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QUALITATIVE ALTERATION IN HEPATIC MICROSOMAL CYTOCHROME P-450 APOPROTEINS ASSOCIATED WITH BILE DUCT LIGATION, AND THE ADMINISTRATION OF ETHINYL ESTRADIOL, PHENOBARBITAL AND 3-METHYLCHOLANTHRENE

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Abstract—We have investigated in rat liver whether different forms of cytochrome P-450 are altered in hepatic disorders associated with impaired drug metabolism. Total hepatic cytochrome P-450 is decreased after either bile duct ligation or the administration of ethinyl estradiol. In contrast, phenobarbital administered alone increases hepatic content of cytochrome P-450, and when administered with ethinyl estradiol the reduction in cytochrome P-450 was prevented. Microsomal ethylmorphine *N*-demethylase activities paralleled changes in cytochrome P-450 content, except in bile duct ligation, where it is diminished to a greater extent. Four forms of microsomal cytochrome P-450 apoproteins, ranging in molecular weight from 50,000 to 58,000, were tentatively identified in a sodium dodecyl sulfate (SDS)–6 M urea polyacrylamide gel electrophoresis system by their responsiveness to pharmacological agents, turnover and benzidine peroxidase staining. Phenobarbital administration increased primarily band IV (50,000 daltons); in contrast only band III (53,000 daltons) was responsive to 3-methylcholanthene. Bile duct ligation was associated with a marked reduction in bands I, III and IV while bands I and III were decreased with ethinyl estradiol administration. Simultaneous administration of phenobarbital and ethinyl estradiol demonstrated a return of band I and an increase in density of bands II and IV. The mechanism of this reversal by phenobarbital was determined by the double-isotope technique and demonstrates that phenobarbital increases the relative synthesis rates of P-450 apoproteins in ethinyl estradiol-treated rats. These studies support the hypothesis that multiple forms of cytochrome P-450 are present in liver microsomal membranes and that alterations in specific apoproteins may be associated with an increase or a decrease in the functional properties of cytochrome P-450.

The hepatic microsomal mixed-function oxidase system has a predominant role in the overall metabolism by the body of a wide variety of endogenous as well as exogenous substances [1]. Not surprisingly, therefore, a variety of hepatic disorders as well as drugs are associated with a reduction in the capacity of the liver to metabolize drugs and other mixed-function oxidase system substrates such as steroids and fatty acids [2—5]. It has been previously reported that, in the rat, extrahepatic biliary obstruction [6] and the administration of pharmacological doses of ethinyl estradiol which reduces bile flow are associated with significantly decreased levels of microsomal cytochrome P-450, the major component of the mixed-function oxidase system [7, 8]. In both situations the mechanism responsible for the reduction in cytochrome P-450 appears primarily to be a reduced rate of synthesis rather than increased degradation of this hemoprotein [8].

Recently a number of reports have demonstrated that different molecular forms of microsomal cytochrome P-450 are present in the liver [9–14]. In the present study we have investigated the possibility that bile secretory failure or cholestasis may be associated with a differential decrease in some but not necessarily all molecular forms of cytochrome P-450. Furthermore, since the mechanism for the decrease in cytochrome P-450 appears to be in the inverse of that operating to increase the level of this hemoprotein with phenobarbital administration [6, 15], we have investigated the effect of simultaneous administration of ethinyl estradiol and phenobarbitol on hepatic cytochrome P-450 and hepatic drug-metabolizing capacity. The data presented below demonstrate that although bile duct ligation and ethinyl estradiol treatment are associated with a similar quantitative decrease in cytochrome P-450, there are qualitative differences. In addition, the ethinyl estradiol alterations are reversed by phenobarbital administration.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (Charles Rivers Breeding Laboratories, Wilmington, MA) weighing 150–200 g were used throughout the study. The animals were maintained in a well-ventilated room at 22° with alternating 12-hr periods of light and dark, and were fed Purina Laboratory Chow and tap water *ad lib.*

One group of animals underwent double ligation and transection of the common bile duct under light ether anaesthesia. A normal feeding schedule was reinstituted after surgery, and the animals were killed 3 days later after a 12-hr fast. A second group of animals received ethinyl estradiol (Wyeth, 5 mg/kg/

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day in propylene glycol) by subcutaneous injection daily for 5 days. These animals were killed by exsanguination on day 6 after a 12-hr fast.

