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POLYMERIC SORBENTS FOR BILE ACIDS. III. HYDROPHILIC AND HYDROPHOBIC MODELS OF CHOLESTYRAMINE

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ABSTRACT

A study is presented of the binding of glycocholate bile salt anions by crosslinked polymeric beads consisting of either an acrylamide or a Merrifield resin. Various amine-containing pendant groups have been synthesized onto the functional sites of the backbones of these resins, thus forming bile acid sorbents. Based on the effects of systematic changes in the structure of the functional groups on the sorption isotherms, it is shown that the extent of binding is favored by changes that increase the basicity, especially quaternization. On the other hand, sorption is inhibited by extensive crosslinking. It is concluded that the sorption of glycocholate by these sorbents involves an ion-exchange mechanism.

INTRODUCTION

In humans, as well as other mammals, bile acids are synthesized from cholesterol. This biosynthesis is the predominant process by which serum cholesterol is consumed. The bile acids are stored in the gallbladder until food is ingested at which time they are injected into the intestines to assist in digestion, particularly the

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digestion of fats. As they pass down the ileum they are absorbed from the intestines and returned to the liver to complete the so-called enterohepatic circulation. The reabsorption is very efficient so that during each cycle only a small percentage of bile acids (~1%) is lost and is excreted via the feces. To maintain a constant pool of bile acids, this loss is compensated for by the synthesis of more bile acids from cholesterol in the liver.

It is now generally accepted that there is a strong correlation between elevated plasma cholesterol levels and the risk of cardiovascular disease, which is still the leading cause of death among North American adults. Furthermore, because the build-up of arterial plaque is reversible [1, 2], there is strong incentive to lower plasma cholesterol levels. In part, this reduction can be achieved by careful control of diet. Further reduction can be attained by ingestion of bile acid sorbents such as cholestyramine. It is a crosslinked, hence nonabsorbable, anionic-exchange resin that sequesters bile acids in the gastrointestinal tract, thereby interrupting the enterohepatic circulation. Oral administration of cholestyramine increases the bile acid loss through fecal elimination that, in turn, results in a decrease in the serum cholesterol level.

The binding of bile acids by cholestyramine is essentially an ion-exchange process. The interaction is largely nonspecific so that an effective reduction in cholesterol levels is attained only with relatively large doses, up to 54 g/d [3]. An urgent need remains for more efficient bile acid sorbents. A previous report from this laboratory describes bile acid sorbents consisting of lysine-containing oligomeric functional groups attached to crosslinked polyacrylamide beads [4].

Schematically, cholestyramine (Fig. 1) can be viewed as a resin of the type $P-R''-C_6H_4-R'-NR_3^+$, where P represents the crosslinked polymer backbone, R″ is a spacer (absent in cholestyramine), R′ is another spacer (CH_2 in the case of cholestyramine), and R is either H or CH_3. Studies of the effects of systematic changes of chemical structure, variation of either the backbone, P, or the position of the spacer groups, R′ or R″, between the benzene ring and the quaternary ammonium group, or the quaternization of the amino group, on the binding characteristics should give valuable information about the interactions of cholestyramine with bile acids.

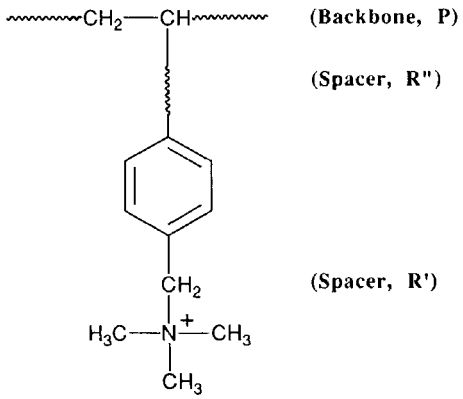

FIG. 1. Structure of cholestyramine.

This paper presents the results of studies made with ion-exchange resins prepared by functionalizing crosslinked polymeric beads with groups that chemically resemble various portions of the active sites of cholestyramine. The first series of resins were beads having a hydrophilic polyacrylamide backbone onto which was grafted 4-(aminomethyl)benzoic acid; the acid was either coupled directly to the backbone or separated from it by a spacer, R″, consisting of three alanine residues. The effect of the presence of the spacer on the *in vitro* sorption of bile acids from aqueous solution was studied; comparison was also made between the behavior of sorbents with primary and quaternary amino groups. The effect of the degree of substitution of the resin was studied using polyacrylamide resins having different functionalities. To determine the role of the methylene group in the sorption, 4-aminophenylacetic acid was also coupled to polyacrylamide, with and without the spacer.

