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Structure and Function of Bile. I. Relation between the Structure of Artificial Bile and Activity of Pancreatic Lipase¹⁾

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An aqueous solution of lecithin, bile salts, and cholesterol (artificial bile) which resembles human bile was studied by means of polarizing microscopy, electron microscopy, spin-probe electron spin resonance (ESR) spectroscopy, and pancreatic lipase treatment to digest fatty esters. The relation between the structure of artificial bile and the digestion of fatty esters by pancreatic lipase was investigated, and the following results were obtained. Artificial bile consists of bile salt micelles and bile salt–lecithin–cholesterol mixed micelles. Fatty esters are solubilized in these micelles. Lipase hydrolyzes fatty esters solubilized in bile salt micelles but not in bile salt–lecithin–cholesterol mixed micelles. Ranging from 0.1 to 0.4 μ in size, the macromolecular complex of bile salt–lecithin–cholesterol can be observed by electron microscopy.

Keywords—bile; artificial bile; bile salt; lecithin; cholesterol; micelle; digestion; lipase activity; ESR; electron microscope

The fact that bile promotes digestion of fatty esters and the absorption of lipid through the intestinal wall is well known.²⁾ This action is caused by the surface activity of bile salts, the principal constituent of bile. In addition, cholelithiasis is well recognized as a disease related to bile. The relation between the formation of gallstones and the physicochemical characteristics of bile has recently been studied. Admirand and Small³⁾ determined the concentrations of bile salts, lecithin, and cholesterol in gall-bladder bile from patients with gallstones and normal gall-bladder bile. They distinguished normal and “abnormal” bile by plotting the concentrations of bile salts, lecithin and cholesterol on triangular coordinates. The work elegantly showed that normal corresponds to liquid phase (micellar) and abnormal to liquid plus crystal phase. In addition, they proposed that the physicochemical characteristics of bile are determined by the ratio of bile salts, lecithin, and cholesterol.

Concerning the structure of bile, Small⁴⁾ speculated that the lamellar liquid crystal phase of lecithin is mixed with bile salts in the liver resulting in bile salt–lecithin mixed micelles which are excreted *via* the bile duct. These mixed micelles are small disk-like sections of bimolecular leaflet in which the hydrophobic surfaces of the lecithin molecules are surrounded by a perimeter of bile salts. By electron microscopy, Howell and co-workers⁵⁾

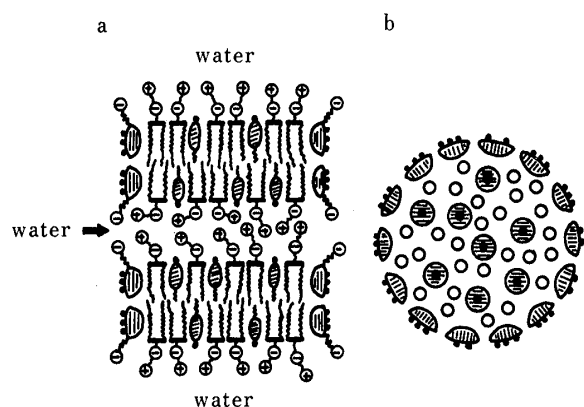


Fig. 1. Structure of Bile Formed from Lecithin, Bile Salt, and Cholesterol in Water^{4,5)}

a) A model of a cigar-shaped macromolecular assembly formed by the association of disk-shaped, mixed micelles of lecithin, cholesterol, and bile salt with intercalated layers of water (longitudinal section).

b) Cross-section of disc-shaped mixed micelles of lecithin, cholesterol, and bile salt.

○, bile salt; ○, lecithin; ○, cholesterol.

investigated calculous and acalculous natural bile and artificial bile ("artificial bile" was composed of aqueous dispersions of cholesterol, bile salts, and lecithin). Cigar-shaped assemblies were observed in gall-bladder bile and artificial bile, and these were interpreted in terms of a specific arrangement of disk-shaped, mixed micelles of lipid and bile salt molecules as shown in Fig. 1.

Despite the many studies of micelle structure and size, there is little information concerning the exact role of bile in lipid digestion. Therefore, the following experiments were carried out to investigate the physicochemical properties of bile.

1) To construct a triangular phase diagram for the artificial bile system, aqueous dispersions of bile salts, lecithin, and cholesterol were observed by polarizing microscopy.

2) To determine micelle shape and size in artificial bile, the artificial biles were studied by electron microscopy.

3) To study the relation between the structure of artificial bile and digestion of fatty esters, activity of pancreatic lipase was measured towards substrate solubilized in the artificial biles.

4) To investigate the physicochemical characteristics of artificial bile, electron spin resonance (ESR) spectra of spin probes solubilized in the artificial biles were observed and analyzed.

