Multiplicity of Hepatic Excretory Mechanisms for Organic Anions

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ABSTRACT Previous studies based upon competition between different organic anions for biliary excretion in vivo have suggested that all organic anions share a common hepatic secretory mechanism. Corriedale sheep with an inherited defect in organic anion excretion by the liver were used to study this problem directly without the need for competition studies, the results of which are difficult to analyze. Maximal biliary excretion of sulfobromphthalein (BSP) in mutant Corriedale sheep was less than 7% of that observed in normal sheep whereas maximal biliary excretion of taurocholate, the major organic anion in sheep bile, was not different in mutant and normal sheep. Taurocholate infusion enhanced maximal hepatic excretion of BSP in normal but not in mutant sheep. These studies of an inheritable disorder which appears to be identical to the Dubin-Johnson syndrome in man, demonstrate that taurocholate excretion requires at least one step in biliary excretion which is not required by other organic anions such as bile pigment, porphyrins, drugs, and dyes.

The organic anions in mammalian bile consist of bile acids, bile pigments, porphyrins, metabolites, dyes, and drugs which are believed to be excreted by the parenchymal liver cell (1–3) and which are present in bile in concentrations very much greater than their simultaneous concentrations in plasma (2). It has been proposed that the organic anions excreted in bile share a common secretory mechanism because of their similarity to the group of compounds secreted by the renal organic anion transport mechanism (4), although bile salts are actively reabsorbed by the kidney tubule whereas phenol red, another organic anion, is actively secreted (5). Parenteral administration of some organic anions depresses the biliary excretion of other organic anions when administered simultaneously (2). Demonstration of competition for biliary excretion in vivo between different organic anions is complicated because many of these compounds may share common binding to serum proteins, mechanisms of entry into liver cells, and various intracellular metabolic trans-

formations. For example, bile salts have been considered to reduce BSP binding to serum albumin (3) and entry into liver cells (3, 6) as well as to enhance BSP excretion in the bile (7). For these reasons, it has not been clearly demonstrated whether one or more hepatic organic anion secretory mechanisms exist and the relationship between such hepatic mechanisms and the renal organic anion transport system is likewise uncertain.

A unique opportunity to investigate this problem was provided by the discovery of Corriedale sheep with an inherited defect in the biliary excretion of various organic anions including BSP, iopanoic acid, phylloerythrin, conjugated bilirubin, and metanephrine glucuronide (8–10). The disorder in sheep appears to be functionally and morphologically identical to the Dubin-Johnson syndrome in man (11).

Sodium taurocholate is the major organic anion in sheep bile (12). In the present study, the maximal biliary excretion of taurocholate and the effect of intravenous infusion of taurocholate on maximal biliary excretion of BSP were determined in normal and mutant Corriedale sheep.

MATERIALS AND METHODS

Three mutant wethers weighing 13.6–20.2 kg and four normal wethers weighing 13.8–23.5 kg were used in these experiments. Wethers were used as these were the only available mutants with this rare disorder although it is recognized that steroid hormones, particularly estrogens, can affect biliary secretion (13, 14). The sheep underwent cholecystectomy under full surgical anesthesia and a special T tube (Davol Rubber Co., Oakland, N. J.) with an external diameter of 4 mm and an internal diameter of 3 mm was inserted into the common bile duct. In the distal limb of the "T," the tube has a small balloon which, following inflation, permits complete collection of bile. With saline irrigation approximately every 10 days, the tube remained in place for from 4 to 14 months and permitted multiple studies in single animals. The external limb of the T tube was occluded except at the time of study. Following surgery, the animals were maintained on hay and goat chow (Purina) for from 3 to 6 wk until the wound healed. The position of the T tube and the completeness of biliary obstruction following inflation of the balloon were demonstrated radiographically following injection of contrast material in the long limb of the tube. The balloon was inflated the night before each experiment and bile was permitted to drain overnight. The animals were placed in a holding stanchion and a polyethylene catheter was inserted into an external jugular vein. In order to establish basal conditions, 5% dextrose and water was infused intravenously for 1 hr using a Harvard infusion pump following which sodium taurocholate and/or sodium BSP was infused intravenously for 1 hr. The rate of infusion of taurocholate or BSP was changed every 40–60 min at which time bile flow had attained a new steady-state level. When taurocholate and BSP were administered simultaneously, they were infused into separate external jugular veins.

