Mechanism of Cholesterol Gallstone Dissolution

II. Correlation between the Effect of the Alkyl Amines as Cholesterol Gallstone Dissolution Rate Accelerators and the Degree of Binding of the Alkyl Amines to the Bile Acid Micelles[1]

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Recent studies have shown that cholesterol monohydrate pellet dissolution rates in real and synthetic bile systems are enhanced dramatically in the presence of small concentrations of amines, quaternary ammonium compounds, and cationic surfactants. Furthermore, these studies have shown that the mechanism of action of these compounds involves reducing the interfacial resistance for cholesterol transfer in some manner. In the present study, data on cholesterol dissolution rates and data on the degree of binding of the amine accelerator to the bile micelle have been correlated. The micellar binding was determined using a dynamic method based upon the silicone rubber diffusion cell membrane system. It was found that the amount of bound amine correlated with the extent of cholesterol monohydrate dissolution rate acceleration and that the charged form of the amine was important. Thus, the data analysis shows that the predominant mechanism by which these amines enhance the dissolution rate involves reducing the electrical charge of the bile acid micelles (i.e., by charge neutralization).

INTRODUCTION

We have recently (1–3) shown that cholesterol monohydrate pellet dissolution rates in real and synthetic bile systems are enhanced dramatically in the presence of small concentrations of amines (primary, secondary, and tertiary) and quaternary ammonium compounds (including cationic surfactants). It appears that only positively charged compounds can function as dissolution rate accelerators since the nonionic and anionic surfactants showed little or negative accelerator activity (3).

Since cholesterol is very insoluble in water, it has been postulated (4–11) that the dissolution of cholesterol is brought about by an actual direct interaction between the bile acid micelles and the cholesterol surface. The exchange or transfer occurs in the resulting "collision complex." The process of transfer of a cholesterol molecule from the pellet surface to the bile acid micelle in the collision complex may be looked upon as a diffusion process in which the molecule moves out of its original environment into the new environment after passing through an intermediate environment which may be the rate limiting one if the interaction between the micelle and the surface is not a favorable one. Based on our observations, the following mechanisms of action of cholesterol dissolution rate accelerators have been proposed (13).

(1) The positively charged accelerator ions may reduce the electrical repulsion between a negatively charged crystal surface and the negatively charged micelle. This may take place as a result of the direct reduction in charge of the micelle when the accelerator ions are solubilized by the

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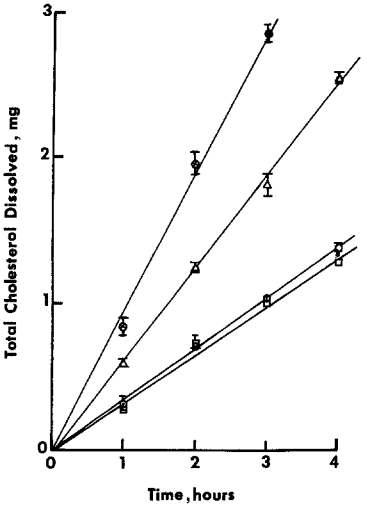

FIG. 1. Effect of pH on dissolution of cholesterol monohydrate at 37°C. System: 58 m*M* chenodeoxycholic acid, 0.1 *M* NaCl, 0.01 *M* phosphate buffer. Key: ○, pH 8.0; ●, pH 9.5; □, pH 11.0. System: 58 m*M* chenodeoxycholic acid, 0.1 *M* NaCl, 0.01 *M* phosphate buffer, 5 m*M* octylamine. Key: ⊗, pH 8.0 and pH 9.5; △, pH 11.0.

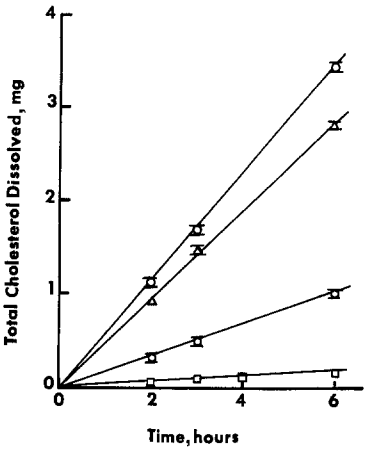

FIG. 3. Effect of octylamine concentration on dissolution of cholesterol monohydrate in 58 m*M* chenodeoxycholic acid + 0.01 *M* phosphate buffer, at 37°C. Key: □, no octylamine; ○, 2 m*M* octylamine; △, 5 m*M* octylamine; ○, 10 m*M* octylamine.

micelles, or as a result of the adsorption of the accelerator ions onto the crystal surface, or by both.