In the present work, the relation between bile structure and lipase activity was examined and it was concluded that artificial bile consists of bile salt micelles and bile salt–lecithin–cholesterol mixed micelles. Lipase hydrolyzes fatty esters solubilized in bile salt micelles but not those solubilized in bile salt–lecithin–cholesterol mixed micelles.

Experimental

Bile Salts and Conjugated Bile Salts—Deoxycholic acid (DIFCO Laboratories) and cholic acid (Sigma Chemical) were decolorized with active charcoal powder and recrystallized several times from MeOH. Each solution of bile acid was neutralized by addition of equivalent NaOH, using a 1 M solution, and the salt was prepared by lyophilization according to the method of Small.⁶⁾ Conjugated bile salts were prepared by the active ester procedure as described by Norman.⁷⁾ The purity of the crystals was confirmed by thin-layer chromatography.

Lecithin—Egg lecithin was prepared by the method of Pangborn and purified on a neutral alumina column (Type IV).⁸⁾ The purity of lecithin was confirmed by thin-layer silicic acid chromatography using a moving phase of chloroform–methanol–water (65:25:4, v/v/v).

Lipase Solution—Pancreatic lipase (25 g, Wako Chemical) was purified according to the method of Boissonnas⁹⁾ and lipase solution was prepared by diluting the purified lipase with isotonic phosphate buffer (pH 8.0) to 25 ml.

Fatty Esters and Spin Probes—2,2,6,6-Tetramethylpiperidinoxyl (TEMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidinoxyl (TEMPO-OH) and 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl (12-NS) were synthesized by the methods of Rozantsev,¹⁰⁾ Mamedova,¹¹⁾ and Waggoner,¹²⁾ respectively. Triolein (Wako Chemical) was purified by alumina and silicic acid column chromatographies with *n*-hexane as the eluent. 4-Oleoyl-

2,2,6,6-tetramethylpiperidinoxyl (TEMPO-OL) and *p*-nitrophenyl oleate were prepared by esterification of the appropriate alcohol with oleyl chloride according to the methods of Waggoner¹³⁾ and Huggins,¹⁴⁾ respectively. These esters were purified prior to use by silicic acid column chromatography with *n*-hexane.

Preparation of Artificial Bile—All artificial biles were prepared according to the method of Small⁶⁾ by using lecithin, cholesterol, and bile salt stock solution. The lecithin was dissolved in EtOH and adjusted to a concentration of 5%. The cholesterol and the bile salt were dissolved at concentrations of 1 and 10%, respectively, in MeOH:benzene = 1:1, v/v. These solutions were mixed in a 30 ml round-bottomed flask with a closing cap to give the desired weight ratio of cholesterol–lecithin–bile salt. The total dry solid per flask was 200 mg. The solvent was evaporated off in a rotary evaporator below 40 °C and the mixture was dried in a vacuum desiccator at 25 °C over P₂O₅. After 24 h of drying, the dry mixture was dissolved to a concentration of 10% in Clark–Lobs buffer solution (pH 10.0) or isotonic phosphate buffer solution (pH 8.0). At observation of the ESR spectrum, phosphate buffer solution containing 3×10^{-4} M spin probe was used. Artificial bile containing 10% solids by weight was employed, because the percent solids of human bile varies between 5 and 25% with an average of 10%.³⁾ The vessel was closed to prevent evaporation of water and shaken at 37 °C for 24 h.

Microscopic Examination—Artificial bile was placed on a slide glass and a cover glass was pressed on top. The slide was examined immediately under ordinary and polarizing microscopes at room temperature as reported by Small.⁶⁾

Electron Microscopy—Artificial bile was diluted with distilled water to a concentration of 1% and the diluted solution was negatively stained with 0.5% potassium phosphotungstate (pH 7.0).⁵⁾ Electron micrographs were taken at 1000–2000 magnification with an electron microscope, HS-7 (Hitachi Ltd., Tokyo).

Hydrolysis of Triolein by Lipase¹⁵⁾—In the Case of Sodium Deoxycholate: Sodium deoxycholate in the range from 0 to 100 mg, 19 ml of phosphate buffer (pH 8.0), and 500 μ l of triolein were placed in a 50 ml round-bottomed flask with a closing cap. After a 15 min preincubation in a thermostat at 37 °C, 1 ml of lipase solution was added to the flask. A portion (2 ml) of the reaction mixture was withdrawn in a 20 ml test tube every 10 min and the test tube was heated in a steam bath to denature the enzyme. The mixture was diluted with distilled water to 20 ml and the diluted mixture was used for the determination of free fatty acid. The free fatty acids were separated as their copper salts by chloroform extraction and the copper was determined colorimetrically with diethyldithiocarbamate according to the method of Duncombe.¹⁵⁾

In the Case of Artificial Bile: Artificial bile (1 ml), 18 ml of phosphate buffer solution, and 500 μ l of triolein were placed in a 50 ml round-bottomed flask. The mixture was shaken for 15 min in a thermostat at 37 °C and 1 ml of lipase solution was added. The resulting free fatty acid was determined in the manner described above.

