Regulation of bile acid synthesis. III. Correlation between biliary bile salt hydrophobicity index and the activities of enzymes regulating cholesterol and bile acid synthesis in the rat

D. M. Heuman, P. B. Hylemon, and Z. R. Vlahcevic

Gastroenterology Section, McGuire VA Medical Center, and Departments of Medicine and Microbiology, Medical College of Virginia, Richmond, VA 23298

Abstract Hepatic bile acid synthesis is thought to be under negative feedback control by bile salts in the enterohepatic circulation, acting at the level of cholesterol 7α-hydroxylase (C7αH), the initial and rate-limiting step in the bile acid biosynthetic pathway. Bile salts also suppress the activity of the rate-limiting enzyme for cholestereol synthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA-R). The mechanisms of these regulatory effects are poorly understood, and one or both may be indirect. Previous data suggest that the hydrophilic–hydrophobic balance of bile salts, a major determinant of their cholesterol solubilizing properties, also determines their potency as regulators of bile acid and cholesterol synthesis. To further evaluate the relationship between the physicochemical and regulatory properties of bile acids, we altered the composition of the bile salt pool of rats by feeding one or more of seven different bile acids (1% w/w for 14 days). We then determined the mean hydrophilic–hydrophobic balance (hydrophobicity index) of the bile salts in bile, and correlated this with the specific activities of C7αH and HMG-CoA-R, and of acyl-CoA:cholesterol acyltransferase (ACAT), a third hepatic microsomal enzyme which regulates cholesterol esterification. In all instances following bile acid feeding, conjugates of the fed bile acid(s) became the predominant bile salts in bile. Highly significant negative linear correlations (each $P < 0.0001$) were found between the hydrophobicity indices of biliary bile salts and the activities of C7αH ($r = 0.79$) or HMG-CoA-R ($r = 0.63$). By contrast, no significant correlation could be demonstrated between ACAT activity and the hydrophobicity index of biliary bile salts. The correlation between activities of HMG-CoA-R and C7αH was also highly significant ($r = 0.81$; $P < 0.0001$). No significant correlation existed between ACAT and either HMG-CoA-R or C7αH. Microsomal free cholesterol was not consistently altered by bile acid feeding. Thus, the potency of circulating bile salts as suppressors of the enzymes regulating bile acid and cholesterol synthesis increases with increasing hydrophobicity. The hydrophobic–hydrophilic balance of the bile salt pool may play an important role in the regulation of cholesterol and bile acid synthesis.—**Heuman, D. M., P. B. Hylemon, and Z. R. Vlahcevic.** Regulation of bile acid synthesis. III. Correlation between biliary bile salt hydrophobicity index and the activities of enzymes regulating cholesterol and bile acid synthesis in the rat. *J. Lipid Res.* 1989. **30:** 1161–1171.

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Regulation of bile acid synthesis has recently become an area of controversy. For many years, it has been generally accepted that conversion of cholesterol to bile acids is regulated by a negative feedback inhibitory mechanism [1–3]. According to this view, bile salts returning to the liver via the portal vein suppress activity of cholesterol 7α-hydroxylase (C7αH), the initial and rate limiting enzyme in the bile acid biosynthesis pathway. Because bile salts have no direct effect on the activity of this enzyme, their action was presumed to be mediated through changes in enzyme synthesis or turnover.

However, this concept fails to account for a number of experimental observations. In particular, several groups of investigators have been unable to demonstrate negative bile acid biofeedback in cultured or freshly suspended hepatocytes following the addition of high concentrations of different bile salts to the culture medium [4–6]. In contrast, manipulations that increase or decrease hepatocyte cholesterol were observed to up- or down-regulate bile acid synthesis in vitro. The latter observations led to the suggestion that bile acids may regulate their own synthesis indirectly by altering the concentration of cholesterol in a critical microsomal regulatory pool [7,8].

Abbreviations: C7αH, cholesterol 7α-hydroxylase; HMG-CoA-R, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ACAT, acyl coenzyme A:cholesterol acyltransferase; HPLC, high performance liquid chromatography; HI, hydrophobicity index; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UCA, ursocholic acid; HCA, hyocholic acid; UDCA, ursodeoxycholic acid; HDCA, hyodeoxycholic acid; MCA, muricholic acids; ALT, alanine aminotransferase.

These in vitro data have stimulated a number of laboratories to reexamine the entire concept of negative bile acid biofeedback, using in vivo systems. Several important points have emerged from these studies. Heuman et al. (9) and Davis et al. (10) reported that intraduodenal infusion of taurocholate to bile fistula rats caused inhibition of bile acid synthesis only when the infusion rates were very high, sufficient to match or exceed the highest transhepatic bile salt fluxes normally observed in rats with uninterrupted enterohepatic circulation. In contrast, infusion of tauroursodeoxycholate, a more hydrophilic bile salt, failed to inhibit bile acid synthesis and cholesterol 7α-hydroxylase activity even at very high, supraphysiological infusion rates (8). Thus different bile salts clearly differed in their effects on bile acid synthesis. These differences were even more clearly evident when seven different bile acids were fed to normal rats with intact enterohepatic circulation. Relatively hydrophilic bile acids (ursocholic, urosodeoxycholic, hyocholic, hyodeoxycholic) caused little or no additional suppression of C7αH, whereas more hydrophobic bile acids (cholic, chenodeoxycholic, deoxycholic) suppressed activity by as much as fivefold. Changes in C7αH were accompanied by parallel changes in activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA-R), the rate-limiting enzyme for cholesterol biosynthesis. In contrast, activity of a third hepatic microsomal enzyme which regulates cholesterol esterification (acyl coenzyme A:cholesterol acyltransferase, or ACAT) was increased to a modest degree by all bile acids fed. It appeared from these studies that inhibitory potency of bile acids on C7αH and HMG-CoA-R was a function of their relative hydrophobicity (11).

