# Formation and secretion of glycolithocholate-3-sulfate in primary hepatocyte cultures

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**Abstract** Bile acid sulfation was studied in primary hepatocyte cultures. The primary hepatocyte cultures formed glycolithocholate-3-sulfate (GLC-S) when glycolithocholate (GLC) was added to the medium. The relative percentage of GLC-S formation increased when the GLC concentration was increased from 10 μM to 100 μM. GLC-S formation was linear to 60 min. GLC-S secretion into the medium was detectable at 75 min and linear to 8 hr. In contrast to the effect of GLC concentration, there was no difference in GLC-S formation or secretion when inorganic sulfate in the medium was increased 16-fold (100 μM–1600 μM). We conclude that the rate of bile acid sulfate formation in cultured primary hepatocytes is primarily controlled by bile acid, but not inorganic sulfate, concentration. — **Kirkpatrick, R. B., and R. A. Belsaas.** Formation and secretion of glycolithocholate-3-sulfate in primary hepatocyte cultures. *J. Lipid Res.* 1985. **26:** 1431–1437.

**Supplementary key words** bile acid sulfates • bile acid sulfation • glycolithocholic acid

The liver is a major site for the detoxification of endogenous and exogenous compounds. Sulfation is a conjugation reaction whereby compounds are converted to their generally less toxic and more water-soluble sulfate esters (1). Sulfotransferases catalyze the formation of these sulfate esters from bile acid (2), hydroxysteroid (3), phenol (4), and estrone (5) substrates. Adenosine-3'-phosphate 5'-phosphosulfate (PAPS) is the sulfate donor in the liver and is rapidly formed from inorganic sulfate and ATP (6).

The sulfation of bile acids is thought to be an important protective mechanism for the liver in the presence of cholestasis (7), during chenodeoxycholate administration for the dissolution of cholesterol gallstones (8), during total parenteral nutrition (9), and during the newborn period (10). Sulfated bile acids have an increased renal clearance (11) and decreased ileal absorption when compared to non-sulfated bile acids. Lithocholate is the most toxic of the bile acids (12) and is the only bile acid significantly sulfated under normal conditions (13).

Primary hepatocyte cultures have proved valuable in the study of hepatic metabolism. Uptake (14), synthesis (15), and secretion (16) of bile acids have been shown to occur. Although several studies in isolated hepatocytes have investigated the sulfation of xenobiotics (17), Lambiotte and Thierry (18) were unable to detect any sulfation of deoxycholate in their primary hepatocyte cultures.

We studied the sulfation of glycolithocholate (GLC) in primary hepatocyte cultures. Glycolithocholate-3-sulfate (GLC-S) was formed when GLC was added to the medium. Total formation of GLC-S was increased when the GLC concentration was increased in the medium. In contrast, changes in medium inorganic sulfate concentration had no effect on GLC-S formation.

## METHODS

### Reagents

Glycolithocholate and glycolithocholate-3-sulfate were purchased from Calbiochem-Behring, San Diego, CA and were greater than 96% pure as judged by high performance liquid chromatography. [24-$^{14}$C]Glycolithocholate (6.5 mCi/mmol) was obtained from Cal Bionuclear, Sun Valley, CA. [$^{35}$S]$Na_2SO_4$ (478 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Fetal bovine serum was from Sterile Systems, Logan, UT. Collagenase was obtained from Cooper Biomedical, Elizabeth, NJ. Swims S77 medium, Waymouth 752/1 medium, penicillin (10,000 U/ml), and streptomycin (10,000 μg/ml) were obtained from KC Biological Company, Lanexa, KS. C-18 μBondapak Sep-Pak was from Waters Associates (Milford, MA). Insulin was obtained from Eli Lilly, Indianapolis, IN. Pyruvate (100 mM) was obtained from Gibco, Grand Island, NY. Heparin sulfate was purchased

Abbreviations: GLC, glycolithocholic acid; GLC-S, glycolithocholate-3-sulfate; PAPS, adenosine-3'-phosphate 5'-phosphosulfate; HPLC, high performance liquid chromatography.

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from Elkins-Sinn, Inc., Cherry Hill, NJ. Rat tail collagen was prepared according to the method of Wood and Keesh (19). Hyaluronidase and rat serum albumin were from Sigma Chemical Co., St. Louis, MO. Anti-rat serum albumin was from Cappel Laboratories, Malvern, PA. Agarose A45 was obtained from Fisher Scientific Co., Fair Lawn, NJ. Glass photographic plates (3½" × 4½") were obtained from Eastman Kodak, Rochester, NY. All other chemicals were reagent grade or better and obtained from commercial sources.

