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Quantification of the Binding Tendencies of Cholestyramine II: Mechanism of Interaction with Bile Salt and Fatty Acid Salt Anions

WILLIAM H. JOHNS and THEODORE R. BATES

Abstract □ The binding of a series of conjugated bile salt and fatty acid salt anions to cholestyramine from aqueous media was investigated and the data were plotted according to the Langmuir adsorption equation. Increases in affinity constants were noted as the number of hydroxy substituents on the bile salt-ring structure decreased. An increase in the chain length of the fatty acid salt caused a corresponding increase in the affinity constant, whereas an increase in the extent of unsaturation in the fatty acid chain produced a reduction in the affinity constants for the fatty acid–cholestyramine interaction. Apparent surface tension-lowering properties of the adsorbate molecules were found to parallel the affinities obtained for both classes of adsorbate molecules, with the exception of the fatty acid anion, linoleate. Based on the results of these studies, it is suggested that the binding mechanism involves a primary electrostatic component reinforced by a secondary nonelectrostatic interaction, the strength of the latter force being dependent on the degree of hydrophobicity of the adsorbate molecule.

Keyphrases □ Cholestyramine binding—quantification □ Bile salts, fatty acid salt anions—cholestyramine interaction mechanism □ Surface tension values–affinity constants, correlation—bile salts □ Hydrophobic character relationship, adsorbate—cholestyramine binding □ UV spectrophotometry—analysis

In previous work with the anionic exchange resin cholestyramine (1), the authors studied the effect of the physiologic electrolytes, sodium chloride and bicarbonate, on the binding process of bile salt anions to cholestyramine. The dihydroxy bile salt anions studied were noted to be insignificantly affected in their extent of interaction with the resin, while the trihydroxy bile salt anion–cholestyramine interactions were markedly reduced in the presence of an added electrolyte. These results suggested that a secondary, nonelectrostatic, type of interaction was taking place at the adsorption site.

The observation that structurally different bile salt anions exhibit varying types, as well as extents, of binding to cholestyramine is of biologic significance in that an appreciation of the possible modes of interaction could ultimately contribute to an enhancement in the efficiency of this pharmacologically important resin.

The purpose of this study was to elucidate the nature of this secondary binding mechanism and the effect of adsorbate structure thereon. In order to accomplish this, the binding tendencies of a selected series of glycine-conjugated bile salts and various physiologic fatty acid salts to cholestyramine were investigated.

EXPERIMENTAL

Materials—The sodium salts of glycocholic acid,[1] glycodeoxycholic acid,[1] glycodehydrocholic acid,[1] glycolithocholic acid,[1] lauric acid,[2] and oleic acid[3] were dried *in vacuo* for at least 48 hr. prior to use. The sodium salt of linoleic acid[3] was prepared by reacting equimolar quantities of the acid with sodium ethylate in absolute alcohol. The resulting salt was washed several times with absolute alcohol, dried at room temperature, and subjected to vacuum desiccation. The cholestyramine[4] employed in this study was of pharmaceutical grade (1). Reagent grade concentrated sulfuric acid, glacial acetic acid, hydrochloric acid, chloroform, sodium hydroxide, copper nitrate, *n*-butanol, and diethyldithiocarbamate were used as received.

Procedure for Adsorption Studies—A series of aqueous solutions of each bile salt and fatty acid salt was prepared over the concentration range of 0.75–5.0 millimolar (m*M*).[5] Twenty-five-milligram samples of cholestyramine were accurately weighed and placed into 50-ml. glass-stoppered conical flasks, together with a 25.0-ml. portion of the adsorbate solution. At each concentration, a control flask was prepared containing a similar quantity of the solution under study but no cholestyramine. These latter control solutions, which were assayed concomitantly with the solutions exposed to cholestyramine, were used to prepare the required Beer's law plots.

[1] Grade A. Obtained from Calbiochem Co., Los Angeles, Calif.
[2] Obtained from Eastman Organic Chemicals, Rochester, N. Y.
[3] Obtained from Fisher Scientific Co., Fair Lawn, N. J.
[4] Supplied by Merck and Co., Inc., Rahway, N. J.
[5] All systems exhibited complete solution over the concentration range studied, with the exception of the higher concentrations of glycolithocholate, which showed slight turbidity. Adsorption, being a dynamic process, would tend to increase the apparent solubility of a relatively insoluble material by removing molecules from solution and thereby promoting solution of any undissolved material.

