Bile Salt-Induced Disintegration of Egg Phosphatidylcholine Liposomes: A Kinetic Study Based on Turbidity Changes

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The disintegration kinetics of egg phosphatidylcholine small unilamellar liposomes in various bile salts (nine species) were investigated by monitoring turbidity changes with a stopped-flow apparatus. The pseudo-first-order rate constants obtained as a function of bile salt concentration (up to 25 mM) were analyzed based on a two-step model in which a penetration–saturation step of bile salt into the bilayer and a lamellar–micellar transition step were assumed for the disintegration mechanism of the bilayer. The order of the rate of the penetration–saturation step, which is assumed to be a measure of the disintegration ability, was as follows: SCDOC > SDOC > STCDOC > STDOC > STC > SC > SGCDOC > SGDOC > SGC. The results indicated that (1) the dihydroxy bile salts have a greater disintegration ability than the corresponding trihydroxy bile salts, (2) the chenodeoxy bile salts have a greater ability than the corresponding deoxy-bile salts regardless of non-conjugated or conjugated form, (3) the taurine conjugates always have a greater ability than the glycine conjugates. The penetration–saturation rate of the bile salts against the lipid bilayer depends considerably on the chemical nature of each bile salt, varying by a factor of about 10^5. In the conjugated bile salts alone, they were in a narrower range of a factor of 10^3. The physical integrity of liposomes can hardly be maintained in the bile salt-rich intestinal tract but the resulting mixed micelles may contribute substantially to solubilization and enhanced delivery of drugs.

Keywords kinetics; membrane disintegration; disintegration model; bile salt; turbidity; egg phosphatidylcholine liposome; stopped-flow monitoring

Liposomes have been extensively examined as a vehicle for improving the delivery of various therapeutic agents.[1] They are usually administered parenterally, but oral administration is also feasible.[2-5] However, the physical integrity of liposomes seems unlikely to be retained in the gastrointestinal tract, in view of the presence of bile salt-rich fluids.[6-8] Bile salts form mixed micelles with phosphatidylcholine, and these play an important role in the absorption of fat-soluble vitamins and cholesterol.[9-12] It is very probable that drug-loaded liposomes are also disintegrated and solubilized by bile salts, critically affecting the drug absorption.

We have recently shown that there is a major difference of ability to disintegrate egg phosphatidylcholine liposomes between sodium deoxycholate (SDOC) and sodium cholate (SC), probably due to their different hydrophobicities. Based on a proposed model containing a penetration–saturation (P–S) step and a lamellar–micellar (L–M) transition step, the disintegration kinetics was analyzed. The results indicated that SDOC penetrates into the bilayer about 500-fold faster than SC.[13]

Because various bile salt species are present in the intestine, the aim of the present study was to extend such an approach to other bile salts including conjugated forms.

Experimental

Materials Phosphatidylcholine (PC) was extracted from egg yolk and purified by column chromatography on silicic acid (Mallinkrodt, St. Louis, MO). Its purity was confirmed by thin layer chromatography.[14] Sodium glycocholate (SGC), sodium taurocholate (STC), sodium glycodeoxycholate (SGDOC) and sodium taurodeoxycholate (STDOC), all Sigma products, were chromatographically pure and were used without purification. Sodium taurochenodeoxycholate (STCDOC), sodium chenodeoxycholate (SCDOC) and sodium glycochenodeoxycholate (SGCDOC) were gifts from Tokyo Tanabe Co., Ltd. and were used without purification.

Preparation of Liposomes PC was dispersed in Tris buffer (pH 7.4) as described elsewhere.[15] Multilamellar liposomes were then sonicated for 60 min by a probe-type sonifier under a nitrogen stream. Titanium fragments from the probe were removed by centrifugation at 10000 × *g* for 10 min. The resulting small unilamellar suspension (SUV) was allowed to equilibrate at room tempeature for several hours and filtered (0.1 μm, cellulose nitrate, Toyo Roshi Co., Tokyo). The PC content was assayed based on phosphorus.[16]

Kinetic Measurement The disintegration kinetics of liposomes in bile salt was followed by monitoring turbidity changes (25 °C), at 400 nm with a stopped-flow apparatus (model RA-401, Union Giken, Osaka) equipped with an ultraviolet-visible detector and a micro-computer (M223 Mark III, Sord Computer Systems). The initial turbidity was 0.114 ± 0.015 at 400 nm. It was assumed that the turbidity changes directly reflect the disappearance rate of the vesicles since the sizes of the resulting mixed micelles are markedly reduced in the presence of bile salts in excess.[17,18] The reaction was carried out in an excess of bile salt so that it could be regarded as pseudo-first-order. An equal volume of the liposome suspension containing 3.0 mM phosphorus was quickly mixed with bile salt solutions at various concentrations up to 50 mM (Tris buffer, pH 7.4). The concentrations of lipid and bile salt are diluted to half in the cell of the apparatus.

