Alteration of Microsomal Biotransformation in the Liver in Cholestasis[1] (34548)

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Recent studies indicate that the pathogenesis of cholestasis is related to structural and functional changes in the hepatocytes (1), rather than to an abnormality in the biliary passages. It has been suggested that the biotransformation system (drug-handling system) of the smooth endoplasmic reticulum of liver is the cellular site first affected in cholestasis (2). The activities of several drug-handling enzymes were reported reduced in animals with cholestasis (3,4). The activity of the biotransformation system may decrease despite increased content of the terminal enzyme, cytochrome P-450, as was shown in dieldrin intoxication (5), resulting in the formation of hypoactive smooth endoplasmic reticulum. In the present study, the effectiveness of the microsomal biotransformation system in relation to the amount of P-450 and P-450 reductase activity was investigated in animals with ligated bile ducts in order to establish the nature of the alteration in the hepatocytes in cholestasis.

Materials and Methods. Sprague-Dawley male rats, with an average body weight of 150 g, were fed Rockland Farm diet and were kept in a constant environment. After a midline abdominal incision, their bile ducts were doubly ligated and a portion of the duct excised and identified histologically. Development of jaundice and an approximately 5 to 10-fold increase of serum bilirubin indicated the success of ligation. A sham-operated group served as controls. Four days after the operation, the animals were killed and a 25% liver homogenate was prepared in 0.25 *M* sucrose containing 1m*M* EDTA, pH 7.5. Microsomes were separated by ultracentrifugation as described before (5). Microsomal protein concentration was measured by the biuret reaction (6). Corrections were made for the turbidity of the microsomal suspension by adding the biuret reagent without copper sulfate to an aliquot of microsomal suspension and subtracting the absorbance from the total absorbance at 540 nm. Aniline hydroxylase and aminopyrine demethylase activity of the microsomes was measured as described by Schenkman (7). Cytochrome P-450 content of microsomes was measured as described previously (5). The samples were processed immediately, since we observed significant reduction of P-450 content at 0° on standing longer than 1 hr. The activity of NADPH-cytochrome *c*-reductase and of P-450 reductase was measured as described (8, 9). For the measurement of P-450 reductase, an anaerobic cell was used. The reaction was started by adding the NADPH-generating system from an air-tight syringe which produced a rapid mixing action. The absorbance difference between 450 and 490 nm was measured against time in an Aminco-Chance dual wavelength spectrophotometer. The amount of NADPH-reducible P-450 and dithionite-reducible P-450 was measured at the end of the reaction. The effect of aniline and hexobarbital on the aerobic differential spectra of P-450 was measured as described by Schenkman (7).

Results. Microsomal protein was 54.5 ± 15.6 mg/total liver/100 g body weight in the control of 11 rats and 47.0 ± 8.9 mg in the ligated group of 11 rats. The difference was not significant ($p > .10$). The activity of aniline hydroxylase was slightly reduced after 4 days

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TABLE I. Effect of Bile Duct Ligation on Microsomal Cytochrome P-450 Content and on the Activities of Aniline Hydroxylase and Aminopyrine Demethylase.

	Control	Ligated
Aniline hydroxylase (nmoles/mg microsomal protein/15 min)	17.6 ± 4.9[a]	14.1 ± 2.3[a]
Aminopyrine demethylase (nmoles/mg microsomal protein/15 min)	100.1 ± 14.8[a]	45.3 ± 5.9[a]
Cytochrome P-450 (nmoles/mg microsomal protein)	0.59 ± 0.15[a]	0.45 ± 0.12[a]
Aniline hydroxylase (nmoles/nmoles cyt. P-450)	29.8	31.4
Aminopyrine demethylase (nmoles/nmoles cyt. P-450)	170.5	110.5

[a] Mean ± SD, n = 8.

of bile duct ligation as compared to controls; the difference was not significant ($p > .10$). A greater reduction of aminopyrine demethylase activity was significant ($p < .01$). The microsomal P-450 content was slightly, and not significantly decreased, ($p > .10$) similar to the decrease in aniline hydroxylase activity, so that aniline hydroxylase activities per nmole of cytochrome P-450 were identical in both groups. The activity of aminopyrine demethylase per nmole of cytochrome P-450 was greatly reduced in the ligated group (Table I).

