

## Bile acid binding to sevelamer HCl

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### Bile acid binding to sevelamer HCl.

**Background.** Clinical studies have shown sevelamer HCl (Renagel®) to be effective for the reduction of serum phosphate in hemodialysis patients. These studies also consistently have demonstrated a significant reduction of low-density lipoprotein (LDL) cholesterol following treatment with sevelamer.

**Methods.** Equilibrium binding of bile acids and oleic acid was determined by incubating sevelamer with ligand containing buffer. Aliquots of the solution were filtered and the free ligand concentrations quantitated by high-pressure liquid chromatography (HPLC). Flow kinetics were determined using a cylindrical flow cell containing trapped sevelamer. Bile acid and oleic acid were pumped through the stirred cell in a manner designed to mimic the in vivo situation. Binding was monitored by HPLC.

**Results.** Sevelamer binds bile acids cooperatively and with high capacity. At low binding densities, the presence of the more hydrophobic bile acids enhances the binding of the less hydrophobic bile acids, and the presence of oleic acid enhances the binding of all bile acids. At saturating oleic acid concentrations, the bile acid binding capacity of sevelamer is reduced by only a factor of two. Moreover, the presence of oleic acid dramatically diminishes the release rate of bile acids from sevelamer.

**Conclusions.** The favorable bile acid binding characteristics of sevelamer provide a compelling explanation for its ability to lower LDL cholesterol in hemodialysis patients and in healthy volunteers.

Sevelamer HCl, marketed under the trade name Renagel® (Genzyme Corp., Cambridge, MA, USA), is a cross-linked, hydrophilic, water-swallowable, cationic hydrogel that has shown efficacy in reducing serum phosphate levels in hemodialysis patients [1–4]. Chemically, sevelamer is a cross-linked polyallylamine polymer (Fig. 1). Clinical studies in hemodialysis patients consistently have shown that, in addition to its primary function as a phosphate sequestrant, sevelamer also significantly reduces low-density lipoprotein (LDL) cholesterol [2, 3, 5–9]. A reduction in LDL cholesterol also was observed in healthy volunteers who were fed a controlled phosphate diet [1]. Since sevelamer shares the non-absorbed cationic character of commercial bile acid sequestrants

[10, 11], it is hypothesized that the cholesterol lowering effects observed for sevelamer involve the binding of bile acids in a manner similar to the commercial bile acid sequestrants cholestyramine, colestipol and colesevelam. Bile acid sequestrants function by binding bile acids in the gut and carrying them through the gastrointestinal tract, resulting in an increase in bile acid excretion in the feces. This removal of bile acids from the entero-hepatic circulation results in an up-regulation of the hepatic enzyme cholesterol 7 $\alpha$ -hydroxylase, which catalyzes the rate limiting step in the synthesis of bile acids from hepatic cholesterol. The resulting depletion of liver cholesterol is accompanied by an increase in hepatic LDL receptor activity, thereby enhancing LDL clearance from circulation. In order to test the hypothesis that sevelamer can function as a bile acid sequestrant, we performed equilibrium and flow kinetic studies of the binding of bile acids to sevelamer. We find that sevelamer has excellent capacity for binding bile acids. Moreover, the cooperative binding of bile acids and of fatty acids, under simulated physiological conditions, can result in (1) substantial total binding of bile acids, (2) an enhanced affinity for less hydrophobic bile acids, and (3) very slow bile acid release kinetics. These results support the hypothesis that the reductions in LDL cholesterol observed in clinical trials with sevelamer reflect the property of this cationic hydrogel to cooperatively bind bile acids and fatty acids.