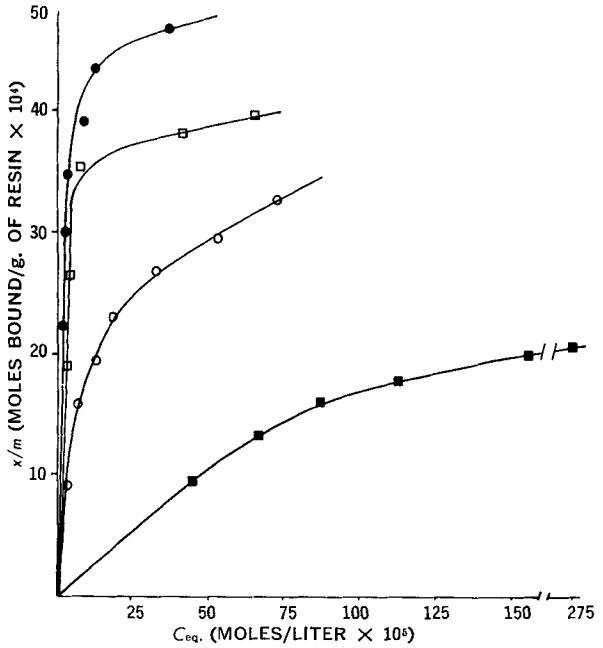

Figure 1—*Adsorption isotherms for the binding of glycine-conjugated bile salt anions to cholestyramine at 25°. Key: glycolithocholate* (●), *glycodeoxycholate* (□), *glycocholate* (○), *and glycodehydrocholate* (■).

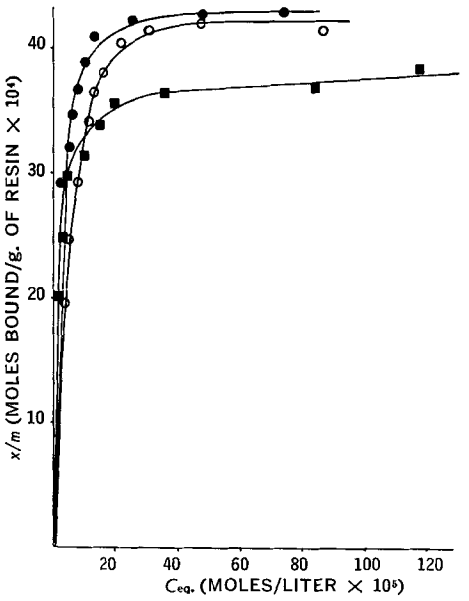

Figure 2—*Adsorption isotherms for the binding of several physiologic fatty acid salt anions to cholestyramine at 25°. Key: oleate* (●), *linoleate* (○), *and laurate* (■).

All containers were closed securely and mechanically shaken[6] at 25° until equilibrium was established; this normally occurred within 24 to 48 hr. The equilibrated samples were subjected to Millipore filtration (0.45-μ pore size), the filtrates suitably diluted or concentrated, and the equilibrium bile salt or fatty acid salt concentration determined (see *Assay Procedures*).

Procedure for Desorption Studies—In order to determine the desorption characteristics of the bound adsorbate molecules, 25.0-ml. quantities of each bile salt or fatty acid salt solution were prepared and shaken with 25.0 mg. of cholestyramine in 125-ml. glass-stoppered conical flasks. After attainment of equilibrium, the samples were diluted with 50.0-ml. portions of distilled water and agitated until equilibrium was again established. After filtration and appropriate dilution or concentration, the filtrates were assayed for free adsorbate concentration.

Surface Tension Determinations—The surface tension-lowering properties of the adsorbate molecules were studied at concentrations well below their respective critical micelle concentrations (CMC). All surface tension measurements were conducted at 25° using a Fisher ring tensiometer. At least eight determinations were performed for each solution and the pure solvent. Apparent surface tension-lowering (π) values were calculated from the difference between the mean surface tension value obtained for distilled water and the adsorbate solutions.

