Mechanism of Cholesterol Gallstone Dissolution

III. Electrophoretic Studies Showing the Correlation between the Bile Micellar Charge and the Effect of Alkyl Amines as Cholesterol Gallstone Dissolution Rate Accelerators[1]

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We have shown that the cholesterol monohydrate dissolution rate acceleration in chenodeoxycholic acid solutions due to the addition of hexylamine and octylamine may be directly related to their ability to bind to the negatively charged chenodeoxycholate micelles. Based on these results, we have proposed that the primary mechanism by which these amines accelerate the dissolution rate is by reducing the micellar charge. An independent test of the above hypothesis was carried out by measuring the electrophoretic mobility of chenodeoxycholate micelles as a function of the amine concentration utilizing the moving boundary electrophoresis method in the presence of and absence of 0.1 *M* sodium chloride. At the concentrations of hexylamine and octylamine which gave the same dissolution rate, J/A, i.e., at equal efficacy, the electrophoretic mobilities were found to be the same. These results verify our hypothesis that the primary mechanism by which these amines accelerate the dissolution rate is by reducing the micellar charge. In addition, particle microelectrophoresis studies showed no significant surface charge variation with cholesterol particles as a function of amine concentration.

INTRODUCTION

For the past several years we have been interested in the mechanisms of cholesterol gallstone dissolution kinetics and how this understanding may lead to new approaches to medical dissolution of cholesterol gallstones (1–4). Our recent efforts have been to examine the possibility of the use of agents which may catalyze or accelerate the rate of dissolution of cholesterol gallstones in unsaturated bile media (5–7). These studies have lead to the finding that alkyl amines and quaternary ammonium compounds are effective in this regard.

In previous communications (8) it was shown that the dissolution rate acceleration due to hexylamine and octylamine may be directly related to their ability to bind to the negatively charged chenodeoxycholate micelles. Based on these studies (8) we have proposed that the primary mechanism by which these amines accelerate the dissolution rates is by reducing the micellar charge. The purpose of the present communication is to investigate this hypothesis more thoroughly. Here an independent test of the hypothesis is presented by measuring the electrophoretic mobility of the chenodeoxycholate micelles as a function of the amine concentration utilizing the moving boundary electrophoresis method. Further, results of a limited number of experiments on microelectrophoresis will be reported to see if there is significant surface charge variation on cholesterol particles as a function of amine concentration.

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EXPERIMENTAL

Materials

Hexylamine,[3] octylamine,[4] sodium chloride,[5] and sodium phosphate[5] (monobasic and dibasic) were reagent grade and were used as received. Chenodeoxycholic acid[6] was recrystallized from *n*-propanol-ethyl acetate mixture. Sodium chenodeoxycholate solution was prepared by titrating an equivalent amount of acid with sodium hydroxide. Cholesterol[3] was recrystallized three times from 95% ethanol.

Moving Boundary Electrophoresis

Preparation of micellar solutions for electrophoresis. Solutions containing 14.5 or 58 m*M* sodium chenodeoxycholate, 100 m*M* sodium chloride, and 10 m*M* sodium phosphate (mixture of di- and trivalent ions at pH 8.0) were prepared, and the specific conductance was measured at 0°C. Since sodium chenodeoxycholate was transported through the usual dialysis tubing, the conventional buffer solution for electrophoresis was prepared by adding 1.0 *M* sodium chloride to a solution containing 100 m*M* sodium chloride and 10 m*M* sodium phosphate (pH 8.0) until the specific conductance was essentially the same (±3%) as that of the micellar solution. Conductivity measurements were made at 0°C in the LKB conductivity cell (K = 4.6 cm^{-1}), using a Type 3216B LKB conductivity bridge.[7]

Electrophoretic analysis. Electrophoresis measurements were made with a Spinco Model H Electrophoresis-Diffusion Apparatus.[8] The experiments were conducted in a 11-ml capacity quartz Tiselius cell at a bath temperature of 1–2°C, using Schlieren optics (λ = 546 nm). Currents were adjusted so that no more than 0.07 W/cm^3 were dissipated in the optical cell, and the cell was measured for acceptable leakage levels before each run. The details of the moving-boundary equipment and procedure have been adequately described elsewhere (9–11).