## METHODS

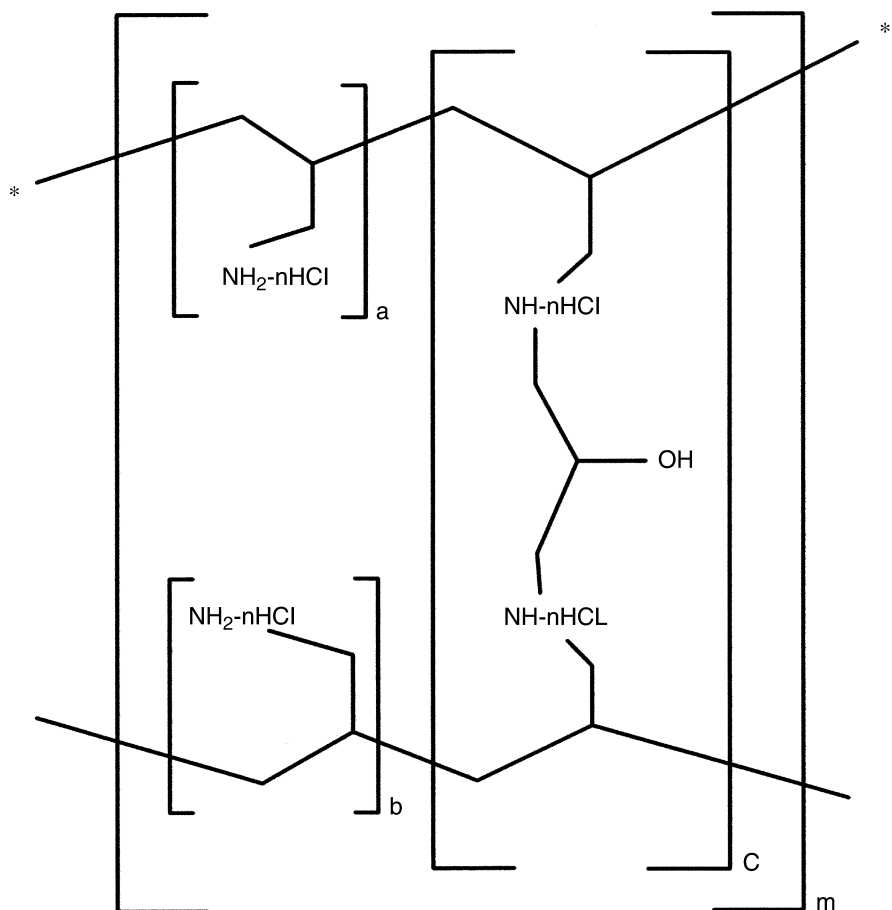
### Equilibrium binding: Experimental

For binding isotherms obtained in the absence of oleate, the binding buffer contained 75 mmol/L sodium bicarbonate, 60 mmol/L sodium chloride, 8 mmol/L potassium chloride, 3 mmol/L sodium phosphate, 2 mmol/L magnesium chloride, and 2.5 mmol/L calcium chloride. The pH of the solution was adjusted into the range of 6.8 to 7.2 using 1 N HCl.

Bile acid stock solution contained 15 mmol/L bile acid. For the individual bile acid isotherms, this solution consisted of a single bile acid. For the mixed bile acid iso-

**Key words:** end-stage renal disease, cholesterol, atherosclerosis, vascular disease, lipid metabolism, hemodialysis, bile acid sequestrants.

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a, b = Number of primary amine groups  
 c = Number of crosslinking groups  
 n = Fraction of protonated amines  
 m = Large number to indicate polymer network

a + b = 9  
 c = 1  
 n = 0.4

**Fig. 1. Structure of sevelamer HCl.** a and b denote the number of primary amine groups; c is the number of cross linking groups; n is the fraction of protonated amines; and m is the large number to indicate the polymer network.

therms, this solution consisted of 7.5 mmol/L cholyl glycine (GC), 3 mmol/L cholyl taurine (TC), 1.5 mmol/L chendeoxycholyl glycine (GCDC), 1.5 mmol/L deoxycholyl glycine (GDC), 0.75 mmol/L chendeoxycholyl taurine (TCDC), and 0.75 mmol/L deoxycholyl taurine (TDC), to give final mole percents of 50:20:10:10:5:5 for GC:TC:GCDC:GDC:TCDC:TDC. This ratio was chosen to mimic the bile acid distribution found in humans.

For binding isotherms performed in the presence of oleic acid, the binding buffer consisted of 100 mmol/L BES (N,N-bis [2-hydroxyethyl]-2-aminoethanesulfonic acid), 160 mmol/L NaCl and 20 mmol/L CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate hydrate}. The addition of CHAPS, a zwitterionic bile acid derivative, ensured the aqueous solubility of oleic acid. This buffer was adjusted to pH 6.8 with NaOH. The sodium salt of oleic acid was used to prepare 10 mmol/L solution of oleic acid (OA) by mixing it in BES buffer containing CHAPS and sonicating for 10 minutes to dissolve the OA.

Binding experiments were performed in the following manner:  $30 \pm 3$  mg of polymer was placed in 50 mL flasks, to which 30 mL of buffer solution containing the appropriate amounts of bile acid, CHAPS and OA were added (except for the CHAPS alone experiment, for which 15 mL was used). The 20% variation in polymer weight is unlikely to be of significant consequence since the bound ligand was normalized against the polymer weight, and the free ligand was determined directly by HPLC. The flasks were then vortexed for approximately 30 seconds and shaken for at least 18 hours at 37°C and 355 rpm to produce a continuous uniform mixture of polymer and bile acid solution. This time frame seemed sufficient, since Benson and colleagues demonstrated that bile acids (GC, TC, GCDC, TCDC, GDC and TDC) equilibrate with cholestyramine within a one-hour time period under conditions similar to those reported here [12]. The pH was adjusted to the range of 6.8 to 7.2 using 1 N HCl and the tube was shaken vigorously at 37°C for an additional two to three hours. Approximately 2 mL

of solution was filtered using a 0.45  $\mu\text{m}$  filter device and used for HPLC quantitation.

The free bile acid concentrations were determined by HPLC. The HPLC system was an HP 1100 (Hewlett-Packard Instruments, Palo Alto, CA, USA) with a Sedex 55 evaporative light scattering detector (Sedere, Alfortville, France). The flow rate was 1.00 mL/min with a column temperature of 50°C and an injection volume of 25  $\mu\text{L}$ . The detector temperature was 40°C with a nitrogen pressure of 1.8 bar. Standard solutions were used to convert detector response into bile acid concentrations. About ten standard bile acid and oleic acid solutions were prepared by half dilution of the stock 15 mmol/L solution. Data points (peak height vs. concentration) were fitted with a straight line passing through the coordinate origin.

Bound ligand,  $r$  (mmoles bound ligand per gram of polymer) was calculated according to the following formula for each individual ligand:

$$r = \frac{(L_i) - (L)}{W_p} * V_t \quad (\text{Eq. 1})$$

where  $[L_i]$  is the initial concentration of ligand (mmol/L),  $V_t$  is the liquid volume in the flask (30 mL),  $(L)$  is the measured concentration of ligand (mmol/L), and  $W_p$  is the dry weight of the polymer (corrected for water loss on drying).

For the measurement of CHAPS alone, an Alltima C18 3  $\mu\text{m}$  column (Alltech, Deerfield, IL, USA) was used. The system was run isocratically with the mobile phase consisting of approximately 15 mmol/L ammonium acetate, 60% methanol, and 3% 1-propanol adjusted to pH 5.3 with acetic acid.