The bile salt used for intravenous infusion was a commercial preparation (California Biochemical Corporation, Los Angeles, Cal.) and bile salt accounted for 80–

92% of the total solids. Approximately 85–90% of the bile salt was present as the taurine conjugate of trihydroxy cholanic acid as determined by thin-layer chromatography. The remaining bile salt was present primarily as taurine-conjugated dihydroxy cholanic acid. A 3% solution was prepared in normal saline immediately before each experiment and passed through a Seitz filter. Taurocholate was also synthesized (15), had the reported melting point characteristics and migration on thin-layer chromatograms, and was used in several experiments in normal and mutant sheep.

In each experiment bile was collected on ice at 10 min intervals; the volume was measured, and taurocholate and BSP concentrations were estimated (5, 6). Recovery of taurocholate added to bile averaged 96 ± 4.1 (SE) % for 16 determinations in the range of 100 to 900 μg/ml. Recovery of BSP added to bile averaged 98 ± 8 (SE) % for 12 determinations in the range of 25 to 750 μg/ml. In four studies in two normal sheep and in four studies in two mutant sheep, free and conjugated BSP were quantitatively separated in plasma and bile by paper chromatography (16).

RESULTS

In 45 studies in 3 mutant sheep and 52 studies in 4 normal sheep, taurocholate was infused intravenously at increasing rates from 19–305 μM/min. Bile flow increased but was not significantly different between normal and mutant sheep at any infusion rate and reached a maximum of 0.049 ± 0.004 ml/min per kg (Table I). Taurocholate excretion in bile also increased during taurocholate infusion but was not significantly different between normal and mutant sheep at any infusion rate and reached a maximum of 14.2 ± 0.8 μM/min per kg (Table II). The same data presented in Table II are presented in Fig. 1 in which mean biliary taurocholate excretion is plotted against taurocholate infused in the same experiments in normal and mutant sheep. χ^2 analysis of the standard error of the means revealed no significant difference in biliary taurocholate excretion between normal and mutant sheep at any taurocholate infusion rate. Further increase in bile salt infusion did not increase, and frequently depressed, bile flow and taurocholate excretion although the animals tolerated large amounts of infused bile salt surprisingly well.

In six studies in four normal sheep and in five studies in three mutant sheep, BSP was infused intravenously at 0.4–0.9 mg/min per kg for 1 hr (Table III). Plasma and biliary dye concentrations were estimated at 10 min intervals and the volume of bile was recorded. Although plasma BSP levels continued to increase in normal and mutant sheep, BSP excretion in bile in normal sheep reached a mean steady-state level of 0.84 ± 0.03 mg/min per kg when mean bile flow was 0.044 ± 0.012 ml/min per kg. In mutant sheep mean maximal BSP excretion in bile was 0.049 ± 0.012 and never exceeded 7% of that observed in normal sheep whereas mean bile flow was 0.039 ± 0.014 ml/min per kg and did not differ significantly from that observed in normal sheep.

In four studies in two mutant sheep and four studies in two normal sheep,

TABLE I

MEAN BILE FLOW IN NORMAL AND MUTANT SHEEP IN RESPONSE TO INTRAVENOUS INFUSION OF SODIUM TAUROCHOLATE

The numbers in parentheses refer to the number of studies performed in each sheep. The weight of each animal increased during the 4–8 month experimental period. Each result is expressed in terms of the appropriate body weight at the time of the particular study. In calculating the standard error of the mean, each study in each animal was considered as a separate entry.