Hydrolysis of *p*-Nitrophenyl Oleate¹⁴⁾—A 2 ml aliquot of phosphate buffer solution containing dispersed *p*-nitrophenyl oleate (8.3×10^{-4} M), 1 ml of artificial bile, and 1 ml of lipase solution were mixed in a test tube. A portion of the mixture was placed in a cell thermostated at 37 °C, and the resulting *p*-nitrophenol was determined colorimetrically at the wavelength of 440 nm.

Hydrolysis of TEMPO-OL and ESR Spectrum—Artificial bile (2 ml) containing 0.6 μ mol of TEMPO-OL and 1 ml of lipase solution were mixed in a test tube. The mixture was placed in a glass capillary tube of 1.5 mm inside diameter and the tube was sealed for ESR measurement. ESR spectra were recorded with a JES-3BS-X (X-band) spectrometer (Japan Electron Optics Laboratory, Tokyo). The temperature of the sample tube was maintained at 37 ± 0.1 °C by using a specially designed thermo-control unit.¹⁶⁾ ESR spectra were analyzed by comparison between the theoretical and observed spectra. The theoretical spectrum was calculated by superposition of various Gaussian shape functions for the spin probes.^{12b)} The spectral simulation was performed by using a DPS-1 microcomputer (Ithaca Audio Inc.) and a MILOT X-Y plotter (Watanabesokuki Inc.).

Results and Discussion

Figure 2 shows a triangular phase diagram based on microscopic analysis of 80 separated mixtures. This diagram shows the physical state of combinations of sodium deoxycholate (SDC), lecithin, and cholesterol in aqueous solution containing 10% total solids of the three components by weight. For example, the single point A represents artificial bile in an isotropic liquid phase (micelles) containing 80 mol% SDC, 15 mol% lecithin, and 5 mol% cholesterol. The crystals are formed from surplus cholesterol in excess of the solubility limits of cholesterol in lecithin lamellae. The liquid crystal consists of an anisotropic droplet or a myelin form. The liquid crystal was observed under a polarizing microscope. It was found that the two-component system of SDC and cholesterol consists of a crystal and liquid phase. The cholesterol crystals disappear as lecithin increases, suggesting that lecithin dissolves the cholesterol. The phase diagram is identical to the result obtained in the lecithin–cholesterol–

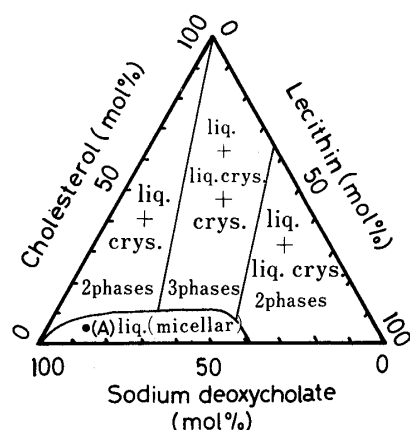


Fig. 2. Three-Component Phase Diagram for SDC-Lecithin-Cholesterol in Water

This phase diagram shows the physical states of SDC, lecithin, and cholesterol in aqueous solutions containing a total of 10% solids and 90% water. The single point A represents a bile containing 80 mol% SDC, 15 mol% lecithin, and 5 mol% cholesterol.

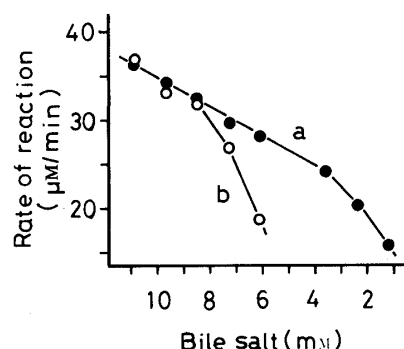


Fig. 3. Relationship between Activity of Pancreatic Lipase and Concentration of SDC

a) Triolein dispersed in SDC solution was used as a substrate.

b) Triolein dispersed in artificial bile solution was used. Artificial bile contained 10% solids by weight and the cholesterol content was kept constant at 5 mol%.

Experimental conditions are identical to those described in Tables I and II.

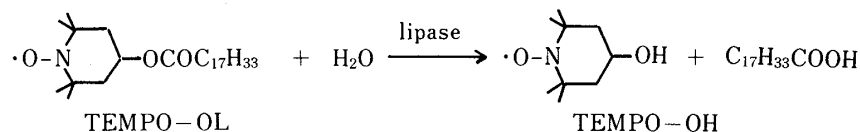


Chart 1

conjugated bile salt mixture (from beef bile) system by Small.^{3,6)} Thus, a compositional change of the bile salt component has little influence on the physical state of the lecithin-cholesterol-bile salt system.