Computer-generated curve fitting gave pseudo-first-order rate constants (k_{obs}). It was confirmed that PC and bile salts have no absorption at 400 nm and the final turbidity becomes zero after complete disintegration. Typical examples of the turbidity changes with time have been given elsewhere.[13]

Kinetic Model The dependency of k_{obs} on the bile salt concentration was analyzed in terms of a kinetic model of the liposome disintegration (see Appendix).

Results and Discussion

The disintegration of the lipid bilayer by surfactants was modeled by Helenius and Simons[17] as follows: Stage I begins with the penetration of surfactant molecules into the bilayer, stage II gives the maximal penetration of surfactant molecules, resulting in lamellar–micellar transition and subsequently in the formation of mixed micelles, and stage III produced further size reduction of mixed micelles due to the increasing ratio of surfactant/phospholipid. Although the above treatment applies to systems in equilibrium, it should be applicable to the kinetic analysis of the disintegration mechanism of liposomes, in which the collapse of the structural integrity of liposomes begins at stage II.

The apparent disintegration rate of liposomes in surfactant solutions is generally dependent on the vesicle size

when the liposome is multilamellar, since it is considered that the bilayers of the liposomes are lost stepwise from the outmost layer.[9] Size-heterogeneous liposomes would further complicate the analysis, if the disintegration rate apparently followed first-order-kinetics.[13,18,19]

In the present experiments the liposomes employed were small unilamellar ones throughout. This has the advantage that the observed rate could be regarded as an ultimate disintegration rate for a single bilayer and serves as a reasonable parameter to compare the disintegration abilities of various surfactants.

Figure 1 shows a plot of k_{obs} against the concentration of SCDOC up to 25 mM. The liposomes remained unchanged turbidimetrically up to about 5 mM, beyond which k_{obs} increased, tending to reach a plateau as observed in the case of SDOC.[13] This result reflects the fact that the disintegration process consists of the P–S step (dependent on the bile salt concentration) and the L–M step (independent of the bile salt concentration) according to the model described in "Appendix." The extrapolated intercept on the bile salt axis is assumed to be the minimal concentration, [MC], of the bile salt required to give the first-order-disintegration kinetics.

Figure 2 shows similar plots for various bile salts (STCDOC is given in Fig. 1). The dependencies of k_{obs} on the bile salt concentration were all linear within the range of bile salt concentrations examined and were much smaller than that of SCDOC.

Only the data of SCDOC could be analyzed with Eq. 11, because of its saturation-type tendency. Figure 3 shows a plot according to Eq. 11, giving the values of k_{lm} from the slope and $k_{b,ps}$ from the extrapolated intercept.

For bile salts other than SCDOC, Eq. 11 is not available and rather $k_{obs}=k_{ps}$ holds because no saturation-type tendency was observed and $k_{lm} \gg k_{ps}$ may be assumed in Eq. 10. The values of $k_{b,ps}$ were therefore calculated from the slopes. The estimated values are listed in Table I.

It is obvious that there are considerable differences of the P–S step against the bilayer among the bile salts. One of the unexpected results was that only SCDOC and SDOC exhibited a saturation-type dependency of k_{obs} on the bile salt concentration, showing an extraordinarily fast disintegration rate.

To compare the disintegration abilities of various bile salts, there are two parameters, *i.e.* $k_{b,ps}$ and k_{lm}, according to the model. The $k_{b,ps}$ appears to be the most important parameter of the disintegration ability since it is assumed to reflect the penetration–saturation step of bile salt molecules into the bilayer, *i.e.* the triggering step for destruction. The observed $k_{b,ps}$ values varied ranging from 1.9×10^2 $mM^{-1} s^{-1}$ for SCDOC to 6.4×10^{-3} for SGC, differing by a factor of about 10^5. The order of magnitude of $k_{b,ps}$ among the various bile salts was as follows:

SCDOC > SDOC > STCDOC > STDOC > STC > SC > SGCDOC > SGDOC > SGC .