For the separation of the bile acids and CHAPS, a Platinum 100A 3- $\mu\text{m}$  column (Alltech) was used. The system was run isocratically with the same mobile phase as before except with a pH of 5.8. The higher pH value was needed to separate the CHAPS peak from the GDC peak, which eluted at a similar time.

For the separation of bile acids, CHAPS, and OA, a Platinum EPS 100A 3  $\mu\text{m}$  column (Alltech) was used. A gradient was run with mobile phase A being the mobile phase as before (pH 5.8) and mobile phase B being 100% methanol. Mobile phase A (100%) was run for 3.5 minutes. From 3.5 to 4.0 minutes a gradient up to 95% mobile phase B was run. This was held constant until 7.0 minutes, at which time a gradient returned the mobile phase to 100% A at 7.1 minutes. The total run time was 10 minutes. The gradient with a higher amount of methanol was needed to elute the oleic acid from the column.

### Equilibrium binding: Data analysis

Equilibrium isotherms were fitted to the Hill equation:

$$r = (S_{\text{max}}) \frac{K^n (L)^n}{1 + K^n (L)^n} \quad (\text{Eq. 2})$$

In this equation,  $r$  is the density of bound sites

**Table 1.** Time course of the flow kinetics experiment

Stage	Mobile phase <sup>a</sup>	Time minutes
1	Bile acids + Oleic acid	30
2	Bile acids	30
3	Physiological buffer	90
4	Flushing solution	60

<sup>a</sup>For the experiments performed in the absence of oleic acid, stages 2 and 3 were combined into a single 60-minute association phase

(mmoles per g of polymer) and  $(S_{\text{max}})$  is the total density of sites (mmoles per g of polymer);  $(L)$  is the free ligand concentration in mmol/L. One physical interpretation of the Hill equation for the current situation is as follows: Bile acids are concentrated within the anionic polymer domain, where they associate as aggregates of size  $n$ . The results of these fittings are given in Table 1, and will be discussed later (**Discussion** section).

### Flow kinetics: Experimental

In the flow experiment, the physiological buffer consisted of 25 mmol/L  $\text{NaHCO}_3$ , 110 mmol/L  $\text{NaCl}$ , 8 mmol/L  $\text{KCl}$ , 3 mmol/L  $\text{NaH}_2\text{PO}_4$ . The pH of the solution was adjusted into the range of 6.95 to 7.05 using 1 N  $\text{HCl}$ . The buffer solution was filtered through a 0.45  $\mu\text{m}$  Nylon filter.

The methanol/water flushing solvent consisted of a 60/40 (vol:vol) mixture of methanol and an aqueous, 30 mmol/L ammonium acetate solution (pH 5.3). The bile acid solution was 10.5 mmol/L GC, and 4.5 mmol/L GDC. To this 15 mmol/L bile acid solution was added 15 mmol/L oleic acid to give the oleic acid mobile phase. The presence of this amount of bile acid sufficed to solubilize the oleic acid.

The cylindrical flow cell had a total volume of about 1.5 mL. Both ends of the cell were stoppered with 70  $\mu\text{m}$  frits. A 25  $\mu\text{m}$  Teflon® membrane (DuPont, Wilmington, DE, USA) was clamped to the outlet side of the cell. A stirring bar in the middle of the cell was used to ensure good mixing during the time-course of the flow experiment. The dry weight of the polymer in the cell varied between 14.7 and 15.3 mg.

The polymers were swollen in physiological buffer for more than one hour before the experiment. During the experiment, the cells were immersed in a water bath at 37°C, and positioned on top of magnetic stirrers. Prior to starting the experiment, physiological buffer was allowed to flow through the cell for 20 minutes.

Each experiment was separated into four continuous stages, as summarized in Table 1. For the experiments performed in the absence of oleic acid, stages 2 and 3 were combined into a single 60-minute association phase.

The mobile phases were controlled by HPLC. The sample collection was controlled by an automated liquid handler (Gilson, Lewis Center, OH, USA). Sample collection started from stage 1. The collection interval for

the first 120 minutes was set at 2 minutes per vial. For the final 72 minutes, this interval was 6 minutes per vial.

For experiments run in the absence of oleic acid, analyses were performed directly from the automated liquid handler. Experiments run in the presence of oleic acid were analyzed by direct injection from the HPLC.

The mobile phase for the analysis was a 60/40 (vol:vol) mixture of methanol and 15 mmol/L aqueous solution of ammonium acetate. For the analysis, we used a 33 mm C18 reverse phase column from Alltech with an inner diameter of 7 mm. The temperature of the column was kept at 47°C. The flow rate of the mobile phase was 1.0 mL/min for internal HPLC injection, and 2.0 mL/min for injection through the auto sampler. The detector was an evaporative light scattering detector, Sedex 55. For this detector, the temperature was set at 40°C, the pressure at 1.7 bar, the manual gain at 7.0.

The injection volume was selected to maximize the oleic acid peak without saturating the detector. It was about 6  $\mu$ L for internal HPLC injection, 25  $\mu$ L for injection through the auto sampler.

Retention times were measured at 2.5 minutes, 3.25 minutes and 5.2 minutes for glycocholic acid (GC), glycodeoxycholic acid (GDC), and oleic acid (OA), respectively. The overall time window was about seven minutes or less.