Hydrolysis of Triolein

To investigate the relation between the structure of artificial bile (liquid phase in Fig. 2, corresponding to normal human bile) and the activity of pancreatic lipase, pancreatic lipase activity was measured in the following manner. The amount of triolein hydrolyzed by pancreatic lipase was determined colorimetrically and the rate of reaction was calculated from the initial velocity of the reaction. The reaction rate data in Tables I and II show the activity of pancreatic lipase on the triolein-SDC system and on the triolein-artificial bile system, respectively. The relations between reaction rate of triolein and concentration of SDC are shown in Fig. 3. As shown in Fig. 3a, hydrolysis without SDC hardly proceeds and the reaction rate is proportional to SDC concentration in the former system. In the case of artificial bile, as shown in Fig. 3b, the reaction rate is lower than in the case of SDC, and deviates from linearity. The activity at the ratio of lecithin:SDC = 1:2 (containing 6 mM SDC) is close to the experimental value (7.2 $\mu\text{M}/\text{min}$) without SDC in Table I. Therefore, it can be presumed that the change of reaction rate on going from the SDC system to the artificial bile system is caused by the structural change of micelles from bile salt micelles to bile salt-lecithin-cholesterol mixed micelles due to the interaction between bile salt and lecithin. Subsequently, the micelle solubilized substrate is less accessible to the enzyme. In order to investigate the micelle structure and the physical state of the substrate solubilized in micelle solution, the following ESR experiments were performed by using the spin-labelled substrate

TABLE I. Relation between SDC Concentration and Activity of Pancreatic Lipase^{a)}

SDC (mM)	v ($\mu\text{M}/\text{min}$)
12.2	39.6 ± 2.2
10.9	36.0 ± 2.6
9.7	34.3 ± 3.6
8.5	32.6 ± 3.6
7.3	29.6 ± 3.1
6.0	28.1 ± 2.2
4.8	25.4 ± 2.5
3.6	24.4 ± 3.9
2.4	20.4 ± 1.9
1.2	15.9 ± 2.1
0.0	7.2 ± 4.3

a) The enzyme activity was determined after mixing the lipase solution and the triolein emulsion with various quantities of SDC. SDC, sodium deoxycholate; \pm , standard deviation.

TABLE II. Relation between Composition of Artificial Bile and Activity of Pancreatic Lipase^{a)}

SDC (mM)	Lecithin (mM)	Cholesterol (mM)	v ($\mu\text{M}/\text{min}$)
10.9	0.3	0.6	36.7 ± 3.4
9.7	1.0	0.6	32.9 ± 2.7
8.5	1.6	0.6	31.6 ± 2.8
7.2	2.3	0.6	26.6 ± 5.6
6.0	2.9	0.6	18.7 ± 5.7

a) Enzyme activity was determined after mixing lipase solution and the emulsion of triolein with various molar ratios of artificial bile. SDC, sodium deoxycholate; \pm , standard deviation.

(Chart 1).

Hydrolysis of TEMPO-OL

The observed ESR spectrum was analyzed by spectral simulation and the calculated spectrum is shown in Fig. 4. The observed ESR spectrum of an emulsion of TEMPO-OL in SDC (Fig. 4a) consists of the superposition of a broad-line spectrum (Fig. 4e) and a three-line spectrum (Fig. 4d),¹³⁾ and the ratio of the broad-line spectrum to the three-line spectrum is 0.42/0.58. The broad-line spectrum is that of oil droplets of TEMPO-OL and the three-line spectrum is that of the bile salt-TEMPO-OL mixed micelles.^{12b)} Next, lipase solution was added to this emulsion of TEMPO-OL, the mixture was allowed to stand for 24 h at 37 °C to complete the lipolysis, and the ESR spectrum was recorded. As shown in Fig. 4b, the spectra of oil droplets and mixed micelles completely disappeared and a highly symmetric three-line spectrum appeared. This ESR spectrum is characteristic of a nitroxide free radical, and was assigned to TEMPO-OH produced by the hydrolysis of TEMPO-OL. In conclusion, lipase hydrolyzes not only a substrate on the interface of an oil droplet¹⁷⁾ but also a substrate solubilized in bile salt micelles.

In the Evaluation of Artificial Bile (Sodium Taurocholate-Lecithin-Cholesterol)—Artificial bile, TEMPO-OL, and lipase were mixed, and a portion of the mixture was sealed in a glass capillary tube. The ESR spectrum was recorded after 10 min and 24 h as shown in Fig.

