Vol. 86, No. 4, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1979 Pages 1089-1095

HYDROXYLATION AND SULFATION OF BILE ACIDS IN RAT HEPATOMA CULTURES UNDER THE INFLUENCE OF A GLUCOCORTICOID

Maurice Lambiotte and Jan Sjövall

Unité de Génétique Cellulaire, Institut de Recherche en Biologie Moléculaire du CNRS, Université Paris VII, place Jussieu 75005, Paris, France, and Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden

Received October 16,1978

Summary : When treated with a glucocorticoid, monolayer cultures of a differentiated rat hepatoma convert added chenodeoxycholic and deoxycholic acids into compounds not formed by untreated cultures. The main metabolites have been identified. Chenodeoxycholic acid was 6β-hydroxylated to yield α-muricholic acid, whereas deoxycholic acid was converted mainly into its 3-sulfate. In addition, deoxycholic acid was hydroxylated in 6β-, 7α- and 1ξ-positions. Thus, glucocorticoids appear to induce one 6β-hydroxylase in particular and a sulfotransferase having a notable substrate selectivity.

As reported previously (1) rat hepatoma monolayers treated with a glucocorticoid convert added dihydroxy bile acids into major metabolites which are not formed by control cultures. The nature of these compounds has not been established. This paper describes the identification of the main metabolites of deoxycholic and chenodeoxycholic acids.

Materials and Methods : Rat hepatoma Faza 967, kindly provided by Dr. M.C. Weiss, was cultivated as monolayers in 100 mm tissue-culture Petri dishes in a mixture, of equal volumes of modified Ham's F12 and NCTC 109 supplemented with 7.5% fetal calf serum (Sorga, Paris, France), previously heat-decomplemented, 0.5 g of bovine serum albumin and 7.1 mg of taurine per liter, 100 i.u. of penicillin and 100 µg of streptomycin sulfate per ml (2). When about $1.2.10^7$ cells per dish had been obtained (from an initial inoculum of 5.10^6 cells), dexamethasone phosphate (1.9 µM) was added and the culture was left for four more days, the medium being renewed every two days. Labelled sodium deoxycholate or chenodeoxycholate were then added to give a final concentration of 15 µg/ml and about 0.25 µCi/ml. After an incubation period of 72 h, the medium was collected and added to three volumes of ethanol. The protein precipitate was removed by centrifugation and the supernatant was evaporated in vacuum at room temperature. The residue was subjected to preparative thin-layer chromatography (TLC).

Solvents were of reagent grade and were redistilled when used in analyses by gas chromatography (GLC) and gas chromatography-mass spectrometry (GC/MS). Chenodeoxycholic (3α,7α-dihydroxy-5β-cholanoic) (Sigma) and deoxycholic (3α, 12α-dihydroxy-5β-cholanoic) (Merck) acids were purified by recrystallization ; their purity was checked by TLC and GLC. The deoxycholic acid contained less than 0.1% of cholic acid. The 24-^{14}C - labelled acids were from the Radiochemical Centre (Amersham) and yielded a single spot upon TLC and autoradiography.

0006-291X/79/041089-07$01.00/0

Preparative TLC was carried out on 2 mm layers of silica gel G (Merck) using the solvent system isoamyl acetate/acetic acid/n-propanol/water, 4:3:2:1 (by vol.) (3). Radioactive zones were located by autoradiography (Kodirex). They were scraped out and eluted with ethyl acetate and ethyl acetate/methanol/pyridine, 10:10:1 (by vol.) for unconjugated metabolites, and methanol/pyridine, 15:1 (v/v), or methanol and butanol for conjugated metabolites. Analytical TLC was carried out on 0.25 mm layers using the above solvent system (system 1) or the following solvent mixtures: isooctane/acetic acid/ethyl acetate, 25:1:125 (by vol.) (system 2); ethylene chloride/acetic acid/water, 10:10:1 (by vol.) (system 3); ethyl acetate/ethanol/25% ammonium hydroxide, 2:7:1 (by vol.) (system 4) (see ref. 4).