### Animals

Random-bred Sprague-Dawley male rats (200–250 g) were obtained from the National Cancer Institute Facility, Frederick, MD, and given free access to pelleted rat chow and water.

### Primary hepatocyte culture preparation

The animals were anesthetized with ether and a midline abdominal incision was made. The portal vein and inferior vena cava were cannulated and oxygenated with Swims S77 medium containing standard additives (1 mM pyruvate, 0.1 U/ml insulin, penicillin 100 μg/ml, streptomycin 100 μg/ml, and cefoxitin 20 μg/ml) plus 10 U/ml heparin and 0.5 mM EDTA perfused through the portal vein at 15 ml/min for 6 min. The liver was removed and perfused for another 10 min with the same medium (except for the EDTA) containing 40 mg/ml collagenase, 100 mg/ml hyaluronidase, and 5 mM $CaCl_2$. The liver was then removed from the perfusion apparatus, rinsed gently in 10 ml of Waymouth 752/1 supplemented with the standard additives and 10% fetal bovine serum, and finely minced. The cell suspension was filtered through fine mesh nylon and centrifuged at 50 *g* for 1 min in 50-ml conical glass tubes. The cells were washed twice and resuspended in the Waymouth 752/1 plating medium at a density of approximately $2 \times 10^6$ cells/ml. Three ml of the cell suspension was plated on rat tail collagen-coated 60-mm plastic petri dishes. The medium was changed at 1 hr after plating to Waymouth 752/1 and standard additives. At 4 hr the plates were individually rinsed with medium using a Pasteur pipet to remove all nonattached cells. The medium was changed at 24 hr after plating and all experiments were initiated with 24-hr cultures.

### Bile acid sulfate determination

After the primary hepatocytes were in culture for 24 hr, the labeled bile acid was added (110,000 cpm/plate). Hepatic bile acid sulfotransferase activity shows the highest in vitro activity with GLC as a substrate (2). GLC was therefore used in all assays. GLC was dissolved in ethanol prior to use. The maximal ethanol concentration of any plate following GLC addition was 0.5%. The addition of ethanol did not affect the formation of bile acid sulfate. The reaction was stopped by removing the medium, immediately adding 0.75 ml of 0.1 M Na citrate, pH 2.8 (4°C), and placing the culture plate on ice. For controls, the plates were kept on ice, the medium was added and then immediately removed.

GLC-S concentration in the medium was determined by high performance liquid chromatography (HPLC) as previously described (20). Medium (2.9 ml) was added to a 12-ml syringe and 10 ml of 0.1 M Na citrate, pH 2.8, was added. The mixture was passed over a C-18 μBondapak Sep-Pak previously sequentially washed with 20 ml of methanol and 10 ml of 0.1 M Na citrate, pH 2.8, buffer. The Sep-Pak was washed with another 20 ml of the citrate buffer and then the bile acid sulfate was eluted with 4 ml of methanol. The methanol was evaporated under a gentle stream of $N_2$ and the bile acid was resuspended in 1 ml of 72% methanol, 0.34 M Na acetate, pH 6.5. Two hundred fifty μl of this solution was chromatographed with a Beckman Model 110A HPLC using a Model 156 Refractive Index detector over an Ultrasphere-ODS column (4.6 mm × 25 cm) (Beckman, Palo Alto, CA) using 72% methanol, 0.34 M Na acetate, pH 6.5, at 0.6 ml. The eluent was collected in 1.8-ml aliquots and the radioactivity was quantified using standard scintillation techniques. Mean retention times for standards are GLC, 18 min and GLC-S, 72 min.

To determine the intracellular GLC-S concentration, the cells were gently scraped from the plates by a rubber spatula and suspended in 0.75 ml of the citrate buffer. The cells were then sonicated for 30 sec and centrifuged (10,000 *g* for 5 min). The supernatant was then treated exactly as the medium. The recovery of labeled bile acid from the HPLC column when compared to the labeled bile acid added to the medium was greater than 93%. The recovery was constant over the concentrations of bile acid added. The coefficient of variation between duplicate culture plates was 3.6% and between primary hepatocyte preparations was 14%. GLC-S formation was defined as the bile acid sulfate in the cells and the medium combined. GLC-S secretion was the bile acid sulfate in the medium. GLC-S was determined by the percentage of radioactivity present as the sulfate compared to the total radioactivity present. Solvolysis was performed by the method of van Berge Henegouwen et al. (21).