5. No further spectral change could be observed after 24 h. From the spectrum after 10 min (Fig. 5a), TEMPO-OL is completely solubilized in artificial bile without residual oil droplets of TEMPO-OL. The spectrum after 24 h (Fig. 5b) was analyzed by the superposition of TEMPO-OH (Fig. 5c) and TEMPO-OL (Fig. 5d) spectra. Other experimental spectra using artificial bile with various component ratios were analyzed in the same way and the calculated spectra agreed well with the observed spectra. The resulting fractional population P_a of TEMPO-OH is plotted against the concentration of bile salt component in artificial bile in Fig. 6. Lipase showed little hydrolysis of the substrate solubilized in artificial bile containing over 35 mol% lecithin. The amount of hydrolyzed TEMPO-OL increased with bile salt component of artificial bile at the ratio of lecithin : bile salt = 1 : 2. This result shows that two

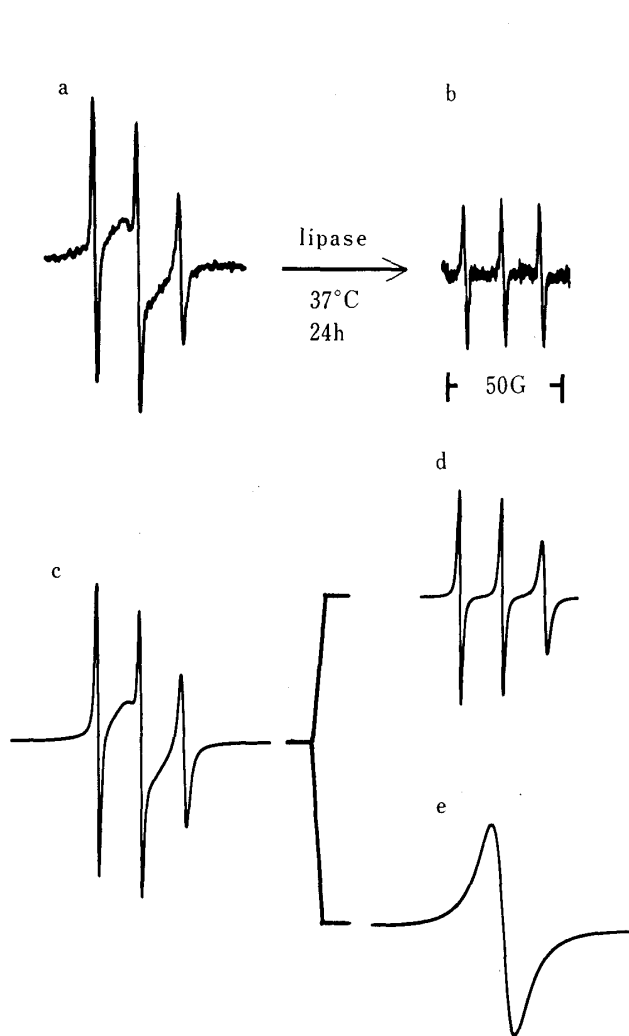


Fig. 4. ESR Spectrum and Hydrolysis of TEMPO-OL in Bile Salt Solution

a) ESR spectrum of 3.0×10^{-4} M TEMPO-OL dispersed in SDC solution.

b) Lipase was added to (a) and the ESR spectrum was measured after 24 h. The ESR spectrum was assigned to TEMPO-OH formed from TEMPO-OL by lipase.

c) Simulation spectrum for (a); this spectrum was calculated by the superposition of (d) and (e).

d) ESR simulation spectrum of TEMPO-OL solubilized in SDC micelles.

e) ESR simulation spectrum of oil droplet TEMPO-OL.

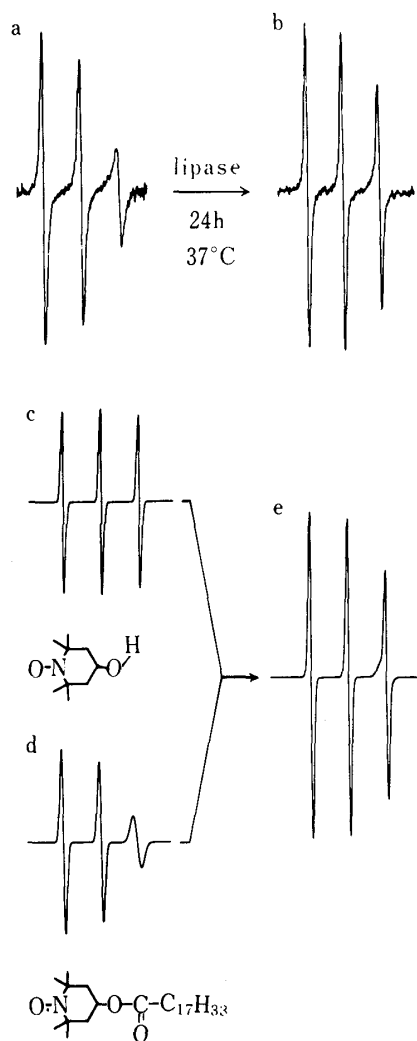


Fig. 5. ESR Spectrum and Hydrolysis of TEMPO-OL in Artificial Bile

a) ESR spectrum of 1.5×10^{-4} M TEMPO-OL solubilized in artificial bile containing sodium taurocholate 90 mol%, lecithin 5 mol%, and cholesterol 5 mol%.