A third group of rats received phenobarbital (Elkins-Sinn Inc., 80 mg/kg/day in 0.9% NaCl) daily by intraperitoneal injection for 5 days. A fourth group of animals was treated with both ethinyl estradiol and phenobarbital daily for 5 days. These animals were killed by exsanguination under light ether anaesthesia after a 12-hr overnight fast. A fifth group of animals received 3-methylcholanthrene (Eastman, 10 mg/kg/day in corn oil) by intraperitoneal injection for 5 days.

Preparation of microsomal fractions. The livers obtained from the above animals were placed in ice-cold 0.9% NaCl, weighed and cut into small sections. They were then homogenized in 0.25 M sucrose, 0.01 M Tris HCl, pH 7.4 (20% w/v), in a Potter-Elvehjem homogenizer with three passes at 500 rev/min. Microsomal fractions were collected by differential centrifugation (600 *g* for 10 min, 12,000 *g* for 25 min, and 105,000 *g* for 60 min) in a Beckman L 65B ultracentrifuge. The microsomal pellets were washed twice in 0.14 M KCl and resuspended in 0.05 M Tris–HCl, pH 7.4. However, for experiments involving the polyacrylamide gel electrophoresis of microsomal fractions, the pellets were washed by the method of Weihing *et al.* [16] to remove adsorbed and intracisternal proteins.

Measurement of cytochrome P-450. Hepatic cytochrome P-450 content was measured in homogenate and microsomal samples diluted with 0.05 M Tris–HCl, pH 7.4 (homogenate, approximately 2–3 mg protein/ml; microsomes, 1 mg protein/ml), in a Cary model 14 double-beam recording spectrophotometer. Microsomal P-450 was measured by the method of Omura and Sato [17] using a molar extinction coefficient of 91 mM^{-1} cm^{-1}. Homogenate P-450 was measured as described by Greim *et al.* [18]. The protein content of the samples assayed was determined by the method of Lowry *et al.* [19], using bovine serum albumin as standard.

Microsomal enzyme assays. Ethylmorphine *N*-demethylase activity was determined by measuring the rate of formaldehyde production by the method of Nash [20]. Assays were performed in duplicate in a 2.025-ml total volume reaction mixture containing 0.05 M Tris HCl, pH 7.4, 150 mM KCl, 10 mM MgCl, 8 mM (DL)-isocitrate, 0.25 units of isocitrate dehydrogenase (Sigma Chemical Co.) and 8 mM ethylmorphine (Merck, Sharpe & Dohme). After preincubation for 1 min at 25°, microsomes were added and incubation at 25° in a shaking water bath proceeded for 5 and 10 min. The reaction was terminated by the addition of 15% trichloroacetic acid, and 1 ml of this mixture was transferred for determination of formaldehyde released [20].

Glucose 6-phosphatase (EC 3.1.3.9) activity in homogenates and microsomal suspensions was assayed by the method of de Duve *et al.* [21], the phosphate released during the reaction being determined by the method of Fiske and SubbaRow [22].

Polyacrylamide gel electrophoresis. Washed [16] microsomal fractions from control and experimental animals were electrophoresed on polyacrylamide vertical slab gels. Microsomal proteins were solubilized in 1% sodium dodecyl sulfate (SDS) and heated at 100° for 2 min with 20% glycerol (v/v), 0.5% β-mercaptoethanol (v/v) and 6 M urea. Pyronin Y (0.001% v/v) was used as a tracking dye. Identical amounts of solubilized protein (varying from 20 to 50 μg, depending on the experiment) from control and experimental microsomes were applied to 15% polyacrylamide 1% SDS–6 M urea slab gels (1.3 mm in thickness) and electrophoresed according to the method described by Laemmli [23] at 5 mA/gel. The gels were fixed and stained in Coomassie blue 0.1% in 12.5% trichloroacetic acid, 50% methanol, and were destained in 10% acetic acid. With each electrophoresis, immunoglobulin G (mol. wt 160,000) bovine albumin (67,000) ovalbumin (45,000) and chymotrypsinogen A (25,000) obtained from Schwarz-Mann were coelectrophoresed and the molecular weights of constituent microsomal proteins were determined graphically as described by Shapiro *et al.* [24]. Coomassie blue-stained polyacrylamide gels were scanned at 580 nm for protein and 405 nm for benzidine peroxidase staining using the Isco model UA-S scanning apparatus with a scanning speed of 7.5 mm/min and a chart speed of 150 cm/hr.

