Microelectrophoresis and Adsorption Studies of Cholesterol Particles in Bile Salt and Alkyl Surfactant Systems

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Abstract □ As part of a continuing program to understand better the behavior of cholesterol particles in physiological situations, an investigation of the interactions of the cholesterol surface with bile salts and alkyl surfactants was undertaken. Microelectrophoretic techniques and adsorption experiments were employed to characterize the adsorption behavior of these agents. In contrast to the alkyl surfactants, the adsorption of bile salts on the cholesterol particle surfaces was much less than expected in the concentration ranges investigated. These results were initially surprising in light of earlier studies where bile salts were found to inhibit greatly the growth of cholesterol crystals. However, the present data are consistent with the idea that the relatively rigid bile salt molecules can adsorb only onto specific sites on the cholesterol surfaces while the more flexible alkyl surfactants can more readily interact.

Keyphrases □ Cholesterol surface, interaction—bile salts, alkyl surfactants □ Electrophoretic mobility—cholesterol particles □ pH-mobility profiles—cholesterol dispersions □ Microelectrophoresis—cholesterol–bile salts, surfactant interactions □ Adsorption studies—cholesterol–bile salts, surfactant interactions

Cholesterol[1] has been implicated as the cause of many diseases, from atherosclerosis to xanthomatoses. While intensive research has been directed toward the understanding of the role cholesterol plays in these disease states, most of it is of a biological or clinical nature. There is surprisingly little reported work on the quantitative physical chemistry of these systems. For example, even the water solubility of cholesterol was an unknown quantity until recently (1). It, therefore, seems that *in vitro* physical-chemical investigations could be used to advantage to solve some of the very pressing problems of cholesterol deposition, namely, gallstone formation and atherosclerosis.

Cholesterol levels in the body are the results of absorption and synthesis on the one hand and excretion on the other. Removal occurs almost exclusively in the feces *via* the gallbladder. The cholesterol being virtually insoluble in water must be rendered soluble for removal. This function is normally performed by the bile. However, the pathological condition in which cholesterol deposits in the gallbladder, cholelithiasis, is widespread; surgical removal of gallstones is one of the most common operations performed today (2). Gallstone operations today outnumber appendectomies (3). A recent study showed that 59.6% of the women and 41.5% of the men (aged 60 to 100) exhibited gallstones at autopsy (4). "As more people live to develop biliary disease at an older age, surgeons can expect to see and treat an increasing number of patients with difficult biliary problems, complicated by all the other medical problems common to older patients." (5)

The mechanisms leading to the formation of gallstones are imperfectly understood. This is due in part to the many conflicting reports which are to be found in the literature. Bile is a supersaturated solution of cholesterol. Factors responsible for increasing cholesterol's concentration in the gallbladder bile may lead to its precipitation as stones (6). Stones will show almost infinite variation in their composition, appearance, and physical properties, dependent upon the factors involved in their formation (7). Stasis of the bile, pH changes, irritation, coagulation, infection, and the bile salt–phospholipid–cholesterol ratio are some of the factors intimated in the cholesterol deposition process. These factors often interact to produce stone formation. For example, infection can produce a change in pH and irritation of the gallbladder wall. This may allow the absorption of a significant amount of bile salt through the gallbladder wall, seriously affecting the bile salt–cholesterol ratio. The high energy of the supersaturated system is then relieved by the precipitation of cholesterol.

Stones have been found during surgery (8) which suggested that they were formed by the aggregation of smaller spherical particles of cholesterol. Frey *et al.* (9) have observed clusters of cholesterol crystals in the gallbladders of mice fed a lithogenic diet. Thus it appears that in the later stages of stone formation, aggregation of cholesterol particles plays an important role.

There have been physical-chemical studies concerned with the interactions of bile salts and cholesterol in micellar solutions (10–12), at interfaces (13), and at crystalline surfaces (14, 15).

Saad and Higuchi approached the problem from the point of view of cholesterol crystal growth. The influence of cholate, taurocholate, and glycocholate on the precipitation behavior of cholesterol in aqueous media was investigated as a function of pH (14). The Coulter counter was utilized to follow the particle-size distribution changes with time in the supersaturated and undersaturated cholesterol suspension systems. With increasing pH, the rates of growth and dissolution became progressively retarded, with complete inhibition of these processes at pH 8 and higher. The bile salts seemed to exhibit specificity of action which suggested strong binding to specific sites on the cholesterol crystal surface. Conventional long-chain surfactants, sodium dodecyl sulfate and myristyl-γ-picolinium chloride, at 1% concentration had no effect on the crystal growth rate.