Based on these findings, we postulated that the overall hydrophilic-hydrophobic balance of the bile salt pool may be an important factor in determining the rates of cholesterol and bile acid synthesis. To permit quantitation of the overall hydrophilic-hydrophobic balance of bile salts in biological samples such as bile, one of us (DMH) recently proposed a bile salt monomeric hydrophobicity index (HI). The index is based on the logarithms of bile salt retention factors in a reverse phase liquid chromatographic system and is weighted according to the mole fraction of each bile salt present in the sample (12). The purpose of the present study was to determine how changes in the overall hydrophilic-hydrophobic balance (HI) of rat biliary bile salts, induced by feeding of bile acids, correlate with changes in the activities of the three key enzymes regulating hepatic free cholesterol content (C7αH, HMG-CoA-R, and ACAT). The results demonstrate a highly significant inverse linear relationship between the activities of HMG-CoA-R or C7αH and the HI of biliary bile salts; in contrast, ACAT correlated poorly with biliary HI. These data indicate that the same physicochemical property of bile salts which determines their detergency may also determine their potency as regulators of bile acid and cholesterol synthesis.

EXPERIMENTAL PROCEDURES

Chemicals

Hyocholic, hyodeoxyhcolic, cholic, chenodeoxycholic, deoxycholic, and ursodeoxycholic acids were purchased from Calbiochem, LaJolla, CA. Ursocholic acid was a generous gift from GIPharmex, S. P. A., Milan, 3-Hydroxy-3-methylglutaryl coenzyme A, $NADP^+$ and NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase and oleoyl coenzyme A were obtained from Sigma Chemical Co., St. Louis, MO. DL-[3-^{14}C]-Glutaryl-3-hydroxy-3-methylglutaryl coenzyme A (57 mCi/mmol), [4-^{14}C]cholesterol (59.4 mCi/mmol), and [1-^{14}C]oleoyl-coenzyme A (57.9 mCi/mmol) were purchased from New England Nuclear, Boston, MA.

Experimental design

Male Sprague-Dawley rats weighing between 200 and 250 g were used in these experiments. They were obtained from Charles River, Cambridge, MA. Rats were housed in wire cages, maintained on a 12-h light-dark cycle (0600-1800 hours light phase) and had free access to laboratory chow (Prolab RMH 3000, Agway Corp., Syracuse, NY). Bile acids (10 g/kg chow [1% w/w]) were added to the powdered chow in ethanol, blended thoroughly with a mechanical mixer, and dried on trays for 24 h. Seven individual bile acids were fed: ursocholic (UCA), hyocholic (HCA), ursodeoxycholic (UDCA), hyodeoxycholic (HDCA), cholic acid (CA), chenodeoxycholic (CDCA), and deoxycholic (DCA). Some bile acids (chenodeoxycholic, deoxycholic) were also fed at lower concentrations (0.5 and 0.1% w/w) in some studies. Combinations of two bile acids (each 0.5% w/w; total 1% w/w) were also fed, including cholic + chenodeoxycholic (CA + DCA), ursodeoxycholic + cholic (UDCA + CA), ursodeoxycholic + chenodeoxycholic (UDCA + CDCA), ursodeoxycholic + deoxycholic (UDCA + DCA), and hyodeoxycholic + deoxycholic (HDCA + DCA). All bile acids were $>95\%$ pure and 99% free of bile acid contaminants; purity was confirmed by gas-liquid chromatography. Each feeding group consisted of four to six rats. Experimental diets were fed to rats for 14 days. Weight and chow consumption were determined periodically. On the 14th day, rats were anesthetized with methoxyflurane between 0800 and 1000 hours; the bile duct was cannulated, and bile was sampled for 20 min, after which the liver was removed.

Analytical methods

Bile samples were extracted with chloroform-methanol 2:1 according to Folch, Lees, and Sloane Stanley (13). The

water-methanol phase containing bile salts was recovered quantitatively. Methanol (20 ml) was added to 1 ml of methanol-water phase together with HPLC internal standard, 0.2 μmol 7α,12α-dihydroxy-5β-cholanoic acid (Calbiochem) and taken to dryness under a stream of air at 70°C (14). Samples were resuspended in 1 ml of HPLC mobile phase solvent. HPLC analysis of conjugated bile salts was carried out as previously described (15).

Rat livers were homogenized in buffer containing: 0.1 M potassium phosphate buffer (pH 7.4), 0.05 M potassium chloride, 0.1 M sucrose, 0.03 M EDTA, 0.005 M EGTA, and 0.003 M dithiothreitol. After centrifugation at 18,000 *g* twice for 10 min, the supernatant fluid was centrifuged for 90 min at 105,000 *g* at 40°C. The resulting microsomal pellet was suspended in fresh buffer to a concentration of 10–20 mg microsomal protein per ml, aliquoted, and frozen once at −20°C. Enzyme activity was determined within 4 weeks of killing the rats. Microsomal protein and cholesterol were determined as described by Bradford (16) and Abell et al. (17), respectively. Activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, and acyl CoA:cholesterol acyltransferase (ACAT) were determined by isotope incorporation methods as described by Shefer, Hauser, and Mosbach (18), Whitehead et al. (19) and Erickson et al. (20), respectively; all assays were run in triplicate. Incubation times (C7αH, 20 min; HMG-CoA-R, 30 min; ACAT, 4 min) were chosen such that the rate of product formation was linear for the duration of the assay; normalized data have been expressed as product formed per hour per mg microsomal protein. The group *t* test was used for statistical analysis of group data. Regression analysis (least squares method) was performed using the SAS/ STAT statistical analysis program for IBM personal computers (SAS Institute, Cary, NC). The mean values for C7αH, HMG-CoA-R, and ACAT for some of the bile acid feeding groups (UCA, HCA, HDCA, UDCA) have been reported previously (11).