Merrifield resin, which consists of a hydrophobic styrene-containing backbone resembling that of cholestyramine, was also used in the syntheses of some resins. Again 4-(aminomethyl)benzoic acid and 4-aminophenylacetic acid were attached to the backbone, either directly or separated by a tri-alanine spacer. Studies were made of the bile acid sorption characteristics in a manner similar to that used for sorbents with the polyacrylamide backbone.

EXPERIMENTAL

Four polymeric backbones were utilized: two hydrophilic polyacrylamide resins and two hydrophobic Merrifield resins. The first polyacrylamide backbone was a copolymer of dimethylacrylamide and *N*-acryl-1,6-diaminohexane reticulated with bisacrylyl-1,2-diaminoethane (11% crosslinked, Chemalog). The functionality of the resin was 0.241 mmol amine/g resin, as determined by potentiometric titration in 0.050 *M* KNO_3 using standardized $AgNO_3$ solution. The second polyacrylamide resin was a copolymer of dimethyl acrylamide and the acryloyl derivative of sarcosine methyl ester reticulated with bis(acrylamido)ethane (1 mmol/g resin, Milligen). This resin was reacted either with ethylene diamine (Aldrich) or tris-(2-aminoethyl)amine (Aldrich) to produce resins with functionalities of 0.835 and 1.40 mmol primary amino groups/g resin, as determined by acid-base titrations and by potentiometric titrations with $AgNO_3$.

The first Merrifield resin was a 1% crosslinked chloromethylated (1.00 mmol/g resin) styrene divinylbenzene copolymer (Sigma). The second Merrifield resin was similar to the first but with a chloromethyl substitution of 4.15 mmol/g resin.

(1) Functionalization of the Resins

In every case the pendant functional groups were constructed by reacting the carboxylic acid function, of an appropriate amino acid, with the amino group of the polymer resin after forming the symmetrical anhydride which reacted to produce a peptide linkage.

In preparation for the coupling reaction, the amino groups of the compounds to be attached to the polymer backbone, generally amino acids, were protected. The spacer *t*-BOC-L-alanine (Vega Biotechnologies) was used without further purifi-

cation. To protect the amino group of 4-(aminomethyl)benzoic acid (Aldrich) and 4-aminophenylacetic acid (Aldrich), di-*tert*-butyl-dicarbonate (*t*-BOC, Aldrich) (2-fold excess) was added to a solution of the acid and triethylamine (freshly distilled, 2-fold excess) (A and C) in tetrahydrofuran (THF)/water (1:1) [5]. The reaction was followed by tlc. Upon completion, the THF/H_2O was distilled off, *in vacuo*, and the residue was dissolved in ethyl acetate; the organic layer was washed with a 5% citric acid solution, followed by water, dried over $MgSO_4$ and evaporated *in vacuo* to yield the *t*-BOC derivative as a white powder, which was recrystallized from methylene chloride.

All couplings were done by solid phase peptide synthesis (SPPS) techniques, using a Vega 250 synthesizer [6]. Unless otherwise specified, all solvents were distilled prior to use. Dichloromethane (DCM) was dried over molecular sieves (0.4 nm).

The polyacrylamide resin was neutralized with 40% diisopropylethylamine (DIEA) in DCM for 20 minutes. Symmetrical anhydrides of alanine, of the protected 4-(aminomethyl)benzoic acid and 4-aminophenylacetic acid, were prepared for the coupling by dissolving at 0°C six equivalents of the *t*-BOC derivative in DCM (alanine) or in dimethylformamide (DMF). Stirring was continued for 20 minutes, and 3 equivalents of 10% dicyclohexylcarbodiimide (DCC)/DCM were then added slowly. The mixture was allowed to stir for 30 minutes at 0°C, during which time dicyclohexylurea (DCU) precipitated. The mixture was then filtered, and the filtrate was added to the resin. The reaction was left to stir until completion of the coupling, as indicated by a negative ninhydrin test [7]. The *t*-BOC protecting groups were then removed using 40% trifluoroacetic acid (TFA) in DCM, followed by neutralization with 5% DIEA in DCM. The ninhydrin test was again used to monitor the deprotection step.