Calculation of mobility. Measurements of the distance of migration were made on the ascending and descending limbs. Although the descending boundaries yield more nearly the correct values of mobilities, the values obtained from the ascending boundaries may also be used since the main purpose of this study is to obtain the relative mobilities rather than the absolute mobilities.

The mobilities may be calculated from the following equation:

$$u = \frac{d \cdot s \cdot \kappa}{i \cdot t} \qquad [1]$$

where

u = mobility ($cm^2\ V^{-1}\ sec^{-1}$)
d = distance traveled
s = cross-sectional area of the optical cell (cm^2)
κ = specific conductance of the micellar solution ($ohm^{-1}\ cm^{-1}$)
t = time (sec)
i = current flowing through the optical cell (amps).

The mobilities were corrected for the magnification factor (1.01).

Microelectrophoresis of Cholesterol Particles

Preparation of dispersions for microelectrophoresis. Dispersions of cholesterol for microelectrophoretic evaluation were prepared by dilution of concentrated stock dispersions. To prepare 100 ml of 0.5% w/v dispersion, 500 mg of the recrystallized cholesterol was first dissolved in 10 ml of hot ethanol. This solution was subjected to ultrasonic irradiation as 10 ml of water was quickly added. The resultant dispersion was then added to 80 ml of water, ultrasonified for 30 sec, treated with nitrogen, and stored in the dark. Twenty-four hours

[3] Eastman Kodak Co., Rochester, N. Y.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Mallinckrodt, St. Louis, Mo.
[6] Canada Packers, Ltd., Canada
[7] LKB Instruments, Inc., Rockville, Md.
[8] Beckman, Spinco Div., Palo Alto, Calif.

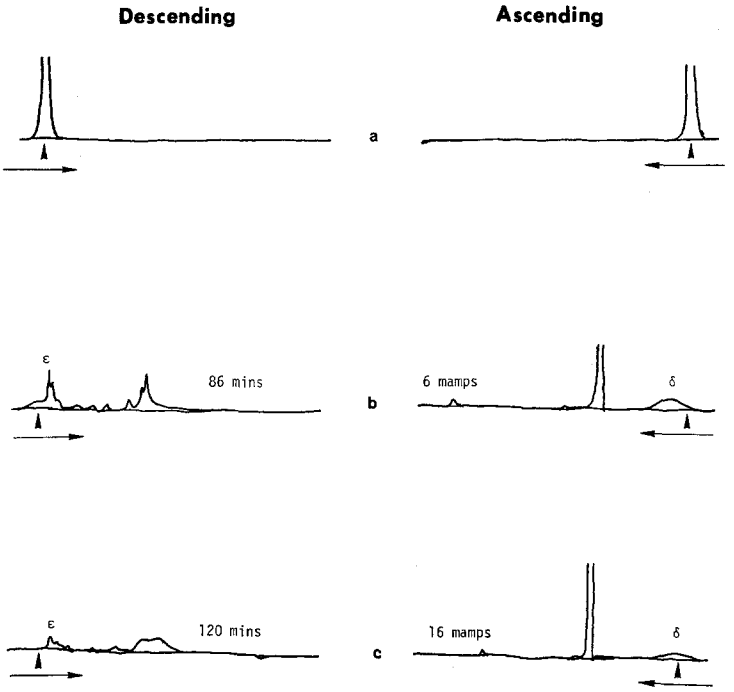

FIG. 1. Electrophoretic patterns. (a) Initial boundaries. (b) 14.5 mM sodium chenodeoxycholate, 0.01 M phosphate buffer, pH 8.0, at 2°C. (c) 14.5 mM sodium chenodeoxycholate, 0.1 M NaCl, 0.01 M phosphate buffer, pH 8.0, at 2°C.

