REDUCED SYNTHESIS OF HEPATIC MICROSOMAL CYTOCHROME P_{450} IN THE BILE DUCT LIGATED RAT

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SUMMARY: Cholestasis produced by bile duct ligation was associated with a 50% reduction in hepatic microsomal cytochrome P_{450} content. Tentative identification of cytochrome P_{450} in washed microsomal fractions was achieved on SDS-urea polyacrylamide gels by its physical properties, half-life and response to pharmacologic agents. The relative synthesis rate of P_{450} apoprotein, determined by a double isotope method, was significantly reduced 3 days after bile duct ligation. P_{450} apoprotein degradation was markedly reduced, with the half-life increasing from 24 to 50 hours. These data indicate that a reduced rate of synthesis, rather than increased degradation, is responsible for reduction of P_{450} in cholestasis.

Heterogeneous turnover of hepatic microsomal membrane bound proteins, and their differential response to phenobarbital stimulation is well established (1,2). Furthermore, there is an apparent relationship between the rate of turnover and the molecular size of membrane-bound polypeptides, in that larger polypeptide chains generally have a rapid rate of turnover (2,3). An exception to this general rule is a microsomal protein of molecular weight 50,000-60,000, tentatively identified as cytochrome P_{450}, which has a relatively rapid turnover (4).

Altered hepatic microsomal biotransformation and lowered levels of cytochrome P_{450} have been implicated in the pathogenesis of cholestasis (5). It has been proposed that increased degradation of microsomal proteins resulting from the solubilizing effect of retained bile salts may be responsible for this phenomenon (6). The data presented in this report indicate that the reduced level of cytochrome P_{450} associated with bile duct ligation results from decreased synthesis rather than increased degradation of the apoprotein.

