Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

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X. X. Zhu[a]; G. R. Brown[b]; L. E. St-pierre[b]
[a] Department of Chemistry, University of Toronto, Mississauga, Ontario, Canada [b] Department of Chemistry, McGill University, Montreal, PQ, Canada

To cite this Article Zhu, X. X. , Brown, G. R. and St-pierre, L. E.(1992) 'Polymeric Sorbents for Bile Acids: II. Oligopeptide-Containing Resins with Quaternary Amine Groups', Journal of Macromolecular Science, Part A, 29: 9, 711 — 721
To link to this Article: DOI: 10.1080/10601329208054111
URL: http://dx.doi.org/10.1080/10601329208054111

J.M.S.—PURE APPL. CHEM., A29(9), pp. 711–721 (1992)

POLYMERIC SORBENTS FOR BILE ACIDS: II. OLIGOPEPTIDE-CONTAINING RESINS WITH QUATERNARY AMINE GROUPS†

X. X. ZHU,‡ G. R. BROWN,* and L. E. ST-PIERRE

Department of Chemistry
McGill University
801 Sherbrooke St. W., Montreal, PQ, Canada H3A 2K6

ABSTRACT

Polymeric sorbents for bile acids have been prepared by attaching lysine-containing peptide sequences onto a water-swellable polyamide resin, by solid phase peptide synthesis, and then attaching a terminal *N,N*-dimethyl glycine residue that was subsequently quaternized. The resins with relatively longer peptide sequences demonstrated a higher binding capacity, on a per active site basis, for bile acids in pH 7.4 aqueous buffer solutions at 20°C than cholestyramine and colestipol when tested under the same *in vitro* conditions. In solutions of low ionic strength, they also have a degree of specificity for bile acid anions. The resins have a higher binding affinity for cholic acid than for glycocholic acid, which indicates the importance of the hydrophobic interactions in the binding.

INTRODUCTION

It is now universally accepted that the risk of coronary heart disease is directly related to the plasma cholesterol level [1]. Recent reports give unequivocal evidence that, in a significant number of cases, the buildup of arterial plaque that results

†Dedicated to Otto Vogl on the occasion of his 65th birthday.

‡Present address: Department of Chemistry, University of Toronto, Erindale Campus, Mississauga, Ontario, Canada L5L 1C6.

from elevated plasma cholesterol levels can be reversed by a combination of drugs and a low fat diet [1–4]. Control of cholesterol levels can be achieved clinically by treatment with: 1) inhibitors for the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which is essential to the biosynthesis of cholesterol by the liver [4, 5]; and/or 2) polymeric sorbents for bile acids [2–4]. Enzyme inhibitors for the biosynthesis of cholesterol are used in the treatment of extreme hypercholesterolemia. They are most effective when used in conjunction with bile acid sorbents. Suitably selected polymer sorbents are also used alone in cases of less severe hypercholesterolemia. They bind bile acids in the intestines, thus preventing reabsorption so that the enterohepatic circulation is interrupted. In response, the conversion of cholesterol to bile acids is accelerated to maintain a constant pool of bile salts [6]. The most frequently used resins for this purpose are cholestyramine and colestipol.

The hydrophobic nature of cholestyramine [2], with its polystyrene backbone and nonselectivity, results in a rather low capacity for bile acids so that an effective treatment requires rather high doses (up to 54 g/day). As a consequence, adverse side effects are experienced by many of the hypercholesterolemic patients using this treatment. Colestipol is a water-swellable anion exchanger with a better biological acceptability; however, it appears to be less effective in the binding of bile salts [7, 8]. Other polymeric sorbents are also limited by either poor biocompatibility or low binding capacity. Dietary fibers, such as lignin [9] and pectin [10], have also been reported to eliminate bile acids by sorption from the gastrointestinal tract.

It is well known that bile acids are bound in heme and liver to a number of proteins such as albumin [11], ligandin [12], and lipoproteins [13]. The binding of deoxycholate by bovine serum albumin involves four principal binding sites and about 14 weaker sites for each ligand [14]. Various bile salts have been reported to bind to a single site on ligandin, with association constants of the order of $10^4\ M^{-1}$ [15]. Although the precise nature of the binding is not known, it has been suggested that the positively charged amino groups on the side chains of the basic amino acids in proteins are attracted by the negatively charged carboxylate groups of the bile salts, while hydrogen bonds may also be formed between the hydroxy-substituted bile salts and the protein [16]. It was proposed that the side-chain amino groups of lysine residues in the protein form electrostatic bonds with the carboxylate groups of bile salts [17].