Assay Procedures—The equilibrium concentration of unbound or free adsorbate was determined by one of the following methods.

Bile Salts—Sodium glycocholate and glycodeoxycholate were determined spectrophotometrically in 65% sulfuric acid as described previously (1).

The spectrophotometric method of Minibeck (2) was modified for the determination of sodium glycolithocholate. The residue from an evaporated filtrate sample was dissolved in a 9:1 mixture of concentrated sulfuric and glacial acetic acids and the resultant solution heated at 60° for 30 min. A quantity of 9:1 acid mixture, treated in the same manner, served as a blank.

For the determination of sodium glycodehydrocholate, an aliquot of the equilibrated, filtered sample was evaporated to dryness and the residue dissolved in 0.1 *N* sodium hydroxide. Spectrophotometric determinations were made using the base as a blank.

Absorbance readings were determined using a Beckman model DB-G recording spectrophotometer. All of the adsorbates were found to obey the Beer-Lambert law relationship at their respective wavelengths of maximum absorbance (*i.e.*, glycodeoxycholate, 385 mμ; glycocholate, 320 mμ; glycolithocholate, 316 mμ; glycodehydrocholate, 282 mμ).

Fatty Acid Salts—The fatty acid salts were assayed by a modification of the method proposed by Duncombe (3). An aliquot of the equilibrated, filtered sample was acidified with 1.0 *N* HCl and the free fatty acid extracted with 15.0-ml. quantities of chloroform. A 5-ml. portion of the chloroform phase, diluted when necessary, was shaken with 2.5 ml. of a copper nitrate reagent for a period of not less than 2 min. The aqueous phase was removed by means of aspiration, and a 3-ml. aliquot of the chloroform solution was reacted with 0.5 ml. of a 0.1% w/v solution of diethyldithiocarbamate in *n*-butanol. The blue color which developed was read on a Bausch and Lomb Spectronic-20 colorimeter at 437 mμ using chloroform, treated in an identical manner, as the blank. All fatty acid salts under investigation followed the Beer-Lambert relationship.

The amount of bile salt or fatty acid salt bound to cholestyramine was calculated from the difference between the initial concentration of adsorbate introduced into the system and the concentration present free in solution at equilibrium.

RESULTS AND DISCUSSION

All adsorption experiments were conducted in aqueous solution. Under these conditions, the pH of the systems after equilibration with cholestyramine was essentially independent of initial adsorbate concentration. Based on reported pKa values (4) for the bile acids employed in this study,[7] at concentrations below their respective critical micelle concentrations, it was previously determined (1) that the bile salts were present essentially in the ionized form.

There is scanty information in the literature pertaining to the pKa values of long-chain fatty acids. Ralston (5) reports that small decreases in dissociation constants of fatty acids are noted with increasing molecular weight. Using a method for apparent pKa determination described by Gumtow (6), the apparent pKa's for

[6] Precision Constant Temperature Shaker Bath, Precision Scientific Co., Chicago, Ill.

[7] Ekwall *et al.* (4) report pKa values of 4.99 for lithocholic acid and 4.91 for dehydrocholic acid. Using the equilibrium pH's of 6.66 for the glycolithocholate system and 6.30 for that of the glycodehydrocholate system, percentages ionized were calculated to be 97.9 and 96.1%, respectively. Since the pKa values of glycine conjugates are normally lower than those of the unconjugated bile salts (9), the use of the reported lithocholic and dehydrocholic acid pKa's is a justifiable approximation.