Although surgical observations have suggested aggregation of cholesterol crystals as a mechanism for gallstone growth, this aspect has not been pursued from a colloidal-chemical point of view. It would seem that

[1] Systematic nomenclature for compounds given trivial names in the text includes: cholest-5-en-3β-ol, cholesterol; 3α,7α,12α-trihydroxy-5β-cholanoyl glycine, glycocholic acid; 3α,12α-dihydroxy-5β-cholanoyl glycine, glycodeoxycholic acid; 3α,7α,12α-trihydroxy-5β-cholanoyl taurine, taurocholic acid; 3α,7α,12α-trihydroxy-5β-cholanoic acid, cholic acid.

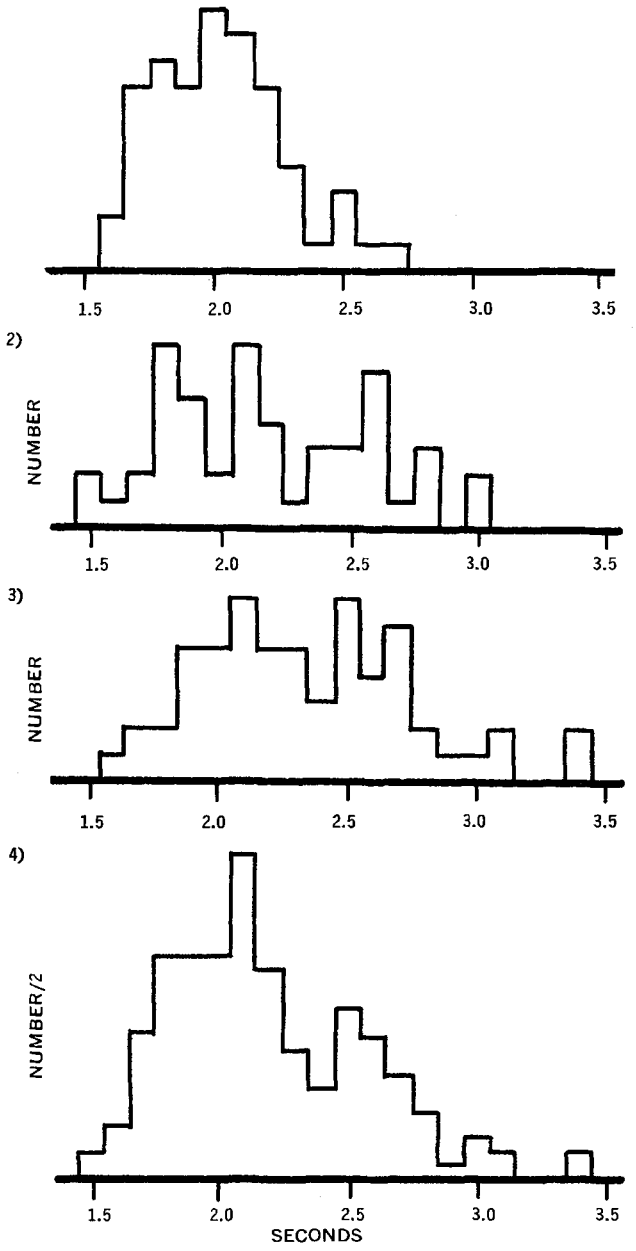

Figure 1—*Histograms of the velocity distribution of cholesterol particles in 10^{-2} M NaCl (time required to travel 0.5 division of Zeta-Meter eyepiece): (1), Experimenter I; (2), Experimenter II; (3), Experimenter II (4-day-old sample); and (4), summation of A, B, and C.*

such a study of the interfacial chemistry of the crystalline cholesterol–bile salt system should be helpful. An investigation of this nature might yield information concerning the etiology and prophylaxis of cholelithiasis.

Microelectrophoresis can be used as a valuable tool to measure interactions at the liquid–solid interface (16–18). Investigations of cholesterol dispersions by microelectrophoretic techniques have been performed. In 1930, Remezov published a voluminous work on the physical-chemical properties of the colloidal state of cholesterol, cholesterol esters, and lecithin (19–23). It is difficult to interpret and correlate his procedures and data. Moyer (24) found Remezov's directions for sol preparation to be too general to reproduce. Douglas and Shaw (25) studied the effect of pH on the electrophoretic mobility of cholesterol, finding a substantial increase in mobility as the pH was raised. Seaman investigated the effects of pH and salts on the mobility of cholesterol particles (26).