### Albumin synthesis

Ten μl of standard or medium from the primary hepatocyte culture plates was placed in 4-mm wells in 1% agarose gels (22). The gels were poured at a dilution of 1:200 (anti-rat serum albumin: 1% agarose) and allowed to stand for at least 1 hr at 4°C prior to use. The plates were electrophoresed at 140 v for 2 hr and the resulting rockets were developed in 1% tannic acid for 10 min to enhance readability.

### Protein determination

Protein concentration of the soluble fraction remaining after sonication and centrifugation of the cells was determined using the biuret method with serum albumin as a standard (23).

## RESULTS

The cells formed a "liver-like" monolayer and synthesized albumin, a liver-specific protein, for at least 96 hr (**Table 1**). At the time of plating, 95% of the cells excluded trypan blue. Concentrations greater than 100 $\mu$M GLC were toxic, with hepatocyte viability decreasing greatly after 60 min of exposure.

GLC was rapidly taken up into the cells from the medium. After 30 min of incubation, over 95% of the added GLC (40 $\mu$M) had been taken up into the cells and 48% was still in the cells in the form of unmetabolized GLC.

The primary hepatocytes rapidly formed GLC-S from the GLC added to the medium. The formation of GLC-S (59 ± 18 pmol $min^{-1}$ mg $prot^{-1}$) (mean ± SEM) was linear to 60 min (**Fig. 1**). When a low concentration of GLC (10 $\mu$M) was added to the medium, 6.0% of the GLC was metabolized to GLC-S for a total amount of 1.8 nmol/$10^6$ cells (**Fig. 2**). At a higher concentration of GLC (100 $\mu$M), the GLC-S formed increased over twofold to 14.1% of the added GLC for a total amount of 42.4 nmol/$10^6$ cells. The highest rate of GLC-S formation was observed at a GLC concentration of 200 $\mu$M; however, hepatocyte viability decreased after 60 min. Although 8.5% of the GLC was metabolized to GLC−S at 40 $\mu$M GLC, the relative amount of GLC-S formed increased over time. During the initial 30 min, 4.5% of the GLC was metabolized to GLC-S. However, the relative percentage metabolized to GLC-S increased to 26% for the GLC remaining at 4 hr to 8 hr. The apparent $K_m$, calculated over the initial 45 min of GLC-S formation, was 82 $\mu$M GLC (**Fig. 3**).

Secretion of GLC-S into the medium was first detectable at 75 min and linear up to 8 hr (**Fig. 4**). The relative and total amounts of GLC-S secreted into the medium

TABLE 1. Albumin synthesis in primary hepatocyte monolayer culture

| Time | μg/hr/mg Protein[a] | μg/hr/$10^6$ Cells |
|---|---|---|
| 4–24 hr | 0.60 ± 0.10[b] | 0.95 ± 0.11 |
| 24–48 hr | 0.68 ± 0.06 | 1.16 ± 0.08 |
| 48–72 hr | 0.96 ± 0.11 | 2.06 ± 0.42 |
| 72–96 hr | 0.60 ± 0.07 | 1.73 ± 0.18 |

[a]Albumin synthesis was assayed as described in Methods.
[b]Mean ± SEM for four animals.

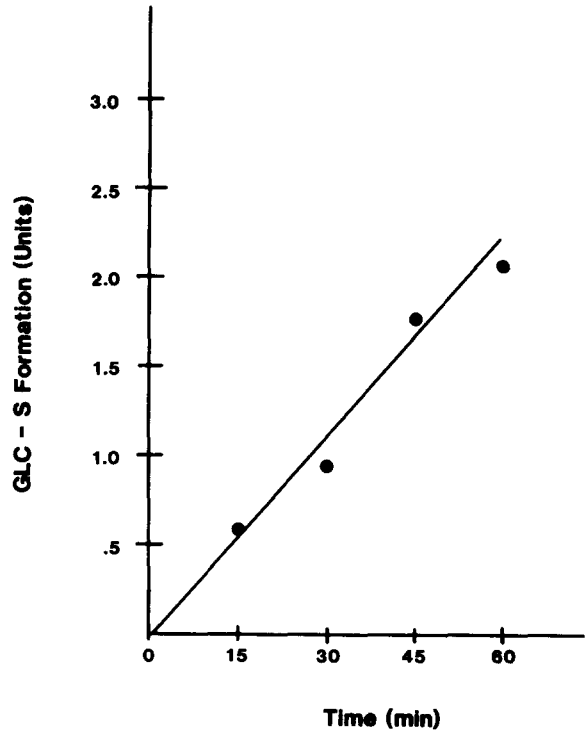


**Fig. 1.** Formation of glycolithocholate-3-sulfate (GLC-S) in primary hepatocyte cultures. In all experiments, glycolithocholate concentration was 100 $\mu$M and sulfate concentration was 800 $\mu$M. GLC-S formation was determined as in Methods. One unit of activity represents 1 nmol of GLC-S formed · mg $prot^{-1}$. Values are the average of two hepatocyte preparations each.

mirrored the total amount of GLC-S formed. Secretion of GLC-S was, however, not as rapid as GLC-S formation and GLC-S tended to accumulate in the hepatocytes.