The more lipophilic bile acids are expected to exhibit a high affinity for the hydrophobic centers of proteins in aqueous solution. Indeed, in the binding of common bile acids, bovine serum albumin exhibits the highest affinity for lithocholate, which has only one hydroxyl group [17, 18]. Similarly, ligandin has a lower affinity for cholate than for deoxycholate. This indicates that the addition of polar groups to the hydrophobic moiety of the bile acid reduces the binding affinity [15].

Improved sorbents with greater specificity, hence, higher capacity, for bile acids are greatly desired to reduce the adverse side-effects of the currently used bile acid sequestrants. Immobilized proteins, which are biocompatible and bind bile acids with high affinity and specificity, should be ideal sorbents. However, since the binding site represents only a small part of the protein, the capacity per unit weight would be unacceptably low. A solution to this problem is to immobolize, on an insoluble polymer backbone, small peptides with a primary structural sequence similar to that of the binding site of the protein. Such materials should retain a biocompatibility similar to that of the protein, but manifest considerably higher binding capacity.

Resins with pendant groups containing lysine and arginine in their sequences have already been proven to be effective sorbents for bilirubin [19, 20]. Thus, the strategy that has been adopted for the studies described in this paper is to chemically graft to a water-swellable resin peptide sequences consisting of amino acids similar to those at the proposed binding sites of proteins. For the polymer support, polyacrylamide resins were used because of their water-swellability. A peptide spacer, usually a short alanine sequence, was added to the polymer resin prior to the coupling of the active units so that the functional groups of these pendants would be more accessible to the binding substrates. Furthermore, based on a consideration of the structure of cholestyramine [8], the quaternary amine group is thought to be important in the binding of bile salts. This has been confirmed by an increase in the extent of bile acid sorption by colestipol when its amino groups are quaternized [21]. Therefore, betaine, a natural biocompound that has a quaternary amine group, was used as the terminal entity of the pendant group. In accordance with this strategy, the synthesis of novel polymeric sorbents with lysine-containing oligopeptide pendant groups is presented in this paper. The isotherms for the sorption of bile salts by these resins are compared with those of cholestyramine and colestipol. It is shown that a combination of ionic and hydrophobic interactions can account for the sorption isotherms of these peptide-containing polymeric resins.

EXPERIMENTAL

A water-swellable polyacrylamide resin (Chemalog), a copolymer of dimethylacrylamide and *N*-acryl-1,6-diaminohexane crosslinked with 11% *bis*-acryl-1,2-diaminoethane, was used as the polymer support. Peptide sequences were synthesized on the support by solid phase peptide synthesis techniques described previously [19]. All of the solvents used in the syntheses were redistilled prior to use, with the exception of dichloromethane and dimethylsulfoxide, which were dried with molecular sieves (Type 4A, Aldrich). The amino acids *N*-α-*t*-BOC-L-alanine and *N*-α-*t*-BOC-*N*-ε-2,4-dichloro-CBZ-L-lysine were purchased from Vega.

The amino acids were added in a stepwise process. The anhydrides of the protected amino acids were prepared by reaction with 1,3-dicyclohexyl-carbodiimide (DCC) in dichloromethane at 0°C for 30 min. After removing the by-product urea by filtration, the anhydride solution was added to the resin. The successful coupling was evidenced by a negative ninhydrin test for free amino groups. The BOC protecting groups were then removed by reaction with 40% trifluoroacetic acid in dichloromethane in preparation for the addition of the next amino acid. The coupling of amino acids was repeated until pendant groups of the desired composition were attained. The protecting groups of the lysine side chains were then removed by reacting the dried resins with anhydrous hydrofluoric acid in the presence of anisole at 0°C for 60 min. The following three resins were synthesized by this method: 1) Lys-Ala_3-Polymer, 2) Lys_3-Ala_3-Polymer, 3) Lys_5-Ala_3-Polymer.

Amino acid analyses, made by an HPLC procedure described previously [20] with L-norleucine (Aldrich) as an internal standard, indicated that the amino acid residues attached as the spacer on the resin was successfully coupled with a yield of >90%.