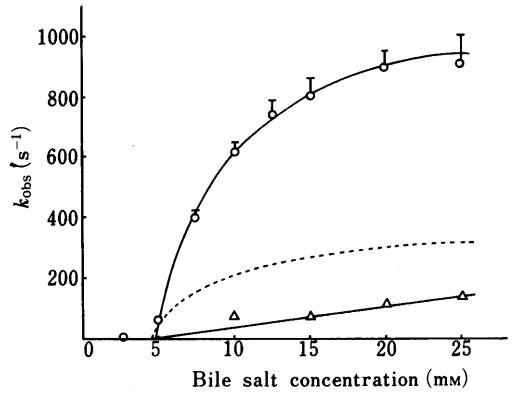

Fig. 1. Effect of Chenodeoxycholate (SCDOC) and Taurochenodeoxycholate (STCDOC) on the Pseudo-First-Order Disintegration Rate Constant (k_{obs}) for Unilamellar Liposomes at 25 °C

Phosphatidylcholine concentration, 1.5 mM. ○, SCDOC; △, STCDOC; broken line; SDOC from reference 13.

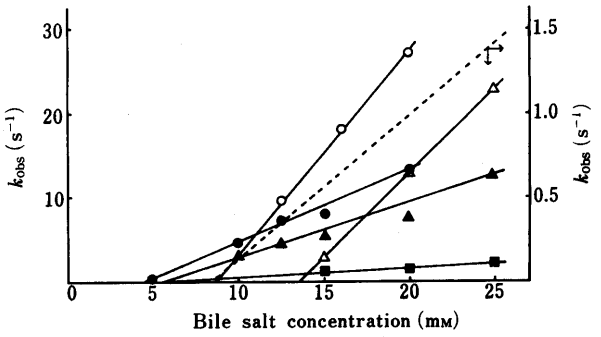

Fig. 2. Effect of Various Bile Salts on the Pseudo-First-Order Disintegration Rate Constant (k_{obs}) for Unilamellar Liposomes at 25 °C

Phosphatidylcholine concentration, 1.5 mM. Open symbols (refer to the left ordinate): ○, STDOC; △, STC. Closed symbols (refer to the right ordinate): ●, SGCDOC; ▲ SGDOC; ■, SGC; broken line; SC from reference 13.

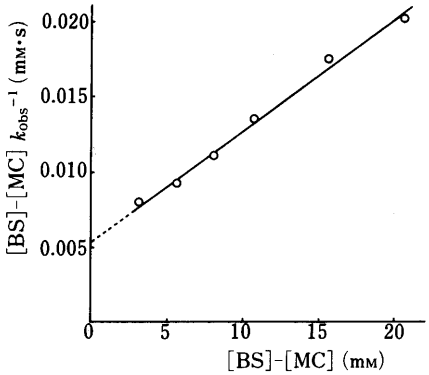

Fig. 3. Plot According to Eq. 11 for the Dependency of the Pseudo-First-Order Disintegration Rate Constant (k_{obs}) on the Chenodeoxycholate (SCDOC) Concentration Shown in Fig. 1

TABLE I. The Values of the Second-Order Rate Constant ($k_{b,ps}$) for the Penetration–Saturation Step and the First-Order Rate Constant (k_{lm}) for the Lamellar–Micellar Transition Step for Various Bile Salts[a] at 25°C

No.	Bile salt	$k_{b,ps}$ ($mM^{-1} s^{-1}$)	k_{lm} (s^{-1})	[MC] (mM)	CMRD[d]
1	SCDOC	1.9×10^2	1.3×10^3	4.4	2.9
2	STCDOC	4.7	— [b]	4.3	2.7
3	SGCDOC	4.2×10^{-2}	— [b]	4.9	3.3
4	STDOC	2.5	— [b]	3.6	2.4
5	SGDOC	2.9×10^{-2}	— [b]	5.5	3.7
6	STC	2.0	— [b]	11	7.6
7	SGC	4.2×10^{-3}	— [b]	6.0	4.0
8	SDOC[c]	5.9×10	3.6×10^2	3.7	2.5
9	SC[c]	8.6×10^{-2}	— [b]	8.7	5.8

a) Liposomes: small unilamellar egg phosphatidylcholine liposomes (1.5 mM phosphorus). *b*) Not determined. *c*) Data from reference 13. *d*) Critical molar ratio for disintegration ([MC]/1.5 mM PC).