In contrast to the effect of GLC on GLC-S formation, there was no effect of inorganic sulfate concentration in the medium on GLC-S formation (**Fig. 5**). When total inorganic sulfate concentration was increased 16-fold (100 $\mu$M to 1600 $\mu$M), there was no effect on the formation or secretion of GLC-S. In order to determine whether PAPS was synthesized under the conditions of our primary hepatocyte cultures, we used unlabeled GLC (40 $\mu$M) and labeled sulfate (800 $\mu$M). Less than 1% of the formed GLC-S was labeled. We were, however, able to demonstrate incorporation of labeled sulfate into GLC-S, by adding unlabeled GLC (40 $\mu$M) to our cultures from 4 hr to 24 hr. At 24 hr, the initiating time for all experiments, the medium was changed and unlabeled GLC (40 $\mu$M) and labeled sulfate (800 $\mu$M) were added. Over 54% of the formed GLC-S was then in labeled form.

The majority (>80%) of the GLC metabolites chromatographed in the region where the di- and tri-hydroxy metabolites of lithocholic acid would be expected to chromatograph. When this peak was collected, solvolyzed, and rechromatographed, the chromatography of

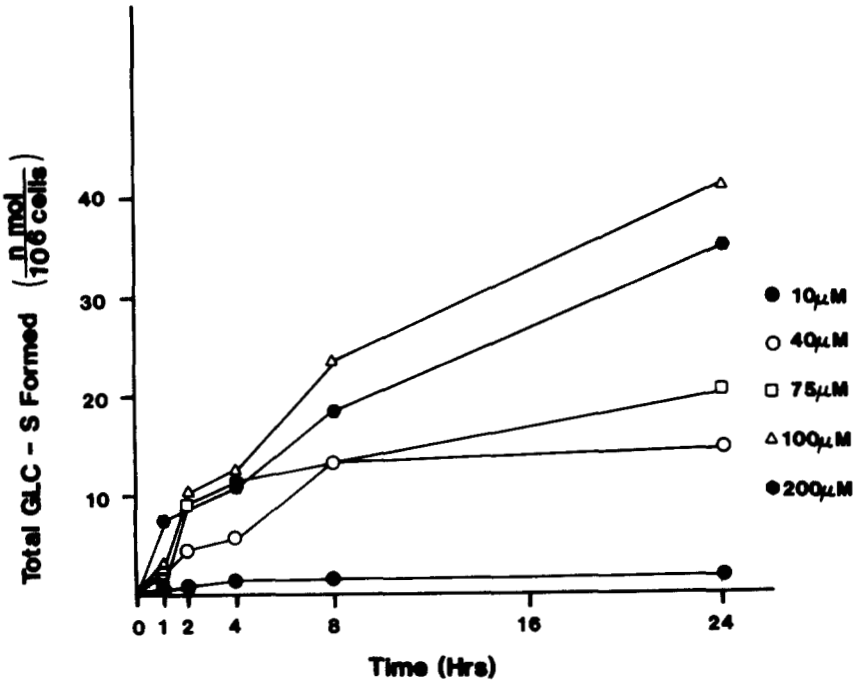


**Fig. 2.** Formation of glycolithocholate-3-sulfate (GLC-S) in primary hepatocyte cultures. Glycolithocholate (GLC) concentration is at right. GLC-S formation was determined as in Methods. Sulfate concentration was 800 μM in all experiments. Values are the average of two hepatocyte preparations each.

the peak was unaffected. In contrast, solvolysis and rechromatography of the peak containing GLC-S resulted in the disappearance of the GLC-S peak and reappearance of the peak corresponding to unmetabolized GLC.

## DISCUSSION

The present data indicate that primary hepatocyte cultures can rapidly sulfate GLC. GLC-S formation was increased when GLC concentration was increased in the medium. GLC-S formation was unaffected by increasing the inorganic sulfate concentration in the medium.