With the Merrifield resins, coupling of the first alanine or 4-(aminomethyl)benzoic acid or 4-aminophenylacetic acid was carried out using the cesium salts to form an ester linkage [8]. Substitution of the resin was determined by a picric acid test [9]. Subsequent couplings were carried out as described above. The resin was then collected, washed with DCM and anhydrous diethylether, and dried *in vacuo* at room temperature (18–20°C).

(2) Quaternization of the Resins

The resins were quaternized by reaction with methyl iodide (Aldrich) (16-fold excess) in methanol or DMF and potassium bicarbonate (Fisher) (10-fold excess), in the absence of light for 2–7 days [10]. At the completion of the reaction, the resin was collected, washed with methanol, and finally with anhydrous diethylether. It was dried *in vacuo* at room temperature (18–20°C). The extent of the quaternization was determined by potentiometric titration with $AgNO_3$. Table 1 lists the resins that were synthesized, their degree of substitution, and the reaction times for the quaternizations.

(3) Sorption Experiments

Studies of the sorption of bile acids by the resins were done essentially as described previously [3, 4] using solutions of sodium glycocholate in aqueous Tris-HCl buffer (0.0025 *M*) at pH 7. A stock solution of bile salt (10–20 mg/dL) was

TABLE 1. List of Synthesized Resins, Their Substitution, and the Reaction Time for the Quaternization

Resin	Substitution, mmol/g	Time, h
P_1[a]-A[b]	0.241	
P_1-Q[c]	0.232	380
P_1-C[d]-M[e]-Ph[f]-A	0.234	
P_1-C-Ph-M-A	0.234	
P_1-C-Ph-M-Q	0.126	243
P_1-S[g]-C-Ph-M-A	0.222	
P_1-S-C-M-Ph-A	0.222	
P_2[h]-C-Ph-M-A	0.747	
P_2-C-M-Ph-M-A	0.358	
M_{f1}[k]-C-Ph-M-A	0.897	
M_{f1}-S-C-Ph-M-A	0.322	
M_{f1}-S-C-M-Ph-A	0.314	
M_{f2}[l]-C-Ph-M-A	2.89	
M_{f2}-C-Ph-M-Q	1.44	171

[a] P_1 = polyacrylamide (0.241 mmol/g).
[b] A = primary amino group ($-NH_2$).
[c] Q = quaternary ammonium group ($-N(CH_3)_3{}^+I^-$).
[d] C = peptide bond ($-NH-CO-$).
[e] M = methylene group ($-CH_2-$).
[f] Ph = phenyl group ($-C_6H_4-$).
[g] S = spacer (3 alanine groups).
[h] P_2 = polyacrylamide (1 mmol/g).
[k] M_{f1} = Merrifield resin (1 mmol/g).
[l] M_{f2} = Merrifield resin (4.15 mmol/g).

prepared in Tris-HCl buffer, and from it a series of standard solutions were prepared by dilution. These solutions were then added to a weighed amount of dried resin (10–15 mg). The mixtures were shaken for 20–24 hours at room temperature (18–20°C) using a mechanical shaker. The solutions containing the resin were then filtered by gravity and the filtrates were analyzed by reverse-phase HPLC [11].

RESULTS

To evaluate the synthesized resins, isotherms were derived for the sorption of glycocholate from aqueous buffer solution. These isotherms are presented in the

form of X, moles of glycocholate bound per mole of functional group, as a function of the equilibrium concentration of bile acid, C_{eq}.

(1) Hydrophilic Backbone with Primary Amino Groups

The most frequently prescribed commercial bile acid sorbent is cholestyramine, crosslinked polymeric particles composed of a hydrophobic, polystyrene-type backbone with quaternary amine functional groups. In view of its well-known inefficiency in sequestering bile acids from the gastrointestinal fluids, it was deemed desirable to synthesize resins with active groups similar to those of cholestyramine but with a more hydrophilic backbone. For this purpose, resins were prepared using two water-swellable polyacrylamide backbones, differing only in functionality.

