Effect of Cholestyramine Particle Size on *in Vitro* Binding of Conjugated Bile Salts (36120)

L. M. HAGERMAN, D. A. COOK, AND D. L. SCHNEIDER
(Introduced by H. P. Sarett)
Mead Johnson Research Center, Evansville, Indiana 47721

Cholestyramine, a nonabsorbable anion-exchange resin, is used to lower blood bile salt levels associated with partial biliary stasis and is currently being widely investigated as a hypocholesterolemic agent. The basis of its pharmacological activity is the sequestering of bile salts in the intestine, thereby increasing fecal bile acid excretion. Factors which affect bile salt binding to resin, such as differing bile salt structures, anion competition and resin particle size, may be important determinants of the overall efficiency of the resin.

Some understanding of the influence of these factors has been gained by *in vitro* studies. A series of experiments by Johns and Bates (1–3) and in this laboratory have shown that the taurine-conjuated bile salts and dihydroxy bile salts are bound more tenaciously to the resin than are the respective glycine conjugates and trihydroxy bile salts. These studies also indicated that binding of trihydroxy bile salts, especially glycocholate, can be markedly reduced by competition from chloride or other anions. Other studies by Blanchard and Nairn (4) showed that reducing particle size of Dowex 1 resin beads increased the availability of exchange sites to cholate and glycocholate.

In the present report the effect of further reduction in resin particle size on binding of single conjugated bile salts from buffered solutions and of mixed-micellar bile salts from gallbladder bile has been examined. With buffered solutions, smaller resin particle size markedly increased the rate of binding of dihydroxy bile salts, but did not improve the binding of trihydroxy bile salts. Bile salts in swine and human gallbladder bile were readily bound to each of the resins and reducing particle size had no effect.

Materials and Methods. Resins. The resins used in these studies were cholestyramine[1] (50–75 μ diameter) and Amberlite XE-234,[2] another strong base polystyrene-type anion-exchange resin of similar particle size. Smaller particle sizes of each resin were also studied using milled cholestyramine[3] (10–30 μ) and Ultrafine Amberlite XE-234 (3–5 μ).[2] Duplicate 20-mg amounts of these resins (anhydrous weight) each having an anion-exchange capacity of approximately 80 μ-moles (4 meq Cl^-/g) were employed in all studies.

Buffered bile salt solutions. The sodium salt[4] of taurocholic acid, glycocholic acid, taurodeoxycholic acid, or glycodeoxycholic acid was dissolved in 0.3 *M* phosphate buffer (pH 6.0) over the concentration range of 1.2 —9.3 m*M*. Twenty milliliters of each bile salt solution was mechanically shaken with cholestyramine in 125-ml Erlenmeyer flasks for 30 min at 25° (5). The suspensions were filtered through Whatman No. 1 filter paper and the filtrates appropriately diluted and assayed by modified Pettenkoffer reactions for trihydroxy bile salt (6) or dihydroxy bile salt (7). Control flasks without resin were treated in the same manner.

In another study, cholestryramine and milled cholestyramine were incubated with 7.0 m*M* bile salt for 15, 30, 60, or 90 min under the same conditions described above.

Swine and human gallbladder bile. Swine gallbladder bile was obtained at cholecystectomy, centrifuged at 10,000*g* for 10 min to remove cellular debris, and stored at —18° until used. Two milliliter aliquots of undi-

[1] Mead Johnson & Co., Evansville, IN.

[2] Rohm and Haas, Philadelphia, PA.

[3] Particle size of cholestyramine further reduced by Jet-Mizer Mill, Fluid Energy Processing and Equipment Co., Philadelphia, PA.

[4] A Grade, Calbiochem., Los Angeles, CA.

luted bile were added to 18 × 100-mm centrifuge tubes containing 20 mg of resin and incubated by mechanical agitation for 30 min at 37°. Control tubes without resin were treated in the same manner. The tubes were centrifuged at 30,000*g* for 10 min and the bile salt concentration in the supernatant fluid determined by the hydroxysteroid dehydrogenase enzyme assay (8). In another study, swine bile was diluted 5-fold with Ringer's solution[5] and 4-ml aliquots were incubated with resin. Bile salt binding was determined in the same manner as that employed above.

Since bile salt micelles may be expanded by the products of pancreatic lipolysis of fat (9), swine bile was also diluted 5-fold with a 10% oil/water emulsion of soybean oil in Ringer's solution and digested for 50 min at 37° by pancreatic lipase[6] (10). The fatty acids liberated were continuously titrated with 0.1 *N* NaOH using a pH-stat[7] to maintain pH at 8.2. Aliquots of this partially hydrolyzed soybean oil–swine bile mixture were incubated with resin and assayed as described above.

Human gallbladder bile[8] was diluted 6-fold with Ringer's solution and a 4-ml aliquot incubated with resin in the same manner as described for swine bile. After centrifugation the taurine and glycine conjugates in a 20-μl aliquot of supernatant fluid were separated on 500-μ thick Silica Gel H[9] glass TLC plates (11). The areas representing the two conjugate types were separately scraped into screw-capped test tubes and eluted with methanol. Aliquots of the methanol eluents were transferred to colorimeter tubes, evaporated under a stream of nitrogen and the bile salts assayed by the enzyme procedure.