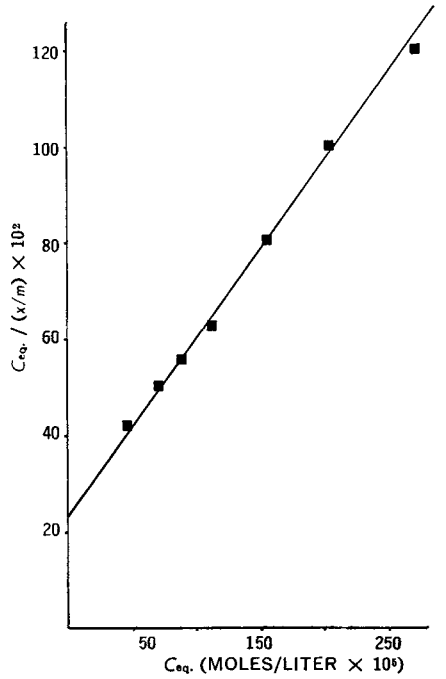

Figure 3—*Langmuir adsorption isotherm for the binding of the glycodehydrocholate anion to cholestyramine at 25°.*

lauric, oleic, and linoleic acids were found to be 4.92, 5.35, and 5.10, respectively. The value obtained for oleic acid is similar to that reported for stearic acid (7) (pKa = 5.75 at 35°), while that of lauric acid is in close agreement with the 4.96 value reported by Markley (8) for nonanoic acid. These experimental values, together with equilibrium pH's (laurate, 6.90; oleate, 6.30; linoleate, 6.10), were used in the Henderson-Hasselbach equation to obtain values of 98.9, 91.3, and 91.0% ionized, respectively. Using this

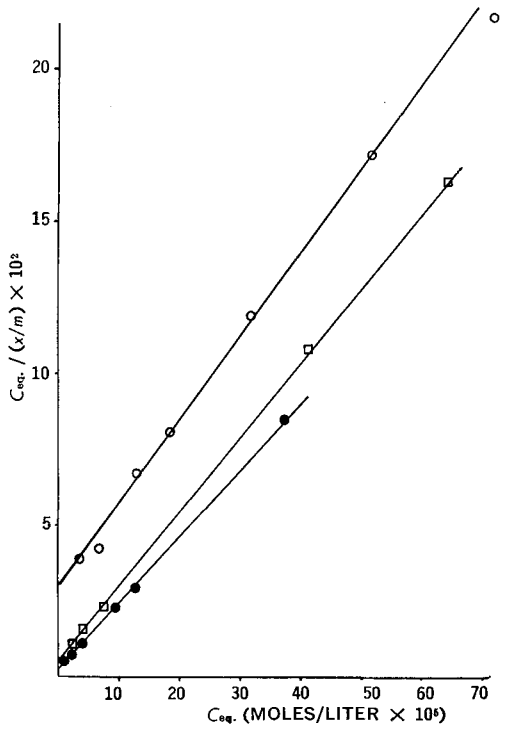

Figure 4—*Langmuir adsorption isotherms for the binding of glycine-conjugated, hydroxy-substituted bile salt anions to cholestyramine at 25°. Key: glycocholate (○), glycodeoxycholate (□), and glycolithocholate (●).*

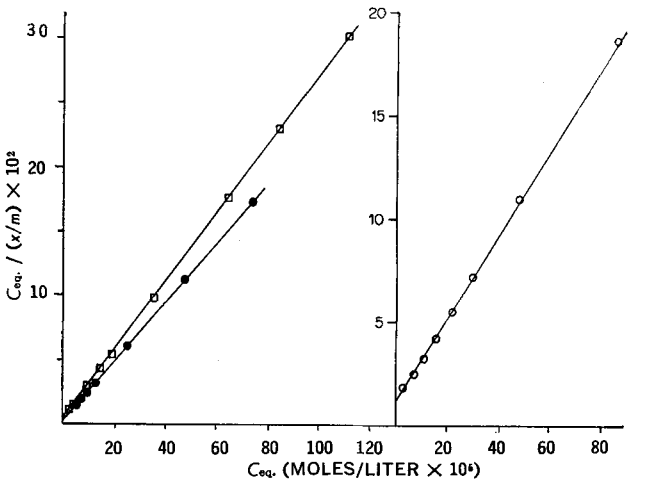

Figure 5—*Langmuir adsorption isotherms for the binding of fatty acid salt anions to cholestyramine at 25°. Key: laurate (□), oleate (●), and linoleate (○).*

as a first approximation, the fatty acids under study were considered to be completely ionized.