Attempts to couple trimethylglycine to the resin by the DCC method proved unsuccessful. Hence, an alternative approach was adopted which involved coupling

of *N,N*-dimethylglycine (DMG) to the resin and subsequently quaternizing the coupled DMG. The symmetrical anhydride was formed by adding DMG, dissolved in DMSO, to a 10% DCC solution. It was added to the resin with the fully deprotected oligopeptide pendant groups, previously swollen in DMF, and was shaken for 8–10 h. After the resins with the coupled DMG had been washed successively with DMF, methylene chloride, and anhydrous ether, they gave a negative ninhydrin test, indicating successful coupling of DMG to all of the available amines.

For quaternization, the resin coupled with DMG was reacted with excess methyl iodide (molar ratio about 200:1) at room temperature for 2–3 days in the dark [22]. A terminal trimethylglycine (TMG) with the betaine structure and iodide counterion was formed. After quaternization the resin was then separated by filtration and was washed successively with DMF, methylene chloride, and anhydrous ether. The resins were dried under vacuum overnight and stored in a vacuum desiccator. The extent of quaternization was determined by potentiometric titration of the iodide ions with silver nitrate solution, previously standardized with standard sodium chloride solution. The results, given in Table 1, indicate that on average there is one quaternary amine per peptide pendant.

The procedure used for the sorption studies was essentially as described previously [8]. Solutions of sodium cholate (Aldrich) and sodium glycocholate (Sigma) in *tris*(hydroxymethyl)-aminomethane-HCl buffer (Tris buffer) and in KH_2PO_4-NaOH buffer (phosphate buffer) were used. In the pH range of the buffers (7.0 to 7.4), the sorption capacities of the resins were not affected by small variations of pH. To each of the resin samples, weighing approximately 10–20 mg, was added an aliquot (about 5 mL) of bile acid solution of suitable concentration. The samples were agitated for 2 h so that equilibrium sorption was attained. Reverse phased HPLC with a refractive index detector was used for the assay of bile acid concentrations before and after sorption, as described previously [23].

RESULTS AND DISCUSSION

The resins with oligopeptide-containing functional groups, synthesized as described above, proved to have somewhat limited capacities on a weight basis for bile acids. "Plateau" values, under optimal conditions, showed that bile acid binding occurred at approximately 10–15% of available functional groups. Nonetheless, the affinities of some of these resins for bile acids are superior to those of cholestyramine [8] under similar sorption conditions. Furthermore, some rather interesting aspects of the bile acid binding are indicated by the isotherms presented below.

Comparison of the Quaternized Peptide Resins

The isotherms for the sorption of sodium cholate in 5.0×10^{-3} *M* Tris buffer by the quaternized peptide-containing resins are shown in Fig. 1. The isotherms are expressed as X, the moles of bile acid bound per molar equivalent of quaternary amine groups, hence peptide pendant groups, as a function of C_{eq}, the equilibrium concentration of bile acid. As indicated by these isotherms, the resins I^-TMG^+-Polymer, I^-TMG^+-Lys-Ala_3-Polymer, and I^-TMG^+-Lys_3-Ala_3-Polymer have essentially identical capacities. Thus, very similar binding behavior is shown by the

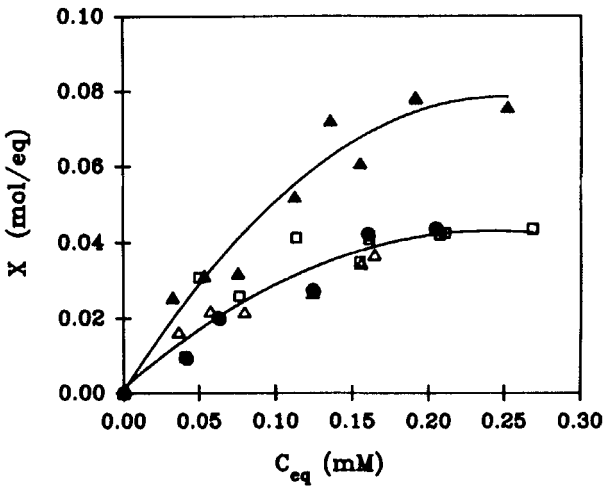

FIG. 1. The sorption isotherms for sodium cholate in 5.0×10^{-3} *M* Tris buffer at 20°C. X: The amount (moles) of bile acids sorbed per molar equivalent of binding active sites; (●) TMG-P; (□) TMG-Lys-Ala_3-P; (△) TMG-Lys_3-Ala_3-P; (▲) TMG-Lys_5-Ala_3-P.

resins having relatively short peptide sequences, suggesting that the binding sites are equivalent. Under these circumstances, the short peptide chain is most likely to retain the randomly extended state. However, the quaternized peptide resin with the lysine-5 sequence demonstrates a significantly greater capacity, suggesting that the longer pendant group is favorable in the binding.