Standard solutions were used to convert detector response into bile acid concentrations. About ten standard bile acid and oleic acid solutions were prepared by half dilution of the stock 15 mmol/L solution. Data points (voltage vs. concentration) were fitted with a straight line passing through the coordinate origin.

### Flow kinetics: Data analysis

If  $t_A$  is the time during which the ligand flows through the cell (the association time), under our experimental conditions,  $t_A = 60$  minutes for all bile acids, and 30 minutes for oleic acid. During this time a concentration  $C_0$  (mmol/L) of ligand flows into the stirred cell at a rate  $F$  (fixed at 0.25 mL/min). In our experiments,  $C_0$  is fixed at 10.5 mmol/L GC, 4.5 mmol/L GDC, and 15 mmol/L oleic acid. This association is followed by  $t_D$  minutes of dissociation ( $t_D = 90$  min for bile acids, and 120 min for oleic acid). After a total time of 150 minutes, a solution of MeOH/NH<sub>4</sub>OAc is pumped through for 30 minutes (150 to 180 min) to displace any remaining ligand. The output is collected as fractions. Each fraction is collected over a time  $\Delta t_i$ , which for our experiments is either 2 or 6 minutes. The mass of polymer within the cell is fixed at 15 mg. The following parameters are defined as:  $N_B$ , total number of micromoles of bile acid bound within the cell;  $N_{IN}$ , total number of micromoles of bile acid that have flowed into the cell;  $N_{OUT}$ , total number of micromoles of bile acid that have flowed out of the cell;

$V_F$ , volume of the reactor cell ( $\sim 1.5$  mL);  $C$ , the instantaneous concentration of bile acid within the cell.

The master equation is that of mass balance:

$$N_B = N_{IN} - N_{OUT} - V_F * C \quad (\text{Eq. 3})$$

where  $N_{IN} = \int_0^t dN_{IN} = C_0 \cdot F \cdot t$  during the association part of the curve, and where  $N_{IN} = N_{IN}^0 = \int_0^t dN_{IN} = \int_0^{t_A} dN_{IN} = C_0 \cdot F \cdot t_A$  during the dissociation part of the curve.

The  $\langle C_i \rangle$  is measured in this experiment, and the concentration averaged from  $t_{i-1}$  to  $t_i$  ( $t_i = t_{i-1} + \Delta t_i$ ). By summation we obtain:

$$N_{OUT} = \sum_{i=1}^Z F * \Delta t_i * \langle C_i \rangle \quad (\text{Eq. 4})$$

where  $Z$  is the total number of time intervals. Note that  $F C dt = dN_{OUT}$  can be written in differential form as:

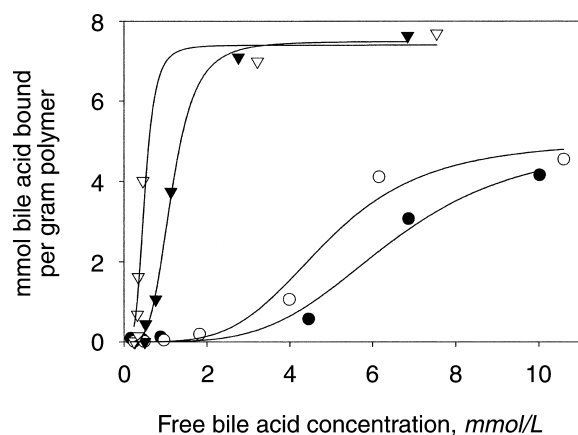
$$C = \frac{1}{F} \left( \frac{dN_{OUT}}{dt} \right) \quad (\text{Eq. 5})$$

Since we knew  $N_{OUT}$  versus  $t$  at intervals of  $\Delta t$ ,  $C(t)$  was obtained by differentiation of this curve. Substitution of  $C$  into equation 3 gave  $N_B$  versus  $t$ . The only unknown at this point was  $V_F$ . Since the polymer was expected to take up a negligible physical volume in the cell, this quantity should be close to the physical cell volume.  $V_F$  was obtained by fitting the concentration versus time curve obtained for the blank (which showed an exponential time constant for mixing that was equal to  $\frac{F}{V_F}$ ), and found values in the range of 1.3 to 1.5 mL.

## RESULTS

### Equilibrium binding

The equilibrium binding of individual bile acids to sevelamer was examined in physiological buffer at 37°C. As observed in our earlier study for bile acid binding to other bile acid sequestrants [13], the binding behaviors of GCDC and GDC to sevelamer are indistinguishable, as are the binding behaviors of TCDC and TDC (data not shown). Fits to equation 2 of the binding data for GC, TC, TCDC and GCDC binding to sevelamer are shown in Figure 2 and are tabulated in Table 2. Also shown in Table 2 are the dissociation constants  $K_d = K_a^{-1}$ , which define the concentration midpoints of the binding curves. The clearly sigmoidal plots of binding density versus free bile acid concentration [(LB) vs. (L)] in equation were best fitted using Hill parameters  $n$  of about 4. For sevelamer, as we have seen for other bile acid sequestrants [13], the order of bile acid binding strength was TDC = TCDC > GDC = GCDC > TC > GC (Table 2). On the basis of saturation capacity ( $S_{max}$ ) in mmoles bound per gram of polymer, the bile acid



**Fig. 2.** Binding of cholyglycine (GC; ●), cholytaurine (TC; ○), chenodeoxycholyglycine (GCDC; ▼), and deoxycholyglycine (GDC; ▽) to sevelamer HCl. The curves are the best fits to the data to the parameters summarized in Table 2.