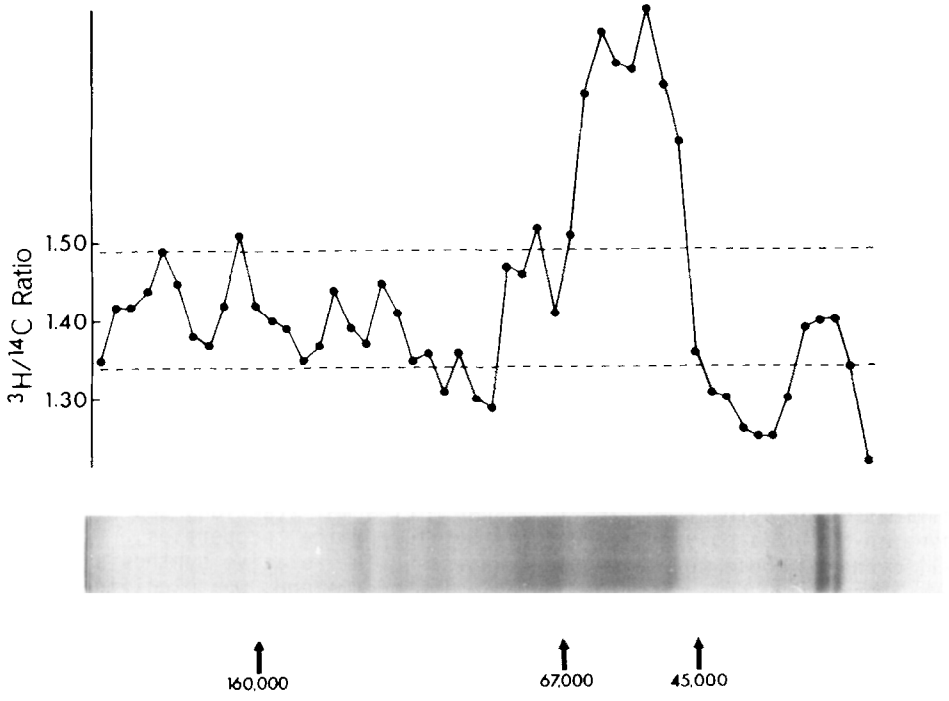

Figure 1. Effect of phenobarbital on the relative synthesis of microsomal protein. A 12 hour fasted control animal was given 50 μCi ^{14}C leucine (311 mCi/mmol, Amersham) intraperitoneally, while simultaneously 200 μCi ^{3}H leucine (1 Ci/mmol, Amersham) was given to a fasted phenobarbital treated animal (80 mg/Kg/day for 3 days). Animals were killed 6 hours later and microsomes prepared and electrophoresed as described under Methods. The ratio dpm ^{3}H/dpm ^{14}C for each 1.5 mm gel slice was divided by the homogenate ^{3}H:^{14}C ratio (origin on left, front on right). Dotted horizontal lines represent 95% confidence limit derived from control v. control; any point outside these limits represents a significant variation from normal. Absolute dpm per slice ranged 320-5000 for ^{3}H and 210-1700 for ^{14}C. The lower figure illustrates the Coomassie-blue stained microsomal pattern in 7.5% gel. Major protein migrated between 50,000-60,000 MW, determined from semi-log plots of standard proteins (15).

MATERIALS AND METHODS: Studies were performed with male Sprague-Dawley rats weighing 200-300 g. Cholestasis was produced by double ligation and transection of the common bile duct under ether anesthesia. Three to five days later the animals were killed, their livers homogenized in 4 volumes of cold 0.25M sucrose - 0.01 M Tris HCl pH 7.4, and microsomal fractions were prepared by differential centrifugation. Pelleted microsomes were washed according to the method of Weihing et al. (7) to remove adherent

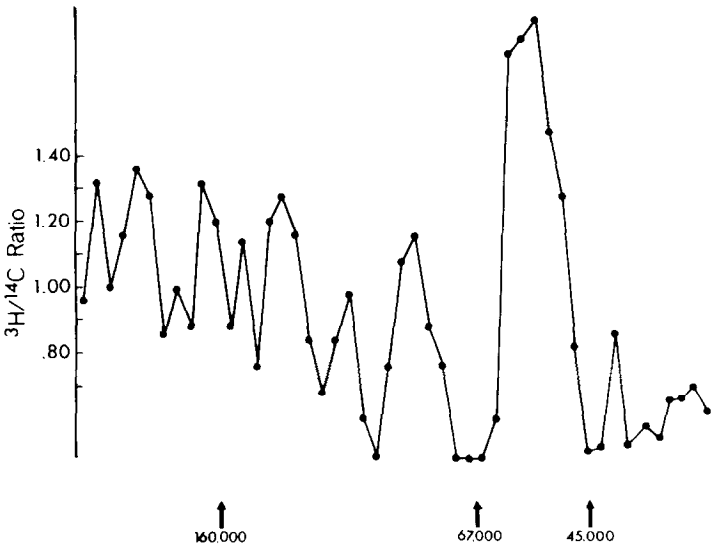

Figure 2. Relative degradation patterns of normal microsomal membrane proteins. Two 12 hour fasted animals were intraperitoneally injected with 75 μCi ^{14}C leucine, and normal diet reinstituted. Forty eight (48) hours later both rats received 200 uCi ^3H leucine intraperitoneally after another 12 hour fast. Animals were killed 6 hours later and microsomes prepared and electrophoresed (see text). The ratio dpm ^3H:dpm ^{14}C for each 1.5 mm slice was divided by the homogenate ratio to yield a turnover index (11). Results as turnover idex for each slice, origin on left.

and cisternal protein, and were suspended in 0.1 M Tris HCl pH 7.4 to a concentration of 1.5 - 2.0 mg protein per ml. Cytochrome P_{450} was assayed in a Carey Model 14 spectrophotometer by the method of Omura and Sato (8), and protein was assayed by the method of Lowry et al. (9).