Identification of apoprotein cytochrome P-450. The tentative identification of apoprotein cytochrome P-450 based on its apparent molecular weight, turnover and pharmacological responsiveness has been described previously [6]. In the present study we sought to further identify the apoprotein moiety of this cytochrome within the polyacrylamide system. Welton and Aust [11] and Welton *et al.* [12] have described the localization of cytochrome P-450 within an SDS-polyacrylamide system by benzidine peroxidase staining. Solubilized microsomal protein (100 μg/gel) obtained from a phenobarbital treated-animal was electrophoresed on 10% polyacrylamide SDS–6 M urea slab gels. One gel was stained for protein with Coomassie blue as described above. A second gel was washed for 30 min in 0.02 M Tris HCl, pH 7.5, at 25° and then placed in 0.25 M acetate buffer, pH 5.0, containing 0.25% benzidine (w/v), 25% methanol (v/v) and 0.75% hydrogen peroxide [11].

Effect of phenobarbital on the relative synthesis rate of cytochrome P-450 in estrogen-treated rats. Both extrahepatic cholestasis produced by bile duct ligation and administration of ethinyl estradiol are associated with a reduction in microsomal cytochrome P-450 content and in the relative rate of synthesis of apoprotein cytochrome P-450 [6, 8]. Since the administration of phenobarbital with ethinyl estradiol results in microsomal cytochrome P-450 levels not significantly different from control [7], we examined the effect of the addition of phenobarbital to ethinyl estradiol treatment on the rate of synthesis of P-450 apoprotein. Changes in the relative rate of synthesis of microsomal proteins were examined by the previously described adaptation [6] of the double-isotope labeling technique of Dehlinger and Schimke [15]. The rate of synthesis of microsomal proteins in ethinyl estradiol plus phenobarbital-treated rats was compared to that in control and that in ethinyl estradiol-treated animals as follows. Two rats treated with both phenobarbital and ethinyl estradiol were each given 200 μCi [4,5-^{3}H]leucine (Amersham Radiochemical Centre, 1 Ci/m-mole) by intraperi-

toneal injection after an overnight fast and killed by exsanguination 6 hr later. A 5-day ethinyl estradiol-treated rat simultaneously received 50 μCi [^{14}C]leucine (Amersham Radiochemical Centre, 348 mCi/mmole) by intraperitoneal injection and was killed 6 hr later. Similarly, a control (0.9% NaCl treated) received 50 μCi [^{14}C]leucine intraperitoneally and was killed 6 hr later. One [^{3}H]leucine-labeled liver was combined with each [^{14}C]leucine-labeled liver for homogenization in 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.4, and microsomes were prepared as described above. Washed [16] microsomal fractions from (a) [^{14}C]leucine-labeled control with [^{3}H]leucine-labeled phenobarbital-treated plus ethinyl estradiol-treated animal and (b) [^{14}C]leucine-labeled ethinyl estradiol-treated plus [^{3}H]leucine-labeled phenobarbital plus estrogen-treated animals were solubilized in 1% SDS and electrophoresed on 7.5% polyacrylamide-SDS–6 M urea disk gels (1.9 × 10 cm). Each gel was sliced into consecutive 1.5-mm sections and depolymerized by the addition of 30% hydrogen peroxide. Protosol and toluene–Omnifluor were added as described previously [6]. Radioactivity was measured in a Packard Tri-carb and dis./min/slice was determined after external quench correction. The range of dis./min/slice for ^{3}H was between 600 and 3000 and that for ^{14}C between 67 and 1200. Standard deviations of control experiments were determined by the method of Weisberg [25]. The ratio ^{3}H/^{14}C for each slice was divided by the appropriate homogenate ^{3}H/^{14}C ratio, and radioactivity was associated with specific Coomassie blue-staining microsomal proteins on the basis of their corresponding R_f within this system [15].

RESULTS

Hepatic cytochrome P-450 and drug-metabolizing capacity. In control animals, total hepatic (homogenate) cytochrome P-450 content was 211.6 ± 5.3 nmoles/100 g of body wieght (Table 1). This value was significantly reduced in both bile duct-ligated ($P < 0.001$) and ethinyl estradiol ($P < 0.05$)-treated animals. In contrast, the administration of phenobarbital for 5 days led to a significant increase ($P < 0.001$) in total hepatic cytochrome P-450. In animals treated for 5 days with both ethinyl estradiol and phenobarbital, the hepatic P-450 content was significantly greater than that seen with ethinyl estradiol alone ($P < 0.01$). Alterations in hepatic cytochrome P-450 were associated with similar changes in microsomal ethylmorphine *N*-demethylase activity, which is also illustrated in Table 1. The reduction in activity associated with ethinyl estradiol administration ($P < 0.001$) was counteracted by the coincident administration of phenobarbital. However, ethylmorphine *N*-demethylase activity was significantly lower with combined estradiol plus phenobarbital therapy than in animals treated with phenobarbital alone.