**Table 2.** Fitting parameters obtained for the binding of individual bile acids to sevelamer HCl

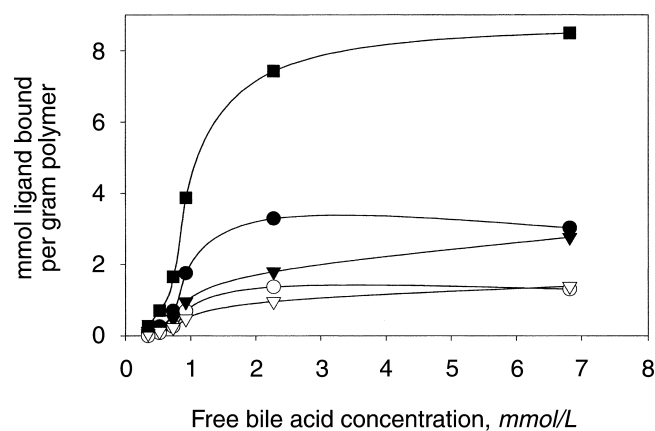
Bile acid	$K_d$ $mmol/L^{-1}$	$N^a$	$S_{max}$ $mmol/g$	$K_d$ $mmol/L$
GC	$0.16 \pm 0.02$	4	$5.1 \pm 0.8$	6.3
TC	$0.20 \pm 0.02$	4	$5.1 \pm 0.6$	5.0
GCDC = GDC	$0.87 \pm 0.03$	4	$7.5 \pm 0.2$	1.1
TCDC = TDC	$2.0 \pm 0.3$	4	$7.4 \pm 0.7$	0.5

<sup>a</sup> $N$  was fixed at 4

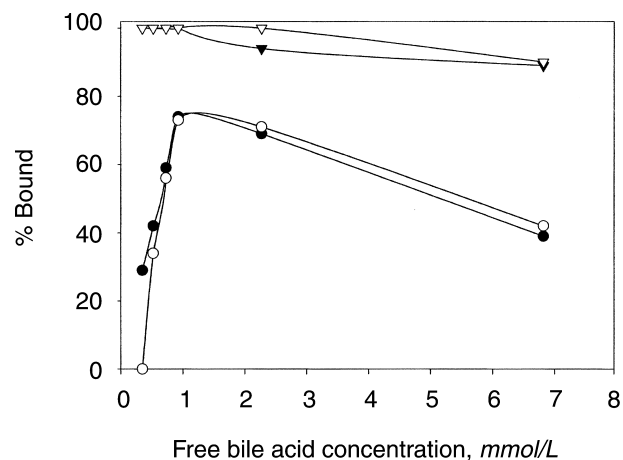
binding capacity of sevelamer met or exceeded that of conventional bile acid sequestrants [13].

Upon comparing Figures 2 and 3, the binding of individual bile acids to sevelamer was quite different from the binding of bile acids in mixed bile acid solution. Whereas the individual bile acids showed distinct concentration midpoints ( $K_d$ ) ranging from about 0.5 mmol/L to about 6 mmol/L when monitored in isolation (Fig. 2 and Table 2), when monitored in a mixture, the concentration midpoints for the binding of the individual bile acids were all around 1 mmol/L (Fig. 3). This point is made more clearly in Figure 4 where the data of Figure 3 were replotted as the percent of a particular bile acid bound as a function of the free bile acid concentration. Note also that in both Figures 3 and 4 the data were plotted against the free bile acid concentration, which was the sum of the free (that is, unbound) bile acid concentrations of each of the individual bile acids. This result demonstrates that the binding of the more hydrophobic dihydroxy bile acids (GDC, GCDC, TDC and TCDC) cooperatively enhances the binding of the more hydrophilic trihydroxy bile acids (GC and TC).

This cooperative effect is even more dramatic for the case of the binding of CHAPS, a zwitterionic bile acid analog. As shown in Figure 5, in the absence of added bile acid, no binding of CHAPS occurred to sevelamer.



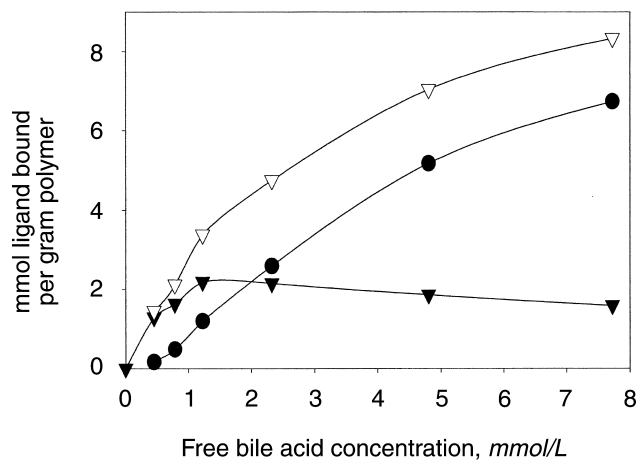
**Fig. 3.** Binding of a physiological mixture of bile acids to sevelamer in physiological buffer. In this experiment, the ratio of GC:TC:GCDC:GDC:TCDC:TDC was 10:4:2:2:1:1. Symbols are: (■) total bile acid; (●) GC, (○) TC; (▼) GCDC + GDC; (▽) TCDC + TDC. The curves are spline fits, and are presented as a visualization aid.



**Fig. 4.** Binding of a physiological mixture of bile acids to sevelamer in physiological buffer (conditions as in Fig. 3), plotted as % bound versus free bile acid concentration. Symbols are: (●) GC, (○) TC; (▼) GCDC + GDC; (▽) TCDC + TDC.