(2) The accelerator ions may not only have an effect from the standpoint of electrical interactions, but these ions, especially those with large hydrophobic tails, may also contribute toward making the intermediate environment of the collision complex less hydrophilic and, therefore, less aqueous.

(3) Finally, an accelerator molecule or ion might have direct specific catalytic effect upon the transfer rate of the cholesterol

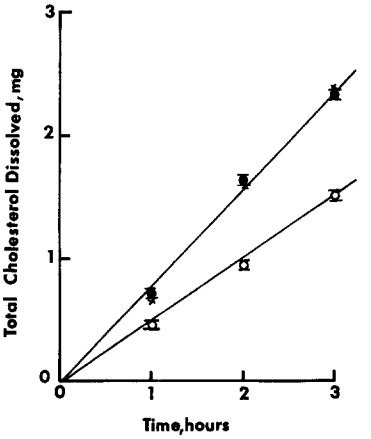

FIG. 2. Effect of pH on dissolution of cholesterol monohydrate in 58 m*M* chenodeoxycholic acid + 0.1 *M* NaCl + 0.01 *M* phosphate buffer + 5 m*M* hexylamine, at 37°C. Key: ●, pH 8.0; ×, pH 9.5; ○, pH 11.0.

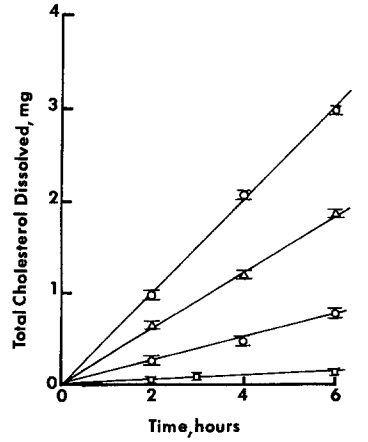

FIG. 4. Effect of hexylamine concentration on dissolution of cholesterol monohydrate in 58 m*M* chenodeoxycholic acid + 0.01 *M* phosphate buffer, at 37°C. Key: □, no hexylamine; ○, 3 m*M* hexylamine; △, 5 m*M* hexylamine; ○, 10 m*M* hexylamine.

TABLE I

Effect of pH on the Dissolution Rate, J/A, and the Solubility, C_s, of Cholesterol Monohydrate

System	pH	$J/A \times 10^4$	C_s
58 m*M* Cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	8.0	0.78	1.10
58 m*M* Cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	9.5	0.75	1.11
58 m*M* Cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	11.0	0.72	1.23
5 m*M* Octylamine, 58 m*M* cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	8.0	2.03	1.04
5 m*M* Octylamine, 58 m*M* cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	9.5	2.07	1.13
5 m*M* Octylamine, 58 m*M* cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	11.0	1.38	1.16
5 m*M* Hexylamine, 58 m*M* cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	8.0	1.75	1.07
5 m*M* Hexylamine, 58 m*M* cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	9.5	1.65	1.14
5 m*M* Hexylamine, 58 m*M* cheno, 0.1 *M* NaCl, 0.01 *M* PO_4	11.0	1.04	1.13

molecule, e.g., by a 1:1 complex in the "transition" state.

In all the past studies, meaningful structure activity relationships could not be considered since the accelerator molecules in question were bound to varying degrees to the micelles and other components of the synthetic bile. Recently, we have developed dynamic membrane transport methods to determine the degree of bound and unbound accelerator in simulated bile (12, 13).

From cholesterol dissolution rate accelerator studies utilizing the static pellet dissolution method (10) and hexylamine and octylamine as the accelerators and using the data from the determination of the degree of bound and unbound amine in simulated bile utilizing the dynamic transport methods, we should be able to gain an understanding of the possible mechanisms by which these amine compounds function as cholesterol dissolution rate accelerators.