Microsomal recovery within each experimental group, determined as the percentage of total homogenate glucose 6-phosphatase activity recovered in the microsomal pellet, is also illustrated in Table 1. In most groups microsomal recovery was approximately 30 per cent; however, with bile duct ligation only 18 per cent of homogenate glucose 6-phosphatase activity was recovered in the microsomal fraction. Microsomal recovery, based on homogenate P-450 recovery, was consistently higher than that based on glucose 6-phosphatase (control 40.1 ± 3.6 per cent compared to 30.7 ± 2.8 per cent), yet again recovery from bile duct-ligated animals was lower than from control. These studies suggest that, in contrast to ethinyl estradiol- and phenobarbital-treated animals, changes in bile duct-ligated rats may in part result from altered microsomal recovery.

Polyacrylamide gel electrophoresis. Scans of microsomal proteins from an animal treated with phenobarbital and separated by 10% polyacrylamide-SDS–6 M urea slab gel electrophoresis is illustrated in Fig. 1. The lower panel shows microsomal proteins stained with Coomassie blue and scanned at 580 nm. The major proteins are located in the mol. wt 50,000–58,000 region of the gel as calculated by the method of Shapiro *et al.* [24]. The R_f of Coomassie blue-stained proteins in this region was 0.63 to 0.76. The upper panel shows a scan at 405 nm from the same microsomal fraction electrophoresed in an identical manner but stained with benzidine peroxidase to demonstrate residual heme, which has not dissociated from cytochrome P-450 by SDS. A major

Table 1. Effect of cholestasis and phenobarbital on cytochrome P-450, ethylmorphine *N*-demethylase and microsomal recovery*

	Hepatic cytochrome P-450 (nmoles/100 g body wt)	Ethylmorphine *N* demethylase (μmoles formaldehyde released/mg protein/hr)	Microsomal recovery (per cent homogenate glucose 6-phosphatase recovered in microsomal fraction)
Control	211 ± 5	1.36 ± 0.09	30.7 ± 2.8
Bile duct ligated	135 ± 20†	0.25 ± 0.04†	17.5 ± 3.7†
Ethinyl estradiol	169 ± 32‡	0.61 ± 0.09†	29.0 ± 1.7
Ethinyl estradiol plus phenobarbital	337 ± 65§	1.48 ± 0.05§	23.2 ± 2.3
Phenobarbital	622 ± 80†	2.14 ± 0.18†	31.9 ± 2.7

* Mean ± 1 S. D.; animals in each group.
† Significantly different from control value, $P < 0.001$.
‡ Significantly different from control value, $P < 0.05$.
§ Significantly different from ethinyl estradiol alone, $P < 0.01$.

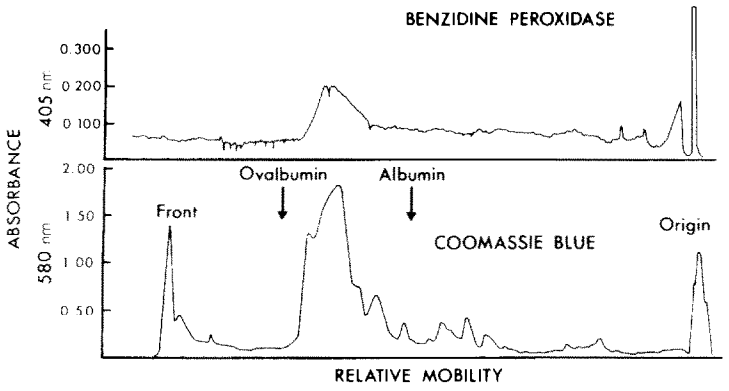

Fig. 1. Scan of 10% polyacrylamide SDS 6 M urea vertical slab gel electrophoresis of microsomal proteins from phenobarbital-treated (80 mg/kg/day × 5 days) rats. Coomassie blue- and benzidine peroxidase-staining and scanning were performed as described in Materials and Methods. Molecular weight markers for ovalbumin (45,000 daltons) and albumin (67,000 daltons) are shown by arrows.

