P-450 Level and Taurochenodeoxycholate 6β-Hydroxylase System of Rat Liver Microsomes (34644)

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The 6β-hydroxylation of taurochenodeoxycholate by rat liver microsomes has been previously reported (1). The participation of cytochrome P-450 in this reaction was subsequently demonstrated by the photochemical action spectrum method (2). In this respect, the enzyme is similar to a number of other mixed function oxidases involved in steroid and drug hydroxylations. These are complex enzymes which introduce one atom of molecular oxygen into the substrate molecule while reducing the other oxygen atom to water. In the process, electrons flow from NADPH through an electron transport system consisting of a flavoprotein at one end and cytochrome P-450 at the other. The steroid 11β-hydroxylase system of bovine adrenal mitochondria has been solubilized and resolved into three components of flavoprotein, nonheme-iron protein, and cytochrome P-450 (3). Mixed function oxidases of liver microsomes have, however, resisted similar attempts at resolution and purification, mainly because of instability of the P-450 towards the various solubilizing agents.

Recently, Lu *et al.* (4) have reported the solubilization of fatty acid ω-hydroxylase system from rabbit liver. Fractionation of the soluble preparation yielded a P-450 containing fraction and a TPNH-cytochrome c reductase fraction. The ratio of P-450 to protein in the former fraction was, however, the same as that of the intact microsomes.

Using our previous method (1), we have prepared an extract of liver microsomes in 1.0 *M* phosphate, active in 6 β-hydroxylation after removal of the 105,000*g* pellet. The ratio of P-450 to protein indicated a partial purification of P-450 over that in the original microsomes. The apparent K_m for taurochenodeoxycholate was estimated at 1.2×10^{-4} *M*.

Since the involvement of P-450 in the 6β-hydroxylase system was established, it became of interest to assess its influence in the overall rate of hydroxylation. We treated the experimental animals with various agents which were expected to alter either the levels of P-450 in the liver, the bile acid pool, or both, and measured in each case the 6β-hydroxylase activity and the concentration of P-450. The agents used were (a) phenobarbital, a known inducer of P-450 (5), (b) cholestyramine, a bile acid sequestering resin which causes an increased production of bile acids (6), (c) thyroxine, which enhances the production of chenodeoxycholic acid (7), and (d) chenodeoxycholate to increase the bile acid pool.

Methods. Four groups of five male Sprague-Dawley rats (2 months old), weighing 200–250 g each, were kept in individual cages during a 5-day treatment period: (a) Control group, the animals were fed the regular Purina rat chow. (b) Cholestyramine group, the regular Purina pellets were pulverized and mixed with cholestyramine (Merck, Sharp and Dohme) in proportion of 1 g of resin to 19 g of chow. Pellets of approximately the same size as the original were then formed. Each rat ingested an average of 1 g of resin/day. (c) Phenobarbital group, each rat was injected intraperitoneally 25 mg of sodium phenobarbital (Winthrop Laboratories)/day for 5 days. (d) Thyroxine group, each rat was given 40 μg of monosodium thyroxide (Sigma Chemical Co.) subcutaneously each day. (e) Chenodeoxycholate group, rats were injected intraperitoneally with 9 mg of sodium chenodeoxycholate/day.

All rats were killed by decapitation at the end of the treatment period and the livers from each group of rats were pooled, rinsed, and homogenized in 0.01 *M* phosphate buffer, pH 7.6. An aliquot of each homogenate equivalent to 15 g of liver was taken for the preparation. Microsomes were obtained after 1-hr centrifugation at 105,000*g* of the 10,000*g* supernatant. The microsomal pellets were washed once and resuspended in the homogenizing medium. Aliquots were then used for incubation and for determination of P-450.

6-β-Hydroxylase activity was assayed by incubating taurochenodeoxycholate-24-^{14}C (0.2 μmole, 1.0 × 10^5 dpm) for 20 min at 37° in the medium described in Fig. 1. The 1.0 M extracts were prepared as described previously (1), and P-450 was measured by the method of Omura and Sato (8).

Results and Discussion. The 6β-hydroxylase activity of the microsomal fractions from the differently treated animals is shown in Fig. 1. As shown, treatment with phenobarbital increases the hydroxylase activity beyond the control values. On the other hand, treatment with either thyroxine, cholestyramine, or chenodeoxycholate caused a slight decrease in the 6β-hydroxylase activity.

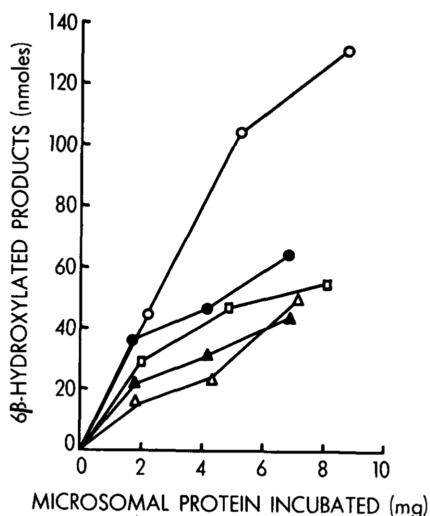

FIG. 1. 6β-Hydroxylase activity of liver microsomes obtained from control rats and rats treated with phenobarbital, thyroxine, cholestyramine, and chenodeoxycholate. Sodium taurochenodeoxycholate-24-^{14}C (1.0 μmole, 1.0 × 10^5 dpm) was incubated with varying amounts of microsomes for 20 min at 37°. The medium was 5 ml of 0.1 M phosphate buffer, pH 7.4, containing 5 m*M* $MgCl_2$, 1 m*M* nicotinamide, 0.5 m*M* glutathione, 0.4 m*M* NADP, 5 m*M* glucose 6-phosphate and 1 Kornberg unit of glucose 6-phosphate dehydrogenase. Control (●), phenobarbital (○), cholestyramine (□), thyroxine (▲) chenodeoxycholate (△).