Addition of aniline to control microsomes produced a typical type II difference spectrum (7). In the ligated group the maximum was shifted to 434 nm from 430 nm, otherwise the shape and magnitude of spectral change was similar to control. The type I spectral change produced by the addition of hexobarbital to microsomes was much smaller in the ligated group than in control (Fig. 1). The spectral dissociation constant, (K_s), with aniline (type II) as substrate, was similar in both control and ligated groups. The addition of taurochenodeoxycholate or bile of a control rat to control microsomes *in vitro* did not influence the K_s for aniline. The K_s with hexobarbital as substrate ranged from 0.090 to 0.140 in eight control rats, and from 2.80 to 68.5 in the ligated group of eight rats. Addition of the bile salt or bile of a control animal to control microsomes *in vitro*, decreased A_{max}, but only bile increased the K_s considerably. Incubation of control microsomes with the soluble fraction obtained from the liver of a ligated rat only slightly elevated the K_s (Fig. 2). Since the magnitude of spectral change produced by equimolar amounts of aminopyrine is less than one half of that produced by hexobarbital (7), measurement of K_s with aminopyrine as substrate could not be done in the ligated group, where the binding capacity of microsomes was severely impaired.

NADPH-cytochrome *c* reductase activity was similar in both groups (Fig. 3). The activity of P-450 reductase is expressed as time (seconds) needed for the half completion of the reaction ($t_{1/2}$). The activity of P-450 reductase without added modifier was,

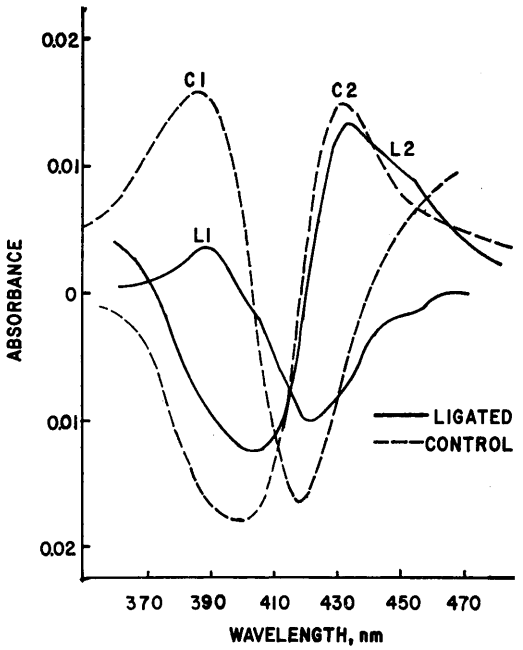

FIG. 1. Spectral changes caused by the addition of hexobarbital (1) or aniline (2) to liver microsomes of control (C) and bile duct ligated (L) rats. Protein concentration was 2 mg/ml, substrate concentration was 1.0 mM in 0.2 M Tris-HCl buffer, pH 7.5. Aerobic difference spectra were obtained in an Amin-co-Chance split-beam spectrophotometer.