In the present studies, the effects of both bile salts and alkyl surfactants on the electrophoretic mobility of cholesterol particles have been determined. Adsorption studies on the same systems were used to complement this data and to allow explanation of some of the phenomena observed.

EXPERIMENTAL

General Considerations—Because the goal of this study was to monitor and interpret the surface properties of cholesterol particles in aqueous media, the presence of minute impurities and the manner of preparation of the dispersant were expected to be important. Cholesterol is known to be unstable towards light, heat, and other radiation in the presence of air. Until the advent of thin-layer chromatography, the widespread nature of cholesterol's autoxidation was not realized (27). Many products are produced during autoxidation, almost all of which are more polar than cholesterol. Oxidation of cholesterol in the presence of UV light has been reported to produce acids of unknown structure (28, 29).

The purity of experimental materials is always of importance in studies of surface properties (30–32). The cholesterol dispersions of some earlier investigations had shown a pH–mobility dependency. This is surprising in light of the nonionogenic nature of cholesterol. The effect of pH on the mobility of cholesterol dispersions of varying purity was therefore initiated prior to the studies of the cholesterol–surfactant systems.

Materials—Purified cholesterol was prepared by subjecting commercial cholesterol (Fisher Scientific Co. and Eastman Organic Chemicals) to the dibromination purification procedure of Fieser (33). Upon the termination of this method, the sample was further recrystallized from ether–methanol, ether–ethanol, and ethanol–water. The purified crystalline sample (m.p. 150°) was stored in a refrigerator under nitrogen. It was found by TLC to be homogeneous. The carbon–hydrogen analysis of this material agreed well with theory: theoretical C, 83.37; H, 12.03; observed C, 84.04; H, 11.99.

The IR spectra[2] of this sample, run in $CHCl_3$, agreed with those in the literature (34–36).

Distilled water of <2 μmho specific conductance was employed in all studies.

All inorganic salts were of analytical reagent quality. Most of the organic additives were used as received unless otherwise noted; *n*-octanol (Fisher Scientific Co.), sodium taurocholate (Mann Research Laboratories, Inc., New York, N. Y., and Calbiochem, Los Angeles, Calif.), sodium cholate (Mann special enzyme grade), cholic acid (Eastman), sodium glycocholate [Calbiochem (A grade)], sodium glycodeoxycholate [Calbiochem (A grade)], sodium dodecyl sulfate-^{35}S (Nuclear-Chicago Corp., Des Plaines, Ill.), sodium dodecyl sulfate (supplied by Dr. K. J. Mysels), cholic-carboxyl-^{14}C acid (Tracerlab, Waltham, Mass.), naphthalene A.R. (J. T. Baker Chemical Co., Phillipsburg, N. J.), 2,5-diphenyloxazole (PPO) (Packard Instrument Co., Inc., Downers Grove, Ill.), octanol-^{14}C (ICN, City of Industry, Calif.).

Buffers used were prepared by titration of the appropriate components in a beaker. The pH was monitored with a Corning model 7 pH meter.[3] Buffers were stored under refrigeration to retard mold growth. Sodium acetate, 1.0 *N*, was added to 10^{-3} *M* acetic acid to produce pH 5 buffer. A pH 6.8 stock buffer was made by dissolving 0.340 g. of potassium phosphate (KH_2PO_4) and 0.254 g. of sodium phosphate dibasic anhydrous (Na_2HPO_4) in enough water to make 100 ml. One milliliter of this stock buffer was used for each 100 ml. of dispersion. Sodium hydroxide (0.1 *N*) was added to 10^{-2} *M* boric acid to produce the pH 9 buffer and to 10^{-4} *M* sodium borate to produce the pH 11 buffer.

Preparation of Dispersions for Microelectrophoresis—Dispersions of cholesterol for microelectrophoretic evaluation were prepared

[2] Perkin-Elmer model 337, Norwalk, Conn.
[3] Corning Glass Works, Corning, N. Y.