Calculation of hydrophobic-hydrophilic balance

The average hydrophobic-hydrophilic balance of biliary bile salts was quantified by means of a bile salt monomeric hydrophobicity index (12). Briefly, the hydrophobicity index HI_x of an individual bile salt x is based on its capacity factor (k_x) in C18 reversed phase HPLC (mobile phase of methanol-water 70:30 (vol/vol) ionic strength 0.15, pH 8.5), expressed relative to the capacity factors of taurocholate and taurolithocholate. It is calculated as follows:

$$HI_x = \frac{\log(k_x/k_{tc})}{\log(k_{tlc}/k_{tc})}$$

where HI_x is the hydrophobicity index of bile salt x, and tc and tlc refer to taurocholate and taurolithocholate, respectively. HI_x values of taurocholate and taurolithocholate by this definition are fixed arbitrarily at 0 and 1, resepectively. Bile salts that are less hydrophobic than taurocholate (free or conjugated ursocholate, ursodeoxycholate, hyocholate, hyodeoxycholate, and α and β muricholate) have negative hydrophobicity indices. Conversely, bile salts that are more hydrophobic than taurocholate (free or conjugated chenodeoxycholate, deoxycholate, and lithocholate), have positive hydrophobicity indices (**Table 1**).

The overall hydrophobicity index of a mixture of two or more bile salts is then calculated as follows:

$$HI = \sum_{x=1}^{n} HI_x F_x$$

where F_x is the mole fraction of bile salt x in a solution containing n different bile salts. The hydrophobicity index is linearly related to the standard free energy change of partition of bile salts between the polar mobile phase and nonpolar column and thus constitutes a rational measure of hydrophilic-hydrophobic balance.

HI values are dependent upon the state of bile acid ionization, which in turn is affected by pH and ionic strength. Values shown in Table 1 were determined at ionic strength 0.15 and at a pH > 2 units above the mean pKa of free bile salts, conditions comparable to those in normal hepatic bile.

TABLE 1. Hydrophobicity indices (HI_x) of individual bile salts (from ref. 12)

Bile Salt	Taurine Conjugate	Glycine Conjugate	Unconjugated Bile Salt
Ursocholic	−0.94	−0.89	
α-Muricholic	−0.84	−0.79	
β-Muricholic	−0.78	−0.73	
Ursodeoxycholic	−0.47	−0.43	−0.31
Hyocholic	−0.39	−0.40	
Hyodeoxycholic	−0.31	−0.26	
Cholic	0.00	+0.07	+0.13
Chenodeoxycholic	+0.46	+0.51	+0.59
Deoxycholic	+0.59	+0.65	+0.72
Lithocholic	+1.00	+1.05	

Hydrophobicity indices (HI_x) were calculated from capacity factors (k_x) determined by reversed phase HPLC (mobile phase methanol-water 70:30 (w/w), ionic strength 0.15 and pH 8.5–9.0; stationary phase octadecyl silane [C18]). HI_x was calculated using the formula:

$$HI_x = \frac{\ln(k_x/k_{tc})}{\ln(k_{tlc}/k_{tc})}$$

where k_{tc} and k_{tlc} refer to capacity factors of taurocholate and taurolithocholate, respectively. See text for further discussion.

RESULTS

The data in **Table 2** show the biliary bile acid composition of rats fed individual or mixed bile acids as compared to control diet. The predominant bile salts in bile of control animals were conjugates of cholic acid (45.1%) and muricholic acids (MCA) (39.5%). In all instances following bile acid feeding, conjugates of the fed bile acids became the predominant bile salts in bile. Feeding of two bile acids similarly resulted in marked enrichment of bile with conjugates of the fed bile acids. These data clearly demonstrate that the bile acids fed were absorbed from the intestine, conjugated, and incorporated into the bile salt pool. Some of these bile acid composition data were included in a previous paper (11).

A certain amount of bile salt biotransformation occurred consequent to bile acid feeding. For example, after administration of chenodeoxycholic acid, α and β muricholic acids were major constituents of the bile salt pool. Also, after deoxycholic acid feeding there was evidence of formation of cholate. These biotransformations occurred as a result of rat liver's capacity to hydroxylate the steroid nucleus at the 6β and 7α positions (21). Administration of ursocholic or cholic acids resulted in an increase of deoxycholate fraction in bile, an effect attributable to 7-dehydroxylation of the fed bile acids by intestinal bacteria (22). In control rats, biliary bile salts were present predominantly as taurine conjugates. Following bile acid feeding, approximately 50% of bile salts was present as glycine conjugates. Ratios of glycine to taurine conjugates were similar in all bile acid-fed groups.

In **Fig. 1**, activities of C7αH, HMG-CoA-R, and ACAT for individual livers from rats fed individual bile salts or a mixture of bile salts have been plotted against

TABLE 2. Biliary bile salt composition after bile acid feeding

Fed Bile Acid	UCA (n = 6)	UDCA (n = 4)	HCA (n = 6)	HDCA (n = 4)	CA (n = 5)	CDCA (n = 5)	DCA (n = 4)
	Individual Bile Acids Fed (1% w/w)						
	mole % of total ± SD						
Biliary bile salts							
UCA	55.8 ± 7.9	1.1 ± 2.3	1.9 ± 4.7	0.0 ± 0.0	0.2 ± 0.2	0.0 ± 0.0	1.5 ± 1.8
AMCA	0.4 ± 0.7	0.2 ± 0.4	1.5 ± 3.8	0.0 ± 0.0	0.2 ± 0.3	16.7 ± 3.3	2.2 ± 3.0
BMCA	2.6 ± 1.2	10.7 ± 2.4	15.4 ± 2.8	10.1 ± 1.9	6.9 ± 1.5	22.6 ± 8.2	15.1 ± 12.7
UDCA	2.2 ± 1.2	84.5 ± 4.9	0.0 ± 0.0	4.1 ± 2.0	1.3 ± 2.4	10.6 ± 2.8	1.3 ± 0.6
HCA	0.0 ± 0.0	0.0 ± 0.0	66.2 ± 7.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
HDCA	0.4 ± 1.1	0.0 ± 0.0	2.4 ± 3.8	65.9 ± 6.6	0.0 ± 0.1	1.4 ± 1.4	0.6 ± 0.3
CA	16.9 ± 6.3	1.9 ± 0.7	10.6 ± 2.1	8.0 ± 2.5	77.1 ± 9.8	3.6 ± 3.6	15.5 ± 3.2
CDCA	0.4 ± 0.9	1.5 ± 1.3	1.9 ± 0.8	2.3 ± 1.1	0.7 ± 0.5	6.9 ± 6.9	2.2 ± 0.9
DCA	21.4 ± 6.2	0.0 ± 0.0	0.1 ± 0.2	9.7 ± 7.3	13.6 ± 8.1	0.0 ± 0.0	61.5 ± 9.5
LCA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2
% Glycine conjugates	63.4 ± 16.4	52.2 ± 5.1	47.2 ± 8.0	44.7 ± 1.6	56.5 ± 9.1	61.3 ± 2.9	43.3 ± 13.1