		Bile flow, *cc/min per kg*					
Taurocholate infusion rate *μM*/min per g		0	19	38	76	152	305
Normal sheep	Weight						
	kg						
A	13.6–18.4	0.002 (4)	0.015 (4)	0.028 (3)	0.040 (4)	0.042 (1)	0.047 (1)
B	18.0–23.6	0.004 (4)	0.018 (4)	0.022 (4)	0.035 (1)	0.046 (1)	0.048 (1)
C	16.2–18.9	0.004 (4)	0.017 (3)	0.024 (1)	0.038 (4)	0.043 (3)	0.049 (3)
D	14.2–18.1	0.001 (4)	0.015 (1)	0.024 (4)	0.039 (3)	0.042 (3)	0.050 (3)
Mean		0.003±0.001 (16)	0.016±0.002 (12)	0.025±0.005 (12)	0.038±0.003 (12)	0.047±0.004 (8)	0.049±0.004 (8)
Mutant sheep							
X	13.6–18.1	0.003 (4)	0.021 (3)	0.026 (4)	0.029 (3)	0.044 (3)	—
Y	14.7–16.8	0.002 (4)	0.016 (3)	0.024 (3)	0.041 (3)	0.045 (3)	0.048 (4)
Z	15.6–20.2	0.003 (4)	0.016 (3)	0.023 (3)	0.038 (2)	0.043 (4)	0.050 (4)
Mean		0.003±0.001 (12)	0.018±0.004 (9)	0.027±0.004 (10)	0.036±0.004 (8)	0.044±0.004 (10)	0.048±0.005 (8)

BSP was infused during the course of maximal bile secretory response to taurocholate infusion and the biliary excretion of taurocholate and BSP was estimated. Results of a representative experiment are presented in Fig. 2 and data from all experiments are presented in Table IV. During infusion of 5% dextrose and water, mean bile flow, expressed as milliliters per minute per kilogram, was 0.009 in the normal sheep and 0.010 in the mutant. Infusion of BSP increased mean bile flow to 0.023 in the normal sheep and to 0.021 in the mutant. Taurocholate infusion enhanced mean bile flow to 0.047 in the

TABLE II

MEAN TAUROCHOLATE EXCRETION IN BILE IN NORMAL AND MUTANT SHEEP IN RESPONSE TO INTRAVENOUS INFUSION OF SODIUM TAUROCHOLATE

The numbers in parentheses refer to the number of studies performed in each sheep. See legend of Table I for further details.

	Taurocholate excreted in bile, *μm/min per kg*					
Taurocholate infusion rate, *μM/min per kg*	0	19	38	76	152	305
Normal sheep						
A	0.6	3.3	4.6	8.3	—	—
	(3)	(4)	(1)	(4)		
B	0.6	3.9	5.2	8.5	—	—
	(3)	(4)	(4)	(1)		
C	0.9	3.6	5.3	6.9	12.4	14.9
	(3)	(3)	(1)	(4)	(3)	(3)
D	1.1	4.0	4.9	9.1	11.3	13.5
	(3)	(1)	(4)	(3)	(3)	(3)
Mean	0.8±0.2	3.7±0.3	5.0±0.6	8.2±0.7	11.9±0.8	14.2±0.8
	(12)	(12)	(10)	(12)	(6)	(6)
Mutant sheep						
X	0.8	3.6	4.1	9.0	11.4	—
	(4)	(3)	(4)	(3)	(3)	
Y	0.4	4.1	4.6	7.9	9.9	15.3
	(4)	(3)	(3)	(3)	(3)	(4)
Z	1.8	3.9	5.4	8.3	10.8	12.3
	(3)	(2)	(2)	(2)	(3)	(3)
Mean	1.0±0.4	3.9±0.5	4.7±0.5	8.4±0.6	10.7±0.7	13.8±0.7
	(11)	(8)	(9)	(8)	(9)	(7)

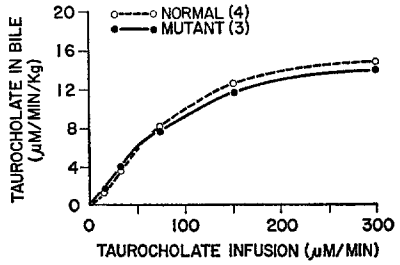

FIGURE 1. The effect of sodium taurocholate infusion on taurocholate excretion in bile in normal and mutant sheep. Each point represents the mean of 6 to 12 separate studies. Data for each study are presented in Table II.

normal sheep and to 0.044 in the mutant. Despite the comparable increase in bile flow in both sheep, BSP excretion increased approximately 2.3-fold in normal sheep but was not significantly affected in mutant sheep. At the conclusion of the BSP infusion, 65 ± 12% (SE) of BSP in plasma was conjugated

in normal sheep and 76 ± 12% of BSP in plasma was conjugated in mutant sheep. Biliary BSP was 82 ± 12% conjugated in normal sheep and 59 ± 17% conjugated in mutant sheep. After taurocholate infusion, biliary BSP was 73 ± 17% conjugated in normal sheep and 48 ± 9% conjugated in mutant sheep.