From these results, several characteristics are apparent: (1) the non-conjugated bile salts, SCDOC and SDOC, show an unusually great disintegration ability (2) the dihydroxy bile salts tend to have a greater disintegration ability than the corresponding trihydroxy bile salts, (3) the chenodeoxycholic acids have a greater ability than the corresponding deoxycholic acids regardless of form (free form or conjugate), (4) the taurine conjugates always have a greater ability than the glycine conjugates.

Bile salts are structurally characterized by the number and position of the OH groups and by being either non-conjugated or conjugated, and these factors are closely related to their hydrophobic–hydrophilic balance. The penetration and subsequent saturation of bile salt molecules into the bilayer is basically comparable to the distribution of a nonpolar lipid or nonswelling amphiphile such as cholesterol into the bilayer, except that bile salts finally break up the extended bilayer into small disk-like fragments and form mixed micelles.[20] Among many factors involved, the number and position of the OH groups seem to be most influential for the P–S step of bile salts, and the difference of the molecular surface available for hydrophobic interaction is likely to be responsible for the dramatic variation of the $k_{b,ps}$. The dihydroxy bile salts generally display lower aqueous solubilities and lower critical micelle concentrations than the trihydroxy bile salts.[21] It is therefore clear that the concentration of monomeric bile salt by itself is not a rate-determining factor in the penetration of bile salt molecules into the bilayer.

As to the taurine and glycine conjugates which predominantly occur in nature, the $k_{b,ps}$ values fall in a narrower range, from $4.7\,mM^{-1}s^{-1}$ for STCDOC to 4.2×10^{-3} for SGC. This indicates that the effects of the conjugation include reduction of the membrane-disintegrating ability, which might be of physiological importance, *i.e.* reducing potential damage to membranes always exposed to bile salts. Since a taurine residue is more polar than glycine, the taurine conjugates have slightly higher critical micelle concentrations than the corresponding glycine conjugates.[22] If the critical micelle concentration were a measure of hydrophobicity or ability to penetrate into the bilayer, such a great difference as about 10^3 times between the corresponding conjugates could not be explained. It is known that the stability of the bilayers is also affected by the head groups of lipid components incorporated: even though they face the aqueous phase, the effect influences the hydrophobic portion, resulting in lipid-packing defects.[9,23] Therefore the hydrophilic moiety of the bile salt molecules may possibly play an important destabilizing role on the ordered structure of the bilayer.

Although only SCDOC and SDOC showed a saturation tendency from which the k_{lm} value could be calculated, the k_{lm} values available here may not be a suitable parameter for comparison. It is, however, interesting that both the bile salts, which are rare in nature, have some common features, *i.e.*, non-conjugated with either glycine or taurine, and a dihydroxy bile salt.

When the bilayers are saturated with bile salt molecules, the bilayer structure seems to be in the most destabilized state, *i.e.* the greatest physical tension. It is therefore probable that the rate of the lamellar–micellar transition is predominantly determined by the physical resistance to the tension rather than the species of bile salts incorporated in the bilayers. From this viewpoint, the magnitude of k_{lm} values obtained for SCDOC and SDOC could be regarded as representative of the rate of this transition process for any bile salts.

As mentioned earlier, the [MC] was estimated as the minimal concentration of a bile salt required to break the bilayer structure. However, mixed micelle formation occurs at much lower bile salt concentrations than those investigated in this study since the molar ratio of bile salts to solubilized PC is generally 1—2 at equilibrium.[12,24] Since the [MC] was simply extrapolated from the relationship between k_{obs} and bile salt concentration, it represents a hypothetical lower limit of the bile salt concentration at which pseudo-first-order kinetics of the turbidity disappearance holds, although it is limited to small unilamellar vesicles.

The molar ratio of [MC] to the liposome concentration (see Table I), which was termed the "critical molar ratio for disintegration" (CMRD),[13] should not be confused with the molar ratios of surfactant to phospholipid, Re_e^{sat} and Re_e^{sol} defined at equilibrium.[9,25] The CMRD represents how much bile salt is minimally required to induce rather rapid breakdown of the liposome on a molar basis. Figure 4 shows the relationship between the $k_{b,ps}$ and the CMRD in Table I for easier comparison. The bile salts seem to form groups according to their chemical structure: one is the dihydroxy group, having a great variation of the $k_{b,ps}$ but a very narrow range of the CMRD (2.5—3.5). The other is the trihydroxy group having a dependency of the $k_{b,ps}$ on the CMRD.