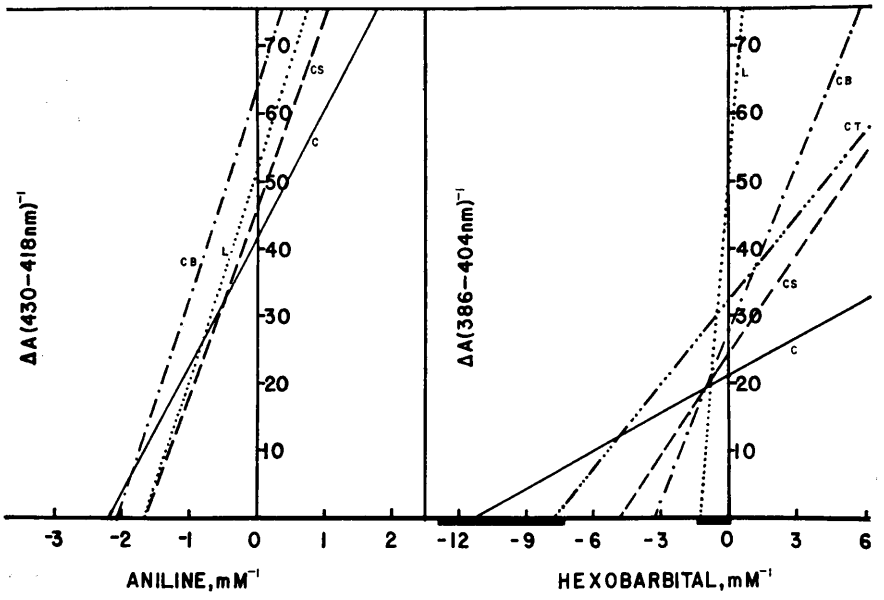

FIG. 2. Aniline and hexobarbital dependent spectral dissociation constants (K_s) of hepatic microsomes of control (C) and bile duct ligated (L) rats. Changes of absorbance at the maximum (430 nm and 386 nm respectively) relative to the isosbestic point (418 nm and 404 nm respectively) was measured in an Aminco-Chance dual-wavelength spectrophotometer. The microsomal protein concentration was 2.0 mg/ml in 0.2 *M* Tris-HCl buffer, pH 7.5 at 37°. B = +200 μl bile; T = +0.66 μm of taurochenodeoxycholic acid (Na salt); S = supernatant of the liver of a bile duct ligated rat; after 30 minutes the microsomes were recentrifuged, washed and resuspended in Tris buffer. Heavy lines on the abscissa indicate the range obtained from eight ligated rats (near 0) and from eight control (further left).

$t_{1/2}$= 2.19 ± 0.69 sec in control and 2.90 ± 0.61 in the ligated group; the difference was not significant ($p > .10$). Addition of aniline as a modifier *in vitro* decreased the rate of P-450 reduction, the $t_{1/2}$ was 3.50 sec for control and 3.59 sec for the ligated group. Addition of hexobarbital as modifier, increased the rate of P-450 reduction in control ($t_{1/2}$=1.50), but failed to do so in the ligated group ($t_{1/2}$= 2.59 (Fig. 4).

Discussion. Bile duct ligation caused, after 4 days, a slight decrease in the content of microsomal protein and cytochrome P-450 of the liver and a proportionate decrease in the hydroxylation of aniline, while the oxidative demethylation of aminopyrine was greatly reduced. The latter cannot be explained by the slight decrease in P-450. This means that the activity of the biotransformation system, with regard to certain substrates was reduced despite adequate amounts of P-450 present.

Two types of substrates are distinguished according to their binding sites on P-450. Type I substrates are presumably bound to the lipoprotein region of the apoenzyme, while type II substrates are presumably bound to the sixth ligand of the iron in the heme moiety (7, 10). Bile duct ligation did not impair the binding of a type II substrate, aniline.

By contrast, the binding of a type I substrate, hexobarbital, is severely restricted in the ligated group. The spectral dissociation constant, K_s, for hexobarbital was increased by more than two orders of magnitude, and the maximal absorbance change, A_{max}, was decreased. Taurochenodeoxycholate alone, in a concentration expected *in vivo* or addition of control bile to control microsomes, or incubation of control microsomes with supernatant obtained from the liver of a ligated rat, produced changes of type I binding *in vitro* which are in the same direction, but of a