Group separation of conjugated bile acids was performed on a 110 x 8 mm column of diethylaminohydroxypropyl Sephadex LH-20 (DEAP-LH-20) (5). Sulfated and non-sulfated bile acids were also separated on a 230 x 8 mm column of Sephadex LH-20 using chloroform/0.02 M NaCl in methanol, 3:2 (v/v), as the solvent (5). Solvolysis was performed in ethanol/acidified ethyl acetate, 3:27 (v/v), at 39° for 16 h (5). The solution was then neutralized and taken to dryness in vacuo. Alkaline hydrolysis was made in 15% NaOH in 50% aqueous ethanol at 110° for 10 h or in 2 M aqueous KOH at 110° for 6 h. After acidification the bile acids were extracted with ethyl acetate or freshly distilled diethyl ether. Methyl esters were prepared with diazomethane in diethyl ether, chromic acid oxidations were carried out in acetone, and trimethylsilyl ethers were prepared with hexamethyldisilazane/trimethylchlorosilane in pyridine (4-6).

GLC analyses were performed using 2-3 m x 3.4 mm columns packed with 1.5% SE-30 or 1.5% Hi-Eff 8 BP on Chromosorb WHP or Gas-Chrom Q, 80-100 mesh (4-6). Column temperatures were 210-240°. Computerized GC/MS analyses were carried out using the same columns in a modified LKB 9000 instrument. Spectra were taken by repetitive magnetic scanning and data were plotted as mass spectra and fragment ion current chromatograms (5,7).

Results: Incubations with both chenodeoxycholic and deoxycholic acids yielded metabolites which had TLC mobilities of taurine conjugates of these bile acids. However, intense spots also appeared at other positions. Chenodeoxycholic acid yielded spots with a mobility of trihydroxy bile acids and their taurine conjugates. The same was true for deoxycholic acid which also yielded a major compound with a mobility different from that of any known metabolite. The rates of formation of these metabolites and other quantitative relationships is the subject of a separate study (8).

Identification of α-muricholic acid (3α,6β,7α-trihydroxy-5β-cholanoic acid) as the major metabolite of chenodeoxycholic acid. This metabolite had a mobility in TLC-system 1 similar to that of cholic acid. In system 2 it was located between chenodeoxycholic and cholic acids. After elution from a TLC plate it was subjected to ion exchange chromatography on DEAP-LH-20. All the radioactivity was recovered in the fraction containing unconjugated bile acids. Following methylation and trimethylsilylation it was analyzed by GLC and GC/MS

both on nonselective SE-30 and selective Hi-Eff 8 BP columns. The retention time on both columns and the mass spectral fragmentation pattern were identical with those of the derivative of α-muricholic acid (4,9). No β-muricholic acid was present and the fragment ion current chromatograms did not reveal the presence of any other trihydroxycholanoate.

Identification of hydroxylated metabolites of deoxycholic acid. In the presence of dexamethasone phosphate, Faza 967 hepatoma cells transformed part of the added deoxycholate into a mixture of trihydroxylated compounds whose taurine conjugates appeared as a single spot in solvent system 1. The unconjugated metabolites had the same mobility as cholic acid in this system. A wide zone corresponding to the cholic and part of the deoxycholic acid spots, was eluted from the plate and the trihydroxy fraction was isolated by chromatography of the methyl esters on Unisil (10). The trimethylsilylated fraction was analyzed by GLC and GC/MS using the SE-30 column. Three peaks were obtained having retention times relative to that of the cholic acid derivative of 1.00, 1.08 and 1.19. The mass spectrum of the compound with the shortest retention time was identical with that of the derivative of cholic acid. The retention time, 1.09, and the mass spectrum of the second product were identical with those of the derivative of 3α,6β,12α-trihydroxy-5β-cholanoic acid (see 5). The mass spectrum of the third product was identical with that of the derivative of a bile acid found in human urine and tentatively identified as 1,3,12-trihydroxycholanoic acid (5). The same bile acid has recently been obtained by microbial 1-hydroxylation of deoxycholic acid (Carlström, K. and Sjövall, J. in preparation). Thus, the third metabolite was identified as 1ξ,3α,12α-trihydroxy-5β-cholanoic acid. In addition to these three hydroxylation products, the fragment ion current chromatograms and mass spectra indicated the presence of small amounts of 3α,7β,12α-trihydroxy-5β-cholanoic acid. The relative amounts of the three major hydroxylation products were calculated from the peak areas in the GLC analyses. Cholic acid constituted about 25%, 3α,6β,12α-trihydroxy-5β-cholanoic acid about 60% and the 1-hydroxylated bile acid about 15%.