The parameters obtained in this study are limited to small unilamellar liposomes, but they suggest that even multilamellar liposomes would be destroyed within a minute after contact with bile salts. The total concentration of bile salts in bile ranges from 5 to 50 mM in human[8] and 360 mM in rabbit.[6] It is not likely that the physical integrity of liposomes is maintained in the bile salt-rich intestinal fluid. But the resulting mixed micelles with reduced sizes are expected to have easier mobility in the intestine and to contribute to the enhanced delivery of drugs, if the liposomally-loaded drugs are well solubilized by the mixed

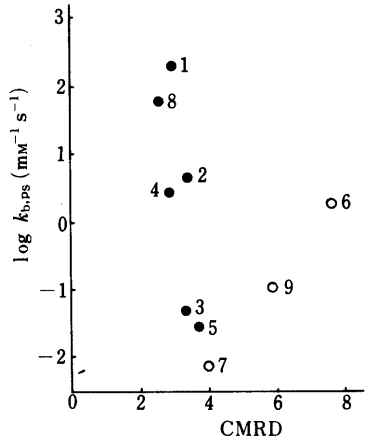

Fig. 4. The Relationship between log $k_{b,ps}$ and the Critical Molar Ratio for Disintegration (CMRD) for Various Bile Salts

The numbers indicated are the same as in Table I. ●, dihydroxy bile salts; ○, trihydroxy bile salts.

micelles.

Appendix

The model applies only to unilamellar liposomes for simplicity. The rate of the reaction of liposomes and bile salt is represented in terms of turbidity change as follows:

$$-\frac{d[T]}{dt}=k_b[BS][T] \tag{1}$$

where [T] is the turbidity and replaces the liposome concentration, [BS] is the concentration of bile salt, and k_b is the second-order rate constant. Under the condition that the [BS] is in excess of the liposome concentration, we have

$$-\frac{d[T]}{dt}=k_{obs}[T] \tag{2}$$

where the effective [BS] is assumed to be its total concentration.[18]

The linear dependence of log [T] on time will give the pseudo-first-order disintegration rate constant (k_{obs}). From the dependency of k_{obs} on the bile salt concentration for SDOC,[13] we assume a kinetic model in which the penetration-saturation step of bile salt molecules (P–S step) and the subsequent lamellar–micellar transition step (L–M step) are involved as follows:

	P–S step		L–M step	
intact liposome $[T]_i$	$\xrightarrow{k_{ps}}$	saturated liposome $[T]_{sat}$	$\xrightarrow{k_{lm}}$	mixed micelles no turbidity

where k_{ps} is the pseudo-first-order rate constant for the P–S step, *i.e.* $k_{b,ps}$ [BS] where $k_{b,ps}$ is the second-order rate constant and k_{lm} is the first-order-rate constant for the L–M step. $[T]_i$ and $[T]_{sat}$ are the turbidities of the intact liposomes and the saturated liposomes, respectively. The total turbidity ($[T]_{tot}$) is given by

$$[T]_{tot}=[T]_i+[T]_{sat} \tag{3}$$

Differentiating Eq. 3 with time gives

$$\frac{d[T]_{tot}}{dt}=\frac{d[T]_i}{dt}+\frac{d[T]_{sat}}{dt} \tag{4}$$

where

$$-\frac{d[T]_i}{dt}=k_{ps}[T]_i \tag{5}$$

and

$$\frac{d[T]_{sat}}{dt}=k_{ps}[T]_i-k_{lm}[T]_{sat} \tag{6}$$

Accordingly, we obtain

$$-\frac{d[T]_{tot}}{dt}=k_{lm}[T]_{sat} \tag{7}$$

Because turbidity changes of $[T]_i$ and $[T]_{sat}$ could not be separately determined, we assumed a quasi-steady state condition for $[T]_{sat}$. Therefore, the following equation is obtained from Eq. 6,