Cholesterol was determined by a modified Liebermann–Burchard reaction (12).

Results. The relative affinities of four conjugated bile salts for cholestyramine (50–75 μ) in the phosphate buffer system are shown in Fig. 1. Taurocholate had a greater affinity

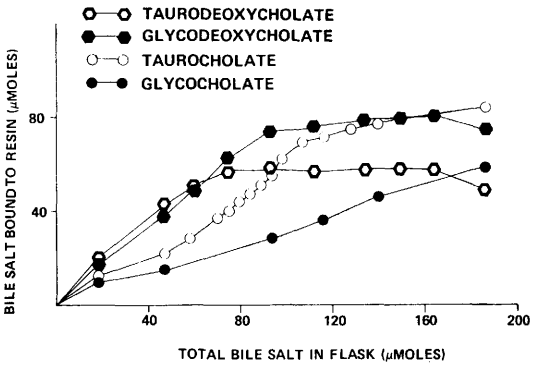

FIG. 1. Relative affinity of conjugated bile salts for cholestyramine (50–75 μ). Each flask contained 20 mg cholestyramine (80 μmoles Cl^-/20 mg), 20 ml 0.3 *M* phosphate buffer (pH 6.0), and was incubated for 30 min at 25°

for the resin than did glycocholate at all levels of bile salt studied. The affinity of the dihydroxy bile salts was greater than that of either trihydroxy bile salt as indicated by the steeper slope of the ascending portion of the conjugated deoxycholate-binding curves. However, under these conditions no more than 60 μmoles of taurodeoxycholate or 80 μmoles of glycodeoxycholate were bound in spite of excess bile salt in the flask. Similar results were obtained with the conjugates of chenodeoxycholate.[10]

Incubation of conjugated bile salts with cholestyramine (50–75 μ) or milled cholestyramine (10–30 μ) for various time intervals indicated that equilibration of the trihydroxy bile salts between resin and solution was achieved within 15 min and reducing particle size had little effect on the amount bound (Table I). By contrast, the binding of dihyroxy bile salts had not reached equilibrium after 90 min and reducing resin particle size markedly increased the rate and extent of their binding.

[5] NaCl, 9.00 g; KCl, 0.40 g; $CaCl_2 \cdot 6H_2O$, 0.25 g; $NaHCO_3$, 0.20 g/liter H_2O.

[6] Steapsin, Sigma Chemical Co., St. Louis, MO.

[7] E. H. Sargent Co., Chicago, IL.

[8] Obtained through the courtesy of Dr. Cecil Bendush of the Clinical Research Division, Mead Johnson Research Center, and Dr. Heinz Zunker, Department of Pathology, Protestant Deaconess Hospital, Evansville, IN.

[9] Kensington Scientific Corp., Oakland, CA.

[10] Unpublished observations, Hagerman, L. M., Cook, D. A., and Schneider, D.L.

TABLE I. Effect of Incubation Time on Binding Conjugated Bile Salts to Cholestyramine (50–75 μ) or Milled Cholestyramine (10–30 μ).[a]

	Resin particle diameter (μ)	Incubation time (min):	(μmoles bile salt bound) 15	30	60	90
Trihydroxy bile salts						
Taurocholate	50–75		74	79	83	81
	10–30		84	90	89	89
Glycocholate	50–75		42	44	42	45
	10–30		43	44	42	44
Dihydroxy bile salts						
Taurodeoxycholate	50–75		26	32	42	48
	10–30		47	54	63	73
Glycodeoxycholate	50–75		48	55	67	76
	10–30		70	78	93	101

[a] Each flask contained 140 μmoles of bile salt.

Contrary to the results with pure solutions of dihydroxy bile salt, reducing resin particle size did not improve the binding of bile salts in undiluted swine gallbladder bile (Table II). Approximately 75% of the anion-exchange sites on each of the resins were occupied by bile salt. Similar amounts of bile salt were bound when the bile was diluted with Ringer's solution to a bile salt concentration within the range normally found in the upper small intestine (Table II).

When partially hydrolyzed soybean oil was added to swine bile to expand the bile salt micelles and to simulate the intestinal milieu during fat digestion, reducing resin particle size again did not improve bile salt binding. However, fatty acids hydrolyzed from soybean oil did decrease binding of bile salt to all four resins (Table II).

In agreement with previous findings, reducing resin particle size again markedly increased binding of taurodeoxycholate in phosphate buffer (Table II) when tested under the same conditions used in the swine bile studies.

Studies with human bile (Table III) showed that all four resins efficiently bound bile salts; reducing resin particle size did not appreciably affect binding. Analysis of the glycine–taurine (G/T) ratio of the conjugated bile salts remaining unbound in the supernatant fluid indicated a preferential binding of taurine conjugates, since this ratio was increased from 1.9/1 in the untreated bile to 3.2/1 in the cholestyramine-treated bile. Under these *in vitro* conditions, there was virtually no binding of human biliary cholesterol to any of the resins (Table III).