The P-450 levels in the different microsomal fractions are shown in Table I. Phenobarbital caused a marked increase in P-450 concentration. This is a well-documented phenomenon (5). Cholestyramine has a slight depressing effect on microsomal P-450. This observation is in line with those of Boyd *et al.* (9) on the effect of biliary drainage on P-450 levels. On the other hand, in our experiments, injection of chenodeoxycholate failed to cause a rise in the level of P-450. Thyroxine treatment caused a large decrease in P-450 similar to that reported by Mitropoulos *et al.* (10).

TABLE I. P-450 Levels in Microsomes from Differently Treated Animals.[a]

Treatment	P-450/mg of protein[b]	% of control
Control	0.0514	100
Chenodeoxycholate	0.0410	80
Thyroxine	0.0187	36
Phenobarbital	0.1170	228
Cholestyramine	0.0484	94

[a] Microsomes were obtained from rat livers homogenized in 0.01 *M* phosphate (pH 7.6) at the end of a 5-day treatment period.

[b] P-450 is expressed in Δ OD units (450–490 nm).

When the 6β-hydroxylase activity was compared with the content of P-450, there was no quantitative correlation. For example, the P-450 level in the thyroxine-treated animals was only one-third that of control animals, while 6β-hydroxylase was decreased just slightly. In this case, it is possible that the effect of decreasing P-450 on the 6β-hydroxylase activity was partially offset by an increase in some other component of the enzymatic system brought about by thyroxine. It has been shown that the activity of

NADPH-cytochrome c reductase is enhanced by thyroxine treatment (10), and it is probably involved in the 6β-hydroxylase system as we have previously indicated (1). On the other hand, phenobarbital, in addition to increasing P-450, has been shown by Orrenius and Ernster (5) to elevate also the levels of other microsmal enzymes including NADPH-cytochrome c reductase. It thus seems from these results that the level of P-450 alone is not the rate-controlling factor in the 6β-hydroxylase system.

Extraction of microsomal pellets with 1.0 *M* phosphate buffer, pH 7.6, yields, upon centrifugation at 105,000*g* for 1 hr, a clear slightly yellow supernatant which is active in 6β-hydroxylation. On the basis of protein content, this extract is twice as effective in 6 β-hydroxylation as the original microsomes (1). Microsomes obtained in two homogenizing media were extracted with 1.0 *M* phosphate and the P-450 level was determined in the orginal microsomes, the extract, and the residual pellet. The data in Table II show that the specific activity of P-450 in the 1.0 *M* phosphate extract is increased 2 to 2.5 times over that of the microsomes and that the specific activity of the residual pellet is less than that of the intact microsomes.

Aliquots of 1.0 *M* phosphate extract were incubated with varying amounts of substrate for 30 min. From a Lineweaver-Burk plot of the data (Fig. 2), an apparent K_m of 1.2×10^{-4} *M* was estimated. This value is of the same order of magnitude as those of some

TABLE II. Extraction of P-450 by 1.0 *M* Phosphate.[a]

	P-450/mg of protein[b]; liver homogenized in:	
	Sucrose	0.01 *M* PO_4
Intact microsomes	0.042	0.042
Residual pellet	0.025	0.036
1.0 *M* PO_4 extract	0.094	0.103

[a] Microsomes were obtained from rat livers homogenized in either 0.01 *M* phosphate (pH 7.6) or 0.25 *M* sucrose, and then extracted with 1.0 *M* phosphate.

[b] P-450 is expressed in Δ OD units (450–490 nm).

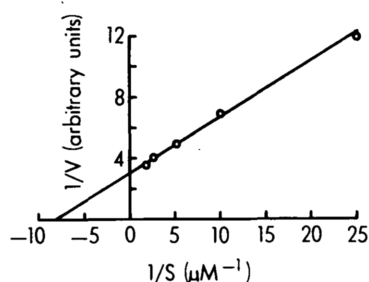

FIG. 2. Lineweaver-Burk plot of the effect of increasing concentrations of taurochenodeoxycholate on the rate of 6β-hydroxylation by 1.0 *M* phosphate extract. Varying amounts of taurochenodeoxycholate were incubated with the extract, in the medium described in Fig. 1. The estimated K_m was 1.2×10^{-4} *M*.

drug oxidases found in rat liver ($\sim 10^{-4} M$) but is greater than some of the steroid hydroxylases ($\sim 10^{-5} M$) (11). The maximal reaction rate for the 6β-hydroxylase was approximately 1 nmole/min/mg of protein.

Summary. The level of cytochrome P-450 and the activity of taurochenodeoxycholate 6β-hydroxylase of rat liver microsomes were determined after the administration of phenobarbital, cholestyramine, thyroxine and chenodeoxycholate. Treatment with phenobarbital caused increases in both the 6β-hydroxylase activity and P-450 level, while the other compounds caused decreases in both. Quantitatively there was a lack of parallelism between the hydroxylase activity and the level of P-450, indicating that P-450 is not a rate-limiting factor in the 6β-hydroxylase system. An extract of the microsomes was prepared in 1.0 *M* phosphate (pH 7.6) as previously described. The extract was active in 6β-hydroxylation, with an apparent Michaelis-Menten constant towards taurochenodeoxycholate of 1.2×10^{-4}M, and a maximum rate of hydroxylation of approximately 1 nmole/min/mg protein. The ratio of P-450 to protein of the extract was 2 to 2.5 times greater than that in the microsomes.

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