Similar behavior is exhibited by the isotherms for the sorption of glycocholate in 5.0×10^{-2} *M* phosphate buffer (Fig. 2), with capacities that follow the order of

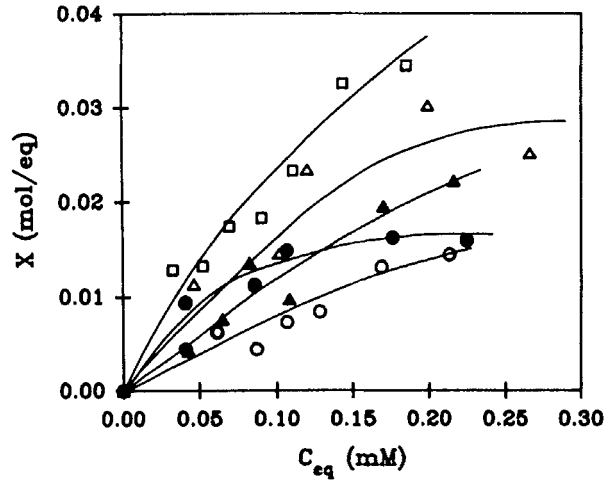

FIG. 2. The sorption isotherms for sodium glycocholate in 5.0×10^{-2} *M* phosphate buffer at 20°C. (○) TMG-P; (●) TMG-Ala_3-P; (△) TMG-Lys-Ala_3-P; (▲) TMG-Lys_3-Ala_3-P; (□) TMG-Lys_5-Ala_3-P.

TMG-Lys_5-Ala_3 > TMG-Lys_3-Ala_3 ≈ TMG-Lys-Ala_3 ≈ TMG-Ala_3 > TMG. The two latter isotherms show that the incorporation of the alanine-3 spacer enhances the binding capacity of the quaternized peptide resins somewhat, perhaps by rendering the binding sites more accessible. It should be noted that comparison with the isotherms given in Fig. 1 shows substantially less sorption in each case, either because of buffer effects or due to selectivity. This will be considered in more detail below.

It is apparent that, beyond some minimum size, the binding sites become more effective as the oligopeptide sequence length increases. For short sequences the binding affinity depends predominantly on the primary structure, probably the ionic charge, of the oligopeptide pendant groups. As the sequence length increases it becomes sufficiently long so that it can adopt a specific conformation. As a consequence, the secondary structure becomes important in the binding process. The interaction with bile salts may very well be facilitated by such conformational changes. The importance of peptide conformation of the longer oligopeptide pendants has also been observed in the binding of bilirubin with peptide resins, where binding capacity increases markedly as the peptide pendant is extended [19].

Effect of Resin Particle Size

The capacities of these resins, even that with lysine-5 pendant groups, to bind bile acids are quite low. Since the polyacrylamide beads used as the support were rather highly crosslinked (11% according to the supplier), experiments were made to determine the effect of decreasing the size of resin particles by grinding them to a powder. The capacity to bind sodium cholate was remarkably enhanced, by roughly a factor of 2, when the ground form of the resin was used (Fig. 3). Studies of the kinetics of sorption indicated that equilibrium capacites were achieved in both cases.