The isotherm for the sorption of glycocholate at 20°C by the unmodified polymeric resin (P_1-A) having a hydrophilic polyacrylamide backbone (P_1) and a primary amine functionality (A) of 0.241 mmol/g resin has the regular Langmuir shape and indicates a low, but significant, affinity for the bile acid (Fig. 2). Modification of the functional groups by coupling 4-(aminomethyl)benzoic acid to the primary amine functional sites to produce a sorbent with the structure P_1-C-Ph-M-A, where C is the peptide linkage, Ph is the phenylene group, M is the methylene group and A is the primary amino group, increases the extent of sorption from 0.05 to 0.10 mol/eq of pendant group at a C_{eq} of 15 mg/dL (Fig. 2). Although the pendant functional group resembles that of cholestyramine, except that it is in the primary amine form, this resin sorbs much less glycocholate, 0.1 compared to 1.1 mol/eq pendant for cholestyramine, under similar experimental conditions [3, 12]. This startling effect, which was entirely unexpected, suggests that quaternization, with the resultant increase in basicity, is of key importance.

In an attempt to improve the accessibility of the glycocholate to the 4-(aminomethyl)benzoic acid, and thus promote sorption, a spacer consisting of three

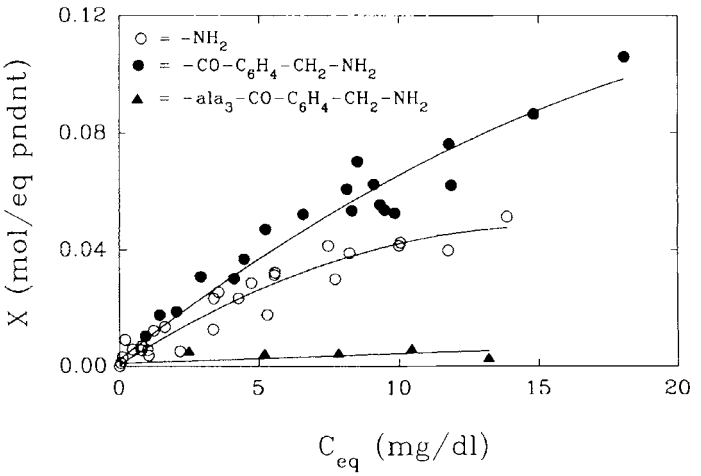

FIG. 2. Isotherms (T = 20°C) for the sorption of glycocholate by P_1-A (○), P_1-C-Ph-M-A (●), and P_1-S-C-Ph-M-A (▲).

alanines was incorporated into the functional group to produce a resin with the structure P_1-S-C-Ph-M-A, where S is the ala_3 spacer. Previous studies with sorbents with pendant groups consisting of basic amino acids showed that the active group sorbs bilirubin more effectively when it is separated from the hydrophilic backbone by this spacer [3]. The choice of the amino acid, alanine, to form the spacer is justified by the fact that it contains no reactive groups in its structure other than the amide linkage, thus excluding the possibility of any reaction with the spacer during the synthesis. Furthermore, this spacer should have a hydrophobic/hydrophilic ratio similar to that of the backbone.

Contrary to expectations, the incorporation of the spacer actually causes a decrease in the sorption capacity of the resin for glycocholic acid, even when compared to the backbone (Fig. 2). This resin shows essentially no sorption of glycocholate. The incorporation of the ala_3 spacer should have little effect on the basicity. Thus, the decrease in sorption may be due to a decrease in the accessible volume for the glycocholate anions when the pendant length is increased. On the other hand, some synergistic effect involving the active site and the backbone is also possible.