	CA + DCA (n = 4)	HDCA + DCA (n = 4)	UDCA + CA (n = 4)	UDCA + CDCA (n = 4)	UDCA + DCA (n = 4)	Chow-Fed Controls (n = 5)
	Mixtures of Two Bile Acids Fed (each 0.5% w/w)					
	% of total ± SD					
Biliary bile salts						
UCA	0.7 ± 0.4	0.1 ± 0.2	0.1 ± 0.1	0.2 ± 0.3	0.2 ± 0.0	0.2 ± 0.1
AMCA	0.4 ± 0.3	0.0 ± 0.0	0.8 ± 1.0	4.4 ± 3.0	0.0 ± 0.0	1.8 ± 0.9
BMCA	6.6 ± 0.9	9.2 ± 3.6	16.6 ± 3.7	18.0 ± 5.4	18.5 ± 2.6	37.7 ± 3.0
UDCA	1.4 ± 0.7	2.5 ± 2.9	41.2 ± 2.1	55.1 ± 2.9	45.9 ± 3.2	2.3 ± 0.3
HCA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
HDCA	3.2 ± 5.2	28.8 ± 11.8	2.9 ± 2.2	0.3 ± 0.5	0.0 ± 0.0	6.7 ± 0.8
CA	44.9 ± 8.2	21.1 ± 10.9	26.7 ± 7.0	0.3 ± 0.6	7.8 ± 2.0	45.1 ± 2.4
CDCA	2.7 ± 2.3	2.0 ± 1.5	1.0 ± 0.7	21.9 ± 2.9	0.4 ± 0.6	4.4 ± 0.3
DCA	40.2 ± 4.2	36.4 ± 5.1	10.8 ± 6.4	0.0 ± 0.0	27.2 ± 2.4	1.8 ± 0.9
LCA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
% Glycine conjugates	53.0 ± 13.2	49.0 ± 9.4	40.6 ± 9.9	43.8 ± 4.1	48.8 ± 4.2	4.8 ± 2.1

Data were obtained by HPLC analysis of bile samples collected via common bile duct cannulation at the time of killing.

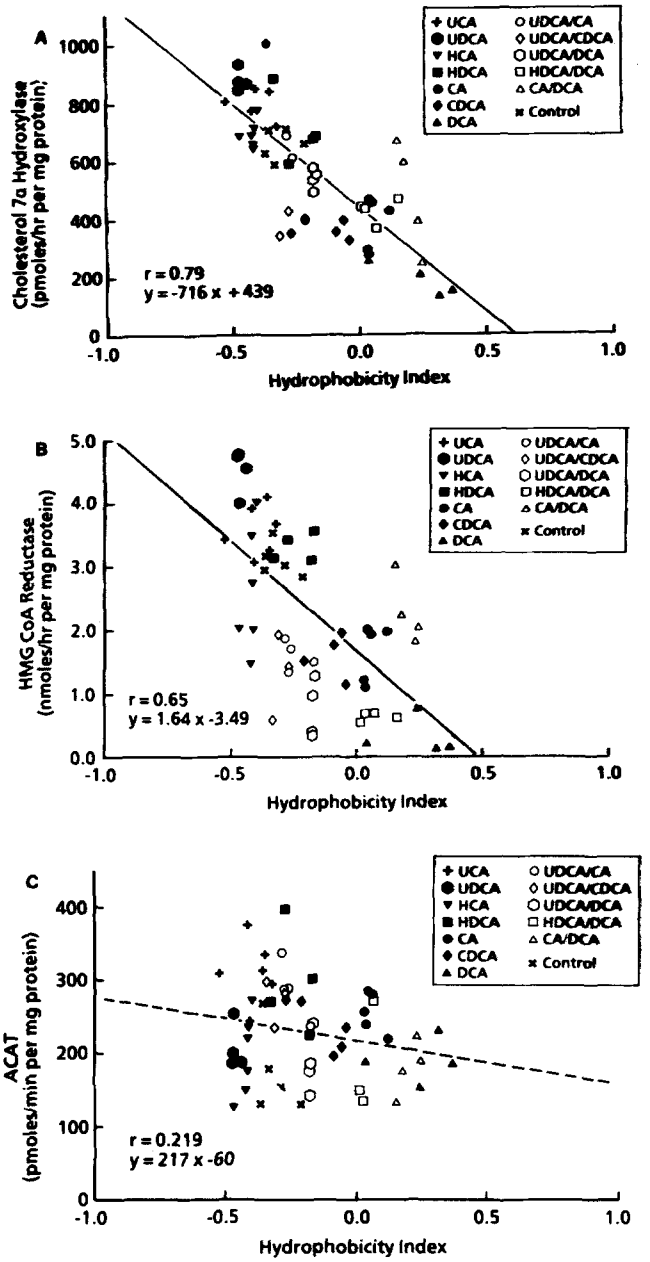

Fig. 1. Correlation between biliary bile salt hydrophobicity index (HI) and activities of three enzymes that regulate hepatic cholesterol metabolism: A) cholesterol 7α-hydroxylase (C7αH); B) HMG-CoA reductase (HMG-CoA-R); and C) acyl CoA:cholesterol acyltransferase (ACAT). Data shown are for individual rats. All bile acids were fed for 14 days, either singly as 1% (w/w) of chow (filled symbols) or as combinations of two bile acids, each comprising 0.5% of chow (open symbols). Control rats received chow only. The hydrophobicity index, a measure of the mean hydrophilic–hydrophobic balance of a mixture of bile salts, was calculated as described in Methods.