b) Lipase was added to (a) and the ESR spectrum was observed after 24 h.

c) ESR simulation spectrum of TEMPO-OH liberated from TEMPO-OL by lipolysis.

d) ESR simulation spectrum of TEMPO-OL solubilized in artificial bile.

e) Superposition of TEMPO-OH (c) and TEMPO-OL (d) spectra. This spectrum coincides well with observed spectrum (b).

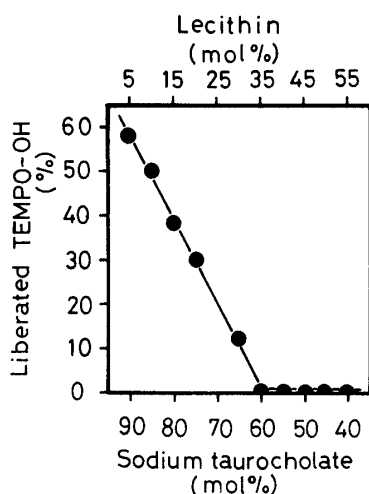


Fig. 6. Effect of Artificial Bile of Various Compositions on the Activity of Lipase

TEMPO-OL solubilized in artificial bile was used as the substrate. Artificial bile containing 10% solids by weight was employed and the cholesterol content was kept constant at 5 mol%.

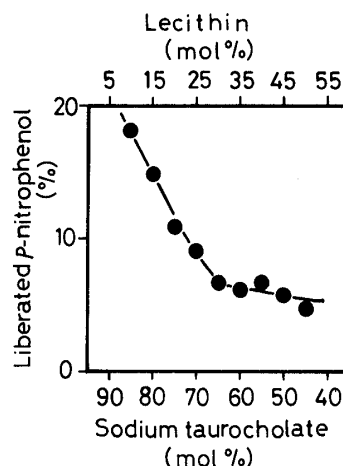


Fig. 7. Effect of Artificial Bile of Various Compositions on the Activity of Lipase

p-Nitrophenyl oleate solubilized in artificial bile was used as the substrate. Experimental conditions were identical to those described in Fig. 6.

micelle systems, one accessible to pancreatic lipase and the other not, are present in artificial bile. The ratio of accessible micelles to unaccessible micelles increases with the ratio of bile salt to lecithin in artificial bile. A similar increase of lipolysis with bile salt content in artificial bile was also observed in the following experiments.

Hydrolysis of *p*-Nitrophenyl Oleate

Artificial bile (lecithin-cholesterol-sodium taurocholate), *p*-nitrophenyl oleate, and pancreatic lipase were mixed. The reaction mixture was incubated for 6 h at 37 °C to complete the hydrolysis, and the resulting *p*-nitrophenol was determined. The relationship between the formed *p*-nitrophenol and the bile salt content in artificial bile is shown in Fig. 7. The hydrolysis of *p*-nitrophenyl oleate proceeds from the ratio of lecithin : bile salt = 1 : 2, and increases with bile salt content in the artificial bile solution.

The results of hydrolysis by lipase indicate that the mixture of artificial bile and substrate consists of two micelle systems. One micelle system is substrate-bile salt mixed micelles in which the substrate in the mixed micelles is hydrolyzed by pancreatic lipase. The other micelle system is substrate-bile salt-lecithin-cholesterol mixed micelles, and the pancreatic lipase cannot react with substrate solubilized in the micelles.

In order to confirm this hypothesis, the following electron microscopy and ESR experiments were performed.