(2) Effect of the Position of the Methylene Group

As suggested above, there is evidence that the basicity of the pendant group is an important factor in determining the sorption capacity. The basicity of resin P_1-C-Ph-M-A can be altered substantially by moving the methylene group to the other side of the phenylene moiety to produce the resin P_1-C-M-Ph-A. For the pendant group produced by coupling with 4-aminophenylacetic acid so that the amino group is directly attached to the aromatic ring, the amine will be much less basic because the positive charge can be stabilized by the phenyl ring. To obtain a measure of the magnitude of this effect, comparison may be made of the basicities of benzylamine, $C_6H_4-CH_2-NH_2$, with $pK_b = 4.67$, and aniline, $C_6H_4-NH_2$, with $pK_b = 9.37$. The isotherms in Fig. 3 show greater sorption of glycocholic acid for the resin P_1-C-Ph-M-A, as expected. In addition, the incorporation of an ala_3 spacer, S, into the functional group of resin P_1-C-M-Ph-A to form P_1-S-C-M-Ph-A again results in a marked decrease in sorption capacity.

The role of the methylene spacer in the sorption of glycocholate was studied further with sorbents consisting of a polyacrylamide backbone (P_2) of higher functionality, 1 mmol/g resin, and pendant groups produced by coupling with 4-(aminomethyl)benzoic acid to yield resin P_2-C-Ph-M-A, and with 4-(aminomethyl)phenylacetic acid to produce resin P_2-C-M-Ph-M-A. While both contain the methylene group between the phenylene group and the active amine, the 4-(aminomethyl)phenylacetic acid pendant groups have an additional methylene group at the 1 position, i.e., between the phenylene group and the carbonyl group attached to the backbone. At a given equilibrium concentration, resin P_2-C-M-Ph-M-A, that has both methylene groups, binds approximately twice as much glycocholate per functional group as P_2-C-Ph-M-A (Fig. 4). The presence of the methylene group between the carbonyl group and the phenyl group apparently allows better accessibility to the binding sites. Furthermore, the increased hydrophobicity may also be of importance. This difference does not seem to be a result of the difference in the density of functional sites since sorption isotherms for the binding of glycocholate by unmodified polyacrylamide beads P_1-A and P_2-A indicated simi-

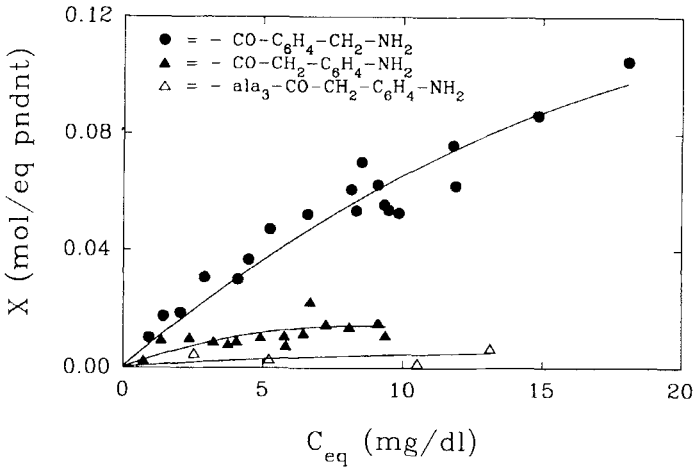

FIG. 3. Isotherms (T = 20°C) for the sorption of glycocholate by P_1-C-Ph-M-A (●), P_1-C-M-Ph-A (▲), and P_1-S-C-M-Ph-A (△).

lar capacities when expressed on the molar basis. Similarly, the active sites of resin P_2-C-Ph-M-A (Fig. 4) are less effective in binding glycocholate than are those of the resin P_1-C-Ph-M-A (Fig. 3) with a lower density of active sites.

(3) Hydrophilic Backbone with Quaternary Amine Groups

It is apparent from the previous sections that basicity plays an important role in the sorption of bile acids. The basicity of the resins can be increased by quaternization of the primary amines of the functional groups. The isotherms in

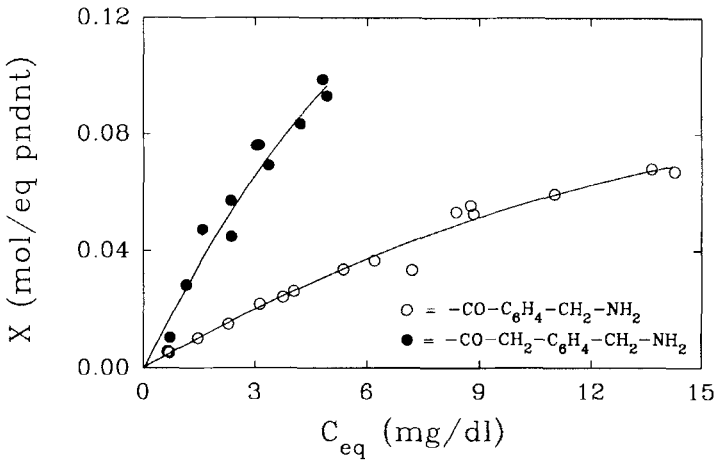

FIG. 4. Isotherms (T = 20°C) for the sorption of glycocholate by P_2-C-M-Ph-M-A (●) and P_2-C-Ph-M-A (○).