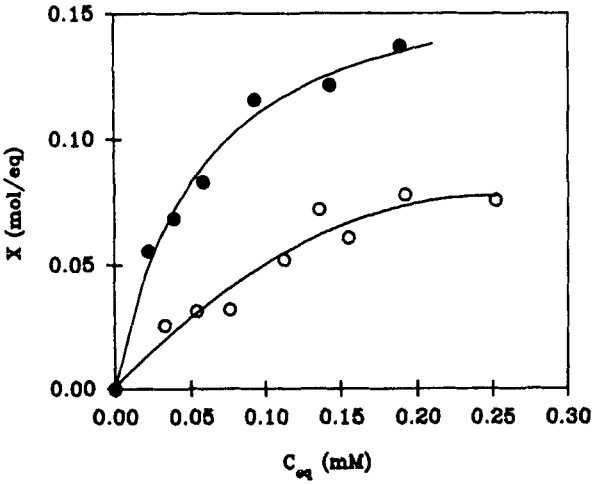

FIG. 3. The sorption isotherms of the resin containing lysine-5 in Tris buffer at 20°C. Sorbate: Sodium cholate in 5.0×10^{-3} *M* Tris buffer. (○) TMG-Lys_5-Ala_3-P; (●) TMG-Lys_5-Ala_3-P (ground).

Thus, these data suggest that, even in the swollen state, the matrix of the beads cannot be penetrated easily by the bile salt anions. In fact, a substantial fraction of the possible binding sites appears to remain isolated from the ligands, simply because it is too difficult for the bile salt anions, which are rigid molecules of fairly large size, to get sufficiently close to these binding sites. The ground form of the resin has a much larger surface area than the bead form. When the surface area increases, the fraction of quaternary amine groups that are located on the surface of the resin is also increased and more binding sites are exposed to the bile salt anions. This, in turn, facilitates the interaction between the resin and the ligands, thus resulting in a higher binding capacity. Furthermore, since the functional groups are located on the surface, the interaction with bile salt carboxyl groups is strengthened because of the shortened distance between the interacting groups. The less accessible groups cannot participate in the interaction or they participate only in weaker interactions. Similar effects of resin surface area were observed in the sorption of unconjugated bilirubin with polymeric resins [24].

Comparison of Different Buffers and Ionic Strength

The bile salt binding characteristics of the peptide-containing resins are different from those of cholestyramine and colestipol, presented in a previous paper [8]. The binding with the peptide-containing sorbents is less affected by changes in the nature of the sorbate. Switching from phosphate buffer to Tris buffer, the sorption capacity of cholestyramine for bile salt is greatly reduced since this anion exchanger is also a good sorbent for inorganic anions, including ions such as $H_2PO_4^-$ and HPO_4^{2-} [8]. As shown in Fig. 4, the binding affinity of the quaternized resin containing oligopeptide pendant groups with lysine-5 in its sequence is essentially identical

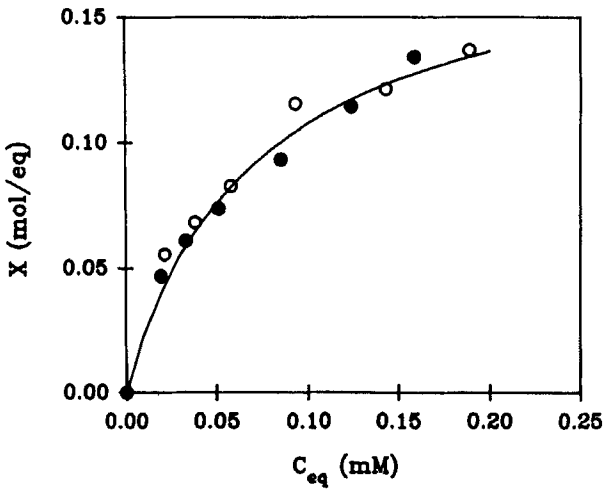

FIG. 4. The sorption isotherms of the quaternized peptide resin containing lysine-5 (TMG-Lys_5-Ala_3-P, ground) in different buffers at 20°C. (○) Sodium cholate in 5.0×10^{-3} *M* Tris buffer ($I = 5.0 \times 10^{-3}$ *M*); (●) sodium cholate in 5.0×10^{-3} *M* phosphate buffer ($I = 7.5 \times 10^{-3}$ *M*).

for cholate in Tris and phosphate buffer systems (ionic strengths are 5.0×10^{-3} and 7.5×10^{-3} M, respectively). This indicates that the specificity of the peptide-containing resins have a certain specificity for bile salts. The quaternized peptide resin containing the lysine-5 sequence is a much better sorbent than cholestyramine in the phosphate buffer. At relatively low ionic strength, the binding affinity of this resin for bile salts remains the same regardless of the existing anions of the buffer system. The specificity of the quaternized peptide-containing resin for bile salts is a further indication that the binding is not merely an ion-exchange process, as in the cases of cholestyramine and colestipol. The peptide-containing resins apparently do not have the same high affinity for the inorganic anions.