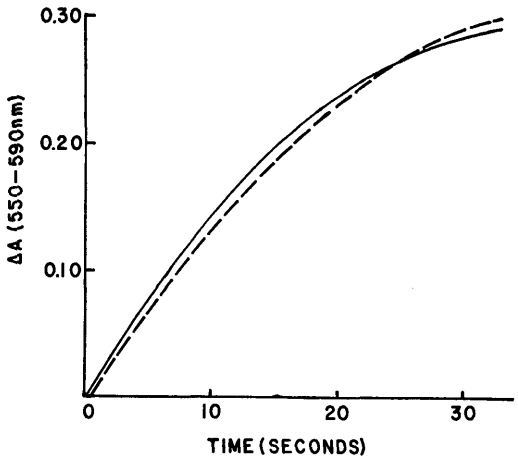

FIG. 3. NADPH-cytochrome c reductase activity was measured in an Aminco-Chance dual wavelength recording spectrophotometer at 37° in a system containing 1.2 μmoles of NADP, 24 μmoles of isocitrate, 0.54 units of isocitrate dehydrogenase, and 10 μmoles of $MgCl_2$. The total volume was 3.0 ml, containing 0.250 mg of microsomal protein/ml in 1.15% KCl-0.02 *M* Tris-HCl buffer, pH 7.5. The reaction was started after the addition of 0.1 μmoles of cytochrome c. Control: — — —, Ligated: ———.

lesser magnitude than those produced by the ligation of bile ducts. The *in vitro* treatments also selectively impaired type I binding; type II binding was unaffected by these treatments. This raises the possibility that the biliary components retained in the liver of rats with ligated bile ducts interfere with type I binding. The exact nature of the responsible components, and the effect of other bile salts, is under investigation. The data suggest that the type I binding site may be irreversibly blocked, or denatured, or removed by these components. Treatment of microsomes with phospholipase C also results in a selective inhibition of type I binding but not type II binding (11), therefore, activation of hepatic phospholipases may also play a role.

In the ligated group, the activity of NADPH-cytochrome *c* reductase was at control levels, and the activity of cytochrome P-450 reductase, which is a measure of electron transfer, was only slightly decreased. This decrease was proportional to the decrease in P-450 content, indicating that electron transfer is normal if no modifiers are added. The activity of P-450 reductase is accelerated by the addition to normal microsomes *in vitro* of type I substrates and slowed by type II substrates (9, 12). Aniline, a type II substrate, decreased the rate of reduction of cytochrome P-450 in both control and ligated groups at approximately equal rates. Type I substrate, hexobarbital, when added as a modifier, stimulated the rate of reduction only in the control group, but not in the ligated group, possibly because of the inability, in the latter group, to form an enzyme-type I substrate complex in sufficient quantity.

These observations suggest that in cholestasis the hypoactivity of the biotransformation system in the smooth endoplasmic reticulum is the result of an impaired type I binding, which, in turn, may decrease the rate of reduction of P-450. It may contribute to the perpetuation of any form of cholestasis (2), by impairing ring hydroxylation necessary for the synthesis of cholate, the prime mover of bile.

Summary. In cholestasis, 4 days after bile duct ligation, the activity of aminopyrine demethylase was greatly decreased, while the content of cytochrome P-450 and the activities of aniline hydroxylase, NADPH-cytochrome *c* reductase, and cytochrome P-

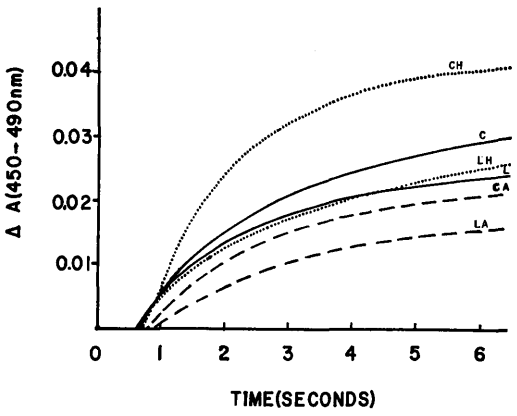

FIG. 4. NADPH-cytochrome P-450 reductase activity. The incubation system was 1.0 mg/ml. The reaction was carried out anaerobically in CO atmosphere C=control; L=ligated; A=addition of 2.0 μmoles of aniline; H=addition of 2.0 μmoles of hexobarbital to the incubation system.

450 reductase were only slightly decreased in the microsomes of the rat livers. Binding of type II substrate to cytochrome P-450 was unimpaired and its modifier effect on P-450 reductase was intact. The binding of type I substrate was greatly decreased, and its stimulating effect on P-450 reductase was abolished, suggesting that the effect of cholestasis on the hepatocellular smooth endoplasmic reticulum is to alter the type I binding sites, which is responsible for the hypoactivity of the biotransformation system.

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