EXPERIMENTAL

Cholesterol Dissolution Rate Determination

Theoretical considerations. A physical model approach has been utilized to study the kinetics of cholesterol monohydrate pellet dissolution *in vitro* in chenodeoxycholic acid solutions (9).

Dissolution of a nonionic, inert solid involves: (a) the contact of the solvent with the solid surface, where (b) an interaction occurs, followed by, (c) the transport of solute molecule away from the interface into the bulk solution. Usually, solvent contact occurs instantaneously, and steps (b) or (c) or both are generally considered to be rate determining.

TABLE II

Influence of Amine Concentration on the Dissolution rate, J/A, and the Solubility, C_s, of Cholesterol Monohydrate

System	pH	$J/A \times 10^4$	C_s
58 m*M* Cheno, 0.01 *M* PO_4	8	0.06	0.93
2.85 m*M* Hexylamine, 58 m*M* cheno, 0.01 *M* PO_4	8	0.29	1.06
4.94 m*M* Hexylamine, 58 m*M* cheno, 0.01 *M* PO_4	8	0.68	1.08
10.08 m*M* Hexylamine, 58 m*M* cheno, 0.01 *M* PO_4	8	1.10	1.10
1.86 m*M* Octylamine, 58 m*M* cheno, 0.01 *M* PO_4	8	0.36	0.97
5.04 m*M* Octylamine, 58 m*M* cheno, 0.01 *M* PO_4	8	0.94	1.01
10.09 m*M* Octylamine, 58 m*M* cheno, 0.01 *M* PO_4	8	1.25	1.08

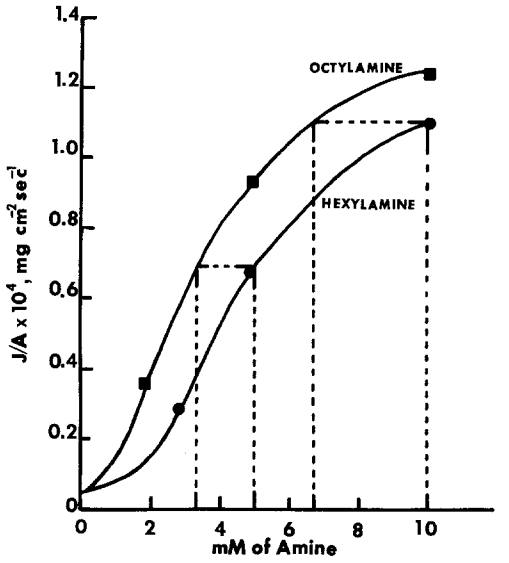

FIG. 5. Effect of amine concentration on the dissolution rate (J/A) in 58 mM chenodeoxycholic acid + 0.01 M phosphate buffer pH 8.0, at 37°C. Key: ●, hexylamine; ■, octylamine.

Berthoud (14) derived the following equation to account for both the interfacial resistance and the resistance for the transport of the solute in solution from the vicinity of the solid surface into the bulk solvent under steady-state conditions (planar case):

$$J = \frac{A(C_s - C_b)}{h/D + 1/P} = \frac{A(C_s - C_b)}{R} \quad [1]$$

where

J = dissolution rate (flux)
A = surface area of dissolving solid exposed to solution
C_s = concentration of solute in solution at saturation
C_b = concentration of solute in bulk
R = total resistance to mass transfer
D = diffusion coefficient of soluble in the solvent
h = Nernst effective diffusion layer thickness
P = effective permeability coefficient of the interfacial barrier.

When interactions at the surface occur rapidly, P becomes very large, and Eq. [1] reduces to Nernst equation (15),

$$J = \frac{AD(C_s - C_b)}{h} \quad [2]$$

and the dissolution process becomes diffusion controlled. However, if surface interactions take place relatively slowly, P becomes very small, $1/P$ becomes much greater than h/D, and Eq. [1] reduces to

$$J = AP(C_s - C_b) \quad [3]$$

and the dissolution process becomes interfacial barrier controlled.

