complete solubilization of the remaining C-17 alkylated Δ^5-3-ketones of the series, the K_m and V_{max} determinations were not extended to the more hydrophobic substrates **1b–e**.

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Cytochrome P-450 in 7α-Hydroxylation of Taurodeoxycholic Acid†

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ABSTRACT: Taurodeoxycholic acid is 7α hydroxylated to form taurocholic acid by rat liver microsomes in the presence of NADPH. This enzymatic reaction has a K_m of 0.03 mM. The reaction is inhibited by $CO:O_2$ mixtures with a K (Warburg's partition constant) of 0.5. The inhibition is maximally reversed by monochromatic light at the wavelength of 450 nm. These observations establish the P-450 dependence of 7α hydroxylation of taurodeoxycholic acid.

The strongly detergent chenodeoxycholic acid is converted to the weakly detergent β-muricholic acid by liver enzymes in the rat (Greim *et al.*, 1972). This conversion involves 6β hydroxylation which has been shown to be cytochrome P-450 dependent (Voigt *et al.*, 1968). The purpose of this study was to determine if the 7α hydroxylation of deoxycholic acid to yield cholic acid likewise requires cytochrome P-450.

The presence of cholic acid was first demonstrated in the bile of bile fistula rats by Bergström and coworkers (Bergström *et al.*, 1953). More recently Einarsson and Johansson (1968) have found that this reaction is catalyzed by enzymes localized in the microsomal fraction of the liver homogenate and requires NADPH and molecular O_2. Furthermore these authors showed that 7α-hydroxylase activity is induced by pretreatment of the rats with phenobarbital. All of these properties suggest that this hydroxylation is catalyzed by cytochrome P-450 (Cooper *et al.*, 1965). To establish the role of P-450 in the deoxycholic acid hydroxylation we have studied the CO inhibition and the efficiency of various wavelengths of monochromatic light of equal intensity, relative to that at 450 nm, in reversing this CO inhibition.

Materials and Methods

Chemicals were obtained from the following sources: sodium salts of taurodeoxycholic acid (3α,12α-dihydroxy-5β-

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cholanoic acid) and taurocholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), Calbiochem (San Diego, Calif.), recrystallized twice from ethanol–ether; disodium salt of $NADP^+$ and isocitrate dehydrogenase (20 mU/μl), Boehringer Mannheim Corp. (New York, N. Y.); trisodium DL-isocitrate, Sigma Chemical Co. (St. Louis, Mo.); clostridial cholylglycine hydrolase, Schwarz–Mann (Orangeburg, N. Y.); calibrated gas mixtures of oxygen and carbon monoxide in nitrogen, Matheson Gas Products (East Rutherford, N. J.).

Male Sprague-Dawley rats (140–160 g) were divided into two groups; one was untreated and the other received daily intraperitoneal injections of phenobarbital (80 mg/kg) for 5 days. Rats were not fed for 24 hr prior to experiments. Homogenates, 20% (w/v), pooled from the livers of four rats in each group were prepared in 0.1 M potassium phosphate buffer (pH 7.4) with a Teflon pestle Homogenizer (A. H. Thomas Co., Philadelphia, Pa). Mitochondria and unbroken cells were removed by centrifugation for 10 min at 800*g* and 10 min at 20,000*g* and the microsomal fraction was sedimented by centrifugation at 105,000*g* for 1 hr. The resultant microsomal pellet was washed in the same buffer and centrifuged at 105,000*g* for 1 hr after which the washed microsomes were suspended in sufficient buffer to obtain a protein concentration of 10 mg/ml. Protein concentration was determined by the biuret reaction (Gornall *et al.*, 1949).

Determination of Taurodeoxycholic Acid Hydroxylation. The incubation system contained sodium taurodeoxycholate in concentrations from 0.013 to 1.0 mM as substrate, 3.3 mg/ml of microsomal protein, 5 mM $MgCl_2$, 0.66 mM $NADP^+$, 16 mM isocitrate, and 20 μl of isocitrate dehydrogenase in 0.1 M potassium buffer (pH 7.4) in a total volume of 3 ml. Incubation was carried out at 37°, except for CO inhibition and light activation experiments where 25 and 30°, respectively, were used. After 15 min the reaction was terminated by adding five volumes of methanol. The combined extracts were evaporated, and the taurine conjugates were hydrolyzed by clostridial cholylglycine hydrolase according to Nair (1969). After acidifying to pH 1, the free bile acids were extracted three times with ether and esterified with diazomethane. The trifluoroacetyl derivatives (Kuksis and Gordon, 1963) and trimethylsilyl derivatives (Chambaz and Horning, 1967) of the bile acid methyl esters were prepared and analyzed in a Hewlett-Packard Model 5750 gas chromatograph. The amount of cholic acid formed and the amount of deoxycholic acid left unchanged were quantitated by peak area integration. Detector response was linear in the range of concentration (0.5–5 μg/sample) used. A zero-time incubation mixture served as a blank. The rate of product formation calculated from the turnover was linear up to 20 min.