the hydrophobicity index of the same rat's biliary bile salt pool. A strong negative linear correlation was observed between the activity of C7αH and the biliary bile salt hydrophobicity index. The correlation coefficient (r) of this inverse relationship was 0.79 ($P < 0.0001$). A similar relationship can be observed between the HMG-CoA-R activities and the hydrophobicity index of the bile acid pool after feeding individual bile acids. Although data exhibit somewhat more scatter ($r = 0.63$), this correlation is also highly significant ($P < 0.0001$). By contrast, the activity of ACAT correlated poorly with the bile salt pool hydrophobicity index ($r = 0.23$; $P = 0.11$; ns).

To determine the influence of individual group data, the regression analyses were repeated after elimination of data for each individual feeding group or for all groups receiving each individual bile acid, singly or in combinations (Tables 3 and 4). In all cases, P remained less than 0.0001. Similarly, elimination of data for rats fed either of the two most hydrophilic bile salts (UCA or UDCA) or either of the two most hydrophobic bile salts (CDCA or DCA) failed to reduce the significance of the relationships noted. Thus the correlations observed are robust and are not dependent upon small numbers of aberrant data.

Table 5 shows group means ± standard deviation for enzyme activity and biliary bile salt hydrophobicity indices. Relatively hydrophilic bile acids (UCA, UDCA, HCA, HDCA) caused little or no change in the hydrophobicity of the rat bile salt pool, as compared to controls; similarly, these bile salts had little effect on activity of C7αH and HMG-CoA-R as compared to controls. More hydrophobic bile salts (CA, CDCA, DCA), by contrast, consistently increased the hydrophobicity index of the bile salt pool and suppressed activity of both enzymes. It is noteworthy that rats receiving chenodeoxycholic acid, either alone or in combination with ursodeoxycholate, exhibited significantly lower activities of both C7αH and HMG- CoA-R than would have been predicted from the overall regression analysis. ACAT in all bile acid-fed groups was equal to or greater than controls and showed no consistent relationship to the hydrophobicity of the fed bile acid.

Table 5 also shows microsomal free cholesterol content for each group of rats. No overall correlation was found between microsomal cholesterol and bile salt pool hydrophobicity, nor were any significant differences found between any of the groups of bile acid fed rats and controls.

Serum alanine aminotransferase (ALT) levels, determined at the time of sacrifice, are shown in Table 5. Isolated elevations of ALT were noted in individual rats in several groups, including most prominently UDC and CDC; however, ALT exhibited no significant correlation with any of the enzyme activities studied. When regression analysis for C7αH or HMG-CoA-R versus HI was repeated following elimination of all data for rats exhibiting ALT > 200 (n = 13), the correlations remained highly significant ($P < 0.0001$).

Bile flow, determined during a 20-min biliary drainage under anesthesia immediately prior to killing, exhibited a great deal of variability (Table 5). Greater bile salt pool hydrophobicity appeared to be associated with higher rates of bile flow, and a modest positive correlation was

TABLE 3. Regression analysis of cholesterol 7α-hydroxylase (verticle axis) versus biliary HI (horizontal axis), performed by the least squares method

	Data Omitted	n	r	Slope	Y-Intercept	P
A) Overall data		53	0.794	−716	439	< 0.0001
B) Omitting individual feeding groups	UCA	47	0.775	−664	433	< 0.0001
	UDCA	49	0.764	−667	439	< 0.0001
	HCA	47	0.792	−751	441	< 0.0001
	HDCA	49	0.801	−707	433	< 0.0001
	CA	48	0.784	−712	442	< 0.0001
	CDCA	48	0.820	−711	457	< 0.0001
	DCA	49	0.726	−661	457	< 0.0001
	UDCA/CA	51	0.793	−715	439	< 0.0001
	UDCA/CDCA	51	0.827	−738	445	< 0.0001
	UDCA/DCA	49	0.796	−716	441	< 0.0001
	HDCA/DCA	50	0.787	−715	440	< 0.0001
	CA/DCA	49	0.843	−828	402	< 0.0001
	Control	48	0.795	−722	439	< 0.0001
C) Omitting individual bile acids (singly or mixed)	UCA	47	0.780	−665	434	< 0.0001
	UDCA	41	0.801	−695	447	< 0.0001
	HCA	47	0.792	−751	441	< 0.0001
	HDCA	46	0.795	−708	433	< 0.0001
	CA	44	0.838	−854	393	< 0.0001
	CDCA	46	0.860	−734	465	< 0.0001
	DCA	38	0.753	−856	394	< 0.0001
D)	UCA alone or UDCA alone	43	0.732	−581	431	< 0.0001
	DCA alone or CDCA alone	44	0.750	−637	483	< 0.0001
	UCA or UDCA (all groups)	35	0.771	−612	437	< 0.0001
	CDCA or DCA (all groups)	31	0.804	−771	457	< 0.0001

To determine the influence of individual groups on the overall result, analysis of all data (A) has been repeated following elimination of data for individual feeding groups (B). In part (C), regression analysis has been repeated with elimination of all data points for rats receiving each individual bile acid (either singly or in combination with other bile acids). In part (D) the effect of eliminating data for rats fed either of the two most hydrophilic bile salts (UCA or UDCA) or either of the two most hydrophobic bile salts (CDCA or DCA) has also been determined. See text for details.

found between bile flow and bile salt pool hydrophobicity index (slope = 0.56; Y-intercept = 1.44 ml/h; $r = 0.25$). However, this trend failed to attain statistical significance ($P = 0.0528$). The wide variability in these bile flow data contrasts with our previous observations in chronic bile fistula rats or rats with exteriorized enterohepatic circulation, in which bile flow was very consistent and reproducible. This suggests that the volume of bile secreted during a brief drainage under general anesthesia is an imprecise estimate of true bile flow in vivo.