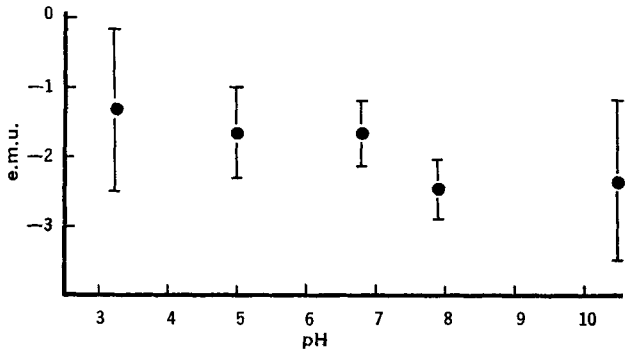

Figure 2—*The pH–mobility profile of cholesterol (bars represent ± 1.96 σ of the population).*

by dilution of concentrated stock dispersions or by precipitation *in situ*.

Stock Dispersion—To prepare 100 ml. of 0.5% w/v dispersion, 500 mg. of the purified 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. Irradiation was continued for 30 sec. 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 was allowed to elapse before this material was used. Two milliliters of this stock was used to prepare 100 ml. of dispersion containing 0.01% cholesterol. The system was ultrasonified prior to use to disperse any aggregates which may have formed.

***In Situ* Precipitation**—Enough purified cholesterol was dissolved in ethanol so that 1 ml. of this solution yielded a 0.01% dispersion when properly reacted; 1 ml. of water was added to an equal quantity of the alcoholic solution. Ultrasonic energy was supplied during mixing. To this dispersion were added the other components of the study, *i.e.*, sodium chloride, buffer, and test additive.

Electrophoretic Mobility Measurements—The electrophoretic mobility of individual particles was measured by the use of a commercial microelectrophoresis apparatus, the Zeta-Meter.[4] Descriptions of the apparatus appeared recently (37, 38) and will not be repeated here.

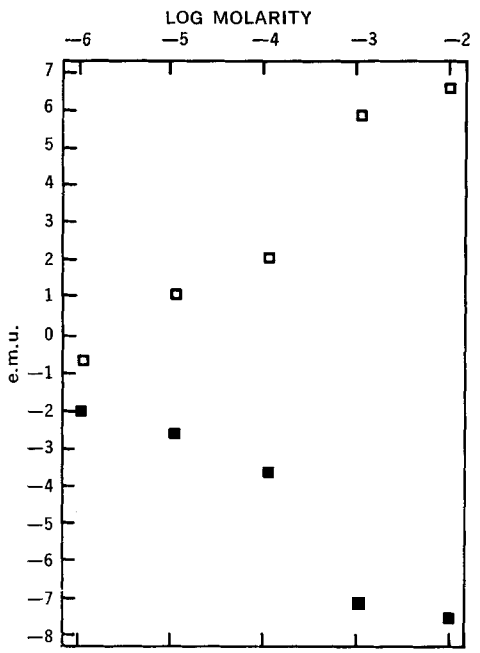

Figure 3—*Concentration–mobility profile of the dodecyl pyridinium chloride–cholesterol (□) and the sodium dodecyl sulfate–cholesterol (■) systems.*

[4] The units of electrophoretic mobility are micron/sec. per volt/cm. Since it is awkward to constantly restate these units, the use of the electrophoretic mobility unit, e.m.u., is proposed; 1 e.m.u. = 1 (micron-cm.)/(volt-sec).

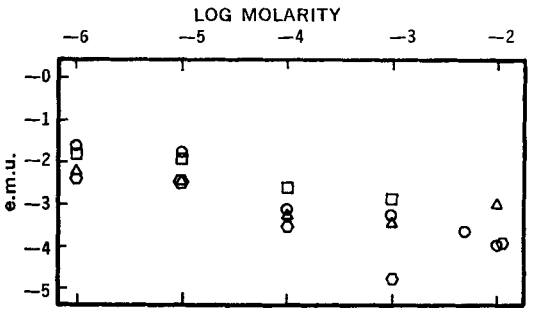

Figure 4—*Concentration–mobility profile of cholesterol–bile salt systems: cholate (○), taurocholate (□), glycocholate (△), glycodeoxycholate (○), at pH 6.8.*

The mobility is determined by measuring the time required for a particle to move over a given distance under a known potential gradient. Twenty particles were observed per experiment. Riddick (39) has stated that: "In tracking colloids to determine *average* EM or zeta potential, one should select *only* particles which appear to be migrating at very close to average velocity—scrupulously avoiding those which approach maxima or minima. However, some systems will show such extreme variation that selection of *average* velocity is difficult to impossible."

