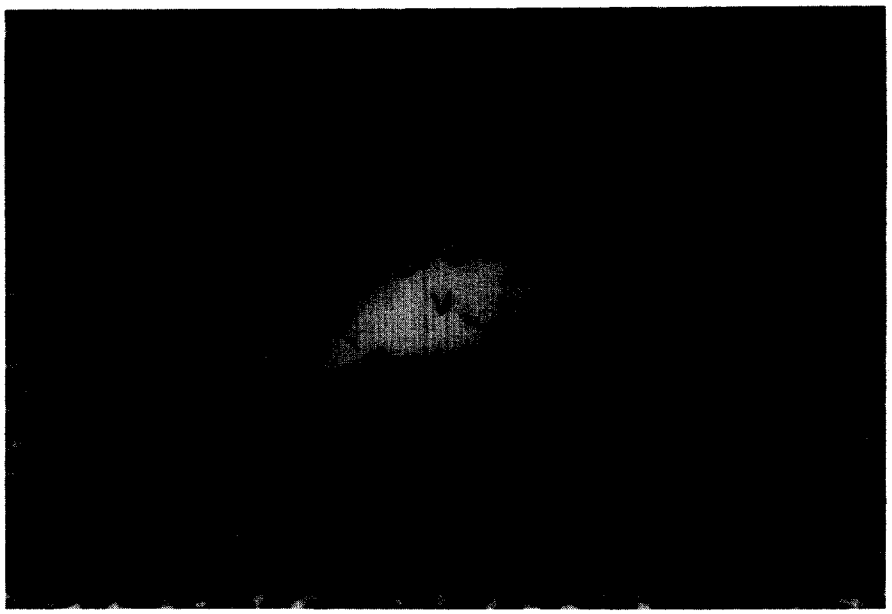

Fig. 5. Immunocytochemical location of CYP7 in methacarn fixed liver from a rat treated with cholestyramine and developed with the anti-peptide antibody. Original magnification ×200. Immunoreactivity was found in all hepatocytes, the arrow indicates a typical hepatocyte. A slightly more intense immunoreactivity was found in hepatocytes surrounding the proximal perivenous regions (V).

the diurnal variation of CYP7 are distinct from bile acid feedback regulation [2, 10]. A recent study has shown that the liver transcription factor DBP (albumin promoter D-site binding protein) may be one such factor [18].

Several groups have produced polyclonal antibodies from purified CYP7 apoprotein [2, 8, 10, 11]. In most cases, while they recognise their target protein in immunoblotting, they also cross-react with several other proteins. As the anti-peptide antibody produced here binds specifically, it was possible to investigate the distribution of CYP7 in rat liver tissue sections. No immunoreactivity was detected in formalin-fixed sections. Formaldehyde fixation involves cross-linkage of amine groups, predominately on the ε-amine of lysine residues. Since the CYP7 epitope contains two lysine residues it is possible that they may have been modified by formaldehyde, causing a loss of antigenicity. In contrast, antigenicity was maintained in tissues fixed with methacarn. In liver sections from cholestyramine treated rats, immunoreactivity was evenly distributed in all hepatocytes except in the proximal perivenous regions where it was more intense. In liver sections from untreated rats, a similar pattern was found to that found in the liver of rats treated with cholestyramine, except that immunoreactivity was less intense. Ugele *et al.* [19] found, using a digitonin/collagenase digestion method, that in cholestyramine-treated rats, all hepatocytes were capable of bile acid synthesis, however, in untreated rats this was restricted to hepatocytes in the perivenous regions. Also, Li *et al.* [20] have shown that HMG-CoA is expressed in all hepatocytes following treatment of rats with a bile acid sequestrant. This is similar to the distribution of CYP7 and indicates that cholesterol synthesis by HMG-CoA reductase is closely linked to the expression of CYP7 as has been suggested previously [21].

This present work has demonstrated a simple method for the production of an anti-peptide antibody of defined specificity to the cytochrome P450 enzyme, CYP7. This antibody has provided the first immunocytochemical evidence of the hepatic distribution of CYP7 in untreated and cholestyramine treated rats.

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