Although the quaternized peptide resins have a certain degree of specificity for bile acids, their affinity for bile salts is still affected by the ionic strength of the buffer. As shown in Fig. 4, for phosphate buffer the binding of the bile salts by quaternized peptide resins is reduced, roughly by a factor of 3, by the increase in the buffer concentration from 5.0×10^{-3} to 5.0×10^{-2} M (ionic strengths 7.5×10^{-3} and 7.5×10^{-2} M, respectively). Since the energy of the electrostatic interactions varies inversely with the dielectric constant of the medium, the ionic bond should be weakened by the higher ionic strength of the solution. The specificity of the peptide-containing resins for bile salts implies only a preference in the binding process. Given other anions in sufficiently high concentrations at high ionic strength, the resin will also interact with the anions that are available in the immediate surroundings. In this case a simple mass action or concentration dependence dominates the binding process.

Comparison of Different Ligands

To obtain further insight regarding the specificity of the oligopeptide resins, separate isotherms were obtained for the sorption of cholate and of glycocholate in 5.0×10^{-3} M phosphate buffer by the quaternized resin containing the lysine-5 sequence. The resin shows a lower affinity for glycocholate than for cholate (Fig. 5). This offers further evidence of the importance of hydrophobic interaction.

The presence of the glycine residue makes glycocholic acid more hydrophilic than cholic acid. This is manifested by the higher water solubility of glycocholic acid in water and by the polarity sequence of bile acids [23]. More hydrogen bonds can be formed between glycocholate and water than between cholate and water. Since binding to the resin requires at least partial desolvation of the bile acid anions, more energy is needed to break the hydrogen bonds between glycocholate and water. The extra energy needed for the binding explains the lower binding affinity for glycocholate. Therefore, the hydrophobicity of the bile acid anions plays an important role in the binding process.

Bile acids are steroid-related compounds. In addition to the isolated hydroxyl groups and the carboxylic acid group on the side chain of the bile acids, the hydrophobicity is one of the most important properties of bile acids. The stereostructure of bile acids indicates that they are rather rigid molecules which have a hydrophilic side and a hydrophobic side. Therefore, hydrophobic interaction between bile salt anion and the polymeric resin is an important factor during the binding process. To bind well to the resin, both the hydrophilic and hydrophobic moieties of the molecule must adjust to have the conformation of lowest energy.

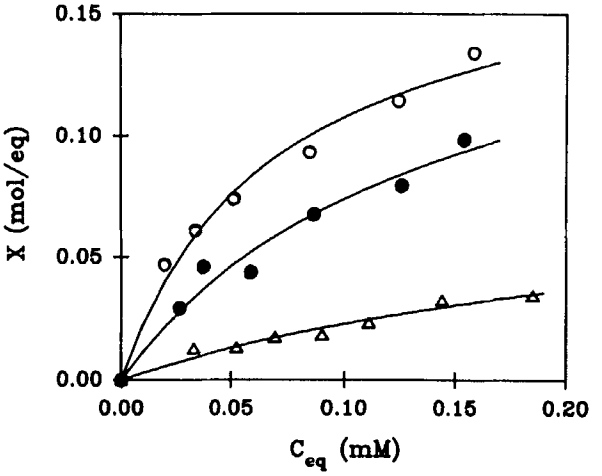

FIG. 5. The sorption isotherms (20°C) of the quaternized peptide resin (TMG-Lys_5-Ala_3-P, ground) showing the effect of ionic strength and of different ligands. (○) Sodium cholate in 5.0×10^{-3} *M* phosphate buffer ($I = 7.5 \times 10^{-3}$ *M*); (●) sodium glycocholate in 5.0×10^{-3} *M* phosphate buffer; (△) sodium glycocholate in 5.0×10^{-2} *M* phosphate buffer ($I = 7.5 \times 10^{-2}$ *M*).