Identification of the major metabolite of deoxycholate: deoxycholate-3-sulfate. In TLC system 1, this compound appeared between glycocholic and glycodeoxycholic acids, quite close to the former compound. Following isolation by preparative TLC the metabolite was studied in different chromatographic systems and after being subjected to different hydrolytic conditions.

Upon chromatography on DEAP-LH-20 the major part of the radioactivity was recovered in the monosulfate fraction. When this fraction was analyzed by TLC, a single radioactive spot was obtained at the site of the metabolite. Alkaline hydrolysis yielded four radioactive spots upon TLC in system 1. Two of these had the mobilities of unchanged metabolite and deoxycholic acid, respectively; a third product travelled near the solvent front. When the metabolite or the sulfate fraction were solvolyzed under conditions used to cleave sulfates, a single radioactive product was formed. This was identified as deoxycholic acid by GC/MS analysis of the methyl ester trimethylsilyl ether derivative.

Since bile acid glucuronides appear close to sulfates in the buffer system used for the DEAP-LH-20 chromatography, a sample of the metabolite was directly derivatized and analyzed by GC/MS using a short SE-30 column at 280° (11). No evidence for the presence of a glucuronide was obtained. Thus, the data indicated that the metabolite was a sulfate. Further support was obtained from the results of a chromatography on Sephadex LH-20. The metabolite had the same mobility as an otherwise unconjugated bile acid sulfate (5). Solvolysis of this fraction yielded deoxycholic acid.

In order to determine the position of the sulfate group, the metabolite was oxidized with chromic acid in acetone and then solvolyzed and derivatized (5). GC/MS analyses using both SE-30 and Hi-Eff 8 BP columns showed that 3α-hydroxy-12-oxo-5β-cholanoic acid (5,9) was formed by the oxidation-solvolysis sequence. This shows that C-3 was the site of conjugation.

These results provide good evidence that the metabolite is the 3-sulfate of deoxycholic acid. A sample of the authentic compound was kindly supplied by

Prof. G.A.D. Haslewood. The TLC mobilities of this compound and the metabolite in systems 3 and 4 were identical, supporting the identification.

Discussion: In the presence of a glucocorticoid, the hepatoma Faza 967 in monolayer culture is able to: 1. hydroxylate chenodeoxycholate in the 6β position to yield α-muricholate as the major metabolite; 2. sulfurylate deoxycholate to yield the 3-sulfate as the major metabolite; and 3. hydroxylate deoxycholate in the 6β, 7α or 1ξ positions. Thus, except for the conjugation with taurine, the metabolic fates of the two dihydroxy bile acids in this system are quite different: deoxycholic acid is mainly sulfurylated whereas chenodeoxycholic acid is exclusively 6β-hydroxylated.

The hydroxylation reactions in the cell cultures resemble those occurring in vivo in the rat liver. The main primary metabolite of chenodeoxycholate in vivo as well as in vitro is α-muricholate (the taurine conjugate may be the preferred substrate in the hydroxylation) (12-14). However, in vivo the 7α hydroxyl group is then epimerized to give β-muricholic acid (12,14). This reaction was not observed in the hepatoma cell cultures.

By far the predominant metabolite of deoxycholate in vivo and in vitro is cholate (conjugated with taurine) (12-14). Hydroxylation in the 6β position is a minor metabolic pathway for deoxycholic acid in rats with ligated bile ducts (15). In the hepatoma cells, 6β-hydroxylation is more active than 7α-hydroxylation. Hydroxylation of bile acids at C-1 as detected in the hepatoma cells has not been described to occur in rats.