peak of staining is seen between R_f 0.64 and 0.76, also corresponding to the 50,000–58,000 region of the gel. Separation of washed microsomal proteins on 10% polyacrylamide gels suggests the presence of several distinct bands which can be satisfactorily separated into four bands by using a 15% concentration of polyacrylamide. The separation of microsomal fractions on 15% polyacrylamide SDS 6 M urea slab gels is shown in Fig. 2, where the only change in Coomassie blue-staining patterns is seen in the 50,000–58,000 dalton region. Thus, on the basis of benzidine staining, pharmacological responsiveness and the previously shown protein turnover [6], we conclude that cytochrome P-450 apoproteins are

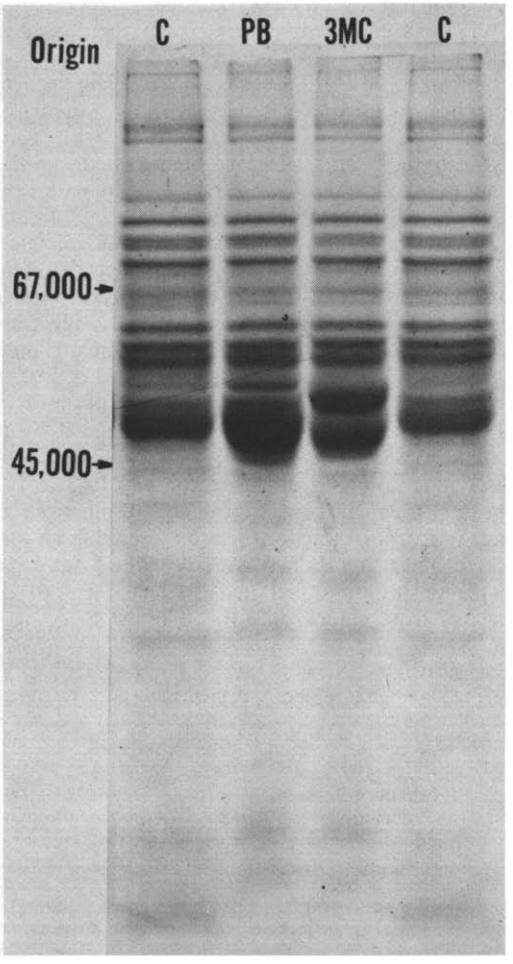

Fig. 2. Electrophoretic patterns of microsomal proteins from control (C), phenobarbital (PB)- and 3-methylcholanthrene (3-MC)-treated rats. Identical amounts of microsomal protein (40 μg) were co-electrophoresed on 15% polyacrylamide SDS 6 M urea slab gel, stained with Coomassie blue (see text). Migration of the molecular weight markers albumin (67,000) and ovalbumin (45,000) are indicated.

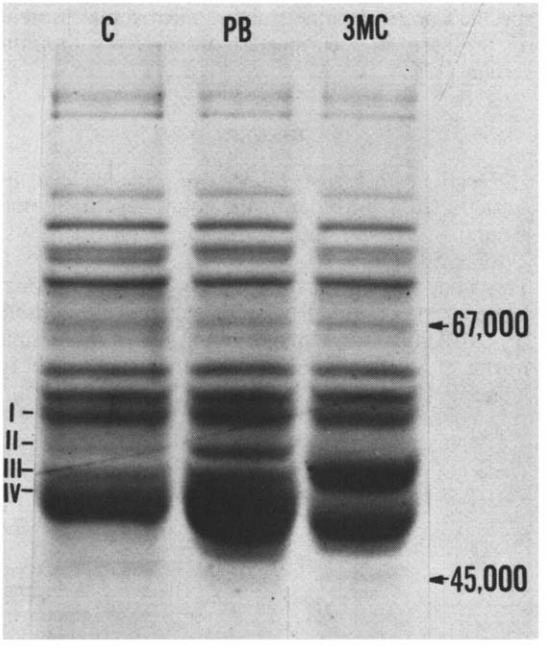

Fig. 3. Fifteen per cent polyacrylamide SDS 6 M urea slab gel electrophoresis of microsomal proteins from control (C), phenobarbital (PB)- and 3-methylcholanthrene (3-MC)-treated rats. Molecular weight markers albumin (67,000) and ovalbumin (45,000) are indicated. Four protein bands, identified as cytochrome P-450 apoproteins, are indicated on L as Bands I, II, III and IV. The differential responsiveness of these bands to phenobarbital or 3-methylcholanthrene is illustrated by the different pattern of Coomassie blue-stained bands within the mol. wt 50,000–58,000 region of the gel. Identical amounts of microsomal protein from each animal were applied.

localized to the 50,000–58,000 dalton region of SDS–polyacrylamide gels.