Both the Berthoud and the Nernst theories represent only semiempirical treatments of the dissolution process and assume a purely diffusional resistance, h/D, for the transfer of solid across the diffusion layer. However, in most dissolution situations, both convec-

TABLE III

Influence of Amine Concentration on the Dissolution Rate, J/A, and the Solubility, C_s, of Cholesterol Monohydrate

System	pH	$J/A \times 10^4$	C_s
58 mM Cheno, 0.1 M NaCl, 0.01 M PO_4	8	0.78	1.10
3 mM Octylamine, 58 mM cheno, 0.1 M NaCl, 0.01 M PO_4	8	1.76	1.07
4.89 mM Octylamine, 58 mM cheno, 0.1 M NaCl, 0.01 M PO_4	8	2.03	1.04
10.30 mM Octylamine, 58 mM cheno, 0.1 M NaCl, 0.01 M PO_4	8	2.53	1.06
3.08 mM Hexylamine, 50 mM cheno, 0.1 M NaCl, 0.01 M PO_4	8	1.56	1.09
4.78 mM Hexylamine, 58 mM cheno, 0.1 M NaCl, 0.01 M PO_4	8	1.75	1.07
10.08 mM Hexylamine, 58 mM cheno, 0.1 M NaCl, 0.01 M PO_4	8	2.29	1.04

tion and diffusion are expected to be important. Thus, for example, in the rotating-disk dissolution situation (2, 16) it can be shown by the Levich treatment (17) that there may be a substantial contribution to the mass transfer by convection, and the dissolution rate is given by:

$$J = \frac{A(C_s - C_b)}{1.612D^{-2/3}\nu^{1/6}\omega^{-1/2} + 1/P} \quad [4]$$

where ν is the kinematic viscosity of the solvent, and ω is the angular velocity of rotation. The corresponding effective diffusion layer thickness may be written as:

$$h = 1.612D^{1/3}\nu^{1/6}\omega^{-1/2}. \quad [5]$$

Design and Considerations

The semiqualitative static pellet dissolution method (10) was chosen over the more quantitative rotary-disk method (2) since only one speed is required with the static pellet method. Despite its semi-empirical nature and its limitations, the static pellet method is more than adequate for the present purposes.

Systems that consist of 58 m*M* chenodeoxycholic acid with or without 100 m*M* sodium chloride and 10 m*M* sodium phosphate buffer at pH 8 were used as the model systems. Hexylamine and octylamine were used as model cholesterol dissolution rate accelerators.

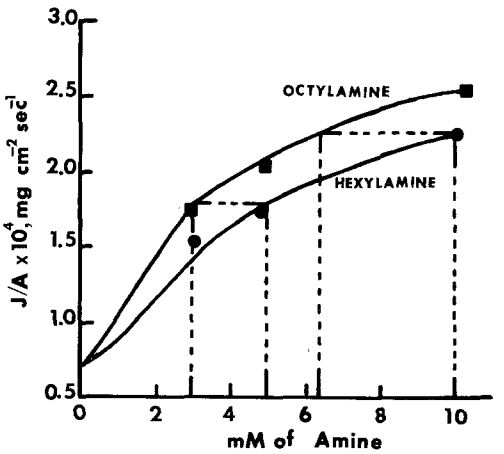

FIG. 6. Effect of amine concentration on the dissolution rate (J/A) in 58 m*M* chenodeoxycholic acid + 0.1 *M* NaCl + 0.01 *M* phosphate buffer, pH 8.0, at 37°C. Key: ●, hexylamine; ■, octylamine.

TABLE IV

Effect of the Total Amine Concentration on the Fraction of Amine Free in 58 m*M* Chenodeoxycholic Acid Solutions Containing 0.01 *M* Phosphate Buffer

System	pH	*ff*
3.0 m*M* Hexylamine, 0.01 *M* PO_4, 58 m*M* cheno	9.0	0.44
4.85 m*M* Hexylamine, 0.01 *M* PO_4, 58 m*M* cheno	9.0	0.45
9.11 m*M* Hexylamine, 0.01 *M* PO_4, 58 m*M* cheno	9.0	0.47
2.88 m*M* Octylamine, 0.01 *M* PO_4, 58 m*M* cheno	8.5	0.18
4.84 m*M* Octylamine, 0.01 *M* PO_4, 58 m*M* cheno	8.5	0.19
9.36 m*M* Octylamine, 0.01 *M* PO_4, 58 m*M* cheno	8.5	0.22

The dissolution rate per unit area, J/A, and the equilibrium solubility, C_s, were experimentally determined.