TABLE III

MEAN PLASMA BSP CONCENTRATION, BSP EXCRETION IN BILE, AND BILE FLOW IN NORMAL AND MUTANT CORRIEDALE SHEEP AT THE CONCLUSION OF BSP INFUSION

Each study consisted of three 1 hr infusion periods at different BSP infusion rates. Results are expressed as mean ± SEM.

BSP infusion rate	Mean plasma BSP concentration		Mean biliary excretion of BSP		Mean bile flow	
Sheep	Normal	Mutant	Normal	Mutant	Normal	Mutant
No. of studies	6	5	6	5	6	5
No. of animals	4	3	4	3	4	3
mg/min per kg	*mg %*		*mg/min per kg*		*ml/min per kg*	
0.4	2.8±0.9	22.6±7.4	0.76±0.08	0.044±0.016	0.033±0.010	0.028±0.010
0.5	—	—	0.72±0.10	0.034±0.011	0.032±0.012	0.031±0.006
0.7	12.7±0.8	52.6±7.9	0.84±0.09	0.048±0.014	0.041±0.007	0.037±0.008
0.9	19.4±0.6	—	0.84±0.03	0.049±0.012	0.044±0.012	0.039±0.014

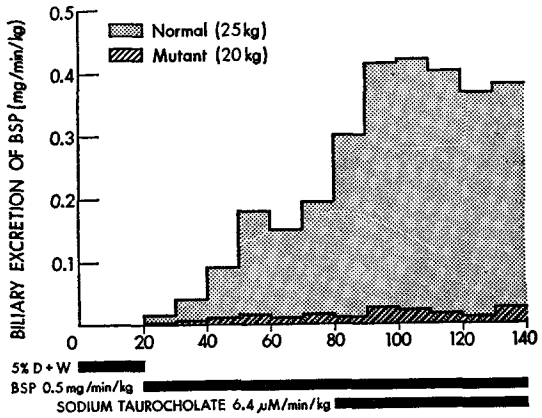

FIGURE 2. Results of a representative experiment illustrating the effect of sodium taurocholate infusion on bile flow and biliary excretion of BSP in a normal and a mutant sheep.

DISCUSSION

Corriedale sheep with inheritable photosensitivity and a black liver have defective biliary excretion of a variety of organic anions (8–10) including BSP as shown in Table III. Despite this defect, the biliary excretion of taurocholate, the major organic anion of human and ovine bile, did not differ from that observed in normal sheep at all taurocholate infusion rates. Although plasma bile acid concentrations were not measured, it is likely that a maximal, or near

maximal, rate of biliary excretion of taurocholate was attained as manifested by the observed changes in bile flow and taurocholate content. Furthermore, increased rates of taurocholate administration usually diminished bile flow and produced clinical toxicity. Therefore, despite a defect in BSP excretion at the level of 7% of normal, taurocholate excretion was unimpaired. Because the mechanism and cellular site of hepatic excretory mechanisms are unknown, only speculation can be offered regarding the locus of the defect in mutant sheep. If one assumes that a series of steps, processes, or carriers may be in-

TABLE IV
EFFECT OF SODIUM TAUROCHOLATE INFUSION ON BILIARY EXCRETION OF BSP IN NORMAL AND MUTANT SHEEP

Four studies were performed in two normal sheep and four studies were performed in two mutant sheep. Results are expressed as mean ± SEM. Details of experiment are provided in text under Methods.