During the course of preliminary work, it was found that the mobility was dependent on location within the cell. As current is passed through the cell, oxidation and reduction take place at the appropriate electrodes. It is then possible for these products to migrate in the cell. The concentration of these materials will be highest in the region of the cell closest to the ends. It is for this reason that all electrophoretic mobility readings were made in the center of the cell. Furthermore, that portion of a sample under investigation that had been used for a mobility measurement was discarded after use. The cell was then thoroughly cleaned and wiped.

All mobility measurements were made at 25 ± 2° unless otherwise stated. Mobility readings were conducted within 72 hr. of sample preparation. Twenty-four hours was allowed for equilibration between cholesterol and the test additives. Electrophoresis systems containing organic liquids, *i.e.*, *n*-octanol, were examined in the glass electrophoresis cell. Earlier tests had shown the glass and plastic cells to yield similar data.

Adsorption Studies—The protocol for adsorption studies was as follows: buffer, sodium chloride, and adsorbate were added to 1.0 g. of cholesterol. The complete adsorption system was shaken on a Burrell shaker at 25 ± 2°. One-milliliter aliquots were withdrawn after the suspensions had been filtered through silver membrane filters[5] (1.2 μ). The 1.0-ml. aliquot was placed into glass scintillation vials containing a dioxane "cocktail" (naphthalene and PPO). The number of counts per minute (c.p.m.) was determined in a Beckman liquid scintillation system.[6]

The cholesterol used for all adsorption studies was prepared by the recrystallization of commercial cholesterol, twice from ethanol and once from ethanol–water. The suspensions used for the adsorption studies were prepared by the following method. The recrystallized cholesterol which had been dried under vacuum was ground in a porcelain mortar. One-gram samples were placed in flasks. The other components of the adsorption system were then added. Grinding was shown not to affect the adsorption properties of the cholesterol.

Throughout all experiments, electrophoretic and adsorption, unless specifically noted otherwise, the ionic strength was maintained at a constant level with 10^{-2} *M* NaCl.

RESULTS AND DISCUSSION

Baseline pH–Mobility Profiles—The distributions of particle velocities in a typical cholesterol dispersion are given in Fig. 1.

[5] Selas Flotronics, Spring House, Pa.
[6] Beckman Instruments, Inc., Fullerton, Calif.

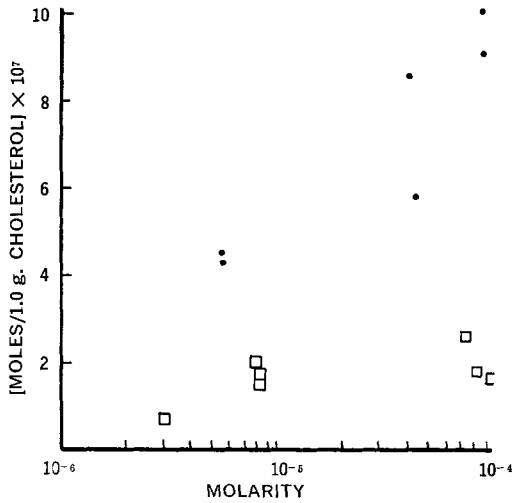

Figure 5—*The adsorption isotherm of sodium dodecyl sulfate* (●) *and sodium cholate* (□) *on cholesterol.*

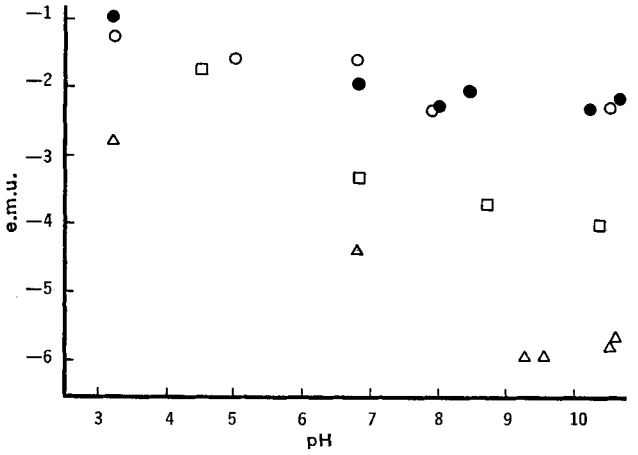

Figure 7—*The effect of octanol* (●), *10⁻³ M sodium cholate* (□), *and octanol–sodium cholate* (△) *on the pH–mobility profile of cholesterol* (○).