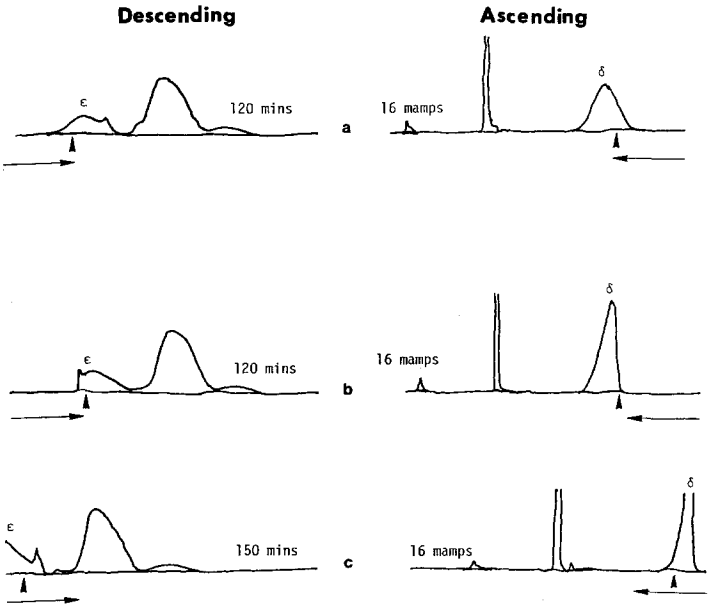

FIG. 2. Electrophoretic patterns. (a) 58 mM sodium chenodeoxycholate, 0.1 M NaCl, 0.01 M phosphate buffer, pH 8.0, at 2°C. (b) 58 mM sodium chenodeoxycholate, 5.1 mM hexylamine, 0.1 M NaCl, 0.01 M phosphate buffer, pH 8.0, at 2°C. (c) 58 mM sodium chenodeoxycholate, 11.0 mM hexylamine, 0.1 M NaCl, 0.01 M phosphate buffer, pH 8.0, at 2°C.

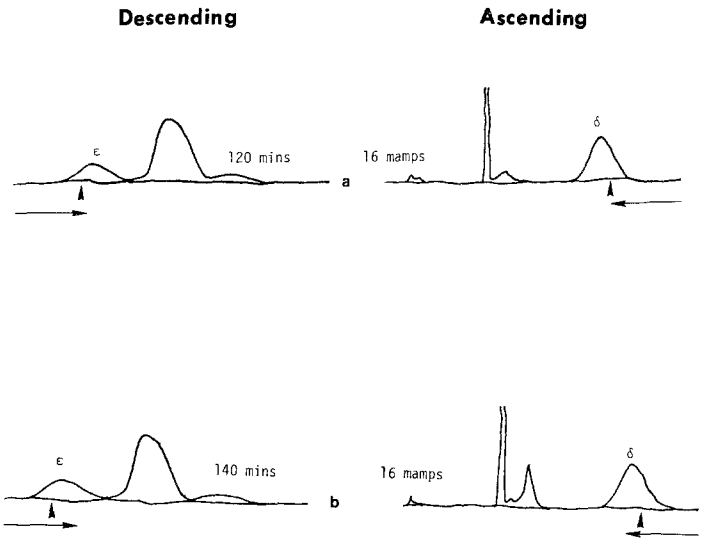

FIG. 3. Electrophoretic patterns. (a) 58 mM sodium chenodeoxycholate, 2.9 mM octylamine, 0.1 M NaCl, 0.01 M phosphate buffer, pH 8.0, at 2°C. (b) 58 mM sodium chenodeoxycholate, 6.5 mM octylamine, 0.1 M NaCl, 0.01 M phosphate buffer, pH 8.0, at 2°C.

was allowed to elapse before this material was used. One milliliter of this stock was used to prepare 50 ml of dispersion containing 0.01% cholesterol. The other components of the study, i.e., sodium chenodeoxycholate, sodium chloride, buffer, and the amines were added to this dispersion. The system was ultrasonified prior to use to disperse any aggregates which may have formed. Twenty-four hours was allowed for equilibration between cholesterol and the amines.