CO Inhibition. Inhibition of the hydroxylation of taurodeoxycholic acid by CO was measured in a Warburg apparatus in the dark as described (Rosenthal and Cooper, 1967). The incubation system was equilibrated with different $CO:O_2$ mixtures for 5 min with constant shaking at 25°. The $CO:O_2$ ratio was varied over a 16-fold range while the O_2 concentration was kept constant at 4%. The manometers were closed and the reaction started by adding the substrate from the side arm. After 15-min incubation, the reaction was stopped and the hydroxylation products were assayed as described above. The partition constant, *K*, between oxygen and CO for the hydroxylation reaction, was calculated from Warburg's partition equation (Warburg and Kubowitz, 1929).

$$K = \frac{n}{1 - n} \times \frac{CO}{O_2} \qquad (1)$$

where $n = V_{CO}/V_{O_2}$ and V_{CO} and V_{O_2} are the rate of hydroxylation in the presence of CO and O_2, respectively. When $(1 - n)/n$ is plotted against the $CO:O_2$ ratio, a straight line should result, the slope is the reciprocal of *K*.

Photochemical Action Spectrum. The apparatus for this reaction has been described earlier (Rosenthal and Cooper, 1967). The incubation flasks were gassed with a $CO:O_2$ mixture of 1:2 at 30°. After addition of substrate, incubation was carried out for 15 min at 30° in the dark, or in a beam of monochromatic light of various wavelengths of approximately equal intensity. The reaction was then stopped and the analysis of reaction products carried out as described above. Calculation of light sensitivity of the hydroxylation reaction was based on the equation (Warburg, 1949)

$$L = \frac{1}{i} \times \frac{\Delta K}{K_d} \qquad (2)$$

where *i* is the light intensity, ΔK is the increase of *K* by light, and K_d is the distribution constant in the dark.

Results

Incubation of taurodeoxycholic acid with microsomes and an NADPH-generating system resulted in the formation of taurocholate by 7α hydroxylation (Figure 1). The reaction product was identified by the gas chromatographic retention times of both F_3CCO_2H and Me_3Si derivatives.

Taurodeoxycholic acid 7α-hydroxylase followed Michaelis–Menten kinetics up to a substrate concentration of approximately 0.3 mM. At higher substrate concentrations the reaction was inhibited. The K_m, calculated by extrapolation of the linear portion of the Lineweaver–Burk plot (Lineweaver and Burk, 1934), was 0.03 mM (Figure 2). In the subsequent studies the substrate concentration was 0.2 mM.

Pretreatment of rats with phenobarbital enhanced the enzyme activity, confirming the observations of Einarsson and Johansson (1968).

The 7α hydroxylation of taurodeoxycholic acid was progressively inhibited with increasing $CO:O_2$ ratios. At a $CO:O_2$ ratio of 12, no hydroxylation product was detected. The partition constant, *K*, at which half-inhibition occurs, was 0.5 (Figure 3). The plot indicates that inhibition of taurodeoxycholic acid 7α-hydroxylase depends on the $CO:O_2$ ratio, and the rate of inhibition follows the law of mass action. Since 7α hydroxylation of taurodeoxycholic acid was about 50% inhibited in the dark at $CO:O_2$ ratio of 0.5, this gas mixture was used to perform the photoreactivation spectrum (Figure 4). Maximal reversal of CO inhibition occurred when the sample was irradiated with light of 450 nm. A second smaller peak was present at 419 nm.

Discussion

Deoxycholic acid is a secondary bile acid which is formed by bacterial dehydroxylation of cholic acid in the intestine and is reconverted, at least in rodents, to cholic acid in the liver by 7α hydroxylation of the steroid nucleus. The 7α-hydroxylase of taurodeoxycholic acid fulfills several criteria of mixed-function oxidases, namely, microsomal localization, NADPH requirement, and increase of enzyme activity after phenobarbital (Einarsson and Johansson, 1968).

