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Studies on Bile-sensitive Lipase. VI[1]. Effect of Bile Salts on *Mucor* Lipase[2]

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In order to clear the mechanism of activation by bile salts, their effect on *Mucor* lipase and substrates was studied. Optimum pH of the lipase was not influenced by the salts. Heat stability of the lipase was slightly deteriorated by their addition. Michaelis constant (Km) and maximum velocity (Vmax) for olive oil in the presence of 5×10^{-3}M of sodium taurocholate (VII) were 275 (mg) and 1.68 (mg/min), respectively, against 403 and 1.22 in their absence. Activation of the enzyme and the accelerating effect on the substrates by bile salts were not recognized in any concentration tested, though the physical properties, viscosity and surface tension, did change. The number of enzyme molecules adsorbed at oil–water interface increased by the addition of bile salts. A good proportional relation was found between the rate of increased relative activity and quantities of the enzyme adsorbed at the interface, and the quantity increased in proportion to the increasing concentration of bile salts. Analogous results were obtained with pancreatic lipase, but adsorption of *Candida* lipase slightly inhibited by the salts decreased by the salts. Diffusion of oleic acid from the interface was markedly increased by the addition of bile salts.

It has been known for a long time that the rate of hydrolysis of triglyceride by pancreatic lipase can be enhanced by bile salts.[4] However, the mechanism of their action has remained unclear. Most of lipase produced by microorganisms was inhibited by bile salts.[5] Sugiura *et al.* found that in one of lipases from *Mucor javanicus* enzymic hydrolysis was accelerated by bile salts.[5b] Some of their influences on the crude enzyme had already been reported.[5b] It would be interesting to know whether bile salts will have any effect on the enzyme itself, or what the mechanism of activation by bile salts is.

In this report, the effect of bile salts on the activity of lipase and physical properties of olive oil emulsion as substrate, and the effect of bile salts on the adsorption of the enzyme and diffusion of fatty acid at oil–water interface are described.

In the present work all experiments were carried out in the polyvinyl alcohol emulsified system (PVA system), where lipolysis is of the zero order.

Materials and Methods

Preparation of Enzyme——The lipase from *Mucor javanicus* was purified according to the method described in the previous paper,[1] and the purified lipase preparation was used throughout this work. Pancreatic lipase was obtained from Tokyo Kasei Kogyo Co., Ltd., and the lipase preparation from *Candida cylindracea* was a gift from Meito Sangyo Co., Ltd.

1) Part V: T. Ogiso and M. Sugiura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1025 (1969).
2) This forms Part XXXXI of "Studies on Enzymes" by M. Sugiura. This work was presented at the Meeting of Tokai Branch, Pharmaceutical Society of Japan, Nagoya, July, 1968.
3) Location: *Mitahora, Gifu.*
4) *a*) O. Fürth and J. Schütz, *Hofm. Beträge.*, **9**, 28 (1906); *b*) M. Shoda, *J. Biochem.* (Tokyo), **6**, 395 (1926); *c*) E.F. Terroine, *Biochem. Z.*, **23**, 460 (1910); *d*) N. Shimizu, *Symposia on Enzyme Chemistry*, **2**, 43 (1949).
5) *a*) M. Sugiura and K. Nagase, *Yakuzaigaku*, **25**, 47 (1965); **26**, 296 (1966); *b*) M. Sugiura, T. Ogiso, S. Narita, K. Nagase, and H. Asano, *Eisei Kagaku*, **13**, 257 (1967).

Bile Salts and Substrates——Glyco-3α, 12α-dihydroxy-7-oxocholanic acid (I) (mp 224—226°, $[\alpha]_D^{20}$: −2.0±0.9 in EtOH), glyco-3α-hydroxy-12-oxocholanic acid (II) (mp 241.5—243°, $[\alpha]_D^{20}$: +93.8±2.6 in EtOH), glycodeoxycholic acid (III) (mp 186—187°, $[\alpha]_D^{20}$: +46.9±0.9 in EtOH), and ursodeoxycholic acid (IV) (mp 198—203°, $[\alpha]_D^{20}$: +63.2±1.9 in EtOH), which were gifts from Shionogi Seiyaku Co., Ltd., were dissolved in equivalent 0.1N NaOH solution or ethanolic NaOH solution, from which EtOH was distilled off in vacuum and bile salts obtained were dissolved in an appropriate volume of water. Sodium deoxycholate (V), sodium glycocholate (VI), sodium taurocholate (VII), and sodium cholate (VIII) were obtained from Tokyo Kasei Kogyo Co., Ltd. Urso powder and ox bile extracts were obtained from Tokyo Tanabe Seiyaku Co., Ltd., and Wako Jun-yaku Kogyo Co., Ltd., respectively. These bile salts and bile derivatives are shown in Chart 1.

