EFFECT OF CARBON MONOXIDE AND PHENOBARBITAL ON HYDROXYLATION OF BILE ACIDS BY RAT LIVER MICROSOMES

K.EINARSSON and G.JOHANSSON
Department of Chemistry, Karolinska Institutet, Stockholm, Sweden

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1. Introduction

The formation and metabolism of bile acids involve a number of hydroxylations. Generally, the hydroxylations of the steroid nucleus are catalyzed by the microsomal fraction of liver homogenate with the addition of NADPH and with the participation of molecular oxygen [1]. The same subcellular fraction catalyzes the hydroxylation of various drugs, aliphatic hydrocarbons, fatty acids and steroid hormones [2]. Many of these reactions have been shown to involve the participation of a flavoprotein, NADPH-cytochrome *c* reductase, and a carbon-monoxide binding pigment, cytochrome P_{450}, as electron carriers between NADPH and oxygen [2]. It has also been shown that administration of drugs, e.g. phenobarbital, induces increased activity of liver microsomes to hydroxylate drugs as well as heptane, laurate and several steroid hormones [2]. These findings have led to the suggestion that a single enzyme system might catalyze the different hydroxylations [3,4]. To examine this possibility and to obtain further information concerning the properties of the hydroxylases involved in the formation and metabolism of bile acids a series of studies has been initiated dealing with the effects of carbon monoxide and phenobarbital administration on these reactions. A recent communication reported the results with the 7α-hydroxylase catalyzing the conversion of cholesterol into cholest-5-ene-3β, 7α-diol and with the 12α-hydroxylase catalyzing the conversion of 7α-hydroxycholest-4-en-3-one into 7α, 12α-dihydroxycholest-4-en-3-one [5]. Evidence was obtained to indicate that these hydroxylases differed from that (those) catalyzing the hydroxylation of drugs and steroid hormones. The present report describes the results with the 7α-hydroxylase catalyzing the conversion of taurodeoxycholic into taurocholic acid and the 6β-hydroxylase(s) catalyzing the 6β-hydroxylation of taurochenodeoxycholic acid and lithocholic (taurolithocholic) acid.

* The following systematic names are given to bile acids referred to by trivial names: cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholanoic acid; α-muricholic acid, 3α, 6β, 7α-trihydroxy-5β-cholanoic acid; β-muricholic acid, 3α, 6β, 7β-trihydroxy-5β-cholanoic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid; deoxycholic acid, 3α, 12α-dihydroxy-5β-cholanoic acid; lithocholic acid, 3α-hydroxy-5β-cholanoic acid.

2. Materials and methods

Tritium-labeled taurodeoxycholic acid (8.3 μC/mg) and taurochenodeoxycholic acid (7.3 μC/mg) were synthesized from tritium-labeled deoxycholic acid and chenodeoxycholic acid as described by Norman [6]. 24-^{14}C lithocholic acid (5 μC/mg) was obtained from New England Nuclear Corp., Boston, Mass. and 4-^{14}C androst-4-ene-3,17-dione (2.4 μC/mg) from Radiochemical Centre, Amersham, England. Pyridine nucleotides, D,L-isocitric acid and isocitric dehydrogenase (type IV) were obtained from Sigma Chemical Co., St. Louis, Mo. This enzyme preparation contains 10 mg of protein per ml and 1 mg of protein converts 4 μmoles of isocitrate into α-ketoglutarate per min.

White male rats of the Sprague-Dawley strain weighing 150–200 g were used. Phenobarbital (100 mg/kg body weight), dissolved in 1 ml of saline, was administered intraperitoneally daily for 6 days. Control

rats were injected with saline. Liver homogenates, 20% (w/v), were prepared in a modified Bucher medium, pH 7.4, and the microsomal fraction was isolated as described previously [5]. The protein concentration of the microsomal fraction was determined with a micro-Kjeldahl method [7].

The incubation mixtures consisted of 0.5–1.5 ml of microsomal fraction, diluted to 3 ml with Bucher medium, and an NADPH-generating system consisting of 0.03 μmoles of $MnCl_2$, 3 μmoles of NADP, 12.5 μmoles of isocitrate and 10 μl of isocitric dehydrogenase solution. The substrates were added as follows: taurodeoxycholic acid, 200 μg in 50 μl of Bucher medium; taurochenodeoxycholic acid, 150 μg in 200 μl of Bucher medium; lithocholic acid, 80 μg in 0.5 ml of 25% aqueous ethanol (v/v); androst-4-ene-3,17-dione, 210 μg in 50 μl of acetone; aminopyrine, 3.6 mg in 1 ml of Bucher medium. Incubations were conducted at 37° for 20 min except those with androst-4-ene-3,17-dione, which were run for 5 min.