These studies demonstrated that the enzyme activity is inhibited by carbon monoxide. This is in contrast to previous observations (Einarsson and Johansson, 1969), and the reason

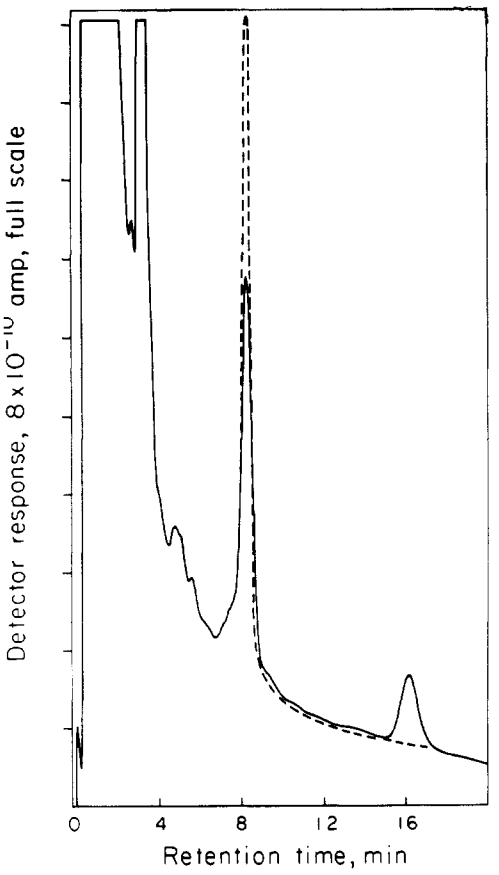

FIGURE 1: Microsomal 7α hydroxylation of taurodeoxycholic acid. Taurodeoxycholic acid was incubated with microsomes and NADPH as described in Methods. The reaction products, as the F_3CCO_2H derivatives of bile acid methyl esters, were separated by gas chromatography. Incubation at 37° in air for 20 min (———), zero-time incubation (– – – – –). Gas chromatography. 2% OV-210 on Gas Chrom Q mesh 100–120 glass column, 6 ft long, 6 mm od. Column: 235° isothermal, injection port: 245°, flame: 245°, flow rate: 60 cm^3 of helium/min. Retention time for deoxycholic acid was approximately 8 min, and for cholic acid, 16 min.

for this discrepancy is not known. The Warburg partition constant, K, was 0.5, a value similar to that of other mixed-function oxidases (Cooper *et al.*, 1965; Estabrook *et al.*, 1963).

The function of P-450 in the 7α hydroxylation of taurodeoxycholic acid suggested by previous findings is more firmly established by these studies which demonstrate that wavelengths of light around 450 nm are the most efficient in reversing carbon monoxide inhibition of 7α hydroxylation. The

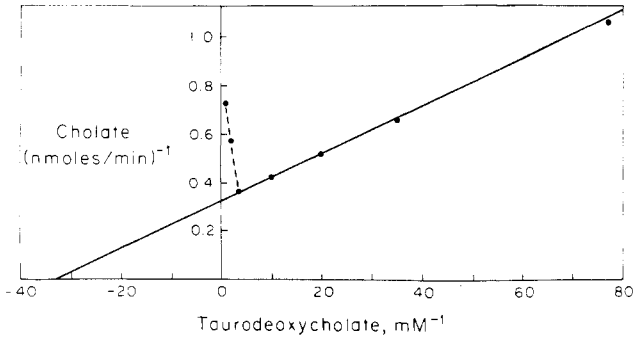

FIGURE 2: Lineweaver–Burk plots for taurodeoxycholic acid 7α-hydroxylase. Incubation system described in Materials and Methods.