Figure 3 illustrates the differential response in cytochrome P-450 apoproteins seen after the administration of the microsomal protein-inducing agents phenobarbital and 3-methylcholanthrene. The approximate molecular weights of the four protein bands tentatively identified as cytochrome P-450 apoproteins, calculated by the method of Shapiro *et al.* [24], are: Band I, 58,000; Band II, 55,000; Band III, 53,000; and Band IV, 50,000. Phenobarbital primarily produced an increase in Band IV (50,000 daltons) but also smaller increases in Bands I and II, while 3-methylcholanthrene increased Band III (53,000 daltons).

Electrophoresis of microsomal fractions obtained after 3 days of bile duct ligation demonstrates the apparent reduction in Bands I, III and IV (Fig. 4), while ethinyl estradiol administration is associated with the apparent reduction in Bands I and III, but Bands II and IV are unaltered. The effect of simultaneous administration of ethinyl estradiol with phenobarbital for 5 days is illustrated in Fig. 5, demonstrating that Band I is present, and the apparent increased density in Bands II and IV.

Cytochrome P-450 turnover. The mechanism underlying the reversal by phenobarbital of the ethinyl estradiol-associated alteration in cytochrome P-450 apoproteins was examined by a double-isotope technique designed to determine alterations in the relative rate of synthesis of proteins. The results are illustrated in Fig. 6. The upper panel, A, demonstrates the application of the double-isotope method to a control situation, where both ^{14}C- and ^{3}H-labeled leucine are given to control animals. The dotted horizontal lines represent 95 per cent confidence limits for variability from the mean microsomal protein $^{3}H/^{14}C$ ratio [25]. In the control situation, no individual $^{3}H/^{14}C$ ratio lies outside these limits. The middle panel, B, represents the relative rate of synthesis of ^{3}H-labeled microsomal proteins in estrogen plus phenobarbital-treated animal compared to that in a ^{14}C-labeled control. The relative rate of synthesis of P-450 apoproteins in the mol. wt 50,000–58,000 region of the gel is greater (increased $^{3}H/^{14}C$) in the treated animal. The increased rate of synthesis of P-450 apoprotein in the combined estrogen plus phenobarbital-treated animal is more marked in the lower panel, C, which compared the rate of synthesis in combined treatment to that in estrogen treatment alone, the latter situation being associated with a reduced rate of synthesis of P-450 apoprotein [6].

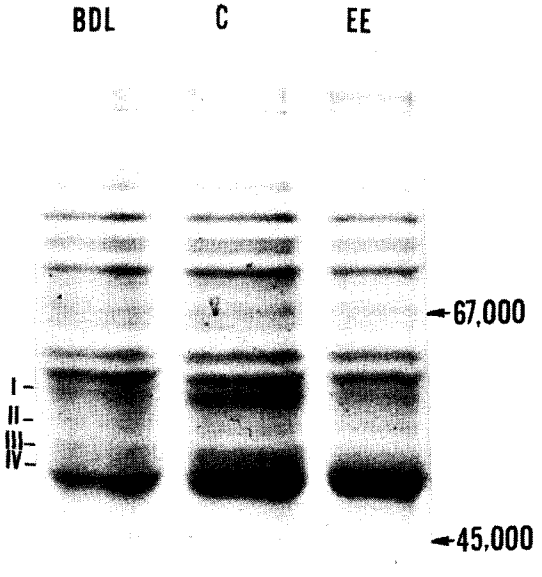

Fig. 4. Identical amounts of protein (40 μg) from microsomal fractions collected from bile duct-ligated (BDL), control (C) and ethinyl estradiol (EE)-treated rats electrophoresed on 15% polyacrylamide–SDS 6 M urea slab gel and stained with Coomassie blue. The migration of molecular markers albumin (67,000) and ovalbumin (45,000) is indicated. The P-450 apoprotein bands are indicated as I, II, III and IV.