Although bile acid sulfate formation is markedly increased in cholestasis (9), studies in cholestatic human liver disease (24) and bile duct-ligated hamsters (25) have demonstrated no increase in in vitro hepatic bile acid sulfotransferase activity. This suggests that the increased bile acid sulfate production observed in cholestasis is due to an increased intracellular availability of the bile acid substrate. Our findings would support this contention, since bile acid sulfate production can be increased by increasing the GLC concentration in the medium. GLC concentrations used in this study are within the normal range for portal vein bile acid concentrations in the rat (14–341 μM) (26) and human (13–22 μM) (27). Cholestasis can result in increased hepatic bile acid concentration up to 600 μM (28) and this increased hepatic bile acid concentration can result in further hepatocellular damage (29).

The failure of varied inorganic sulfate concentration to affect bile acid sulfate production was unexpected. Sulfate availability has been shown to be important in in vitro studies (30) and isolated hepatocyte studies of xenobiotic

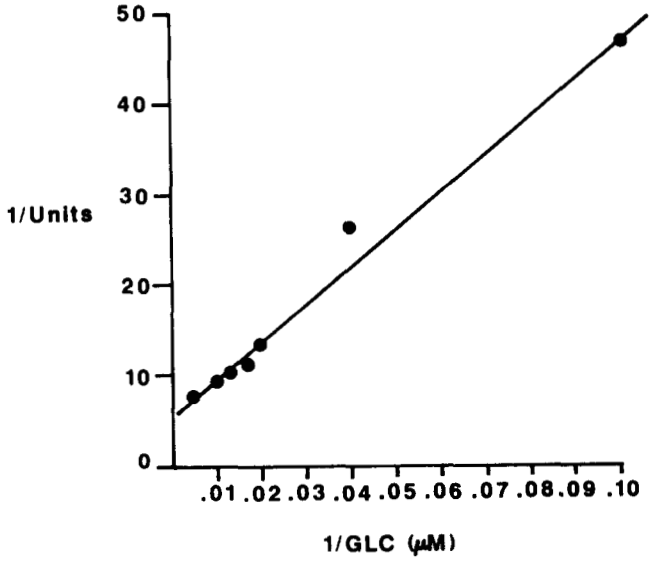


**Fig. 3.** Lineweaver-Burk plot of glycolithocholate-3-sulfate (GLC-S) formation in primary hepatocyte culture. GLC-S formation was determined at 45 min as in Methods. Sulfate concentration was 800 μM in all experiments. One unit of activity represents 1 nmol of GLC-S formed $min^{-1} \cdot mg\ prot^{-1}$.

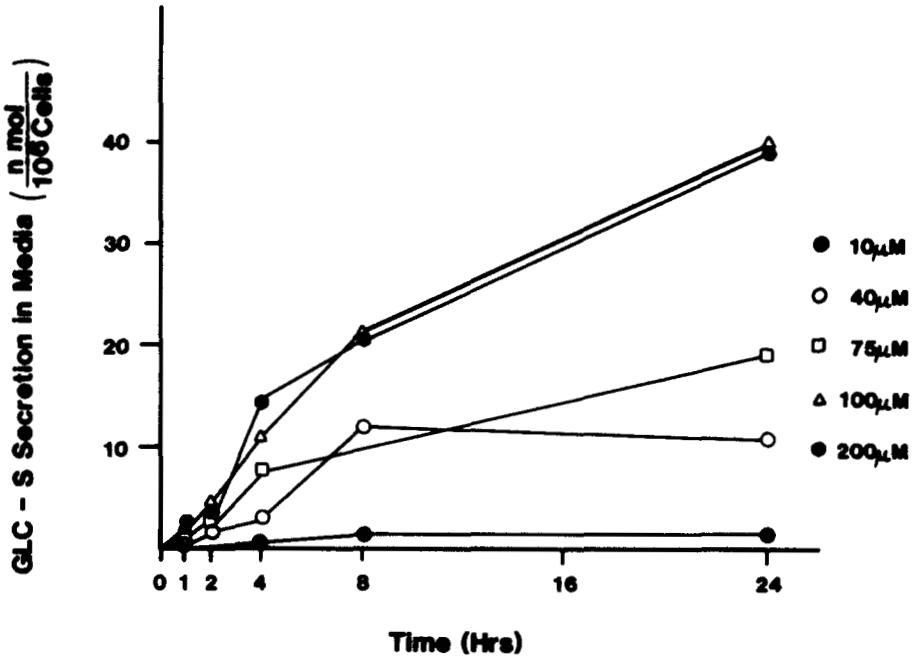


**Fig. 4.** Secretion of glycolithocholate-3-sulfate (GLC-S) into the medium in primary hepatocyte culture. Glycolithocholate concentration is at right. GLC-S secretion was determined as in Methods. Sulfate concentration in all experiments was 800 μM. Values are the average of two hepatocyte preparations each.