Incubations with taurodeoxycholic and taurochenodeoxycholic acid were terminated by the addition of an equal volume of 95% aqueous ethanol (v/v). After filtration the solution was hydrolyzed with 1 M KOH for 12 hr at 110°. The mixture was acidified with 6 M HCl and extracted with ether. The ether extract was washed with water until neutral and the solvent was evaporated. The residue of the ether extract was chromatographed on a column of hydrophobic Hyflo SuperCel with a phase system consisting of methanol-water (150 ml: 150 ml) as moving phase and chloroform-heptane (45 ml: 5 ml) as stationary phase [8]. Incubations with lithocholic acid were terminated by the addition of an equal volume of 95% aqueous ethanol (v/v). After filtration the mixture was acidified with 0.2 M HCl and extracted with ether. The ether extract was washed with water until neutral and the solvent was evaporated. The residue was chromatographed on a column of hydrophobic Hyflo SuperCel with phase system F 2 [8]. Incubations with androst-4-ene-3,17-dione and aminopyrine were analyzed as described recently [5].

Radioactivity was determined with a methane gas flow counter except in the case of incubations with androst-4-ene-3,17-dione where radioactivity was measured with a Packard scintillation counter model 4322.

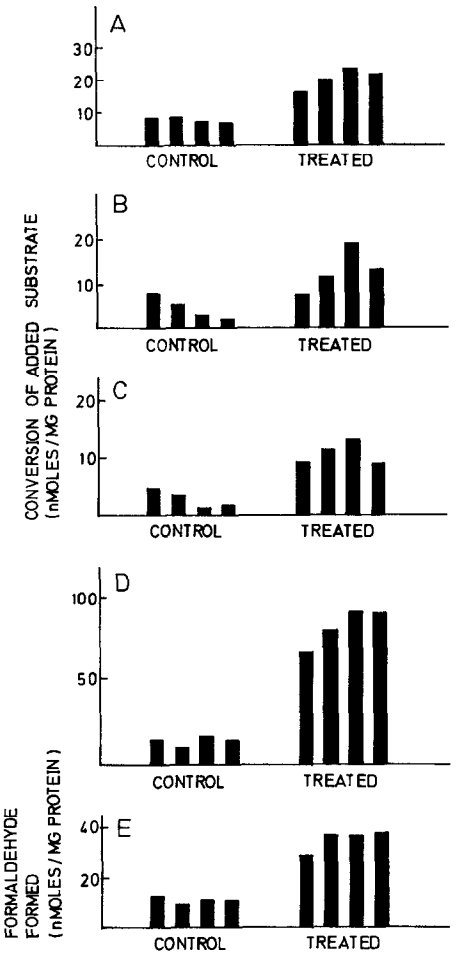

Fig. 1. Effect of phenobarbital treatment on the hydroxylation of taurodeoxycholic acid, taurochenodeoxycholic acid, lithocholic acid and androst-4-ene-3,17-dione and on the oxidative demethylation of aminopyrine. Control, control rat; treated, phenobarbital-treated rat. A, 7α-hydroxylation of taurodeoxycholic acid; B, 6β-hydroxylation of taurochenodeoxycholic acid; C, 6β-hydroxylation of lithocholic acid; D, conversion of androst-4-ene-3,17-dione into polar products. E, formation of formaldehyde from aminopyrine. The values listed below each other were obtained with liver homogenate from the same animal.

3. Results

The hydroxylation of taurodeoxycholic acid, taurochenodeoxycholic acid and lithocholic acid, catalyzed by the microsomal fraction of liver homogenate fortified with an NADPH-generating system,

was studied in control rats and phenobarbital-treated rats. The hydroxylated products formed were the same in both groups of animals. Taurodeoxycholic acid was hydroxylated in the 7α-position to yield taurocholic acid. The identification was carried out after hydrolysis to cholic acid, which was crystallized to constant specific radioactivity after addition of authentic cholic acid. Taurochenodeoxycholic acid was converted into a mixture of tauro-α-muricholic acid (80–90%) and tauro-β-muricholic acid (10–20%). These metabolites were identified after hydrolysis by their chromatographic properties only. Extracts of incubations with taurochenodeoxycholic acid were hydrolyzed and the hydrolyzed mixture was chromatographed to separate chenodeoxycholic acid from α- and β-muricholic acids. The fractions containing the muricholic acids were chromatographed together with unlabeled cholic acid with phase system C 1 [5]. The radioactivity was eluted in two peaks, one appearing slightly before and one after the titration peak of cholic acid. These elution patterns are typical of α- and β-muricholic acid, respectively. Lithocholic acid was converted into 3α, 6β-dihydroxy-5β-cholanoic acid, identified by its chromatographic behaviour in thin layer chromatography with phase system S 11 [9]. When taurolithocholic acid was incubated,, the same product (as taurine conjugate) was formed to the same extent, Practically no hydroxylation of the bile acids was obtained when NADH was used instead of NADPH.