Electrophoretic mobility measurements. The electrophoretic mobility of individual particles was measured by the use of a commercial microelectrophoresis apparatus, the Zeta-Meter.[9] The apparatus has been described adequately elsewhere (12) and the description will not be repeated here.

The mobility was determined by measuring the time required for a particle to move over a certain given distance under a known potential gradient. The cell has a cylindrical electric path and particles are observed at a distance of 0.65 mm from the cell wall to correct for solvent movement. The electrophoresis cell is so designed as to make this initial depth coincide with the counting

[9] Zeta-Meter Inc., New York, N. Y.

TABLE I

Micellar Mobilities as a Function of Hexylamine and Octylamine Concentrations

System	Mobility[a] × 10^{-4} (cm^2 sec^{-1} V^{-1})
58 mM Chenodeoxycholic acid, 0.1 M NaCl, 0.01 M PO_4	−1.74
58 mM Chenodeoxycholic acid, 0.1 M NaCl, 0.01 M PO_4, 10.95 mM hexylamine	−1.25
58 mM Chenodeoxycholic acid, 0.1 M NaCl, 0.01 M PO_4, 6.5 mM octylamine	−1.33
58 mM Chenodeoxycholic acid, 0.1 M NaCl, 0.01 M PO_4, 5.14 mM hexylamine	−1.52
58 mM Chenodeoxycholic acid, 0.1 M NaCl, 0.01 M PO_4, 2.9 mM octylamine	−1.49

[a] Mobilities calculated from the ascending boundary.

TABLE II

Micellar Mobilities as a Function of Hexylamine and Octylamine Concentrations

System	Mobility[a] × 10^4 (cm^2 V^{-1} sec^{-1})
58 m*M* Chenodeoxycholic acid, 0.1 *M* NaCl, 0.01 *M* PO_4	−1.29
58 m*M* Chenodeoxycholic acid, 0.1 *M* NaCl, 0.01 *M* PO_4, 10.95 m*M* hexylamine	−0.91
58 m*M* Chenodeoxycholic acid, 0.1 *M* NaCl, 0.01 *M* PO_4, 6.5 m*M* octylamine	−0.98
58 m*M* Chenodeoxycholic acid, 0.1 *M* NaCl, 0.01 *M* PO_4, 5.14 m*M* hexylamine	−1.14
58 m*M* Chenodeoxycholic acid, 0.1 *M* NaCl, 0.01 *M* PO_4, 2.9 m*M* octylamine	−1.14

[a] Mobilities calculated from the descending boundary.

line on the eyepiece of the stereoscopic microscope. Twenty particles were observed per experiment. In determining the average electrophoretic mobility one should select only particles which appear to be migrating at very close to average velocity avoiding those which approach maxima or minima. However, in certain systems the selection of average velocity is difficult to impossible due to large variations in the velocity (13).

Mufson *et al.* (14) found that mobility of cholesterol particles in bile acid systems was often dependent on the linear location within the cell. As a result, all electrophoretic readings were made mid-length in the cell. All mobility measurements were made at 25 ± 2°C unless otherwise stated.

