PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 92–95 (1974)

Bile Acids XLI. Hepatic Microsomal 12α-Hydroxylation of Allochenodeoxycholate to Allocholate[1] (38049)

ROBERT J. BLASKIEWICZ, GEORGE J. O'NEIL, JR., AND WILLIAM H. ELLIOTT[2]

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

Earlier studies from this laboratory (1, 2) have demonstrated the ability of the rat with a cannulated bile duct to convert allochenodeoxycholic acid[3] to allocholic acid in a limited manner. Similar studies with microsomal preparations from rat liver have confirmed the 12α-hydroxylation of the substrate to form allocholic acid (3, 4). Since 12α-hydroxylation of a preformed dihydroxycholanic acid to a trihydroxy acid (5) is an unusual sequence, the ability of liver microsomal preparations of other species to effect this transformation was investigated; preliminary comparative studies were conducted with such preparations from the rabbit, chicken, and human.

Materials and Methods. Bile acids and chromatography. [3β-^{3}H]-Allochenodeoxycholic acid (2) with a specific activity of 2.97×10^8 dpm/mg was purified by acetic acid partition chromatography (6). Allocholic acid and its methyl ester were prepared from methyl cholate by allomerization with Raney nickel (7, 8). Thin layer chromatography (tlc) was performed on plates of silica gel G (Brinkmann Instruments, Inc.) (20 cm × 20 cm × 0.25 mm) as described (9); plates were developed in solvent 1 (acetone–benzene, 7:3) for methyl esters, or solvent 2 (chloroform–methanol–acetic acid, 80:12:3) (10) for free acids. The dried plates were sprayed with 1% phosphomolybdic acid solution; the zones of interest were scraped from the plates and the material suspended in Bray's solution (11) for radioassay in a liquid scintillation counter (2).

Incubations. Livers were excised from adult rabbits (3–4 kg) after anesthesia with sodium pentothal, and suspended in modified Bucher's medium (12) at 4°, pH 7.4. Microsomal fractions were prepared according to the method of Einarsson (13). Microsomal preparations of chicken liver were obtained similarly from an adult chicken except that ether was used as anesthetic for removal of the liver. A sample of human liver was obtained through the Department of Surgery at cholecystectomy of a 48 yr old female patient with reported normal liver function at the time of surgery. Protein of the microsomal fractions was determined by the biuret method with bovine serum albumin as a standard. Incubations were carried out in air with shaking at 37° with 3 ml of the microsomal preparation, 3 μmoles of added NADPH in 0.1 ml of homogenizing medium, and 127 nmoles of substrate (6.7 μCi) in 50 μl of

[1] These studies were supported by Grant HL-07878, National Institutes of Health; R. J. B. and G. J. O. were supported by Grant FR-5388.

[2] Author to whom correspondence should be directed.

[3] The following trivial names are defined: allochenodeoxycholic acid, 3α,7α-dihydroxy-5α-cholanic acid; allocholic acid, 3α,7α,12α-trihydroxy-5α-cholanic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanic acid; cholic acid, 3α,7α,12α-trihydroxy-5β-cholanic acid; lithocholic acid, 3α-hydroxy-5β-cholanic acid; ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanic acid.

methanol for periods from 10–60 min. With each series of incubations a heated control was included; the microsomal preparation was heated at 100° on a steam bath for 15 min before addition of substrate and NADPH. Incubations were terminated by addition of ten volumes of 95% ethanol; the precipitate was removed by filtration, and the samples were dried *in vacuo* and redissolved in 25 ml of ethanol. An aliquot (0.1 ml) was removed for radioassay. NADPH was obtained from Sigma Chemical Co.; the constituents of Bucher's medium (12) were Analytical Reagent Grade (Mallinckrodt Chemical Works).

Identification of products of incubation. An aliquot (10 μl) from each incubation was analyzed by tlc with authentic allocholic and allochenodeoxycholic acids in an adjacent lane; the appropriate regions were scraped and assayed for radioactivity (2). To establish the identity of the product, the material was subjected to acetic acid partition chromatography. Allocholic acid in fractions[4] 60-4 through 80-2 was converted to the methyl ester, diluted with authentic ester and crystallized to constant specific activity.

Results. Recovery of tritium from the incubations was greater than 90%. Analysis of the products of incubation by tlc consistently showed no significant radioactivity in the region of allocholic acid from those samples in which the microsomal preparation had been heated at 100° prior to addition of the substrate, demonstrating the necessity of the enzyme system for the conversion. On the other hand, for example, about 24% of the chromatographed ^3H was found in the zone of mobility of allocholic acid from incubation of the microsomal preparation from female rabbit liver. Table I shows the identification by isotopic dilution of allocholic acid from a 60 min incubation of the liver microsomal preparation from a male rabbit. Similar results were obtained with other fractions of allocholic acid from the remaining incubations. The effect of time on the amount of product formed with and without added NADPH is shown in Fig. 1.