sulfation (50–800 μM sulfate) (31). The observed differences in the effect of sulfate availability between xenobiotic and bile acid sulfation may reflect their relative activities, since the specific activity of rat liver phenol sulfotransferase is over 20-fold greater than the specific activity of rat liver bile acid sulfotransferase activity. The concentrations of sulfate used (100–1600 μM) in this study are within the normal serum range for the human (270

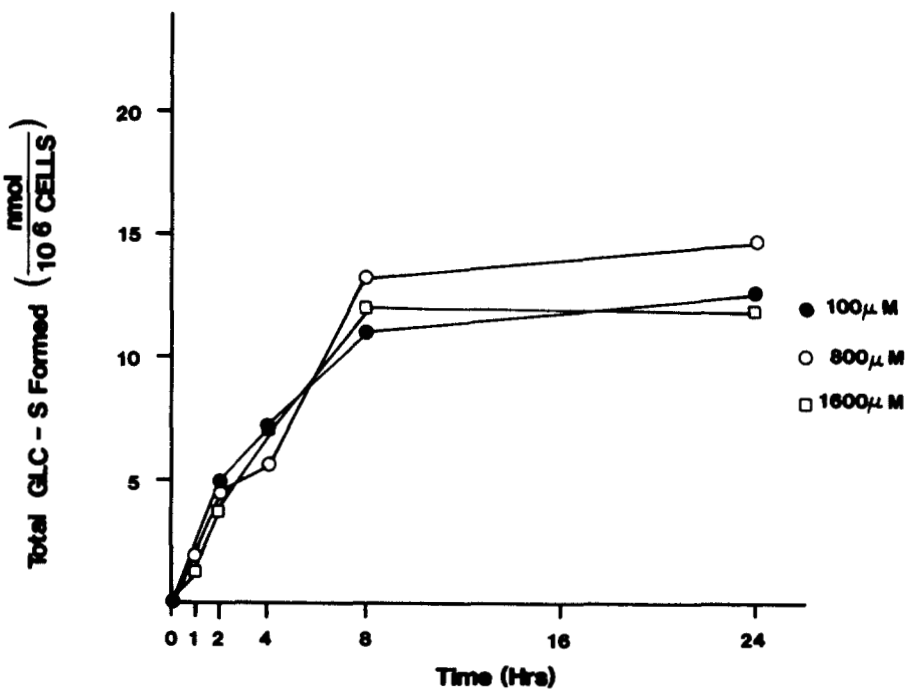


**Fig. 5.** Formation of glycolithocholate-3-sulfate (GLC-S) in primary hepatocyte culture. Sulfate concentration is at right. GLC-S formation was determined as in Methods. Glycolithocholate concentration in all experiments was 40 μM. Values are the average of two hepatocyte preparations each.

$\mu$M) (32) and rat (800 $\mu$M) (32). It is unlikely that sulfate uptake into the hepatocytes is a limiting factor because, at these concentrations, inorganic sulfate is taken up into the cells within minutes (33). Serum and hepatic inorganic sulfate are also in rapid equilibrium with hepatic PAPS, the sulfate donor for bile acid sulfate formation (6, 30).

The failure of the primary hepatocyte cultures to utilize labeled sulfate indicates that extracellular sulfate is not affecting GLC-S formation. This may be due to inability of the cultures to synthesize PAPS, the $K_m$ for sulfate may actually be low, or the concentrations of GLC used do not deplete available intracellular PAPS. The finding that preincubation of the cells with GLC results in utilization of extracellular sulfate suggests that the culture can synthesize PAPS and may also indicate that the $K_m$ for sulfate is low.

GLC-S formation occurred rapidly after GLC uptake into the cells. GLC uptake did not appear to be rate-limiting since there was an initial accumulation of unmetabolized GLC in the cells. GLC-S formation in the primary hepatocytes (59 pmol $min^{-1}$ mg $prot^{-1}$) was similar to the in vitro specific activity we have previously observed for hepatic bile acid sulfotransferase (12 pmol $min^{-1}$ mg $prot^{-1}$) (34). Secretion of GLC-S was less rapid than GLC-S formation, and GLC-S tended to accumulate in the cells. The fact that GLC-S formation occurs at a greater rate than GLC-S secretion may explain why serum bile acid sulfate levels are a sensitive indicator of cholestasis (35).