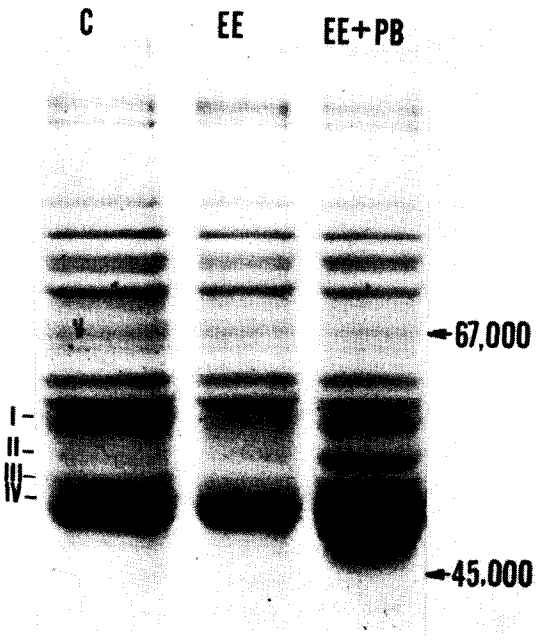

Fig. 5. Electrophoretic pattern of microsomal proteins from control (C), ethinyl estradiol (EE)-treated and ethinyl estradiol plus phenobarbital (EE + PB)-treated rats on 15% polyacrylamide–SDS–6 M urea slab gel stained with Coomassie blue. Molecular weight markers albumin (67,000) and ovalbumin (45,000) are indicated. The pattern of protein band staining in the molecular weight region 50,000–58,000 is shown. Identical amount (40 μg) of protein from each microsomal fraction was applied to gel.

DISCUSSION

A number of recent reports indicate that multiple forms of hepatic microsomal cytochrome P-450 exist, each with possibly distinct substrate specificity and substrate inducibility [9–14]. In the present study we have utilized two models of bile secretory failure, previously shown to be associated with reduced drug metabolism and cytochrome P-450 levels [2, 6, 7], in order to examine the possibility that the alteration in cytochrome P-450 content may reflect a reduction in some but not necessarily all forms of this heterogeneous moiety.

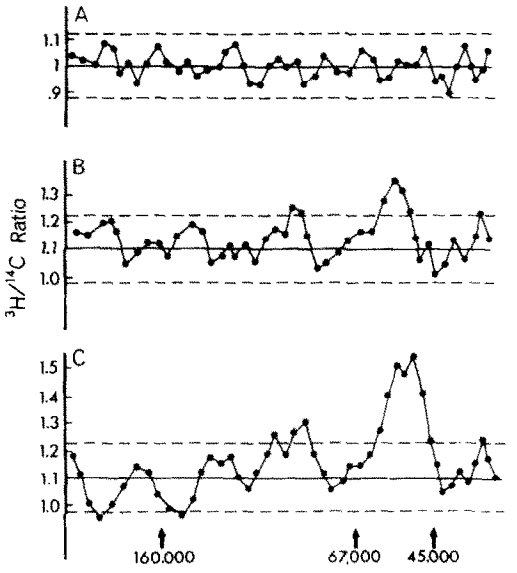

Fig. 6. Double-isotopic determination of the effect of combined ethinyl estradiol (EE) plus phenobarbital (PB) treatment on the relative rate of synthesis of microsomal proteins compared to that in control (C) and EE-treated rats (see text). The ratio $^3H/^{14}C$ for each slice was divided by the homogenate $^3H/^{14}C$ ratio. Dotted horizontal lines represent 95 per cent confidence limits for variation from mean $^3H/^{14}C$ ratio. Origin is shown on left and front on right; molecular weight markers: gammaglobulin (160,000), albumin (67,000) and ovalbumin (45,000) are indicated on horizontal. (A, upper panel): Relative rate of synthesis of microsomal proteins in control situation. (B, middle panel): Relative rate of synthesis of microsomal proteins in EE- and PB-treated animals (^3H-labeled leucine) compared to control (^{14}C-labeled leucine). (C, lower panel): Relative rate of synthesis of microsomal proteins in EE- and PB-treated rat (^3H-labeled leucine) compared to EE-treated rat (^{14}C-labeled leucine).

Decreased cytochrome P-450 levels in liver homogenates (see Table 1) confirm the previously observed alterations in microsomal fractions [6]. However, reduced *N*-demethylation of ethylmorphine in bile duct-ligated animals (18 per cent of control) is out of proportion to the reduced activities of the mixed-function oxidase system activities (63 per cent of control) and suggests that additional factors such as the hepatic accumulation of bile acids [26] may contribute to altered drug metabolism. In contrast, ethinyl estradiol-induced bile secretory failure which has normal hepatic bile acid concentrations [27] has a proportional decrease in both microsomal drug oxidases and *N*-demethylation of ethylmorphine. Thus, reduced drug metabolism and cytochrome P-450 levels after bile duct ligation are due to a complex of secondary events, including: (1) reduced isolation of the microsomal fraction, (2) competitive and non-competitive inhibition of drug oxidation reactions by bile acids [26, 28], and (3) decreased cytochrome P-450 levels due to decreased *de novo* synthesis [6].