ESR Spectrum

The ESR spectrum of TEMPO dissolved in artificial bile solution is shown in Fig. 8a. The spectrum is analogous to that of TEMPO dissolved in lecithin-cholesterol liposomes.¹⁸⁾ The spectrum is a composite of two spectra. One is a spectrum of TEMPO solubilized in lecithin lamellae and the other is that of TEMPO dissolved in water. Similarly, a TEMPO solubility parameter, *f*, approximately equal to the mole fraction of spin probe dissolved in the fluid hydrophobic region of lipids, was measured from the partially resolved high-field nitroxide hyperfine line as a function of lecithin content. In Fig. 8b, this TEMPO parameter is plotted as a function of lecithin content for artificial bile solution containing various mole fractions of lecithin. The results indicate that the amount of spin probe in lecithin lamellae increases with

concentration of lecithin component up to about 30 mol% lecithin, then levels off. On the other hand, the phase transition temperature of the artificial bile from fluid phase to solid phase was determined from the change of TEMPO solubility parameter with temperature. The relation of the phase transition temperature to the bile salt content in artificial bile with a constant ratio of lecithin:cholesterol=1:1 is illustrated in Fig. 9b, and the relation of the phase transition temperature to cholesterol content in artificial bile with a constant ratio of bile salt:lecithin=2:1 in Fig. 9a. These results on temperature dependence indicate that the phase transition temperature of artificial bile depends on the ratio of lecithin to cholesterol, and the physicochemical characteristics of artificial bile are similar to those of the lamellar structure of lecithin-cholesterol liposomes.¹⁸⁾

The ESR spectrum of 12-NS dissolved in artificial bile solution is shown in Fig. 10. This spectrum is a superposition of a three-line spectrum and a broad-line spectrum in the slow-motional region for spin probe which is solubilized in lecithin lamellae.^{12b, 19)}

The ESR experiments demonstrate that artificial bile contains a system of lecithin-

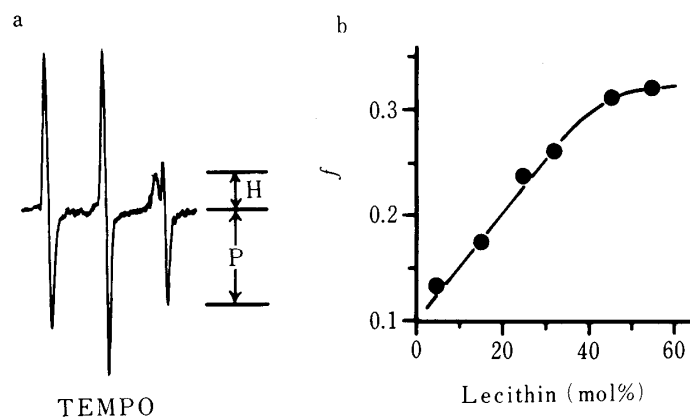


Fig. 8. ESR Spectrum of TEMPO in Artificial Bile of Various Compositions

- a) ESR spectrum of TEMPO dissolved in artificial bile, lecithin:cholesterol:sodium taurocholate=55:5:45 (mol%). The ESR spectrum was measured at 20°C.
b) TEMPO solubility parameter, $f=H/(H+P)$, as a function of lecithin content of artificial bile. Artificial bile containing 10% solids by weight was employed and the cholesterol content was kept constant at 5 mol%.

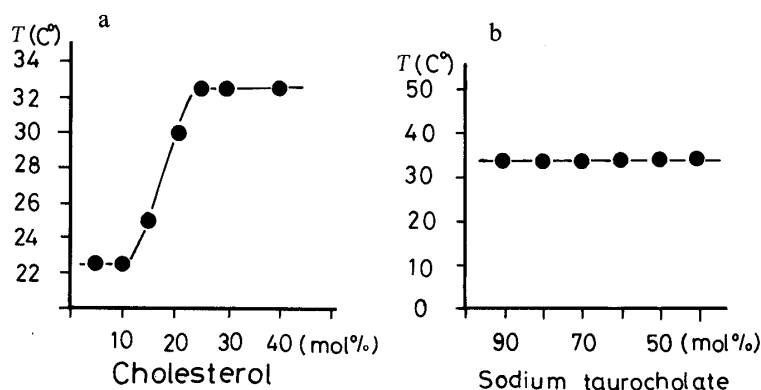


Fig. 9. Relation between Phase Transition Temperature and Components in Artificial Bile

The phase transition temperature of artificial bile (fluid phase to solid phase) was determined from the abrupt change of the TEMPO solubility parameter, f , with temperature.

a) Relation between the phase transition temperature and cholesterol content at a constant ratio of bile salt:lecithin=2:1.

b) Relation between the phase transition temperature and bile salt content (sodium taurocholate) at a constant ratio of lecithin:cholesterol=1:1.

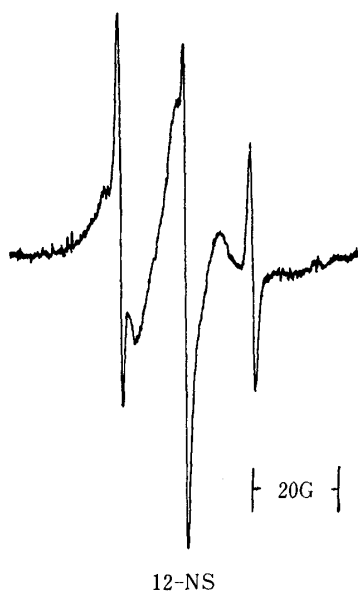


Fig. 10. ESR Spectrum of 12-NS

Measurement was done on artificial bile solution containing $3.0 \times 10^{-4} \text{ M}$ 12-NS at 37°C . The molar ratio of artificial bile was lecithin : cholesterol : sodium taurocholate = 35 : 5 : 60 (mol%).