In **Fig. 2**, activities of C7αH, HMG-CoA-R, and ACAT in livers of bile acid-fed and control rats have been plotted pairwise against each other. There was a very strong positive linear correlation between the activities of C7αH and HMG-CoA-R ($r = 0.81$; $P < 0.0001$). By contrast, ACAT exhibited no significant correlation with either C7αH or HMG-CoA-R.

DISCUSSION

The relative balance of hydrophobic and hydrophilic properties of the common C-24 bile salts is determined by the state of ionization, by the number, position and orientation of hydroxyl groups, and by the presence and nature of ring or side chain esters (12). Data from several recent publications suggest that the hydrophilic-hydrophobic balance of bile salts, as quantified by reversed phase HPLC, may be an important factor in determining a number of their physiologic functions. Specifically, several studies have shown that hydrophobic bile salts are better solubilizers of cholesterol (23). Carulli et al. (24) and Loria et al. (25) demonstrated in man that hydrophobic bile salts also are more potent stimulators of biliary cholesterol and phospholipid secretion than hydrophilic bile salts. Also, more hydrophobic bile salts exhibit greater hepatic and intestinal toxicity (26, 27).

The data presented in this report suggest that the hydrophilic-hydrophobic balance of bile salts may play a role in the regulation of cholesterol and bile acid synthesis. A highly significant inverse linear relationship was observed between the activities of C7αH and HMG-CoA-R and the hydrophobicity index of the biliary bile salt pool after feeding of bile acids. Rat bile is normally rich in cholate and muricholates and is almost devoid of chenodeoxycholate, deoxycholate, and lithocholate; consequently,

TABLE 4. Regression analysis of HMG-CoA reductase activity versus biliary HI, performed by the least squares method

	Data Omitted	n	r	Slope	Y-Intercept	P
A) Overall data		53	0.653	−3.49	1.64	< 0.0001
B) Omitting individual feeding groups	UCA	47	0.614	−3.24	1.62	< 0.0001
	UDCA	49	0.593	−2.91	1.63	< 0.0001
	HCA	47	0.690	−3.84	1.66	< 0.0001
	HDCA	49	0.659	−3.45	1.59	< 0.0001
	CA	48	0.650	−3.62	1.60	< 0.0001
	CDCA	48	0.662	−3.51	1.70	< 0.0001
	DCA	49	0.567	−3.20	1.74	< 0.0001
	UDCA/CA	51	0.663	−3.55	1.67	< 0.0001
	UDCA/CDCA	51	0.667	−3.57	1.67	< 0.0001
	UDCA/DCA	49	0.695	−3.48	1.77	< 0.0001
	HDCA/DCA	50	0.630	−3.28	1.74	< 0.0001
	CA/DCA	49	0.731	−4.39	1.35	< 0.0001
	Control	48	0.640	−3.40	1.67	< 0.0001
C) Omitting individual bile acids (singly or mixed)	UCA	47	0.614	−3.24	1.62	< 0.0001
	UDCA	41	0.684	−3.14	1.80	< 0.0001
	HCA	47	0.690	−3.84	1.66	< 0.0001
	HDCA	46	0.639	−3.25	1.67	< 0.0001
	CA	44	0.748	−4.83	1.18	< 0.0001
	CDCA	46	0.678	−3.58	1.73	< 0.0001
	DCA	38	0.607	−3.74	1.70	< 0.0001
D)	UCA alone or UDCA alone	43	0.513	−2.38	1.59	< 0.0004
	DCA alone or CDCA alone	44	0.567	−3.16	1.82	< 0.0001
	UCA or UDCA (all groups)	35	0.620	−2.75	1.75	< 0.0001
	CDCA or DCA (all groups)	31	0.595	−3.39	1.99	< 0.0001

To determine the influence of individual groups on the overall result, analysis of all data (A) has been repeated following elimination of data for individual feeding groups (B). In part (C) regression analysis has been repeated with elimination of all data points for rats receiving each individual bile acid (either singly or in combination with other bile acids). In part (D) the effect of eliminating data for rats fed either of the two most hydrophilic bile salts (UCA or UDCA) or either of the two most hydrophobic bile salts (CDCA or DCA) has also been determined. See text for details.

it is relatively hydrophilic (hydrophobicity index −0.32 ± 0.05). This may explain why basal rates of cholesterol and bile acid synthesis in the rat are high compared to other species such as the hamster (28). Feeding of hydrophilic bile acids (UCA, UDCA, HCA, HDCA) changed the hydrophobicity index of the rat biliary bile salt pool only modestly, and consequently the C7αH and HMG-CoA-R activities in these animals were not significantly altered. In contrast, feeding of more hydrophobic bile salts (CA, CDCA, and DCA) increased the hydrophobicity of the bile salt pool and led to highly significant suppression of C7αH and HMG-CoA-R activities.

In this study, CDCA, a more hydrophobic bile salt than CA, paradoxically caused less reduction of the biliary hydrophobicity index than did CA. However, the inhibition of enzyme activity seen with CDCA was slightly greater than that seen with CA. The explanation for this anomaly may lie in the fact that CDCA is extensively transformed on a single pass through the liver to very hydrophilic α- and β-muricholic acids (21, 29). The hydrophobicity index of bile salts in portal blood or hepatocytes of CDCA-fed rats therefore must be significantly greater than the hydrophobicity index of bile salts in bile. We postulate that a hydrophobicity index determined for bile salts in the portal blood or in the hepatocyte itself might yield a more precise correlation with C7αH and HMG-CoA-R activities. However, such determinations are technically much more difficult to carry out and were not done in the present study.