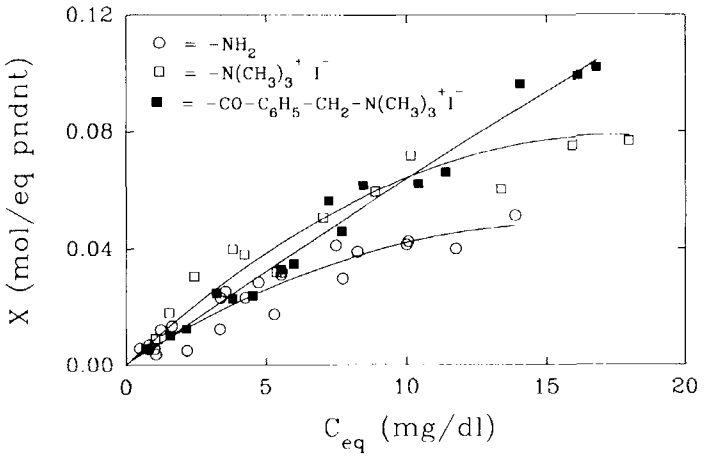

FIG. 5. Isotherms (T = 20°C) for the sorption of glycocholate by P_1-A (○), P_1-Q (□), and P_1-C-Ph-M-Q (■).

Fig. 5 demonstrate that within the range of bile acid concentrations used in these experiments, the quaternized resin P_1-Q has a distinctly higher sorption capacity than the unquaternized resin P_1-A [13]; however, binding occurs at less than 10% of the available sites. For all of the resins, a similar increase in sorption capacity resulted from quaternization.

The higher affinity of the quaternized pendant groups for glycocholate is consistent with the suggestion that the primary interaction is electrostatic in nature. The primary amine resins are weaker bases than the corresponding quaternized resins so that the interaction with the bile acid anion is less favored. In addition, Donnan considerations require an increased pH of the resin phase relative to the glycocholate solution (by as much as 2 pH units), so that the primary amine sites will be only partially protonated. Furthermore, the results indicate that hydrogen bonding, which cannot occur with the quaternary amines, appears to be unimportant.

The most surprising aspect of the sorption of bile acids by these resins is that although the quaternized 4-(aminomethyl)benzoic acid functional sites are *identical* to the pendant groups of cholestyramine, -Ph-M-Q, when they are coupled to a hydrophilic backbone the sorption capacity for glycocholate is very much lower (Fig. 5). Thus, the use of a water-swellable backbone causes a decrease in the sorption rather than the expected increase. This suggests that the hydrophobic character of the backbone must be an important feature.

(4) Hydrophobic Backbones

The previous results give strong evidence for the importance of hydrophobic interactions in the binding of glycocholate by polymeric resins. To consider this effect further, the backbone was changed from hydrophilic to hydrophobic. In these experiments, 4-(aminomethyl)benzoic acid was coupled to a Merrifield resin (polystyrene crosslinked with divinyl benzene, functionality = 1 mmol/g), to form

resin M_{f1}-S-C-Ph-M-A. Sorption of sodium glycocholate by this hydrophobic resin was too small to measure. Similarly, the resin M_{f1}-S-C-M-Ph-A, produced by coupling of 4-aminophenylacetic acid, sorbed only negligible quantities of glycocholate. In the aqueous Tris-HCl buffer, the Merrifield resin swells very little when compared to the polyacrylamide backbone. Therefore, the decreased sorption capacity probably reflects poor accessibility of the binding sites of the resin.