Although the relative importance of 7α- and 6β-hydroxylation of deoxycholate is different in rat liver and the hepatoma cells, the major metabolic difference lies in the extensive sulfurylation in the hepatoma cells. Formation of sulfates is of little quantitative importance in rat liver in vivo (16). The sulfates of deoxycholic and chenodeoxycholic acids both occur in small amounts in rat bile (16) whereas only deoxycholic acid is sulfurylated by the hepatoma cells. This supports the existence of multiple steroid sulfotransferases (17) and shows that these enzymes may exhibit a marked substrate specifi-

city. The selective induction in the hepatoma cells of an enzyme catalyzing sulfurylation of deoxycholic acid could be of value for the characterization of a single type of sulfotransferase.

In vivo, the adrenals appear to regulate the activity and distribution of different forms of glucocorticoid sulfotransferases in rat liver (18). Administration of cortisol and deoxycorticosterone to adrenalectomized female rats increases the activities of three sulfotransferases. However, there is a complex interaction of several regulatory systems in the intact rat (18,19) and induction or stimulation of a specific sulfotransferase activity as in the case of the hepatoma cells has not been observed.

Hydroxylation of bile acids in 6β and 7α positions are catalyzed by cytochrome P-450 dependent microsomal systems (see 13). To our knowledge this work provides the first clearcut evidence of a hormonal induction of such activity in a cloned hepatoma strain in culture.

Acknowledgements: The technical help of Mrs. N. Thierry and Mrs. G. Alvelius is gratefully acknowledged. This work was supported by grants from Ligue Francaise contre le Cancer and the Swedish Medical Research Council (grant no. 3X-219).

References

1. Lambiotte, M. (1977) Bile Acid Metabolism in Health and Disease. G. Paumgartner and A. Stiehl eds., pp. 33-47, MTP Press Ltd,. Lancaster.
2. Lambiotte, M., Vorbrodt, A., and Benedetti, E.L. (1973) Cell Differentiation 2, 43-53.
3. Hofmann, A. (1962) J. Lipid Res. 3, 127-128.
4. Eneroth, P., and Sjövall, J. (1969) Methods in Enzymology, Vol. XV, R.B. Clayton ed., pp. 237-280, Academic Press, New York.
5. Almé, B., Bremmelgaard, A., Sjövall, J., and Thomassen, P. (1977) J. Lipid Res. 18, 339-362.
6. Makita, M., and Wells, W.W. (1963) Anal. Biochem. 5, 523-530.
7. Reimendal, R., and Sjövall, J. (1972) Anal. Chem. 44, 21-29.
8. Lambiotte, M., and Thierry, N. J. Biol. Chem. submitted for publication.
9. Sjövall, J., Eneroth, P., and Ryhage, R. (1971) The Bile Acids, Vol. I. P.P. Nair and D. Kritchevsky eds., pp. 209-248, Plenum Press, New York.
10. Matern, S., Sjövall, J., Pomare, E.W., Heaton, K.W., and Low-Beer, T.S. (1975) Med. Biol. 53, 107-113.
11. Almé, B., Nordén, A., and Sjövall, J. (1978) Clin. Chim. Acta 86, 251-259.
12. Bergström, S., Danielsson, H., and Samuelsson, B. (1960) Lipide Metabolism. K. Block ed., pp. 291-336, Wiley & Sons, New York.
13. Danielsson, H., and Sjövall, J. (1975) Ann. Rev. Biochem. 44, 233-253.
14. Hsia, S.L. (1971) The Bile Acids, Vol. I. P.P. Nair and D. Kritchevsky eds., pp. 95-120, Plenum Press, New York.

15. Ratliff, R.L., Matschiner, J.T., Doisy, E.A., Jr., Hsia, S.L., Thayer, S.A., Elliott, W.H., and Doisy, E.A. (1959) J. Biol. Chem. 234, 3133-3136.
16. Eriksson, H., Taylor, W., and Sjövall, J. (1978) J. Lipid Res. 19, 177-186.
17. Roy, A.B. (1971) Handbook of Experimental Pharmacology New Series, Vol. 28:2. B.B. Brodie and J. Gillette eds., pp. 536-563, Springer Verlag, Berlin.
18. Singer, S.S. (1978) Endocrinology 103, 66-73.
19. Carlstedt-Duke, J., and Gustafsson, J.-Å. (1973) Eur. J. Biochem. 36, 172-177.