However, as a bile acid mixture was added to sevelamer containing 20 mmol/L CHAPS, binding of CHAPS increased with bile acid binding and then leveled off and decreased slightly at higher bile acid concentrations. Note that the total CHAPS concentration was constant at 20 mmol/L, whereas the total bile acid concentration varied from 0 to 15 mmol/L. Hence, even in the presence of fatty acids, the bile acids bound with much greater affinity to sevelamer than did CHAPS.

The rationale for using CHAPS was to solubilize oleic acid in aqueous solution so that the effect of oleic acid on bile acid binding could be monitored. As shown in Figure 6, the presence of oleic acid dramatically enhanced bile acid binding at lower binding densities without greatly reducing the total bile acid binding capacity.



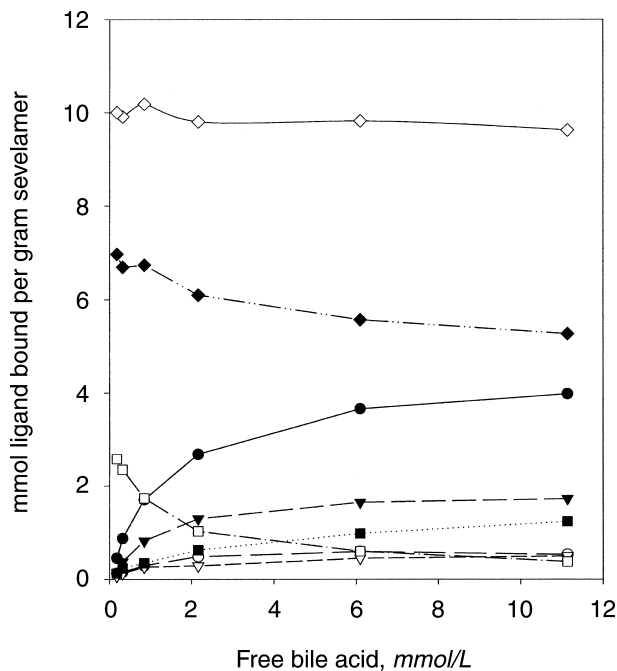
**Fig. 5. Binding of a physiological mixture of bile acids to sevelamer in 100 mmol/L BES in the presence of 20 mmol/L CHAPS.** As before, the ratio of GC:TC:GCDC:GDC:TCDC:TDC was 10:4:2:2:1:1. Symbols are: (●) total bile acid bound; (▼) CHAPS bound; (▽) CHAPS + bile acid bound. The curves are spline fits, and are presented as a visualization aid.

#### Flow kinetics: Fixed bile acids, no fatty acid

Flow cell studies were performed to examine the net association and dissociation rates of bile acids under conditions designed to mimic those encountered by the polymers as they traverse the gastrointestinal tract. The chosen conditions were based on experimental results of the concentrations of bile salts and fatty acids in the gut [14–16]. Clearly, gastrointestinal transit times were highly variable in normal humans, and concentrations of fatty acids and bile salts may have varied with diet. The chosen conditions nonetheless allowed us to examine trends in behavior, even though we did not expect them to be quantitatively predictive.

During the association part of this experiment, a solution of mixed bile acid (15 mmol/L total bile acid, 70% GC, 30% GDC) in physiological buffer was pumped at 0.25 mL/min through a 1.3 mL volume mixing chamber containing either buffer plus polymer gel or buffer alone (blank). The chamber was stirred and maintained at 37°C. Association occurred during the first 60 minutes. After 60 minutes, the dissociation phase began. At the start of this phase, bile acid flow was stopped and buffer flow was started. Buffer was pumped through the cell for 90 minutes. At 60 + 90 = 150 minutes, the remaining bound bile acid was removed from the polymer by flushing the cell with methanol/ammonium acetate. Hence, at 20 minutes we were in the middle of the association phase. At 60 minutes, the association phase was completed, and the dissociation phase began. At 150 minutes, mass balance was verified by displacing bound bile acids (displacement phase). Results of these experiments are shown in Figure 7, for selected time points.