Note that the average velocities are quite similar, yet the distributions are broad, and in agreement with the observations of Seaman (26) who found the mobilities of alcohol-precipitated cholesterol particles in a given batch to exhibit considerable scatter. Electrophoretic data reported in the literature usually signify the average mobility of 20 particles in a single experiment. Due to the broad distributions encountered in the cholesterol system, it was decided to repeat each experiment several times and record the mean of the mean mobilities. (The standard deviation was generally ≤ 0.5.)

Figure 2 shows the influence of pH on the electrophoretic mobility. As can be seen, there appeared to be little or no influence of buffer concentration upon the mobility at constant pH. It is of interest to compare these results with those of Seaman (26), Moyer (24), and Douglas and Shaw (25). At high pH's, Douglas and Shaw found e.m.u. values as high as −5. These are contradictory to the authors' results and to those of Seaman. The large mobility observed by Douglas and Shaw may have been related to the presence of oxidation products of cholesterol. Aging and heating of cholesterol were found to increase the pH–mobility dependence of cholesterol (40).

Effects of Bile Salts and of Alkyl Surfactants on the Electrophoretic Mobility of Cholesterol Particles—Figure 3 shows the concentration–mobility profiles for cholesterol particles in the sodium dodecyl sulfate–cholesterol and the dodecyl pyridinium chloride[7]–cholesterol systems for which substantial mobility dependencies upon concentration were noted. Similar experiments with hexadecyl pyridinium chloride and dodecyltrimethylammonium bromide showed that the large concentration dependencies are typical of the alkyl surfactants.

Figure 4 gives the concentration mobility profiles of cholesterol particles in bile salt systems. It is of interest to note that although these salts have different side chains and varying numbers of hydroxyl groups on the four-ring nucleus, they all have similar concentration effects on electrophoretic mobility. This similarity might be related to the fact that although the hydrophilic portions are different, the hydrophobic "backs" of all these bile salts are nearly the same (42).

It was, however, surprising that the electrophoretic mobility produced by all the bile salts remained rather small even at the highest bile salt concentrations. These results initially appeared contradictory to the earlier findings based upon the studies of cholesterol crystal growth inhibition by cholates which suggested strong interaction of bile salt anions with the cholesterol surface. However, as will be seen, these data are consistent with the idea that cholates may exhibit limited adsorption on cholesterol surfaces while conventional long-chain alkyl surfactants are able to adsorb more generally.

Adsorption Studies—Figure 5 represents the data on the adsorption of sodium dodecyl sulfate and of sodium cholate from aqueous solutions onto cholesterol. Experiments at 1×10^{-3} *M* cholate were not meaningful because of the large uncertainties in the calculated amounts adsorbed at high concentration.

These results show that cholate reaches an adsorption plateau at around 10^{-5} *M*. This plateau is reached at the same concentration levels used previously in the Coulter counter studies of crystal growth inhibition. These results are also consistent with the micro-

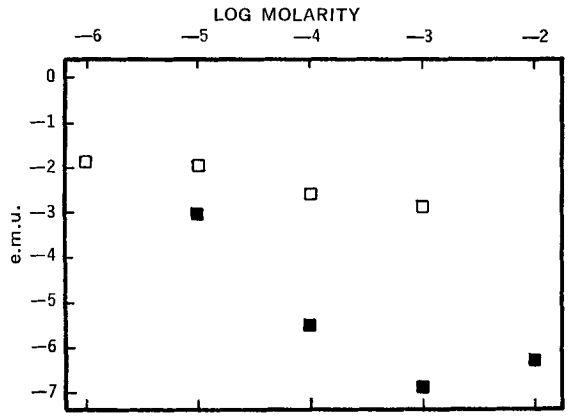

Figure 6—*Concentration–mobility profile of cholesterol–sodium taurocholate systems at pH 6.8 showing the effect of purity of commercial samples: Sample* A (□) *and Sample* B (■).

[7] Purified by extraction and recrystallization (41).