TABLE I. Identification of Allocholic Acid in Fractions 60-4; 80-1, 2–3.

Crystallization	Wt. mg.	Sp. Act. dpm/mM × 10^{-7}
Me ester:		
calculated[a]	50.0	6.56
1) 1:1 *A-M*	22.6	5.96
2) 20:1 *A-M*	15.0	5.80
3) *A*	9.5	5.72
Acid:		
1) *M*	7.1	5.71
2) *M*	2.6	5.93

[a] Methyl allocholate (50.0 mg) was added to 7.5×10^6 dpm of tritiated material. *A* = acetone; *M* = methanol.

The percentages of tritium found in allocholic acid from 10 to 60 min incubations for male and female rabbits, chicken, and the human are shown in Fig. 2; the reaction was linear in each case from 10 to 60 min. Table II compares the results of 60 min incubations in these 3 species in terms of nmoles of substrate per mg of microsomal protein and nmoles of product formed per mg of protein. In a 60 min incubation the

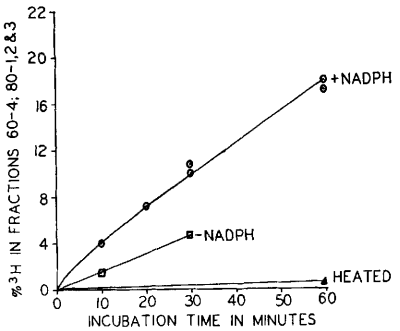

FIG. 1. NADPH requirement of male rabbit liver microsomal hydroxylase. Rabbit liver microsomes (3 ml) were incubated for various periods of time with 127 nmoles of [3β-^{3}H]allochenodeoxycholic acid with or without addition of 3 μmoles of NADPH. [^{3}H]-allocholic acid was identified in fractions 60-4, 80-1, 2 and 3.

[4] The fractions collected in this system (6) are designated as follows: the first number refers to the percent benzene in hexane and the second to the fraction in the respective series; thus, Fraction 60-4 refers to the fourth fraction eluted with 60% benzene in hexane.

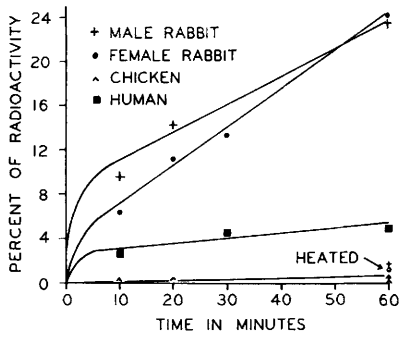

FIG. 2. Percent of tritium in allocholic acid derived from [3β-^{3}H]allochenodeoxycholic acid. The substrate (127 nmoles) was incubated with liver microsomes and 3 μmoles of NADPH for various periods of time.

rabbit microsomal fraction afforded 3–4.5 times as much product as that from the rat (3, 4). A sex difference may be present, but additional experiments are necessary to establish this point. By comparison 12α-hydroxylation of the substrate was very weakly demonstrated by the chicken and was rather limited with the human.

Discussion. These results demonstrate the ability of microsomal preparations of the rabbit, chicken, and human to hydroxylate allochenodeoxycholate at C-12 to form allocholate. The data clearly suggest that the rabbit is the species of choice for further study of this interesting phenomenon. In the rabbit, the predominant bile acids are cholic and deoxycholic acids, the latter derived from the former by intestinal microorganisms (14, 15, 5). Allocholic acid is a component of bile from the germ-free rabbit (16) and is a precursor of allodeoxycholic acid preesnt in bile and gallstones of the conventional rabbit (17, 18). Chenodeoxycholic acid has not been found in rabbit bile (19), but it has been detected in low concentrations in fistula bile (20), in feces (21), and in bile of the germ-free rabbit (16). The rabbit metabolizes chenodeoxycholic acid to lithocholic acid, 7-ketolithocholic acid and ursodeoxycholic acids (19). To ascertain whether cholic acid may be formed from chenodeoxycholic acid with a rabbit liver microsomal preparation fortified with NADPH, tritiated chenodeoxycholic acid was incubated as described for 60 min; no significant activity identifiable with cholic acid was found. Allochenodeoxycholic acid has not been identified in rabbit bile.

In chicken bile chenodeoxycholic acid is the predominant bile acid, although cholic acid is also present. Chenodeoxycholic acid is metabolized by the chicken to cholic acid only to the extent of 0.7% (22). In man the predominant bile acids are chenodeoxycholic, cholic, and deoxycholic acid, the latter a product of intestinal bacterial action on cholic acid. Chenodeoxycholic acid is not metabolized to cholic acid in this species, but is converted by bacteria to lithocholic acid (5). The activity of the microsomal 12α-hydroxylase on allochenodeoxycholic acid has been attributed (2) to the coplanarity of the nucleus of the 5α-derivatives, in contrast to the 5β-derivatives.