Adsorption Studies—The adsorption isotherms describing the binding of the investigated bile salt and fatty acid salt anions to cholestyramine were plotted according to the following Langmuir equation (10) and are shown in Figs. 1 and 2.

$$x/m = \frac{k_1 k_2 (C_{eq.})}{1 + k_1 (C_{eq.})} \qquad \text{(Eq. 1)}$$

where $C_{eq.}$ = the concentration of adsorbate molecules remaining in solution at equilibrium, x/m = the number of moles of adsorbate bound per gram of adsorbent, k_1 = the association or affinity constant, and k_2 = the capacity constant. The general shape of the x/m *versus* $C_{eq.}$ plots is representative of only monolayer adsorption (11). The curves show a tendency to plateau at high $C_{eq.}$ values, indicating that the system is approaching the limiting monomolecular-exchange capacity of cholestyramine for the particular adsorbate molecule.

Figures 3–5 represent the data plotted according to a rearranged form of Eq. 1:

$$\frac{(C_{eq.})}{x/m} = \frac{1}{k_1 k_2} + \frac{(C_{eq.})}{k_2} \qquad \text{(Eq. 2)}$$

The linearity observed with all adsorbate anions under study demonstrates the adherence of the binding process to the Langmuir-type adsorption isotherm (Eq. 2). The adsorption constants, k_1 and k_2, were obtained from the least-squares intercept and slope values of a regression line drawn through the data points and are reported in Table I.

Except for the glycodehydrocholate anion, the capacity constants, k_2 (expressed as the number of moles of anion adsorbed per mole equivalent of cholestyramine), all demonstrate a tendency toward unity. As a possible explanation of glycodehydrocholate's behavior, it is suggested that hydration, expected of the polar substituents on the phenanthrene ring system, would be more extensive with this bile salt due to the greater polarity induced by the presence of the three keto substituents (12). This could lead to enhanced steric hindrance and possible occlusion of neighboring binding positions, thus resulting in the reduced capacity observed.

An examination of the affinity constants, k_1, for the bile salt series shows an increase in the following order: glycodehydrocholate (triketo) ≪ glycocholate (trihydroxy salt) < glycodeoxycholate (dihydroxy salt) < glycolithocholate (monohydroxy salt). Since all of the bile salt anions are glycine conjugates, the observed differences cannot be related to changes in the structure of the side chain. Reduction in steric hindrance in the ring structure when going from tri- to monohydroxy salts cannot, in and of itself, be responsible for the observed affinity differences, nor is it reasonable to assume that substitution of hydroxy groups by keto functions can cause any appreciable effects on the anionic charge strength.

Table I—Langmuir Adsorption Constants for the Binding of Conjugated Bile Salt and Fatty Acid Salt Anions to Cholestyramine at 25°

Anion	k_1 (l./mole of Adsorbate, $\times 10^{-4}$)	$(k_2)^a$ (Moles of Adsorbate Bound per Mole Equivalent of Resin)	Apparent Surface Tension Lowering (dynes/cm.)
Bile salt			
Glycodehydrocholate	0.163	0.593	7.9[b]
Glycocholate	0.891	0.863	12.0
Glycodeoxycholate	4.25	0.941	23.7
Glycolithocholate	5.70	1.12	36.6
Fatty acid salt			
Laurate	5.23	0.892	10.8[c]
Oleate	7.39	1.01	38.0
Linoleate	1.92	1.03	31.8

[a] Based on a monomer equivalent weight for cholestyramine of 230. [b] Based on surface tension determinations of 2.0 m*M* bile salt solutions. [c] Based on surface tension determinations of 0.25 m*M* fatty acid salt solutions.