There is a general agreement that the subunits of hepatic microsomal cytochrome P-450 have molecular weights between 47,000 and 59,000 [9–13]. We have confirmed that proteins migrating in the molecular weight range of 50,000–58,000 daltons in a 10% SDS-polyacrylamide slab gel system retain some peroxidase activity (see Fig. 1) and have a selective affinity for hematin [27]. However, while cytochrome P-450 apoproteins are demonstrably contained within this molecular weight region of the gel, one cannot conclude that all polypeptides in this region represent cytochrome P-450 apoprotein. For example, epoxide hydrase, a phenobarbital-inducible heme-portein migrates in this region of the gel [30, 31]. However, in salt-washed microsomes [16] from control animals, the proportion of total protein represented in the molecular weight 50,000–58,000 region of the gel is 15.6 ± 1.0 per cent, and cytochrome P-450 apoproteins constitute 65 per cent of this protein region.*

We have tentatively identified four bands within the molecular weight region 50,000–58,000 of 15% polyacrylamide gels as representing cytochrome P-450 apoproteins. Phenobarbital, a potent inducer of a wide variety of microsomal hydroxylation reactions [1, 32], produced an increase primarily in the lower molecular weight region (Band 4) but also smaller increases in Bands I and II. In contrast, 3-methylcholanthrene, a more limited inducer of microsomal hydroxylation [1, 32, 33], increases staining density principally in the higher molecular weight region (Band III). The different patterns of stimulation may be equitable with the different pharmacological effects of these two compounds. Moreover, differential induction of multiple cytochrome P-450 apoproteins by 3-methylcholanthrene and phenobarbital have been described by several other laboratories using polyacrylamide gel separation [10–13]. These studies differ from the present study chiefly with respect to the responsiveness of the apoproteins to inducing agents, for rather than demonstrating specific band responsiveness to phenobarbital, we have found a different pattern of response with some overlap between the effect of these two agents. In general, all workers agree that phenobarbital stimulates a lower and 3-methylcholanthrene a higher molecular weight polypeptide. Our results are more consistent with Haugen *et al.* [13], who demonstrated that phenobarbital has a more general effect. However, there is little agreement regarding molecular weight designation of cytochrome P-450 apoproteins, probably resulting from the different mobility of membrane proteins in various polyacrylamide gel concentrations [34], and also to different species and stains of rats examined.

The major purpose of the present study was to examine the possibility that with cholestasis there may be a preferential loss of some cytochrome P-450 apoproteins, since previous reports [3, 35, 36] have demonstrated preferential loss of certain microsomal hydroxylations. This hypothesis was confirmed by the demonstration of a more generalized reduction in bands in bile duct-ligated animals, while in contrast, ethinyl estradiol administration reduced only Bands I and III. It can therefore be concluded that the multiple forms of cytochrome P-450 apoproteins respond differently to pharmacologic induction and cholestatic depression.

The reduction in cytochrome P-450 levels in ethinyl estradiol-treated rats is associated with a reduction

* A. M. Mackinnon, E. Sutherland and F. R. Simon, unpublished results.

in the relative rate of synthesis of cytochrome P-450 [8], and phenobarbital increases this relative synthesis rate [14, 15]. Not surprisingly, therefore, in the double-isotope labeling experiments we demonstrate that the rate of synthesis of cytochrome P-450 apoprotein is very much greater in the combined phenobarbital and ethinyl estradiol-treated animals when compared to the depressed rate in the ethinyl estradiol-treated rats. However, we are currently unable to separate by slicing of preparative gels the radioactivity in different cytochrome P-450 apoprotein bands, and therefore can only assume that the reduction by ethinyl estradiol and correction by phenobarbital result from specific changes in the synthesis of cytochrome P-450 apoproteins.

These studies support the accumulating evidence that multiple cytochrome P-450 forms are present in liver microsomal membranes, and suggest that substrate specificity may be conferred by particular molecular forms, accounting for the differential reduction or stimulation in some hydroxylation reactions. In particular, these studies have demonstrated that alteration in specific apoproteins may be associated with either an increase or a decrease in the level and functional properties of cytochrome P-450. Decreased drug metabolism in cholestasis is complex, involving altered levels of cytochrome P-450 apoproteins as well as the direct effects of bile acids on microsomal membranes.

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