The Stoichiometric Binding Constants and Free Energy Changes

The thermodynamic aspects of the binding of bile acid anions to the active sites of resins have been described in a previous paper [8]. The affinity of the binding may be expressed by the stoichiometric binding constant, *K*, which is the equilibrium constant for the binding process. The stoichiometric binding constant *K* can be derived from the best fit to the sorption isotherm assuming simple, independent binding of a single bile acid anion to a given identical site of the resin [8]. These binding constants can then be related to the free energy change by

$$\Delta G^\circ = -RT \ln K$$

The results of the calculations are shown in Table 1. It can be seen from this table: 1) The binding affinities of the quaternized peptide-containing resins generally follows the order TMG-Lys_5-Ala_3 > TMG-Lys_3-Ala_3 ≈ TMG-Lys-Ala_3 > TMG; 2) compared with cholestyramine, the affinities of these resins are less sensitive to the ionic strength of the buffer solution. This is an indication of their specificity or selectivity for bile acids anions in the presence of other anions; 3) the hydrophobicity of the bile acids contributes favorably to the interaction, as indicated by the higher binding affinity for cholate than for glycocholate.

As part of the attempt to obtain biocompatible sorbents for bile acids, the water-swellable polymer sorbents consisting of oligopeptide sequences and quaternary amine groups have been prepared and tested in aqueous buffer solutions for the sorption of bile acids. In both Tris and phosphate buffers, the new sorbents with relatively long peptide chains (especially that with lysine-5 in its sequence) have shown a greater capacity, on a per active site basis, for bile acids than cholestyramine and colestipol. Unlike other polymer sorbents, these resins were less affected

TABLE 1. The Binding Constants and Free Energy Changes for the Sorption of Bile Acids at 20°C

Resin	Functionality, mmol/g	$K \times 10^{-3}$ M^{-1}	$\triangle G°$, kJ/mol	Note
I^-TMG^+-P	0.16	0.24	−13	(NaC, Tris $I = 5.0 \times 10^{-3} M$)
		0.071	−10	(NaGC, phos $I = 7.5 \times 10^{-2} M$)
TMG-Ala_3-P	0.16	0.094	−11	(NaGC, phos $I = 7.5 \times 10^{-2} M$)
TMG-Lys-Ala_3-P	0.18	0.22	−13	(NaC, Tris $I = 5.0 \times 10^{-3} M$)
		0.13	−12	(NaGC, phos $I = 7.5 \times 10^{-2} M$)
TMG-Lys_3-Ala_3-P	0.19	0.23	−13	(NaC, Tris $I = 5.0 \times 10^{-3} M$)
		0.11	−12	(NaGC, phos $I = 7.5 \times 10^{-2} M$)
TMG-Lys_5-Ala_3-P	0.17	0.43	−15	(NaC, Tris $I = 5.0 \times 10^{-3} M$)
		0.22	−13	(NaGC, phos $I = 7.5 \times 10^{-2} M$)
TMG-Lys_5-Ala_3-P (ground)	0.17	1.1	−17	(NaC, Tris $I = 5.0 \times 10^{-3} M$)
		1.1	−17	(NaC, phos $I = 7.5 \times 10^{-2} M$)
		0.47	−15	(NaGC, Tris $I = 5.0 \times 10^{-3} M$)
		0.76	−16	(NaGC, phos $I = 7.5 \times 10^{-2} M$)

[a]TMG: the iodide form of trimethylglycine; P: polymer support; NaC: sodium cholate; NaGC: sodium glycocholate; Tris: Tris buffer, phos: phosphate buffer; I: ionic strength of the buffer.

by the sorption media, indicating that the resins containing peptide sequences may have a certain specificity for bile salt anions. The resins with longer peptide sequences would be preferred since they had a greater affinity for bile acids. This increased affinity may be due to the conformational arrangement of the longer peptide chains. The higher binding capacity of the ground form of the resin indicated the water-swellable resin matrix could not be penetrated by the bile acid molecules and that the binding was dependent on the availability of the active sites. Therefore, resin sorbents of smaller bead size or of larger pore size would make the binding sites more accessible.

ACKNOWLEDGMENTS

The authors are grateful to Mr. J. A. Rochemont and Mr. N. Franco from the Clinical Research Institute of Montreal for their kind help in the analysis of the amino acid content of the peptide resins. X.X.Z. thanks McGill University for the Clifford C. F. Wong and the Dalbir Bindra Fellowships. Financial support from the Natural Sciences and Engineering Council of Canada and the Quebec Government (Fonds FCAR) is gratefully acknowledged.

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Received August 21, 1991