RESULTS AND DISCUSSION

Figures 1–3 represent the electrophoretic schlieren patterns of solutions used in this study. It will be seen in these figures that two peaks or maxima are evident in the patterns of both the ascending and descending boundaries but that patterns are far from being mirror images of each other. This is expected since the electrophoresis experiments were carried out under conditions which were far from "ideal" conditions. In the ideal case electrophoresis should be carried out with very dilute micellar solutions in buffers of rather high electrolyte concentration so that conductivity of the solution will be determined almost entirely

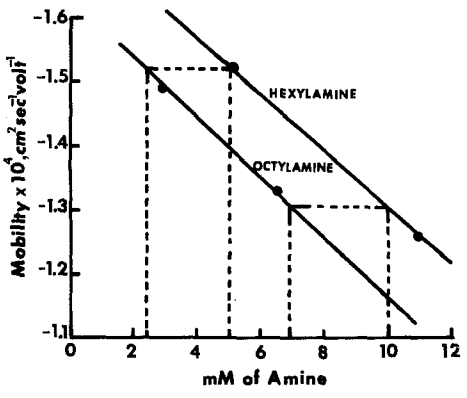

FIG. 4. Effects of hexylamine and octylamine concentrations on ascending micellar mobilities in 58 m*M* sodium chenodeoxycholate solutions containing 0.1 *M* NaCl and 0.01 *M* phosphate buffer, pH 8.0, at 2°C.

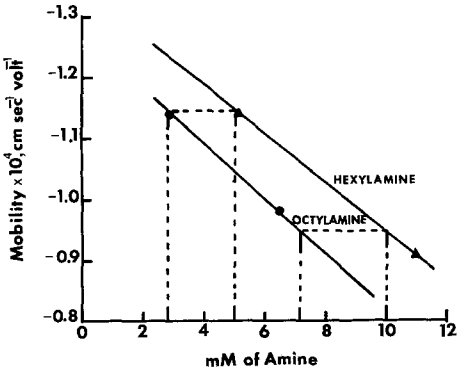

FIG. 5. Effects of hexylamine and octylamine concentrations on descending micellar mobilities in 58 m*M* sodium chenodeoxycholate solutions containing 0.1 *M* NaCl and 0.01 *M* phosphate buffer, pH 8.0, at 2°C.

TABLE III

The Concentrations of Hexylamine and Octylamine at a Given Micellar Mobility in 58 m*M* Sodium Chenodeoxycholate Solutions Containing 0.1 *M* NaCl and 0.01 *M* Phosphate Buffer at pH 8.0

Mobility[a] (cm^2 sec^{-1} V^{-1} × 10^4)	Concentration	
	m*M* of Hexylamine	m*M* of Octylamine
A. −1.52	5	2.4
B. −1.31	10	6.9
C. −1.15	5	2.8
D. −0.95	10	7.15

[a] Mobilities calculated from the ascending (A, B) and descending (C, D) boundaries.

by the buffer ions because of the low mobility and low equivalent concentration of the micellar ions. Thus the buffer ions ensure throughout the u-tube a uniform pH and a uniform electric field through which the micellar ions migrate (15).

From Figs. 1b and 1c it would be possible to identify the "false" boundaries, namely, the δ boundary and the ϵ boundary. At low micellar concentration, these boundaries are expected to become smaller as the electrolyte concentration is increased. Clearly the last boundaries of the ascending and descending patterns become smaller as the electrolyte concentration is increased and hence these are the false boundaries.

TABLE IV

Data Showing Good Correlation Between the Dissolution Rate Data and the Electrophoresis Data on the Proposed Role of Micellar Charge in Dissolution Rate Acceleration

Hexylamine concentration (m*M*)	Equivalent octylamine concentration (m*M*)	
	From electrophoresis[a]	From dissolution rate data
A. 5	2.4	3.0
B. 10	6.9	6.4
C. 5	2.8	3.0
D. 10	7.15	6.4

[a] Based on ascending (A, B) and descending (C, D) boundaries data.

The small, most rapidly moving peaks, sharper and smaller on the ascending side would appear to be due to molecularly dispersed chenodeoxycholate. The small multiple peaks in the descending patterns, in the 14.5 m*M* chenodeoxycholate system, are due to convection currents. Qualitative evidence of a small amount of material of positive mobility can be seen in the two solutions containing hexylamine (descending peak, especially at 10 m*M*) which is most likely free hexylamine.