These experiments demonstrated that, in the absence



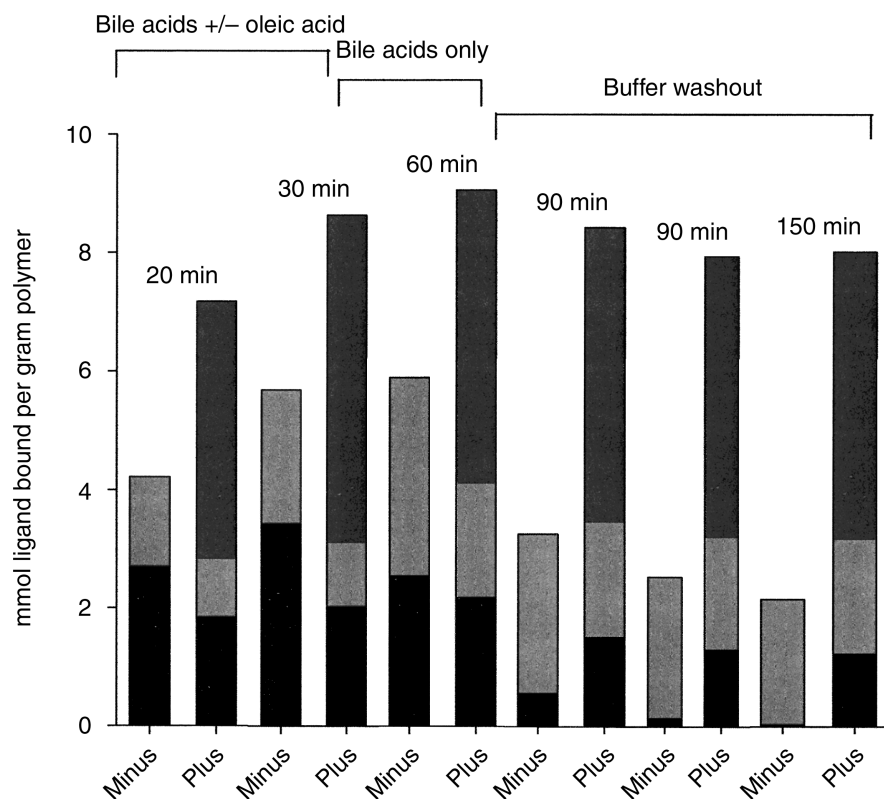
**Fig. 6. Binding of a physiological mixture of bile acids to sevelamer in 100 mmol/L BES in the presence of 20 mmol/L CHAPS and 10 mmol/L oleic acid.** As before, the ratio of GC:TC:GCDC:GDC:TCDC:TDC was 10:4:2:2:1:1. Symbols are: (●) total bile acid bound; (◆) oleic acid bound; (◇) oleic acid plus bile acid bound; (□) CHAPS bound; (○) TC bound; (▼) GC bound; (▽) TDC + TCDC bound; (■) GDC + GCDC bound. CHAPS was added to ensure the aqueous solubility of oleic acid. The curves are spline fits, and are presented as a visualization aid.

of fatty acid, bile acid association kinetics were rapid for both GC and GDC binding to sevelamer. Dissociation of GC also was rapid, so that by the end of the dissociation period, very little GC remained bound to sevelamer.

#### Flow kinetics: Mixed bile acids in the presence of fatty acid

To more closely mimic in vivo conditions, flow cell studies were performed to examine the effect of oleic acid on the kinetics of bile acid binding. The above experimental methodology was modified in the following manner:

During the association part of the experiment, a solution of mixed bile acid (15 mmol/L total bile acid, 70% GC, 30% GDC) in physiological buffer was pumped at 0.25 mL/min through a 1.3 mL volume mixing chamber containing either buffer plus sevelamer or buffer alone (blank). The chamber was stirred, and maintained at 37°C. During the first 30 minutes, association occurred in the presence of 15 mmol/L OA. During the next 30 minutes, the cell was flushed with 15 mmol/L bile acid alone. After 60 minutes, the dissociation phase began. At the start of this phase, bile acid flow was stopped, and buffer flow was started. Buffer was pumped through the cell for 90 minutes. At 60 + 90 = 150 minutes, the



**Fig. 7. Time course of bile acid binding and oleic acid binding to sevelamer HCl in a stirred flow experiment.** The 'minus' plots show binding in the absence of oleic acid, whereas the 'plus' plots show binding in the presence of oleic acid. In this experiment the input ratio of GC:GDC was 70:30. During the first 30 minutes, a solution containing either 15 mmol/L total bile acid (minus) or 15 mmol/L bile acid plus 15 mmol/L oleic acid (plus) flowed into the cell. From 30 to 60 minutes, in both cases, 15 mmol/L bile acid—but not oleic acid—flowed into the cell. From 60 to 150 minutes, the cell was flushed with buffer. In order to verify mass balance, the cell was then flushed with methanol/sodium acetate from 150 to 240 minutes, in order to displace residual bound bile acid. The length of the black bars gives the amount of GC bound, and the length of the light gray bars gives the amount of GDC bound. The total bound bile acid is equal to the sum of these two lengths. The dark gray bars correspond to the amount of oleic acid bound. Hence, the total ligand bound (bile acid plus oleic acid) is equal to the sum of all three lengths.

remaining bound ligand (bile acid + oleic acid) was removed from the polymer by flushing the cell with methanol/ammonium acetate.

The results of this experiment are shown also in Figure 7. It is clear from comparing the curves obtained in the presence of OA to those in the absence of OA that OA competed for binding to sevelamer during the association phase, and thus modestly reduced the total amount of bile acid bound during association. The most dramatic effect, however, was that the otherwise rapid dissociation of GC was significantly retarded by the presence of oleic acid. As a consequence of this effect, by the end of the dissociation phase there was a significant amount of GC bound in the presence of oleic acid, but no measurable GC bound in the absence of oleic acid. As a consequence of the enhanced binding of GC, the total amount of bile acid bound at the end of the dissociation phase also was significantly greater in the presence of oleic acid than in the absence.

## DISCUSSION

### Sevelamer binds bile acids cooperatively and with a high capacity

The sigmoidal binding curves shown in Figure 2 demonstrate that sevelamer binds bile acids cooperatively. When fitted to the Hill model (equation 2), a cooperative

unit of about 4 provides a good fit to the data. The size of this cooperative unit is comparable to that of a typical bile acid micelle [17]. Hence, the picture arises of the association and cooperative binding of bile acid aggregates within the polymer domain. As illustrated in Figure 2, at between 5 and 8 mmole bile acid per gram of polymer, the binding capacity of sevelamer for bile acids is quite high. For comparison, the intrinsic binding capacity of cholestyramine has been estimated at about 3 to 6 mmoles per gram [18–20]. However, the total binding capacity is only one of several parameters that may ultimately determine the biological efficacy of a bile acid sequestrant. In fact, it has been argued that the efficacy of a bile acid sequestrant may reflect in large part its ability to bind the less hydrophobic bile salts, in particular GC [12, 21, 23]. This hypothesis is supported by the clinical data of Dam et al [23]. These data showed that, for six patients treated with 400 mg/kg per day of cholestyramine, the molar percentage of GC to total bile acid in human bile increased greatly in all cases. On average, this percentage increased from 27 to 55% after three weeks of treatment, and further to 61% after six weeks of treatment. Furthermore, for these six patients, the average ratio of trihydroxy to dihydroxy bile acid increased from 0.63 to 1.6 after three weeks of treatment, and to 2.1 after six weeks of treatment. If binding trihydroxy bile acids is important, then there are distinct ad-