Einarsson (13) has detailed optimal conditions for the activity of rat liver 12α-hydroxylase with several Δ^4- and 5β-sterols as potential intermediates in the formation of cholic acid. He and others (23) have demonstrated the capacity of microsomal liver preparations fortified with NADPH to effect hydroxylation of steroids. The rate of 12α-hydroxylation of 7α-hydroxycholest-4-

TABLE II. Formation of Allocholic Acid by Three Species.

	Rabbit			
	Male	Female	Chicken	Human
nmoles substrate/mg microsomal protein	4.32	6.05	9.8	10.5
mg Protein/ml	9.8	7.00	4.32	4.03
nmoles product/60 min/mg microsomal protein	1.04	1.57	0.052	0.56

en-3-one was maximal with phosphate buffer at pH 7.4; the present experiments were carried out successfully at pH 7.4, but no product was detected at pH 5.0. A more detailed investigation (3) of the properties of microsomal 12α-hydroxylase activity with allochenodeoxycholic acid will be reported elsewhere.

Summary. Liver microsomal preparations of male and female rabbits, chicken and human fortified with added NADPH promote 12α-hydroxylation of 3α, 7α-dihydroxy-5α-cholanic acid, allochenodeoxycholic acid, to form allocholic acid. The rabbit is superior to the chicken and human in formation of greater quantity of product. These data support the hypothesis that the coplanarity of the 5α-sterol nucleus is important for the sterochemical specificity of the 12α-hydroxylase system.

The authors are pleased to acknowledge the very capable assistance of Dr. Mei Mei Mui in these studies.

1. Mui, M. M., Noll, B. W., and Elliott, W. H., Fed. Proc. **29**, 3778 (1970).

2. Mui, M. M., and Elliott, W. H., J. Biol. Chem. **246**, 302 (1971).

3. Mui, M. M., Ph.D. Dissertation, St. Louis University (1972).

4. Elliott, W. H., Blaskiewicz, R. J., O'Neil, Jr., G. J., and Mui, M. M., Fed. Proc. **32**, 479 Abs (1973).

5. Danielsson, H., and Einarsson, K., in "The Biological Basis of Medicine," (E. E. Bittar and N. Bittar, eds.), p. 279, Academic Press, New York (1969).

6. Matschiner, J. T., Mahowald, T. A., Elliott, W. H., Doisy, E. A., Jr., Hsia, S. L., and Doisy, E. A., J. Biol. Chem. **225**, 771 (1957).

7. Mitra, M. N., and Elliott, W. H., J. Org. Chem. **33**, 175 (1968).

8. Elliott, W. H., in "The Bile Acids" (P. P. Nair and D. Kritchevsky, eds.) Vol. 1, p. 47. Plenum Publishing Corp., New York (1971).

9. Ziller, S. A., Jr., Doisy, E. A., Jr., and Elliott, W. H., J. Biol. Chem. **243**, 5280 (1968).

10. Siegfried, C. M., and Elliott, W. H., J. Lipid Res. **9**, 394 (1968).

11. Bray, G. A., Anal. Biochem. **1**, 279 (1960).

12. Bergström, S., and Gloor, V., Acta Chem. Scand. **9**, 34 (1955).

13. Einarsson, K., Eur. J. Biochem. **5**, 101 (1968).

14. Ekdahl, P. H., and Sjövall, J., Acta Physiol. Scand. **34**, 287 (1955).

15. Linstedt, S., and Sjövall, J., Acta Chem. Scand. **11**, 421 (1957).

16. Hofmann, A. F., Mosbach, E. H., and Sweeley, C. C., Biochim. Biophys. Acta **176**, 204 (1969).

17. Hofmann, A. F., Bokkenheuser, V., Hirsch, P. L., and Mosbach, E. H., J. Lipid Res. **9**, 244 (1968).

18. Hofmann, A. F., and Mosbach, E. H., J. Biol. Chem. **239**, 2813 (1964).

19. Hellström, K., and Sjövall, J., Acta Chem. Scand. **14**, 1763 (1960).

20. Gregg, J. A., and Poley, J. R., Amer. J. Physiol. **211**, 1147 (1966).

21. Danielsson, H., Kallner, A., and Sjövall, J., J. Biol. Chem. **238**, 3846 (1963).

22. Ahlberg, J. W., Ziboh, V. A., Sonders, R. C., and Hsia, S. L., Fed. Proc. **20**, 283 (1961).

23. Talalay, P., Ann. Rev. Biochem. **34**, 347 (1965).

Received Dec. 6, 1973. P.S.E.B.M., 1974, Vol. 146.