Although GLC sulfation was saturable, the relative percentage of GLC-S formed from GLC increased over twofold from 6.0% to 14.1% when the GLC concentration in the medium was increased 10-fold from 10 $\mu$M to 100 $\mu$M. This increase may be a result of the increased percentage of sulfate formed over time in our hepatocyte cultures. A similar increase in the relative percentage of lithocholate sulfate excreted over time has previously been observed in vivo in man (13). Hydroxylation, the other major metabolic pathway for GLC, probably is also saturated by increasing the GLC concentration in the medium and this results in more intracellular GLC being available for sulfation.

In this report, presence of bile acid sulfation has been demonstrated in primary hepatocyte cultures. It is unclear why Lambiotte and Thierry (18) were unable to detect any definite deoxycholate sulfate formation in their primary hepatocyte cultures. However, GLC is sulfated to a much greater extent than deoxycholate in vitro (2) and HPLC is a more sensitive method for detecting bile acid sulfate formation. The differences may also reflect the conditions of the primary hepatocyte cultures. Future studies will utilize the controlled conditions in primary hepatocyte cultures to study factors that modify and affect this detoxification pathway.

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## REFERENCES

1. Jakoby, W. B., R. D. Sekura, E. S. Lyon, C. J. Marcus, and J-L. Wang. 1980. Sulfotransferases. *In* Enzymatic Basis of Detoxification. Volume II. W. B. Jakoby, editor. Academic Press, New York. 199–228.
2. Chen, L-J., R. S. Bolt, and W. H. Admirand. 1972. Enzymatic sulfation of bile salts: partial purification and characterization of an enzyme from rat liver that catalyzes the sulfation of bile salts. *Biochim. Biophys. Acta.* **480:** 219–227.
3. Marcus, C. J., R. D. Sekura, and W. B. Jakoby. 1980. A hydroxysteroid sulfotransferase from rat liver. *Anal. Biochem.* **107:** 296–304.
4. Sekura, R. D., and W. B. Jakoby. 1979. Phenol sulfotransferases. *J. Biol. Chem.* **254:** 5658–5663.
5. Roy, A. B. 1980. Sulfotransferases. *In* Sulfation of Drugs and Related Compounds. G. J. Mulder, editor. CRC Press, Boca Raton, FL. 83–130.
6. Mulder, G. J., and K. Keulmans. 1978. Metabolism of inorganic sulfate in the isolated perfused rat liver. *Biochem. J.* **176:** 959–965.
7. Makino, I., S. Nakayama, K. Shimozaki, and K. Mashimo. 1972. Sulfated and nonsulfated bile acid in human serum. *Lipids.* **11:** 750–752.
8. Allan, R. N., J. L. Thistle, and A. F. Hofmann. 1976. Lithocholate metabolism during chemotherapy for gallstone dissolution. 2. Absorption and sulfation. *Gut.* **17:** 413–419.
9. Foulen-Fortunet, H. L., L. LeQuernes, S. Erlinger, E. Lerebrouns, and R. Colen. 1982. Hepatic alterations during total parenteral nutrition in patients with inflammatory bowel disease: a possible consequence of lithocholate toxicity. *Gastroenterology.* **82:** 932–37.
10. Watkins, J. B., and E. R. Brown. 1977. Conjugation and sulfation of lithocholic acid in fetal liver. *In* Bile Acid Metabolism in Health and Disease. G. Paumgartner and A. Stiehl, editors. MTP Press, Lancaster, England. 65–70.
11. Stiehl. A., D. L. Earnest, and W. H. Admirand. 1975. Sulfation and renal excretion of bile salts in patients with cirrhosis of the liver. *Gastroenterology.* **68:** 534–544.
12. Palmer, R. H. 1972. Bile acids, liver enzymes, and liver disease. *Arch. Intern. Med.* **130:** 606–617.
13. Cowen, A. E., M. G. Korman, A. F. Hofmann, and O. W. Cass. 1974. Metabolism of lithocholate in healthy man. I. Biotransformation and biliary excretion of intravenously administered lithocholate, lithocholylglycine, and their sulfates. *Gastroenterology.* **69:** 59–66.
14. Van Dyke, R. W., J. E. Stephens, and B. F. Scharschmidt. 1982. Bile acid transport in cultured rat hepatocytes. *Am. J. Physiol.* **243:** 6486–6492.
15. Davis, R. A., W. E. Highsmith, M. Malone-McNeal, J. Archambault-Schexnayder, and J-C. W. Kuan. 1983. Bile acid synthesis by cultured hepatocytes. Inhibition by mevinolin, but not by bile acids. *J. Biol. Chem.* **258:** 4079–4082.
16. Davis, R. A., P. M. Hyde, J-C. W. Kuan, M. Malone-McNeal, and J. Archambault-Schexnayder. 1983. Bile acid