	Normal sheep	Mutant sheep
Mean bile flow, *ml/min per kg*		
At conclusion of 5% dextrose and water infusion (20 min)	0.009±0.005	0.010±0.006
At conclusion of BSP infusion 0.5 mg/min per kg (60 min)	0.023±0.009	0.021±0.011
At conclusion of taurocholate infusion 6.4 μM/min per kg (60 min)	0.047±0.011	0.044±0.009
Biliary BSP excretion, *mg/min per kg*		
At conclusion of BSP infusion 0.5 mg/min per kg (60 min)	0.18±0.06	0.029±0.011
At conclusion of taurocholate infusion 6.4 μM/min per kg (60 min)	0.41±0.10	0.036±0.009

volved in the hepatic excretion of organic anions, then the mutant sheep has a defect in some excretory step which is not required for bile salt excretion. It is theoretically possible that bile salts and other organic anions may share some steps or carriers unaffected by the mutation.

O'Maille et al. (7) and Gronwall and Cornelius (17) have demonstrated in dog and sheep respectively that BSP excretion in bile is limited by the concentration of BSP in bile and not by a true Tm_{BSP}. O'Maille et al. (7) suggest that increased bile flow rate at the canalicular site of BSP excretion produced by bile salt permits an increase in "maximal BSP excretion" to occur without exceeding the limiting biliary dye concentration. This mechanism could be altered membrane permeability, increased dissociation of a carrier: BSP complex at the biliary surface of the cell, or enhanced BSP excretion in a different physical form (i.e., micelle). As anticipated, taurocholate infusion in normal sheep enhanced BSP excretion approximately 230%; however, taurocholate infusion in mutant sheep increased mean BSP excretion by less than 30%. If

taurocholate enhances membrane permeability to the dye, carrier:BSP dissociation, or micelle formation, the defect in mutant sheep must occur prior to such steps in the over-all pathway of hepatic excretion of organic anions. The results in mutant sheep indicate that enhanced BSP-micelle formation is unlikely although this was not specifically analyzed.

Neither selective back-diffusion of BSP nor impaired hepatic blood flow is likely to represent the basic defect in the mutant sheep or the response to taurocholate infusion although methods are not available for quantitation of the former process.

Gutstein et al. (18) studied the effect of dehydrocholate infusion on biliary BSP excretion in a human being with the Dubin-Johnson syndrome who had a virtually complete biliary fistula. Impaired biliary excretion of BSP was substantiated by direct measurement in this patient. In addition, dehydrocholate infusion increased bile flow but did not significantly alter biliary BSP excretion. These observations in man are comparable to those reported here in mutant sheep and suggest that hepatic organic anion excretion in man may also represent multiple excretory mechanisms. Plasma bile acid levels are not increased in mutant sheep or in patients with the Dubin-Johnson syndrome[1] and there are no histologic manifestations of bile secretory failure (cholestasis) (11).

Analysis of BSP conjugates in plasma at the conclusion of BSP infusions revealed that 65 and 70% of plasma BSP was conjugated in normal and mutant sheep, respectively. Presumably the excretory mechanism of the liver for the dye was exceeded and BSP is more readily excreted as a conjugate with glutathione than in the free form. Biliary BSP in normal and mutant sheep also revealed a high proportion of conjugated BSP comparable to that observed in other animals (19). After taurocholate infusion, 82 $\pm$ 12% of biliary BSP in normal sheep was conjugated whereas only 59 $\pm$ 17% of biliary BSP in mutant sheep was conjugated. This observation suggests some specificity of the excretory mechanism or carrier for conjugated BSP and is in contrast to the suggestion that increase in bile flow rate produced by bile salt administration would enhance the transfer of both conjugated and unchanged BSP from the liver cell into the bile (7).

Studies in progress reveal that mutant sheep have the same renal Tm_{PAH} as normal sheep and, in two experiments, hepatic excretion of an organic cation, procaine amide ethobromide, was not different in normal and mutant sheep. These observations suggest that the renal and hepatic mechanisms for organic anion excretion may differ and that organic anions may be excreted by the liver by different mechanisms than those involved in organic cation excretion. The latter conclusion was initially suggested by the studies of Schanker (20, 21).

[1] Arias, I. M. Unpublished observations.