Washed microsomal fractions were solubilized with 1% sodium dodecyl sulphate (SDS) in 0.1 M Tris HCl pH 7.5 and heated at 100°C for 2 minutes. Solubilized microsomes with 20% glycerol (V/V), 0.5% mercaptoethanol (V/V) and 0.001% Pyronin Y as marker were applied to preparative (1.9 x 10 cm) or analytical (0.5 x 10 cm) 7.5% polyacrylamide -SDS- 6M urea gels and electrophoresed according to the method of Laemmli (10). The apparent molecular weight of constituent membrane proteins was determined by the coincident electrophoresis of standard calibrating proteins (Schwarz/Mann). Analytical gels were fixed and stained in Coomassie blue 0.1% in 12.5% trichloracetic acid, 50% methanol and were destained with 10% acetic acid. Preparative gels were frozen overnight

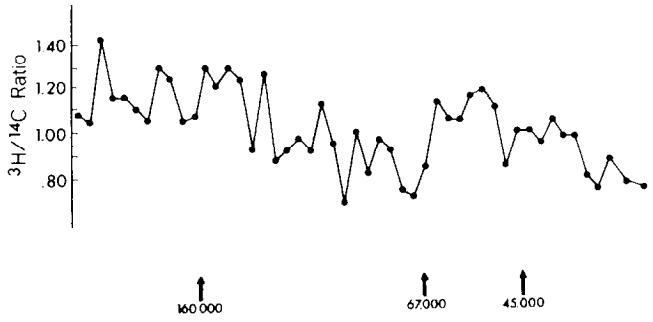

Figure 3. Relative degradation pattern of microsomal proteins from bile duct ligated rats. Three days after bile duct ligation 75 μCi ^{14}C leucine was administered to two 12 hour fasted animals. Experiment then continued as described in legend to Figure 2.

and sliced transversely into consecutive 1.5 mm slices from origin to front. Each slice was solubilized with 0.5 ml 30% hydrogen peroxide in counting vials and 24 hours later 0.5 ml of Protosol (New England Nuclear) was added. Radioactivity was counted in Toluene-Ominfluor scintillant (New England Nuclear) and a Packard Tri-carb counter.

The relative degradation rates of microsomal proteins were determined in control and bile duct obstructed animals by the double isotope technique of Arias et al. (1). Specific details of the ^3H and ^{14}C labelling procedure are given in the figure legends. The effect of bile duct ligation on the relative synthesis rate of microsomal proteins was studied by adapting the method of Dehlinger and Schimke (2). Leucine ^{14}C (50 μCi) was administered intraperitoneally to a 12 hour fasted control rat, while ^3H leucine (200 μCi) was simultaneously administered to a rat 3 days following bile duct ligation. Both animals were killed 6 hours later, and microsomal fractions prepared from the combined liver homogenates. The washed microsomal pellet containing microsomes from control and cholestatic rats was solubilized and electrophoresed as described above, and the ratio dpm $^3H/^{14}C$ for each 1.5 mm gel slice from origin to front was determined.

RESULTS AND DISCUSSION: After 3 days of bile duct ligation, hepatic microsomal

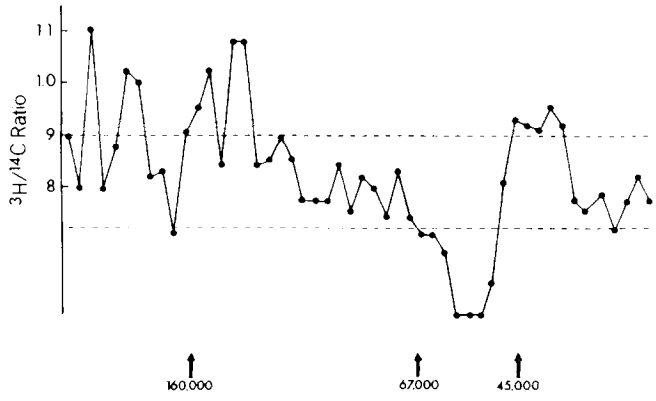

Figure 4. Effect of bile duct ligation on relative synthesis rate of microsomal proteins. A 12 hour fasted control animal was injected intraperitoneally with 50 μCi ^{14}C leucine, while 200 μCi ^{3}H leucine was simultaneously given to a 12 hour fasted rat 3 days (72 hours) post bile duct ligation. Animals were killed 6 hours later, and microsomal fractions processed as described in text and legend to Figure 1.