When a Merrifield resin of higher functionality (1.44 mmol/g resin) was used as the backbone, and 4-(aminomethyl)benzoic acid was coupled to it to form the resin M_{f2}-C-Ph-M-A, the primary amine form still did not sorb measurable amounts of sodium glycocholate. However, when it was quaternized to form M_{f2}-C-Ph-M-Q, the sorption could be quantified, but the capacity was only about 0.01 mol/eq of pendant group. Therefore, it would appear that for the hydrophobic Merrifield resin, the ion-exchange occurs only with difficulty; for the purpose of quantification, a higher number of active sites per gram of resin is required.

DISCUSSION

The striking, and probably most important, result of these experiments is that all of the synthesized resins have a lower capacity for the sorption of glycocholate than does cholestyramine under similar experimental conditions. For the synthesized resins, the most effective sorption was obtained with resins consisting of the polyacrylamide backbone bearing a pendant group identical to that of cholestyramine. However, the extent of binding by this sorbent, measured on a molar basis, is only 15% that of cholestyramine. This behavior is contrary to the original hypothesis, namely, that a sequestrant with a cholestyramine-like active site attached to a hydrophilic backbone would exhibit an increased sorption capacity for glycocholate because of improved compatibility with the aqueous environment.

The limited capacity of the resins with the hydrophilic polyacrylamide backbone to bind glycocholate from aqueous solution, in spite of the extensive swelling in the aqueous solution, may well reflect the extensive crosslinking (11%). Increasing crosslinking interferes with diffusion within the resin beads [14]; indeed, the diffusion paths can become so small that the entry of large ions to some of the sites at the interior of the beads is impossible.

While it might be expected that the resins with the hydrophobic Merrifield backbone would have sorption characteristics similar to those of cholestyramine, the amounts sorbed were too small to be quantified by HPLC. Since they exhibited minimal swelling, or even wetting, in an aqueous environment this result might be anticipated.

The ion-exchange process was confirmed by the sorption isotherms obtained for the hydrophilic resins for which the position of the methylene group was changed (Fig. 3). Increase in the basicity favors the interaction of the functional groups of the resin with the counterion as well as with the bile acid anion so that the net result is an increase in the effectiveness of the ion-exchange process. The occurrence of ion exchange is also in keeping with the observation that the quaternized resins have higher affinities for glycocholate than the corresponding unquaternized resins. When the internal environment of the beads has a pH in the 8–9 range, which is a realistic value when the pH of the solution is 7, the unquaternized resins

may be incompletely protonated. In this case the nonprotonated pendant groups would not be involved in the ion-exchange process, thus decreasing the capacity. Furthermore, for the resin P_1-C-M-Ph-Q, consisting of a polyacrylamide backbone with 4-aminophenylacetic acid as the pendant group, sorption of glycocholic acid in phosphate buffer was about half that with the Tris-HCl. This decrease is due to the presence of phosphate anions which compete with the bile acid anions for the quaternary ammonium group.

The incorporation of the ala_3 spacer between the backbone and the benzylammonium group consistently decreased the sorption capacities of those resins. The spacer has a deleterious effect, perhaps due to a decrease in the space available inside the resin pores which makes it more difficult for the bile acid anion to penetrate the matrix and displace the counterion. Molecular modeling studies suggest that conformational changes in the active site, resulting from the presence of the spacer, may also be of importance [12].

SUMMARY

The effects of systematic changes of the chemical composition of cholestyramine-like sorbents on their capacity to sorb bile acids confirm that the interaction between the bile acids and cholestyramine, *in vitro*, involves an ion-exchange process. Increase in basicity of the functional groups attached to the hydrophilic polyacrylamide resin favors the binding of glycocholate from aqueous buffer solution.

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[13] To account for incomplete quaternization, the isotherms have been normalized as follows: The difference between the functionality of the original resin, in the amine form, and that of quaternized form was assumed to represent unquaternized sites. Thus, the data from the sorption experiments reflected the total amount of bile acid bound by the pendant groups, both unquaternized and quaternized. Using the isotherm for the corresponding unquaternized resin, the moles of bile acid bound by these unquaternized pendant groups, at a particular C_{eq}, was subtracted so that the remaining bound bile acid yielded the isotherm for the sorption of glycocholate by the quaternized pendant groups. This normalization process may somewhat oversimplify the actual sorption behavior since interactions between adjacent sites are completely ignored.
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