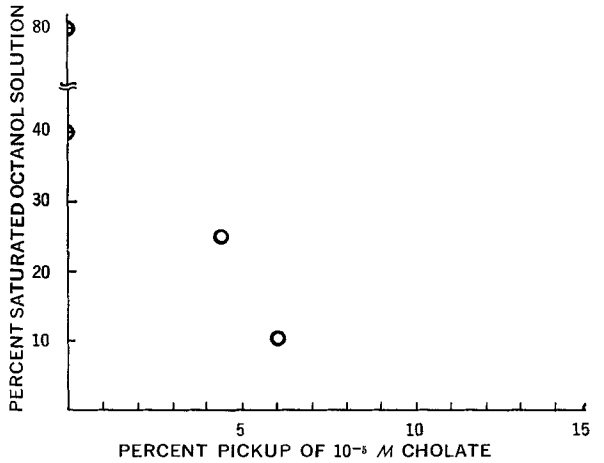

Figure 8—*The effect of octanol concentration on the adsorption of 10^{-5} M sodium cholate on cholesterol (80% saturated octanol solution signifies that 80 ml. of saturated solution has been added to an electrophoretic or adsorption system which contains a total of 100 ml.).*

Table I—Effect of Octanol on the Mobility of the Sodium Taurocholate–Cholesterol and Sodium Dodecyl Sulfate–Cholesterol Systems at pH 6.8

	− e.m.u.
Taurocholate (10^{-3} *M*)	1.92
Taurocholate (10^{-3} *M*) + octanol	4.44
Sodium dodecyl sulfate (10^{-3} *M*)	6.86
Sodium dodecyl sulfate (10^{-3} *M*) + octanol	7.22
Sodium dodecyl sulfate (10^{-4} *M*)	2.35
Sodium dodecyl sulfate (10^{-4} *M*) + octanol	2.65

electrophoretic data in which the bile salts showed very little change in effect on mobility over the range of concentrations studied.

For sodium dodecyl sulfate, adsorption increased sharply between 10^{-5} and 10^{-4} *M*, and reference to Fig. 3 indicates that adsorption should continue to increase up to about 10^{-3} *M*. Thus it appears that the adsorption plateau of sodium cholate occurs at a relatively low surface coverage and is consistent with the microelectrophoretic data present in Fig. 4.

Influence of Octanol and Other Additives on the Adsorption Behavior of Cholates—The low electrophoretic mobility of cholesterol particles in cholate solutions (Fig. 4) and the low extent of adsorption of cholates onto cholesterol (Fig. 5), in contrast to the behavior of the alkyl surfactants, suggested that cholates, probably because of their rigid nuclear structures, cannot be accommodated conveniently by all sites on cholesterol particle surfaces. This idea suggested that an investigation of the influence of additives upon the adsorption behavior of the cholates might be worthwhile, particularly in light of the observation that an unpurified sample of sodium taurocholate gave much higher limiting electrophoretic values than the purified sample (Fig. 6).

Thus the influence of a large number of compounds upon the electrophoretic mobility of cholesterol particles in cholate solutions was investigated (40). It was found that only octanol substantially altered the electrophoretic behavior at 10^{-3} *M* cholate. As can be seen in Fig. 7, octanol did not increase the electrophoretic mobility of the cholesterol particles. However, in combination with millimolar cholate, electrophoretic mobility values as large as those obtained with the alkyl surfactants were observed. It is noteworthy that the influence of octanol upon the electrophoretic mobility was negligible at 10^{-4} *M* cholate.

Experiments were also carried out to determine the influence of octanol on the adsorption of cholate. However, due to large uncertainties, meaningful data were obtained only for cholate concentrations up to 10^{-4} *M* cholate. Figure 8 shows that at 10^{-5} *M* cholate, octanol actually reduced the adsorption of cholate upon the surface of cholesterol. Similar results were obtained at 10^{-4} *M* cholate.

These findings suggest strongly that octanol is an inhibitor for cholate adsorption at low concentrations, but at high cholate concentrations it is able to assist in the adsorption of cholate. At low cholate concentrations, octanol may compete with cholate for those sites that would normally accommodate cholate; at high cholate concentrations ($> 10^{-3}$ *M*), octanol may act as a mediator.

The extent of the mediation which occurs at the higher cholate concentration might be expected to be greatest in a system where both the adsorbent and the adsorbate are rigid molecules as in the case here. Indeed it was found (Table I) that octanol had relatively little effect upon the electrophoretic mobility of cholesterol particles in sodium dodecyl sulfate solutions. It is worthwhile to recognize that many biological situations can involve such "three body" interplay, *e.g.*, cholesterol–protein interactions might be enhanced by the presence of flexible lipid molecules.

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