$$[T]_i=\frac{k_{lm}[T]_{sat}}{k_{ps}} \tag{8}$$

Combination of Eqs. 3, 7 and 8 and rearrangement give

$$-\frac{d[T]_{tot}}{dt}=\frac{k_{ps}k_{lm}}{k_{ps}+k_{lm}}[T]_{tot} \tag{9}$$

and therefore, we have

$$k_{obs}=\frac{k_{ps}k_{lm}}{k_{ps}+k_{lm}} \tag{10}$$

The k_{obs} is represented by two parameters: k_{ps} which is dependent on the bile salt concentration and k_{lm} which is independent of the surfactant concentration. Equation 10 can be rearranged in a manner analogous to the Langumuir-type equation to give Eq. 11. Since k_{ps} is the pseudo-first-order rate constant and the minimal surfactant concentration [MC] required to disintegrate the bilayer, the bile salt concentration is replaced by [BS]−[MC]. Equation 10 can be rearranged to give a linear plot of $\{[BS]-[MC]\}/k_{obs}$ against [BS]−[MC].

$$\frac{[BS]-[MC]}{k_{obs}}=\frac{[BS]-[MC]}{k_{lm}}+\frac{1}{k_{b,ps}} \tag{11}$$

References

1) D. Papahadjopoulos, "Medical Application of Liposomes," ed. by K. Yagi, Japan Scientific Societies Press, Tokyo, 1986, pp. 1—9.
2) T. Yotsuyanagi, *Ann. Rept. Pharm. Nagoya City Univ.*, **31**, 1 (1983).
3) T. Kimura, K. Higaki and H. Sezaki, *Pharm. Res.*, **5**, 189 (1984).
4) D. L. Schwinke, M. G. Ganesan and N. D. Weiner, *Pharm. Res.*, **6**, 256 (1984).
5) V. V. Nadtochii, R. A. Brodskii, G. A. Popov and A. M. Poverennyi, *Tsitologia*, **26**, 908 (1984).
6) R. Coleman, S. Iqbal, P. P. Godfrey and D. Billington, *Biochem. J.*, **178**, 201 (1979).
7) "Scientific Tables," ed. by L. Lentner, Ciba-Geigy Limited, Basle, 1982, pp. 134—144.
8) T. Yamakawa, "Data Book of Biochemistry I," ed. by T. Yamakawa, Tokyo Kagakudojin, Tokyo, 1979, p. 992.
9) D. Lichtenberg, R. J. Robson and E. A. Dennis, *Biochim. Biophys. Acta*, **737**, 285 (1983).
10) P. Walde, J. Sunamoto and C. J. O'Conner, *Biochim. Biophys. Acta*, **905**, 30 (1987).
11) J. A. Barrowman, "Pharmacology of Intestinal Permeation I," ed. by T. Z. Csáky, Springer-Verlag, Berlin, 1984, pp. 647—689.
12) M. Nagata, T. Yotsuyanagi and K. Ikeda, *J. Pharm. Pharmacol.*, **40**, 85 (1988).
13) M. Nagata, T. Yotsuyanagi and K. Ikeda, *Chem. Pharm. Bull.*, **36**, 1508 (1988).
14) A. E. Brandt and W. E. M. Lands, *Biochim. Biophys. Acta*, **144**, 605 (1967).
15) A. D. Bangham, M. W. Hill and N. G. A. Miller, "Methods in Membrane Biology," Vol. 1, ed. by E. D. Korn, Plenum Press, New York, 1974, pp. 1—68.
16) R. J. Mrsny, J. J. Volwerk and D. H. Griffith, *Chem. Phys. Lipid*, **39**, 185 (1986).
17) A. Helenius and K. Simons, *Biochim. Biophys. Acta*, **415**, 29 (1975).
18) D. Lichtenberg, Y. Zilberman, P. Greenzaid and S. Zamir, *Biochemistry*, **18**, 3517 (1979).
19) T. Yotsuyanagi, J. Mizutani, M. Iwata and K. Ikeda, *Biochim. Biophys. Acta*, **731**, 304 (1983).
20) N. A. Mazer, G. B. Benedek and M. C. Carey, *Biochemistry*, **19**, 601 (1980).
21) M. C. Carey and D. M. Small, *Arch. Int. Med.*, **130**, 506 (1972).
22) J. P. Kratohvill and H. T. DelliColli, *Can. J. Biochem.*, **46**, 945 (1968).
23) R. Nayar and A. J. Schroit, *Biochemistry*, **24**, 5967 (1985).
24) D. M. Small, "The Bile Acids," Vol. 1, ed. by P. P. Nair and D. Kritchevsky, Plenum Press, New York, 1971, pp. 249—356.
25) E. A. Dennis, *Adv. Colloid Interface Sci.*, **26**, 155 (1986).