secretion by cultured rat hepatocytes. Regulation by cholesterol availability. *J. Biol. Chem.* **258:** 3661–3667.
17. Anderson, B., M. Berggrenard, and P. Moldens. 1978. Conjugation of various drugs in isolated hepatocytes. *Drug Metab. Dispos.* **6:** 611–616.
18. Lambiotte, M., and N. Thierry. 1980. Hydroxylation, sulfation, and conjugation of bile acids in rat hepatoma and hepatocyte cultures under the influence of glucocorticoids. *J. Biol. Chem.* **255:** 11304–11331.
19. Wood, G. C., and M. K. Keech. 1960. The formation of fibrils from collagen solutions. I. The effect of experimental conditions: kinetics and electron microscopic studies. *Biochem. J.* **75:** 588–598.
20. Kirkpatrick, R. B., L. Lack, and P. G. Killenberg. 1980. Identification of the 3-sulfate isomer as the major product of enzymatic sulfation of chenodeoxycholate conjugates. *J. Biol. Chem.* **255:** 10157–10159.
21. van Berge Henegouwen, G. P., R. N. Allan, A. F. Hofmann, and P. Y. S. Yu. 1977. A facile hydrolysis-solvolysis procedure for conjugated bile acid sulfates. *J. Lipid Res.* **18:** 118–122.
22. Laurell, G. C. 1966. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Anal. Biochem.* **15:** 45–52.
23. Gornall, A. G., C. J. Bardwill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:** 751–766.
24. Lööf, L., and A. Nyberg. 1983. Bile salt sulphation in man. *Upsala J. Med. Sci.* **88:** 1–8.
25. Barnes, S., P. G. Burhol, R. Zander, and B. I. Hirschowitz. 1979. The effect of bile duct ligation on hepatic bile acid sulfotransferase activity in the hamster. *Biochem. Med.* **22:** 165–174.
26. Cronholm, T., and J. Sjövall. 1967. Bile acids in portal blood of rats fed different diets and cholestyramine. *Eur. J. Biochem.* **2:** 375–383.
27. Ahlbery, J., B. Anyelin, I. Björkhem, and K. Einarsson. 1977. Individual bile acids in portal venous and systemic blood serum of fasting man. *Gastroenterology.* **73:** 1377–1382.
28. Greim, H., D. Trülzsch, J. Roboz. K. Dressler, P. Czygan, F. Hutterer, F. Schaffner, and H. Popper. 1972. Mechanism of cholestasis. 5. Bile acids in normal rat livers and in those after bile duct ligation. *Gastroenterology.* **63:** 837–845.
29. Hutterer, F., P. G. Bacchin, H. Derk, J. B. Schenkman, F. Schaffner, and H. Popper. 1970. Mechanism of cholestasis. 2. Effect of bile acids on the microsomal electron transport system *in vitro*. *Life Sci.* **9:** 1150–1166.
30. Weitering, J. G., K. R. Krijgsheld, and G. J. Mulder. 1979. The availability of inorganic sulfate as a rate limiting factor in the sulphate conjugation of xenobiotics in the rat. Sulphation and glucuronidation of phenol. *Biochem. Pharmacol.* **28:** 757–762.
31. Schwarz, L. R. 1984. Sulfation of 1-napthol in isolated rat hepatocytes. Dependence on inorganic sulfate. *Hoppe-Seyler's Z. Physiol. Chem.* **365:** 43–48.
32. Krijgsheld, K. R., E. Scholtens, and G. J. Mulder. 1980. Serum concentration of inorganic sulfate in mammals: species differences and circadian rhythm. *Comp. Biochem. Physiol.* **67A:** 683–686.
33. von Dippe, P., and D. Levy. 1982. Analysis of the transport system for inorganic anions in normal and transformed hepatocytes. *J. Biol. Chem.* **257:** 4381–4385.
34. Kirkpatrick, R. B., and P. G. Killenberg. 1980. Effects of ethinylestradiol on enzymes catalyzing bile acid conjugation and sulfation. *J. Lipid Res.* **21:** 895–901.
35. Balistreri, W. F., F. J. Suchy, M. K. Farrell, and J. E. Heubi. 1981. Pathologic versus physiologic cholestasis: elevated serum concentration of a secondary bile acid in the presence of hepatobiliary disease. *J. Pediatr.* **98:** 399–402.