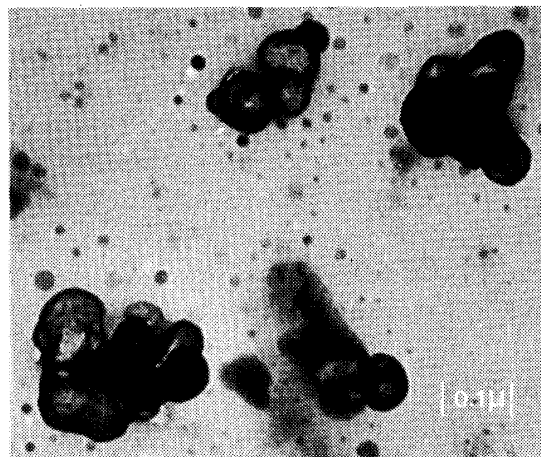


Fig. 11. Electron Micrograph of Artificial Bile

Artificial bile was diluted with distilled water to a concentration of 1% and the diluted solution was negatively stained with 0.5% potassium phosphotungstate (pH 7.0). $\times 10000$.

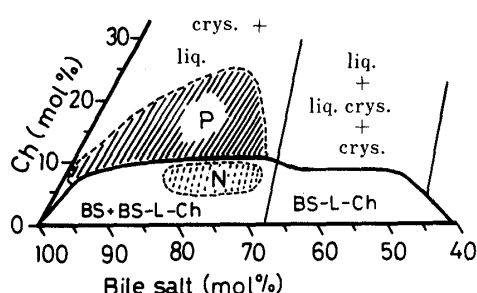


Fig. 12. Phase Diagram of Artificial Bile

Liquid phase as shown in Fig. 2 is separated into a bile salt micelles plus bile salt-cholesterol-lecithin mixed micelles phase and a bile salt-cholesterol-lecithin mixed micelles phase. Composition of bile from a normal subject falls into area N of the liquid phase and that of bile from patients with gallstones falls into area P of the liquid plus crystal phase. Ch, cholesterol; BS, bile salt; L, lecithin.

cholesterol mixed micelles of lamellar structure and the mixed micelles become stable at more than 30 mol% lecithin. This molecular assembly was observed by electron microscopy.

Electron Microscopy

An electron micrograph of artificial bile is shown in Fig. 11. The molecular assembly could be observed in all artificial bile solutions. The size of the molecular assembly in artificial bile was in the range from 0.1 to 0.4μ , but the fine structure of the assembly could not be observed owing to damage by the electron beam. This molecular assembly is the same size as the cigar-shaped assembly (of the order of 0.1μ in length) observed in calculous natural bile and artificial bile by Howell *et al.*⁵⁾

From the results of ESR and enzyme reaction, the following conclusions can be drawn.

a) The micelle phase in Fig. 2 is separated into two phases as shown in Fig. 12. One phase consists of bile salt micelles plus bile salt-lecithin-cholesterol mixed micelles. The other is of bile salt-lecithin-cholesterol mixed micelles.

b) Fatty ester is solubilized in the form of bile salt-ester mixed micelles and bile salt-lecithin-cholesterol-ester mixed micelles.

c) Pancreatic lipase hydrolyzes the substrate present at the surface of oil droplets and the substrate in the substrate-bile salt mixed micelles. However, substrate solubilized in bile salt-lecithin-cholesterol mixed micelles cannot be hydrolyzed.

d) Physical properties of artificial bile can be interpreted in terms of the model of bile proposed by Small *et al.*⁴⁾

From these conclusions and on the basis that bile salt leaves the liver as lecithin-cholesterol lamellae, we consider that normal human bile is present in the phase containing excess bile salt but not in the phase lacking bile salt, as shown in Fig. 12. Therefore, human bile may enhance the hydrolysis of fatty esters by pancreatic lipase.

In the present work, the micelles were studied by the combined use of the ESR spin probe technique and enzyme reaction, so that the biochemical and physical properties of artificial bile could be studied. In particular, the relation between the structure of bile and activity of lipase was analyzed in detail by the use of an ESR spin probe as a substrate. The physical state of human bile was thus made clearer. A simple method for calculation of the lithogenic index of bile will be presented in a subsequent paper.

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References and Notes

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