Fig. 1 summarizes the effect of phenobarbital on hydroxylation of the three bile acids. For comparison the oxidative demethylation of aminopyrine and the hydroxylation of androst-4-ene-3,17-dione were measured. On average, the 7α-hydroxylation of taurodeoxycholic acid was 2.7 times more efficient in phenobarbital-treated rats than in control rats. The 6β-hydroxylation of taurochenodeoxycholic acid was 2.8 times and the 6β-hydroxylation of lithocholic acid 3.8 times more efficient in phenobarbital-treated rats. The increase in oxidative demethylation of aminopyrine and in hydroxylation of androst-4-ene-3,17-dione was 3.2 and 5.7 times, respectively.

The rate of hydroxylation of taurodeoxycholic acid, taurochenodeoxycholic acid and lithocholic acid as well as the rate of oxidative demethylation of aminopyrine were found to be the same in an atmosphere of 4% oxygen and 96% nitrogen as in air. In an atmosphere of 4% oxygen, 56% nitrogen and 40% carbon monoxide the 6β-hydroxylation of taurochenodeoxycholic acid and of lithocholic acid was inhibited by, on average, 83 and 62%, respectively, whereas the 7α-hydroxylation of taurodeoxycholic acid was inhibited only by 14%. The oxidative demethylation of aminopyrine was inhibited almost completely, 95%. The inhibition of the 6β-hydroxylation by carbon monoxide was reversed about 50% by light.

4. Discussion

The results of the present work and that reported recently [5] show differences between the hydroxylation reactions in the biosynthesis and metabolism of bile acids. With respect to the effect of phenobarbital the two hydroxylations involved in the *de novo* synthesis of bile acids, the 7α-hydroxylation of cholesterol and the 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one, were unaffected or inhibited, whereas a two- to four-fold stimulation was observed for the other three hydroxylations, the 7α-hydroxylation of taurodeoxycholic acid and the 6β-hydroxylation of taurochenodeoxycholic acid and lithocholic acid. These reactions are involved in the interconversion of bile acids. In parallel experiments the oxidative demethylation of aminopyrine and the hydroxylation of androst-4-ene-3,17-dione were found to be stimulated three- to six-fold by phenobarbital administration in agreement with previous work by others [10,11].

Carbon monoxide was found to affect differently the three hydroxylations stimulated by phenobarbital administration. The 7α-hydroxylation of taurodeoxycholic acid was practically unaffected, whereas the 6β-hydroxylation reactions were inhibited markedly. Recently, Voigt et al. [12,13] have also found that the 6β-hydroxylation of taurochenodeoxycholic acid is inhibited by carbon monoxide. The 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one has been reported to be partly inhibited [14] or not at all inhibited [15]. With respect to the effect of carbon monoxide on the 7α-hydroxylation of cholesterol no conclusive information is available at present.

The drug-metabolizing enzyme system is characterized by being stimulated by phenobarbital administration and inhibited by carbon monoxide. Of the

five hydroxylations in the formation and metabolism of bile acids studied in the present and previous report [5], two, the 6β-hydroxylations, showed the same properties as the drug-metabolizing enzyme system with respect to effects of phenobarbital and carbon monoxide. In fact, Voigt et al. [12,13] have recently provided evidence that NADPH-cytochrome *c* reductase as well as cytochrome P_{450} are involved in the 6β-hydroxylation of taurochenodeoxycholic acid. The other three hydroxylations, the 7α-hydroxylation of cholesterol, the 12α-hydroxylation of 7α-hydroxycholest-4-en-3-one and the 7α-hydroxylation of taurodeoxycholic acid, were found to differ from each other and from the drug-metabolizing enzyme system in response to phenobarbital administration and carbon monoxide. These differences appear to be explained most easily by assuming the participation of different hydroxylating systems. Such an interpretation of the results would not support the concept of a single hydroxylase system in liver microsomes catalyzing the hydroxylation of steroids and drugs.

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