Materials

Commercial cholesterol[2] was recrystallized three times from 95% ethanol. Radioactive cholesterol monohydrate was prepared by mixing 5 g of the recrystallized cholesterol with 100 μc of a benzene solution of [4-^{14}C]cholesterol[3] (radiopurity checked by thin layer chromatography) in 400 ml of 95% ethanol at 60°C. This solution was filtered while hot and the filtrate allowed to stand for 48 hr at room temperature. Only crystals obtained from slow crystallization were used (where the first crystals were observed after 12 hr). Then the cholesterol monohydrate crystals were filtered and dried *in vacuo* for 24 hr. The crystals obtained were stored in the dark in a desiccator saturated with water vapor at room temperature.

NMR studies quantitatively confirmed the monohydrate nature of the crystals. Thin layer chromatography studies indicated the absence of any impurities (18). X-Ray crystallography[4] studies indicated that they are

[2] Eastman Kodak Co., Rochester, N. Y.

[3] New England Nuclear Corp., Boston, Mass.

[4] Kindly performed by Dr. C. Nordman, Dept. of Chemistry, The University of Michigan, Ann Arbor, Mi.

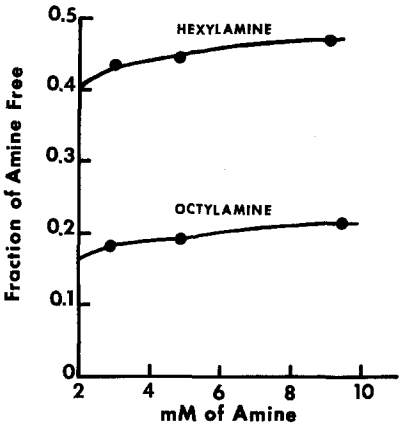

FIG. 7. Effect of amine concentration on the fraction free of the amine in 58 m*M* chenodeoxycholic acid + 0.01 *M* phosphate buffer, pH 8.0, at 37°C.

indeed cholesterol monohydrate crystals, and that they have a lattice system similar to that of cholesterol found in human biliary calculi (19).

Chenodeoxycholic acid[5] was purified by recrystallizing from *n*-propanol-ethylacetate. Sodium chenodeoxycholate solutions were prepared by titrating equivalent amounts of chenodeoxycholic acid with sodium hydroxide and the pH was then adjusted to 8.0. Hexylamine,[2] octylamine,[6] sodium monobasic phosphate,[7] sodium dibasic phosphate,[7] and sodium chloride[7] were all of analytical grade and were used as received.

[5] Canada Packers Ltd., Canada.
[6] Aldrich Chemical Co., Milwaukee, Wis.
[7] Mallinckrodt, St. Louis, Mo.

Dissolution Rate Determination (10)

Pellets of [^{14}C]cholesterol monohydrate were prepared by directly compressing about 100 mg of the material in a die, 1.27 cm internal diameter, under a force of 3000 lb/in.^{2} using a laboratory press.[8] The exposed surface area of the resulting pellets was 1.267 cm. The pellet was held firmly in die by covering the bottom with melted paraffin. The die was kept overnight in a desiccator saturated with water vapor at room temperature. This die was then placed on the bottom of a water-jacketed cylinder, with the pellet facing a stirring paddle inserted at the top of the cylinder. The stirring speed was maintained at 150 rpm during dissolution by a constant-speed motor.[9]

Exactly 10 ml of the dissolution medium preequilibrated at 37°C was added into the cylinder. Immediately the first 0.25-ml sample was withdrawn using a pipet. Four other samples were taken at suitable time intervals. The ^{14}C-labelled samples were subsequently counted using a liquid scintillation counter,[10] and the amount of cholesterol dissolved in the solvent was plotted against time.

Solubility Determination

The solubilities of cholesterol monohydrate in various solvent media were deter-

[8] Model B, Fred Carver Inc., Summit, N. J.
[9] Model CA, Hurst, Princeton, Ind.
[10] Model LS200, Bekman Instruments, Fullerton, Calif.