As stated earlier, the presence of three keto groups in the glycodehydrocholate system confers a greater hydrophilic character to this anion (12). This increased hydrophilicity would lead to a more extensive association of glycodehydrocholate with water molecules, thus producing an anion possessing larger molecular dimensions than the less hydrated hydroxy derivatives. The increased bulkiness of the glycodehydrocholate molecule could effectively prevent the close proximity of this adsorbate to the binding positions on cholestyramine and hence reduce the possibility for a significant degree of interaction. This results from the fact that the strength of the forces involved in the adsorbate-adsorbent interaction would tend to decrease with increasing separation of the two reactants. The results obtained in the present investigation are consistent with ion-exchange phenomenon in general, in that ion-exchange resin selectivity, which is related directly to the strength of adsorbate-adsorbent interaction (13), decreases as the extent of hydration of the adsorbate increases (14). In addition, the fact that the triketo-substituted bile salts exhibit negligible tendencies to undergo micelle formation as compared with hydroxy-substituted derivatives (15) would seem to indicate that their participation in nonpolar interactions, in general, is rather limited. The interrelated effects of an increase in the molecular dimensions due to the increased hydration of this adsorbate molecule and a decrease in its capability of significant nonpolar interactions with the resin matrix are probably responsible for the diminution in the affinity observed with the glycodehydrocholate–cholestyramine interaction.

The data clearly suggest that the primary electrostatic interaction (*i.e.*, between the anionic carboxyl group of the bile salt and the cationic quaternary ammonium group of cholestyramine) is being reinforced by secondary, nonelectrostatic binding forces existing between the hydrophobic portions of the bile salt and resin molecules. As the degree of polarity of the ring system, and hence the degree of hydration, increase (glycolithocholate < glycodeoxycholate < glycocholate ≪ glycodehydrocholate), the hydrophobicity, and hence the strength of the nonelectrostatic component of the interaction, diminish. These results parallel those obtained by Rudman and Kendall (16) who investigated the interaction of a series of bile salts with the protein, albumin. This phenomenon was also qualitatively alluded to in the work of Gordon *et al.* (17), in which the hydroalcoholic, chromatographic elution sequence of conjugated bile acids from a column composed of the acetate form of an anion-exchange resin (Dowex 1-X2) was studied.

The adsorption characteristics of a series of physiologic, long-chain fatty acid salts were examined to establish whether or not a similar secondary, nonelectrostatic binding mechanism was operable. The k_1 values for the laurate and oleate anions, listed in Table I, show an increase in affinity with increasing chain length. Consistent with these results, the studies of Boyer *et al.* (18) and Ballou *et al.* (19) demonstrated an intensification of fatty acid interaction with albumin as the chain length of the adsorbate molecule increased. In connection with fatty acid-albumin binding, Goldstein (20) states that "... the primary bond is presumably electrostatic, but the resulting complex is probably stabilized by van der Waals' forces through the close approximation of the nonpolar residue to similar portions of adjacent protein surfaces." The increase of six carbons, in the case of the oleate anion over that of the laurate anion, confers to the former anion a higher degree of hydrophobic character (even in the presence of the hydrophilic double bond) which results in a magnification of the strength of interaction.

The introduction of a second double bond into the adsorbate molecule, as in the linoleate system (9,12-diene), produced a marked decrease in the affinity constant relative to the oleate anion. The increase in the hydrophilicity of the carbon chain, and thus a weakening of the strength of the nonelectrostatic component of the interaction, could account for the observed reduction in the affinity. In addition, the presence of a second alkene linkage might contribute to this reduction by placing steric restrictions on the mobility of the linoleate molecule; the *cis-cis* configuration of the molecule prohibiting the proximity of adsorbate and adsorbent molecules required for significant nonionic interaction.

Desorption Studies—Under the experimental conditions employed, the studies designed to determine the dissociation or desorption characteristics of the adsorbent-adsorbate complexes showed the binding process to be essentially nonreversible.

Surface Tension Studies—The bile salt and fatty acid anions employed in this investigation all possess surface-active properties in aqueous solution and, as a result, have some facility to concentrate at interfaces. In general, the degree of surface activity elicited is dependent on the existence of a proper balance between the hydrophilic and hydrophobic regions of the molecule, normally increasing with increasing hydrophobicity (21). Since it was the objective of this investigation to establish the role of a secondary, nonelectrostatic type of interaction in the binding process, it was of interest to determine the air-water surface adsorption tendencies of the various bile salt and fatty acid anions. The purpose, therefore, of these surface tension studies was to ascertain whether a parallelism existed among the degree of hydrophobicity of the adsorbate molecules, their binding affinity, and their surface tension-lowering properties.[8]

In Table I are listed the apparent surface tension-lowering (π) values obtained for the various salts under investigation. A comparison of the bile salt values with their respective affinity constants shows that an excellent rank order correlation exists between the two parameters. Likewise, the surface activity of the laurate and oleate systems compares well with their respective k_1 values.