While the present study represents the first extensive investigation of the relationship between the physicochemical properties of bile salts and the activities of enzymes regulating cholesterol and bile acid synthesis, several previous studies support these findings. Spady et al. (30) reported that hydrophobic bile salts (cholate and chenodeoxycholate) inhibited cholesterol synthesis. Hall, Kok, and Javitt (31) and Stange et al. (32) reported strong inhibitory effects on bile acid synthesis with lithocholate and deoxycholate, respectively. Moreover, reduction of deoxycholate formation by colectomy resulted in a subsequent increase of bile acid synthesis (32). Stange and his collaborators (32) concluded that the physiologic regulator of bile acid synthesis in man and possibly other species is deoxycholic acid.

One potential confounding variable in these studies is bile salt toxicity, which could artefactually depress activi-

TABLE 5. Effects of bile acid feeding on enzyme activities

Group	n	HI	C7αH (pmol/h per mg protein)	HMG-CoA-R (nmol/h per mg protein)	ACAT (pmol/min per mg protein)	Microsomal Free Cholesterol (nmol/mg protein)	Serum ALT (IU/ml)	Bile Flow (mg/h)
Control	5	−0.311 ± 0.063	659 ± 52	3.13 ± 0.27	173 ± 58	59.5 ± 11.5	109 ± 11	1.04 ± 0.36
Bile acid								
UCA	6	−0.397 ± 0.072	832 ± 97	3.57 ± 0.41	311 ± 44	47.0 ± 10.4	97 ± 26	1.87 ± 0.33
UDCA	4	−0.464 ± 0.017	879 ± 39	4.52 ± 0.36	207 ± 32	63.6 ± 14.3	400 ± 342	1.03 ± 0.20
HCA	6	−0.423 ± 0.024	691 ± 48	2.61 ± 0.98	195 ± 56	47.7 ± 13.0	58 ± 8	1.10 ± 0.11
HDCA	4	−0.238 ± 0.078	709 ± 125	3.32 ± 0.22	298 ± 72	50.8 ± 17.7	92 ± 13	1.27 ± 0.12
CA	5	0.058 ± 0.037	381 ± 91	1.66 ± 0.45	256 ± 27	68.3 ± 10.7	200 ± 112	1.38 ± 0.22
CDCA	5	−0.133 ± 0.101	368 ± 32	1.57 ± 0.32	239 ± 35	59.2 ± 18.1	743 ± 789	1.06 ± 0.23
DCA	4	0.241 ± 0.147	188 ± 58	0.33 ± 0.29	190 ± 32	75.6 ± 19.3	235 ± 263	1.92 ± 0.68
CA + DCA	4	0.204 ± 0.045	467 ± 193	2.25 ± 0.53	179 ± 38	50.8 ± 11.0	320 ± 364	1.86 ± 0.75
HDCA + DCA	4	0.067 ± 0.065	426 ± 43	0.06 ± 0.07	185 ± 75	62.3 ± 24.1	141 ± 18	1.24 ± 0.13
UDCA + CA	4	−0.250 ± 0.052	647 ± 59	1.59 ± 0.02	287 ± 40	49.4 ± 15.3	108 ± 22	1.05 ± 0.50
UDCA + CDCA	4	−0.317 ± 0.031	385 ± 62	1.31 ± 0.68	270 ± 33	35.1 ± 13.0	136 ± 94	1.62 ± 0.96
UDCA + DCA	4	−0.173 ± 0.006	537 ± 36	0.75 ± 0.45	186 ± 41	41.4 ± 6.5	98 ± 27	0.99 ± 0.18

Mean data, individual feeding groups. All groups except for controls received bile acid as 1% of diet for 14 days. Rats were then anesthetized, the bile duct was cannulated, and bile was collected for 20 min, after which the liver was removed and microsomes were prepared. Included are biliary bile salt hydrophobicity indices, activities of cholesterol 7α-hydroxylase, HMG-CoA reductase, and ACAT, and hepatic microsomal free cholesterol content, serum alanine aminotransferase, and bile flow in bile acid-fed rats (mean ± SD for each feeding condition).

ties of hepatic enzymes. During a previous study (33) in which intraduodenal infusions of conjugated bile salts were administered to bile fistula rats, taurodeoxycholate was observed to induce cholestasis at 9 and taurochenodeoxycholate at 18 μmol/100 g · h^{-1}. In contrast (9), both tauroursodeoxycholate and taurocholate were well tolerated at rates up to 60 μmol/100 g · h^{-1} (approximately twice the normal flux of bile salts in the enterohepatic circulation of the rat (34)). Attili et al. (35) have suggested that the hydrophilic–hydrophobic balance of bile salts may determine their potential to cause hepatotoxic injury.

However, we believe that toxicity cannot account for the correlation between bile acid pool hydrophobicity index and activities of C7αH and HMG-CoA-R found in these studies. No elevations of serum bilirubin were noted. The effect of bile salts on HMG-CoA-R and C7αH was specific; ACAT activity in hepatic microsomes from all bile acid feeding groups was equal to or greater than control. When animals fed CDCA and/or DCA, the two bile acids with established potential for toxicity, were eliminated from the regression analysis, the correlation between biliary bile salt hydrophobicity index and activities of either C7αH or HMG-CoA-R remained highly significant ($P < 0.0004$). ALT elevations were observed in some individual animals from several bile acid feeding groups, but eliminating all rats whose ALT exceeded 200 from the regression analysis similarly did not reduce the significance of the correlation between biliary bile salt hydrophobicity index and activities of C7αH or HMG-CoA-R. Rats also have been fed CDCA and DCA at 10-fold lower concentrations (0.1% w/w). Even at these lower concentrations, DC (n = 4) profoundly suppressed C7αH to 199 ± 60 pmol/mg · h^{-1} and HMG-CoA-R to 0.92 ± 0.32 nmol/mg · h^{-1}; similarly, CDC (n = 2) suppressed C7αH and HMGR activities to means of 383 and 1.54, respectively. Finally, bile flow actually exhibited a modest increase with increasing bile salt pool hydrophobicity, though this trend failed to achieve statistical significance.