TABLE V

Effect of the Total Amine Concentration on the Fraction of Amine Free in 58 m*M* Chenodeoxycholic Acid Solutions Containing 0.1 *M* NaCl and 0.1 *M* Phosphate Buffer

System	pH	*ff*
2.37 m*M* Hexylamine, 0.1 *M* PO_4, 0.1 *M* NaCl, 58 m*M* cheno	9.0	0.42
4.94 m*M* Hexylamine, 0.1 *M* PO_4, 0.1 *M* NaCl, 58 m*M* cheno	9.0	0.54
11.05 m*M* Hexylamine, 0.1 *M* PO_4, 0.1 *M* NaCl, 58 m*M* cheno	9.0	0.57
2.58 m*M* Octylamine, 0.1 *M* PO_4, 0.1 *M* NaCl, 58 m*M* cheno	8.5	0.12
4.92 m*M* Octylamine, 0.1 *M* PO_4, 0.1 *M* NaCl, 58 m*M* cheno	8.5	0.13
10.10 m*M* Octylamine, 0.1 *M* PO_4, 0.1 *M* NaCl, 58 m*M* cheno	8.5	0.14

mined by introducing an excess of [^{14}C]cholesterol monohydrate, about 20 mg, into 2 ml of a solvent in a test tube. The tube was flushed with nitrogen, capped, and then shaken by a wrist-action shaker[11] in a water bath at 37°C. After 4–5 days, a sample was taken using a glass wool-wrapped, long tipped pipet preequilibrated at 37°C. Exactly 0.2 ml of the filtrate was then assayed for cholesterol using a liquid scintillation counter.[10] More samples were taken every 2 days and assayed for cholesterol. The solubility of cholesterol monohydrate in the medium was obtained when the concentration reached a constant level.

Determination of free and bound accelerator molecules in simulated bile solutions using the silicone rubber system. Theoretical considerations, materials, preparation of membrane, diffusion cell and the permeation procedure were described previously (12, 13).

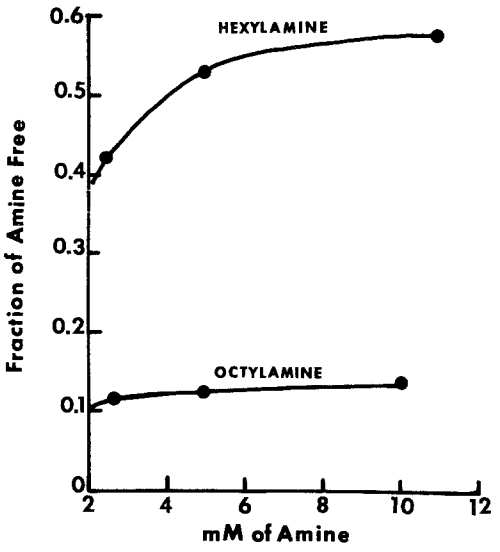

FIG. 8. Effect of amine concentration on the fraction free of the amine in 58 m*M* chenodeoxycholic acid + 0.1 *M* NaCl + 0.01 *M* phosphate buffer, pH 8.0, at 37°C.

RESULTS

Figures 1–4 show representative plots of the results of the dissolution rate experiments. As can be seen, all the plots are very linear, indicating that the solvent media remained very much undersaturated with cholesterol during the dissolution period. In order to prevent saturation with cholesterol, the faster dissolving systems were run for shorter periods. The dissolution rates were obtained by taking the slopes of the dissolution lines. The bar gives the actual spread for duplicate experiments carried out for each condition. Typically, about 5% variation was observed for the faster dissolving systems; for systems with slower rates a greater variation was observed (around 10%). At the end of the run, no noticeable change was observed in the appearance of the pellet surface, as was expected, since less than 3% of the pellet is dissolved.