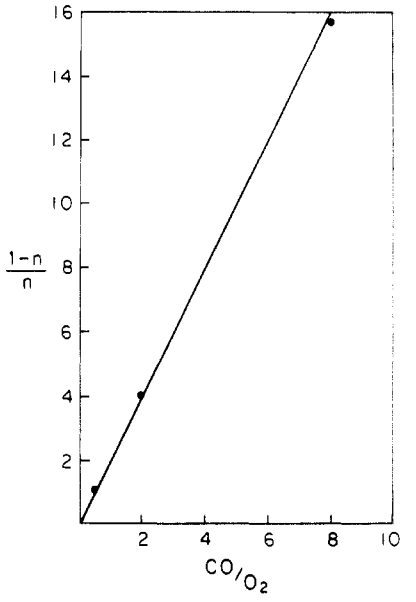

FIGURE 3: CO inhibition of taurodeoxycholic acid 7α-hydroxylase at 25°; 5-min incubation. Incubation system described in Methods. Plot of $(1 - n)/n$ *vs.* $CO:O_2$. The gradient is $1/K$. $n = V_{CO}/V_{O_2}$, where V_{CO} (nmol/min per mg of protein) is the rate in the presence of CO, V_{O_2} (nmol/min per mg of protein) the rate in the presence of air.

significance of the smaller light reversal observed at 419 nm is not at present known and requires further study.

The enzyme activity follows Michaelis–Menten kinetics only with low substrate concentrations. At higher concentrations the Lineweaver–Burk plot shows the characteristics of

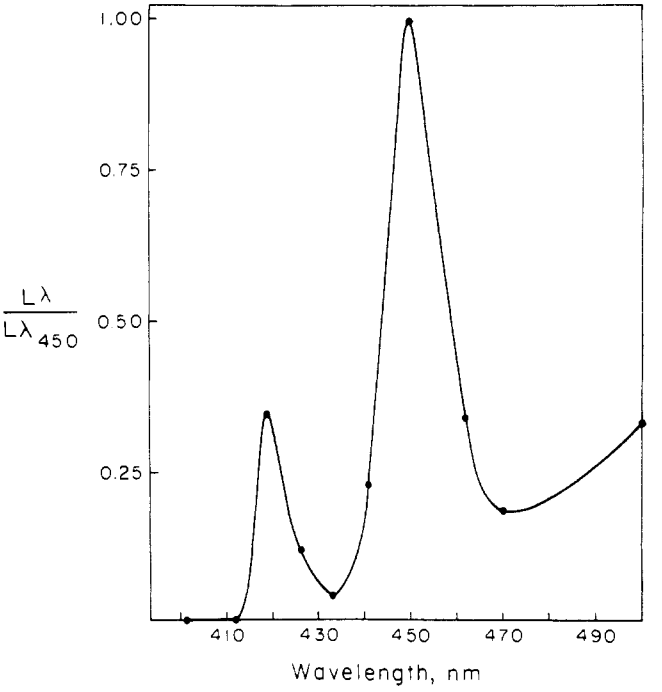

FIGURE 4: Photochemical action spectrum of the taurodeoxycholic acid 7α-hydroxylase at 30°; 15-min incubation. Incubation system described in Methods. Light sensitivity is defined by $L = (1/i) \times (\Delta K/K_d)$, where i is light intensity in terms of (mol quanta)/cm^2 per min, K_d the distribution constant in darkness, and ΔK the increase in K produced by light of a given intensity.

substrate inhibition (Westley, 1969), probably as a consequence of detergent action of the substrate on P-450 (Denk *et al.*, 1971).

The 7α hydroxylation is involved in both the interconversion of bile acids and in the formation of bile acids from cholesterol. The two hydroxylation reactions have some similarities. Both are localized in the microsomes, require NADPH, and are sensitive to CO (Einarsson and Johansson, 1968). However, the phenobarbital induction of cholesterol 7α-hydroxylase is at present controversial (Einarsson and Johansson, 1968; Shefer *et al.*, 1972; Wada *et al.*, 1969), and the reversal of carbon monoxide inhibition with monochromatic light at 450 nm has not been published.[1] Therefore, the role of P-450 in cholesterol hydroxylation has not been established. By contrast, this study on 7α hydroxylation and earlier observations on 6β hydroxylations (Voigt *et al.*, 1968) indicate that P-450 is involved in the interconversion hydroxylation of bile acids. The reaction products, the trihydroxy bile acids, are much weaker detergents than the substrates, the dihydroxy bile acids. P-450 may decrease the hepatic concentrations of strongly detergent dihydroxy bile acids by hydroxylation of the steroid nucleus, thereby protecting the liver cells from injury.

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[1] Subsequently it has been called to our attention that CO inhibition and light reversal of CO inhibition of 7-α-hydroxylation of cholesterol was reported by Boyd *et al.* (1971). Details of these studies will appear in a monograph of the Biochemical Society entitled "Biological Hydroxylation Mechanisms," whlch is in press.