The deviation of the linoleate system from the parallelism found to exist with the other fatty acid salts is thought to be due to the molecular structure of that fatty acid salt. The adsorption process, being heavily dependent on proper steric alignment for maximum nonelectrostatic interaction, would be seriously hindered by the *cis-cis* configuration offered by the linoleate anion. However, the surface tension properties of this anion would not be as drastically affected, since the nonpolar portions of the molecule would be capable of assuming any packing arrangement at the air-water interface commensurate with maximum thermodynamic stability. In addition, it is possible that a minor quantity of contamination was present in the linoleate system. Such an occurrence could produce a significant change in the surface tension-lowering capabilities of the salt below the CMC (22), while at the same time having only negligible effects on the binding characteristics of the anion.

The results of this investigation indicate that the affinity with which fatty acid and bile salt anions bind to cholestyramine is, in part, dependent on the extent of hydrophobic character of the adsorbate anion. A relationship was found to exist between the surface activity of adsorbate molecules, with the exception of linoleate, and the strength of the adsorbate-cholestyramine interactions as reflected by the magnitudes of the association constants for the respective complexes.

Apparently a similar interaction mechanism is functioning in both the bile salt and fatty acid salt series. It would not be unrealistic, therefore, to imagine an *in vivo* situation where a competition existed between endogenous bile salts and exogenous fatty

[8] It should be pointed out that the authors do not intend to imply that possession of surface activity is a necessary prerequisite for significant interaction with cholestyramine.

acids, the latter being present either in food or generated in the small intestine by the action of digestive enzymes on dietary triglycerides. Such a competition, depending on the nature and relative concentration of the fatty acid, could cause a reduction in the therapeutic efficiency of cholestyramine to sequester certain bile salt anions. Studies to determine the nature and extent of any competition which may exist between bile salt anions and other physiologic substances for the binding positions on cholestyramine are in progress and will be the subject of subsequent communications.

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Flexible Nonisothermal Stability Studies

H. V. MAULDING and M. A. ZOGLIO

Abstract □ A method is described which allows *ad libitum* temperature adjustment during the course of a nonisothermal kinetic study. The data obtained are compared to theoretical degradation patterns to obtain from a single experiment activation energy, reaction rates, and stability predictions at any desired temperature. The inversion of sucrose and the hydrolysis of ethyl acetate are studied to demonstrate the validity of the theory and the advantages of the method.

Keyphrases □ Stability studies—flexible, nonisothermal □ Kinetic equations—flexible nonisothermal stability studies □ Sucrose inversion—nonisothermal stability methodology □ Ethyl acetate hydrolysis—nonisothermal stability methodology □ Polarimetry—analysis

The field of nonisothermal kinetics has grown considerably in popularity since the classic treatment by Rogers (1) was published in 1963. Since then numerous publications have appeared in the literature utilizing nonisothermal techniques (2, 3). Others have introduced new ideas and techniques to the field (4, 5). The objective of this study is to eliminate the need for a fixed time-temperature profile during the course of a nonisothermal study. The advantages of such an approach lie in the freedom to change temperature at a rate consistent with analytical findings and also in minimizing experimental requirements. The method involves the subjection of a solution of the substance for study to changing temperature to provide sufficient breakdown for calculation of activation energy, reaction rates, and stability predictions. The degradation is controlled by adjusting the rate of change of temperature according to analytical findings during the experiment. The time–temperature data are fitted to a polynomial expression of sufficient degree to describe the changes. This relationship and the experimental data are then combined and utilized to synthesize a series of degradation pathways corresponding to different levels of activation energy. The curves are compared to the experimental analytical data to obtain the correct energy of activation for the reaction. Utilizing this activation energy and the analytical data, reaction rate and stability calculations can be made.

THEORETICAL

Consider a drug in solution degrading according to some unchanging reaction order as in Fig. 1. Drug concentration can then