cytochrome P_{450} was significantly reduced from a control level of 0.62 ± 0.04 to 0.31 ± 0.11 m μ moles per mg microsomal protein ($p < 0.025$, mean ± S.D., 4 animals each group). To determine the mechanism by which cytochrome P_{450}, the major microsomal protein, is reduced in cholestasis, we initially identified this protein within an SDS-urea polyacrylamide gel system. Since heme is disrupted from the hemoprotein complex by SDS (12), and immunological identification of cytochrome P_{450} has not been described, unequivocal identification of P_{450} was not possible. However, in agreement with established data, the major microsomal protein migrated with an apparent molecular weight of between 50,000 and 58,000 (Fig. 1). When equal amounts (100 μg) of microsomal protein from control and from phenobarbital and 3 methylcholanthrene (3MC) treated animals were applied to analytical gels, increased density of this Coomassie blue-stained protein band was demonstrated with phenobarbital and 3MC stimulated microsomes. The relative synthesis rate of this protein was markedly increased by phenobarbital administration (Fig. I). In addition the half life of the protein in the molecular weight range 50,000 - 58,000 was estimated, by the method

of Glass and Doyle (13) to be 24 hours. This value is very similar to that reported by Greim et al. (14) for the heme moiety of cytochrome P_{450}. These data indicate that, within this analytical system, the protein of molecular weight 50,000 - 58,000 is predominantly cytochrome P_{450}.

The relative rates of degradation of microsomal membrane-bound proteins from a control animal are illustrated in Fig. 2. In general, the higher molecular weight polypeptides have a more rapid rate of degradation than smaller polypeptides. A marked exception to this general rule is the protein of molecular weight 50,000-58,000, tentatively identified as cytochrome P_{450}. Since the estimated half life of the P_{450} apoprotein in this system (24 hours) is similar to that previously obtained for the P_{450} prosthetic group (14), these data suggest that under normal conditions the cytochrome P_{450} heme and protein moieties are degraded as a single unit. However, following 3 days of bile duct ligation, the relative degradation rate of the P_{450} apoprotein is markedly reduced (Fig. 3). The estimated half life of this protein of 50 hours is now indistinguishable from the turnover of total microsomal membrane protein.

Since the postulated increased degradation rate of microsomal cytochrome P_{450} was not demonstrated, the effect of cholestasis on the synthesis of microsomal proteins was determined. The effect of 3 days of bile duct obstruction on the relative synthesis rate of microsomal membrane bound proteins is illustrated in Fig. 4. The synthesis of a number of higher molecular weight proteins was apparently increased. In contrast, the relative synthesis rate of protein migrating in the cytochrome P_{450} region was markedly reduced.

These data demonstrate that the reduction in hepatic cytochrome P_{450} level associated with bile duct ligation results from decreased synthesis of cytochrome P_{450}. Although the double isotope method employed in this study does not provide an index of the absolute rate of protein synthesis, it does indicate increased or decreased incorporation of amino acid into individual proteins following bile duct ligation or phenobarbital administration. Cholestasis increased the synthetic rate of several higher molecular

weight proteins, suggesting that the decreased synthesis of microsomal cytochrome P_{450} protein is a specific effect and not a reflection of generalized microsomal membrane damage. We conclude that cholestasis, like certain pharmacologic agents, exerts a differential effect on the synthesis of microsomal membrane-bound proteins and specifically reduces the synthetic rate of cytochrome P_{450}.

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