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REFERENCES

1. HANZON, V. 1952. Liver cell secretion under normal and pathologic conditions studied by fluorescence microscopy on living rats. *Acta Physiol. Scand. Suppl.* **28**:101.
2. SPERBER, I. 1963. Organic anion excretion in bile. *In* Proceedings of the First International Pharmacology Meeting. B. Brodie, editor. Pergamon Press, Inc., New York. **4**:137.
3. ANDREWS, W. H. H., and T. G. RICHARDS. 1960. The activity of bile salts and certain detergents on the hepatic storage and protein-binding of sulphobromophthalein. *Quart. J. Physiol.* **45**:273.
4. SPERBER, I. 1959. Secretion of organic anions in the formation of urine and bile. *Pharmacol. Rev.* **11**:109.
5. WEINER, I. M., J. E. GLASSER, and L. LACK. 1964. Renal excretion of bile acids: taurocholic, glycocholic, and cholic acids. *Amer. J. Physiol.* **207**:964.
6. WHEELER, H. O., J. I. MELTZER, and S. E. BRADLEY. 1960. Biliary transport and hepatic storage of sulfobromophthalein sodium in the unanesthetized dog, in normal man, and in patients with hepatic disease. *J. Clin. Invest.* **39**:1131.
7. O'MAILLE, R. L., T. G. RICHARDS, and A. H. SHORT. 1966. Factors determining the maximal rate of organic anion secretion by the liver and further evidence on the hepatic site of action of the hormone secretin. *J. Physiol.* **186**:424.
8. ARIAS, I., L. BERNSTEIN, R. TOFFLER, C. CORNELIUS, A. NOVIKOFF, and E. ESSNER. 1964. Black liver disease in Corriedale sheep: A new mutation affecting hepatic excretory function. *J. Clin. Invest.* **43**:1249.
9. ARIAS, I. M., L. BERNSTEIN, R. TOFFLER, and J. BEN EZZER. 1965. Black liver disease in Corriedale sheep: Metabolism of tritiated epinephrine and incorporation of isotope into the hepatic pigment *in vivo*. *J. Clin. Invest.* **44**:1026.
10. CORNELIUS, C., I. M. ARIAS, AND B. I. OSBURN. 1965. Hepatic pigmentation with photosensitivity: A syndrome in Corriedale sheep resembling Dubin-Johnson syndrome in man. *J. Amer. Vet. Med. Ass* **146**:709.
11. DUBIN, N., and F. B. JOHNSON. 1954. Chronic idiopathic jaundice with unidentified pigment in liver cells. *Medicine.* **33**:155.
12. PERIC-GOLIA, L., and H. SOCIC. 1968. Biliary bile acids and cholesterol in developing sheep. *Amer. J. Physiol.* **215**:1284.
13. MUELLER, M. N., and A. KAPPAS. 1964. Estrogen pharmacology. I. The influence of estradiol and estriol on hepatic disposal of sulfobromophthalein (BSP) in man. *J. Clin. Invest.* **43**:1905.
14. SCHERB, J., M. KIRSHNER, and I. M. ARIAS. 1963. Studies of hepatic excretory function. The effect of 17α-ethyl-19-nortestosterone on sulfobromophthalein sodium (BSP) metabolism in man. *J. Clin. Invest.* **42**:404.
15. NORMAN, A. 1956. Preparation of conjugated bile acids using carboxylic acid anhydrides. *Ark. Kemi.* **8**:331.
16. COMBES, B., and S. STAKELUM. 1960. Conjugation of sulfobromophthalein sodium with glutathione in thio-ether linkage by the rat. *J. Clin. Invest.* **39**:1214.
17. GRONWALL, R., and C. E. CORNELIUS. 1966. Biliary excretion of sulfobromophthalein in sheep. *Fed. Proc.* **25**:576.
18. GUTSTEIN, S., S. ALPERT, and I. M. ARIAS. 1968. Studies of hepatic excretory function.

IV. Biliary excretion of sulfobromophthalein sodium in a patient with the Dubin-Johnson syndrome and a biliary fistula. *Israel J. Med. Sci.* **4**:36.

19. PHILP, J. R., G. M. GRODSKY, and J. V. CARBONE. 1961. Mercaptide conjugation in the uptake and secretion of sulfobromophthalein. *Amer. J. Physiol.* **200**:545.
20. SCHANKER, L. 1962. Short communications. Concentrative transfer of an organic cation from blood into bile. *Biochem. Pharmacol.* **11**:253.
21. SCHANKER, L. 1967. Hepatic Transport of Organic Cations. Blackwell Scientific Publications, Ltd., Oxford.