Discussion. These *in vitro* studies were conducted to examine the effect of reducing resin particle size on binding of individual bile salts from buffered solutions as well as mixed bile salts from gallbladder bile. In buffered solutions dihydroxy bile salts had a greater affinity for cholestyramine than did trihydroxy bile salts but a longer time was needed to achieve maximum binding equilibrium between resin and dihydroxy bile salt. Reducing resin particle size had little effect on binding of trihydroxy bile salt, but markedly increased the rate of binding of dihydroxy bile salts.

Parkinson (13) suggested that the physical size of bile salt micelles could limit their binding by cholestyramine. The diameter of taurodeoxycholate micelles in phosphate buffer is reported to be 48 Å (14) whereas the pore width of cholestyramine is about 10–20 Å (15). Since dihydroxy micelles are larger than trihydroxy micelles (16), it may be postulated that the dihydroxy micelles diffuse more slowly into the resin particle because of "sieve exclusion" by the resin matrix (15). When sieve exclusion imposes limi-

TABLE II. Binding of Mixed Bile Salts in Swine Bile to Anion-Exchange Resins of Different Particle Sizes.

	Total bile salt in tube (μmoles)	Aliquot volume in tube (ml)	Cholestyramine	Milled cholestyramine	Amberlite XE-234	Ultrafine Amberlite XE-234
			(μmoles bile salt bound)			
Resin particle diameter (μ):			50–75	10–30	50–75	3–5
Swine gallbladder bile						
Undiluted	289	2	64	73	67	66
Diluted with Ringer's Solution	109	4	64	68	57	63
Diluted with partially hydrolyzed soybean oil	84	4	37	34	31	32
Bile salt solution						
Taurodeoxycholate in 0.3 *M* PO_4 buffer	96	4	69	85	50	90

TABLE III. Bile Salts and Cholesterol Remaining in Solution After Incubation of Human Gallbladder Bile with Anion-Exchange Resins.

Resin (diameter)	Unbound bile salts		G/T ratio	Unbound cholesterol (mg)
	Conjugate type			
	Taurine (μmoles)	Glycine (μmoles)		
None	43	82	1.9/1.0	2.4
Cholestyramine (50–75 μ)	15	48	3.2/1.0	2.4
Milled cholestyramine (10–30 μ)	10	44	4.4/1.0	2.0
Amberlite XE-234 (50–75 μ)	13	49	3.8/1.0	2.3
Ultrafine Amberlite XE-234 (3–5 μ)	11	47	4.3/1.0	2.1

tations on ion exchange, reduction of resin particle size can increase the rate and extent of binding (4).

The results found with gallbladder bile do not support this hypothesis. Although the mixed bile salt micelles in bile are much larger than taurodeoxycholate micelles (16), the biliary bile salts were rapidly and efficiently bound to the resins and reducing resin particle size had no effect. Furthermore, even with the artificially expanded gallbladder bile salt micelles, there was no advantage in reducing particle size.

The efficient binding of bile salt in gallbladder bile may be explained by the fact that bile salt molecules in micelles are in rapid equilibrium with monomer bile salt in solution (16). As monomer bile salt is bound, the equilibrium favors dissociation of bile salt from micelles, thereby making them available to be bound.

The finding that cholestyramine did not bind the cholesterol in human bile provides additional evidence that bile salts are not bound by cholestyramine as intact micelles, since biliary cholesterol is a major micellar component. Furthermore, the increase in G/T ratio of the bile salts after resin treatment indicated that the taurine conjugates were being preferentially bound.

The mechanism whereby decreasing resin particle size increased the rate of binding of pure dihydroxy bile salt is not clear but is probably related to the relatively high affinity of these bile salts for the resin. A strong interaction between dihydroxy bile salt, especially taurodeoxycholate, and binding sites near the surface of the resin particle may impede the diffusion of dihydroxy bile salt through the particle to the interior binding sites. Therefore, decreasing resin particle size may have increased the rate of binding of dihydroxy bile salt by increasing the availability of binding sites.

Summary. Cholestyramine (50–75 μ in diameter) was incubated with graded levels of taurocholate, glycocholate, taurodeoxycholate, or glycodeoxycholate in 0.3 *M* phosphate buffer. The resin showed a preference for taurine conjugates over glycine conjugates and dihydroxy bile salts over trihydroxy bile salts. Decreasing resin particle size to 10–30 μ in diameter permitted a more rapid saturation of the resin's binding sites with dihydroxy bile salt but did not affect binding of trihydroxy bile salt.

Reducing particle size of cholestyramine or a similar anion-exchange resin did not affect bile salt binding from swine and human gallbladder bile. The findings suggest that bile salts are bound by resins in the monomer rather than micellar form, and sieve exclusion of bile salt micelles by the resin matrix does not impede the binding process. Each of the resins preferentially bound the taurine-conjugated bile salts in human bile.

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