In Figs. 2 and 3 we may note a number of differences between the ascending and descending patterns: (a) the distances moved by the boundaries are greater in the ascending limb than in the descending limb; (b) the ascending boundaries are sharper than the descending boundaries; and (c) the areas of the δ boundaries are greater than that of ϵ boundaries. However, since the aim of this study is to test a hypothesis under definite conditions and only the relative mobilities are required, the results of these experiments are sufficient and valid for the present purpose.

Tables I and II and Figs. 4 and 5 show the micellar mobilities calculated from the ascending and descending boundaries. The descending mobilities are lower than the ascending mobilities, as expected. In general, the mobilities decrease with an increase in the amine concentration and smaller amounts of octylamine are required than hexylamine to bring about the same change in the micellar charge.

From Table III and Figs. 4 and 5, it can

TABLE V

Influence of Ionic Strength on Micellar Mobilities

System	Mobility[a] (cm^2 sec^{-1} V^{-1} × 10^4)
14.5 m*M* Chenodeoxycholic acid, 0.01 *M* PO_4	−1.21
14.5 m*M* Chenodeoxycholic acid, 0.01 *M* PO_4, 0.1 *M* NaCl	−0.99

[a] Mobilities calculated from the ascending boundary.

TABLE VI

Electrophoretic Mobility of Cholesterol Particles as a Function of Hexylamine and Octylamine Concentrations

System	Electrophoretic mobility × 10^4 (cm^2 sec^{-1} V^{-1})
58 m*M* Cheno, 0.01 *M* PO_4, 0.01% cholesterol, 3 m*M* octylamine	−3.3 ± 0.7
58 m*M* Cheno, 0.01 *M* PO_4, 0.01% cholesterol, 5 m*M* octylamine	−3.6 ± 0.3
58 m*M* Cheno, 0.01 *M* PO_4, 0.01% cholesterol, 10 m*M* octylamine	−3.6 ± 0.3
58 m*M* Cheno, 0.01 *M* PO_4, 0.01% cholesterol, 5 m*M* hexylamine	−3.1 ± 0.8
58 m*M* Cheno, 0.01 *M* PO_4, 0.01% cholesterol, 10 m*M* hexylamine	−3.3 ± 0.4
58 m*M* Cheno, 0.01 *M* PO_4, 0.01% cholesterol	−2.7 ± 0.9

be seen that for ascending mobility of -1.5×10^{-4} cm^2 V^{-1} sec^{-1} one requires 5 m*M* hexylamine or 2.4 m*M* of octylamine. Similarly, for ascending mobility of -1.3×10^{-4} cm^2 V^{-1} sec^{-1} one requires 10 m*M* hexylamine or 6.9 m*M* octylamine. Similar results were obtained for descending mobilities (Table III).

From the dissolution rate data and the binding studies (8) we had determined that 5 m*M* hexylamine and 3 m*M* octylamine neutralized the micelles to the same extent and that 10 m*M* hexylamine and 6.4 m*M* octylamine neutralized the micelles to the same extent. If this were true, then the electrophoretic mobilities at these amine concentrations should be the same. Table IV shows that, indeed, the values obtained from the electrophoretic studies are nearly the same as predicted.

Table V shows the influence of ionic strength on ascending mobilities. The mobility in presence of 0.1 *M* sodium chloride is about 18% lower than the mobility in absence of sodium chloride. This is clear evidence for the binding of Na^+ ions to the negatively charged bile acid micelles.

Table VI shows the effect of amine concentration on the mobility of cholesterol particles in the chenodeoxycholate system. The average cholesterol particle mobilities are quite similar, although the distributions are broad. Thus, there appears to be little or no influence of amine concentration or amine type upon the mobility of cholesterol particles.

The results of this study verify our hypothesis that the primary mechanism by which these amines accelerate the cholesterol gallstone dissolution rates is by reducing the micellar charge.

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