vantages to a hydrophilic cooperatively-binding polymer such as sevelamer, compared to a more hydrophobic sequestrant such as cholestyramine. A comparison of Figures 2, 3 and 4 shows that, although the intrinsic binding affinity of sevelamer for GC is rather weak, the presence of even trace amounts of more hydrophobic (dihydroxy) bile acids can dramatically enhance GC binding at low binding densities. The picture that emerges is of cooperative binding of mixed bile acid micelles. Even more dramatic is the ability of saturating quantities of fatty acid to facilitate the binding of all bile acids, including GC (Fig. 6). In contrast, fatty acids compete with bile acids for binding to more conventional bile acid sequestrants, and may play a dominant role in limiting the efficacy of cholestyramine [24].

Other factors present in the GI tract also may influence bile acid binding to sevelamer. For example, phosphatidylcholine could compete with bile acids for binding. Variations from neutral pH are possible. However, since active transport of bile acids occurs at the terminal ileum, pH variations would have to occur prior to passage into the colon from the terminal ileum in order to influence significantly the efficacy of sevelamer as a bile acid sequestrant. In any case, the effective pKa of sevelamer should be about 9.5, based on unreported measurements that we have performed on a closely related cross-linked polyallylamine polymer, and even at pH 8.0, which could occur in the distal colon [25], about 75% of the amines on this polymer should be fully protonated.

#### **In the absence of fatty acids, sevelamer releases GC very rapidly**

As Benson and colleagues have shown, trihydroxy bile acids are released rapidly from cholestyramine on the time-scale of transit through the gastrointestinal tract [12]. Combining this observation with the intrinsic poor affinity of cholestyramine for trihydroxy bile acids provides a plausible hypothesis for the relatively poor clinical potency of this bile acid sequestrant. As clinical studies have shown, prolonged treatment with cholestyramine would over time result in a perturbation of the biliary bile acid pool toward one richer in trihydroxy bile salts, GC in particular [23]. Since cholestyramine binds GC with weak affinity, and releases it rapidly, this perturbation should result in a decrease in the ability of a given dose of cholestyramine to bind bile acid over time, and thus in reduced clinical efficacy. The experiment shown in Figure 7 and described above was designed to mimic the time-dependent concentrations to which a sequestrant would be exposed as it traversed the gastrointestinal tract [26]. As shown in Figure 7, in the absence of fatty acid, there was a very rapid release of GC from sevelamer. Hence, to the extent that GC release kinetics are dominant, our results suggest that sevelamer might

also show a similar reduction in clinical efficacy over time as is seen for cholestyramine.

#### **Fatty acids slow the release of GC from sevelamer**

Nonetheless, it should be recognized that in vivo, GC is likely to be released from a polymer that has been pre-loaded with fatty acids. As also shown in Figure 7, under such conditions in vitro, GC release from sevelamer is dramatically slowed. In contrast to the situation in the absence of fatty acid, where no residual GC binding could be discerned following the dissociation period, in the presence of oleic acid, GC binding is comparable to GDC binding. Hence, the data suggest the intriguing hypothesis that the presence of fatty acid can actually enhance the ability of sevelamer to hold on to trihydroxy bile acids in vivo as well as in vitro.

#### **Conclusions**

*Equilibrium binding properties.* As for previously studied bile acid sequestrants, the binding strength of the naturally occurring bile acids to sevelamer follows the order: TCDC = TDC > GCDC = GDC  $\gg$  TC > GC. This ordering reflects a dominant preference of all sequestrants for more hydrophobic bile acids (dihydroxy vs. trihydroxy), and a minor but still significant preference for taurine-conjugated bile acids compared to glycine-conjugate bile acids. However, for sevelamer, this preference is essentially nullified in mixed bile salt solution due to the cooperative nature of bile acid binding.

*Cooperative interactions.* Sevelamer shows cooperative binding isotherms that are well fitted by equation 2 with  $N = 4$ . In mixed solutions of bile acids, at low binding densities, the presence of more hydrophobic bile acids facilitates the binding of more hydrophilic bile acids. Most dramatically, the presence of saturating quantities of oleic acid strongly facilitates the binding of bile acids at low binding densities, without greatly diminishing total binding capacity.

*Bile acid binding dynamics.* In the absence of oleic acid, sevelamer binds bile acids rapidly, and releases GC very rapidly. In contrast, flow measurements performed in the presence of oleic acid show a marked decrease in the GC dissociation rate. Under these conditions, even at the end of the dissociation period, the amount of GC bound per gram of polymer is comparable to the amount of GDC bound.

*Comparison with clinical results.* These studies demonstrate that sevelamer effectively binds bile acids in vitro, under a variety of conditions. The high binding capacity and the favorable cooperative interactions among bile acids and fatty acids for binding to this polymer suggest a significant potential as a bile acid sequestrant. This potential may in turn explain the favorable lipid lowering effects of sevelamer in hemodialysis patients and in healthy volunteers [1–3, 5–9].



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