Table I and Figs. 1 and 2 show the effect of pH on dissolution rate in presence and absence of the amines. In the absence of the amines, no significant effect of pH on the dissolution rate was found. However, in the presence of the amines, the dissolution rate at pH 11 was found to be about 40% slower than that at pH 8.0 and 9.5, for both hexylamine and octylamine. At pH 8.0 and 9.5, the predominant species of hexylamine (pK_a 10.56) and octylamine (pK_a 10.65) is the protonated species, whereas at pH 11, for both amines, the unprotonated species predominates. This further supports

TABLE VI

The Total Amine Concentration and the Concentration of Bound Amines at a Given Dissolution Rate, *J*/*A*, of Cholesterol Monohydrate in 58 m*M* Chenodeoxycholic Acid Solutions Containing 0.01 *M* Phosphate Buffer at pH 8.0[a]

J/*A* × 10^4	m*M* of Amine (total conc.)		m*M* of Amine bound	
	Hexylamine	Octylamine	Hexylamine	Octylamine
1.10	10	6.7	5.3	5.33
0.695	5	3.3	2.75	2.69

[a] System: 58 m*M* chenodeoxycholic acid, 0.01 *M* PO_4.

[11] Burrell Corp., Pittsburgh, Pa.

TABLE VII

The Total Amine Concentration of the Bound Amine Concentration at a Given Dissolution Rate, J/A, of Cholesterol Monohydrate in 58 mM Chenodeoxycholic Acid Solutions Containing 0.10 M NaCl and 0.01 M Phosphate Buffer at pH 8.0[a]

J/A $\times 10^4$	mM of Amine (total conc.)		mM of Amine bound	
	Hexylamine	Octylamine	Hexylamine	Octylamine
2.25	10	6.4	4.30	5.58
1.80	5	3.0	2.33	2.64

[a] System: 58 mM chenodeoxycholic acid, 0.1 M NaCl, 0.01 M PO_4.

the earlier findings (1–3) that the charged form of the amine is involved in the acceleration mechanism.

Table II and Fig. 5 show the effects of the concentration of the amines upon the dissolution rate in 58 mM chenodeoxycholic acid in 0.01 M phosphate buffer (pH 8). Table III and Fig. 6 show the effect of the amine concentration on the dissolution rate in 58 mM chenodeoxycholic acid, 0.1 M sodium chloride in 0.01 M phosphate buffer. In all cases, the dissolution rates increase with increasing amine concentration. Also, it can be seen that octylamine is more effective than hexylamine at the same concentration. In the absence of sodium chloride the rates are significantly lower. Similar effects of electrolytes were previously noted in bile acid-lecithin systems (20). Also, the effect of the amines is more pronounced in the system without sodium chloride.

Table IV and Fig. 7 show data on the fraction free (or unsolubilized) of the amines in 58 mM chenodeoxycholic acid in 0.01 M phosphate buffer taken from a recent study involving the transport of the amines across synthetic membranes. The greater solubilization of octylamine is clearly seen. Table V and Fig. 8 show similar results in the system with 0.1 M sodium chloride.

Using Figs. 5 and 6, it is possible to obtain the concentrations of hexylamine and octylamine required to give the same dissolution rates. Also, for the same conditions, one may calculate from Figs. 7 and 8 the amount of amine that is bound to the micelles and the amount that is free.

Table VI shows the results for the situation when sodium chloride was absent. When the dissolution rate is 1.1×10^{-4} mg cm^{-2} sec^{-1}, the total hexylamine is 10 mM and the total octylamine is 6.7 mM; however, as can be seen, the bound concentrations for hexylamine and octylamine are essentially the same. Similarly when the dissolution rate is 0.695×10^{-4} mg cm^{-2} sec^{-1}, the total hexylamine is 5 mM and the total octylamine is 3.3 mM but the bound amine concentrations are again essentially the same. Thus, it is clearly seen that at a given J/A the amounts of amine bound to the micelles are the same, even though the total amine concentrations may be different. Similar results were obtained with the system with 0.1 M sodium chloride (Table VII).

These experiments and the data analysis show that the predominant mechanism by which these amines enhance the dissolution rate may involve reducing the electrical charge of the bile acid micelles (i.e., by charge neutralization). Thus, the abilities of hexylamine and octylamine to enhance the dissolution rate may be directly related to their ability to bind and thereby to reduce the micellar charge.

An independent examination of the above hypothesis may be made by measuring the micellar charge (electrophoretic mobility) at equal efficacies, i.e., when equal amounts of the amines are bound to the micelles. In accompanying manuscript results of moving boundary electrophoresis experiments are reported and, indeed, it is found that the electrophoretic mobilities are essentially in quantitative agreement with the present hypothesis.

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