Our finding of a close correlation between the activities of C7αH and HMG-CoA-R suggests that regulation of these two enzymes may be linked. Parallelism between C7αH and HMG-CoA-R activities has been noted previously under a variety of conditions including chronic bile fistula drainage or cholestyramine feeding (1, 36, 37), portacaval anastomosis (38), lymphatic drainage (39), diurnal variation (40), and fasting (41). Under certain conditions such as cholesterol feeding, the two activities may diverge; however, this may be a nonspecific effect, as it has been reported that cholesterol feeding stimulates several cytochrome P-450-dependent monooxygenase enzyme activities in liver and intestine (42). Gavey, Trujillo, and Scallen (43) and Scallen and Sanghvi (44) have suggested that regulation of ACAT activity may also be coordinated with that of C7αH and HMG-CoA-R. This did not ap-

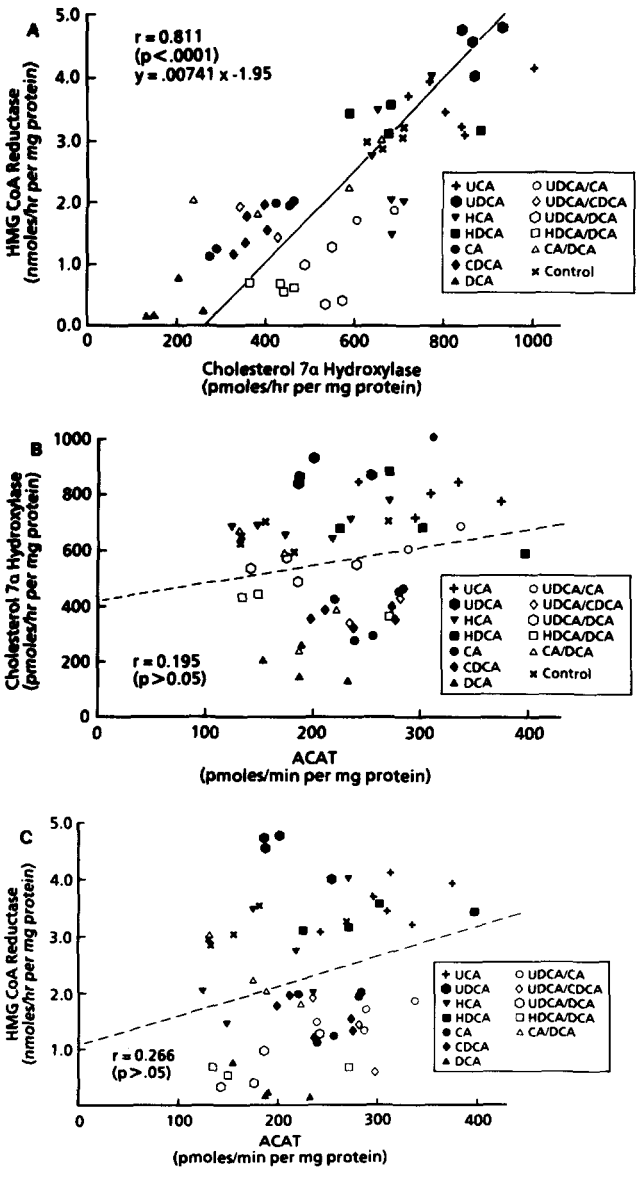

Fig. 2. Pairwise correlation between activities of C7αH, HMG-CoA-R, and ACAT in bile acid-fed rats. Symbols are identical to those used in Fig. 1. Data shown are for individual rats. Activities of HMG-CoA-R and C7αH exhibited a strong positive linear correlation over the entire spectrum of bile acids fed (Fig. 2A). In contrast, neither C7αH nor HMG-CoA-R exhibited a significant correlation with ACAT following bile acid feeding (Fig. 2B and 2C).

pear to be the case in the current study, however, as ACAT activity exhibited no significant correlation with either C7αH or HMG-CoA-R in livers of rats fed different bile acids.

The mechanism by which the hydrophilic-hydrophobic balance of bile salts determines their potency in the regulation of HMG-CoA-R and C7αH has yet to be resolved. Weiss and Dietschy (45) postulated that the inhibitory effect of bile salts on cholesterol synthesis may be related to their ability to promote absorption of the cholesterol from the intestine. Enhanced absorption of cholesterol would lead to the suppression of HMG-CoA-R by chylomicron-remnant cholesterol. More hydrophobic bile salts may be more effective promoters of cholesterol absorption than hydrophilic bile salts and hence more potent inhibitors of HMG-CoA-R. Alternatively, results of Hamprecht et al. (46) and Shefer et al. (37) suggest that bile salts may inhibit HMG-CoA-R directly.

Similarly, down-regulation of C7αH by bile salts could result from direct effects (altered enzyme synthesis and/or degradation, covalent modification) or from indirect effects on some other critical regulatory signal. We propose that newly synthesized cholesterol, or possibly some other product derived from mevalonate (47), may be the proximal regulator. In this model, direct or indirect suppression of HMG-CoA-R by bile salts leads to decreased mevalonate production and decreased cholesterol synthesis, and this in turn leads to down-regulation of C7αH activity. In support of this hypothesis, we have found recently that acute inhibition of HMG-CoA-R causes a rapid decline in C7αH activity in the bile fistula rat, an effect which is abolished by simultaneous administration of exogenous mevalonate (48).

The finding that bile salt HI correlates more closely with C7αH than with HMG-CoA-R suggests that down-regulation of C7αH is the more direct or immediate effect, and that down-regulation of HMG-CoA-R may be an indirect consequence, possibly related to accumulation of cholesterol in a critical regulatory pool. However, this remains speculative. Neither our previous data (11,12) nor those of Einarsson, Åkerlund, and Björkhem (49) have shown any change in microsomal free cholesterol following manipulations which cause bile acid synthesis to vary over a 10-fold range. Moreover, Davis et al. (50) have found that bile acids fail to alter microsomal membrane fluidity. Additional studies are needed to further clarify the interesting interrelationship between HMG-CoA reductase and cholesterol 7α-hydroxylase.

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