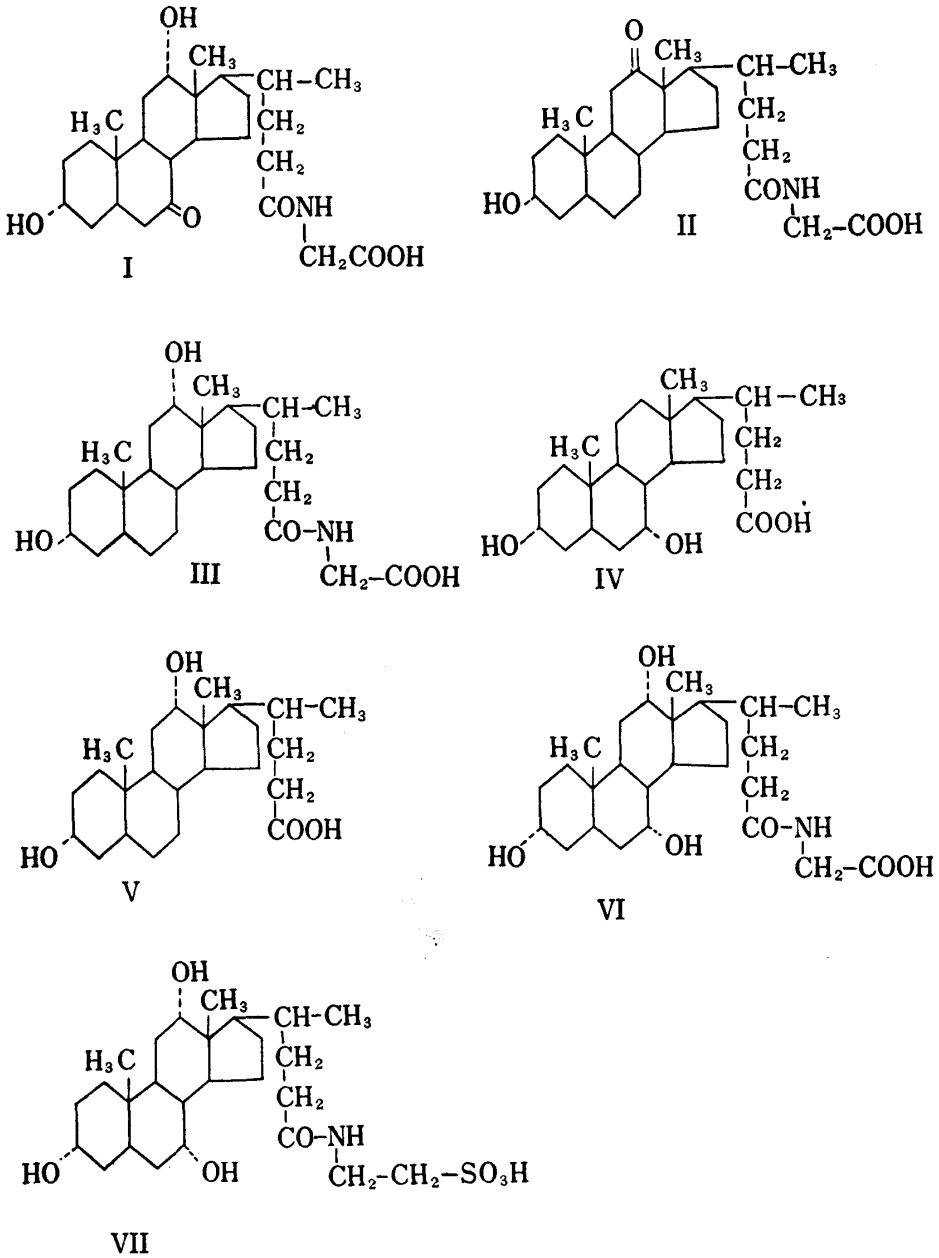

Chart 1

PVA-117, PVA-210, olive oil, and triglyceride were obtained from Kurashiki Rayon Co., Ltd., Iwaki Seiyaku Co., Ltd., and Tokyo Kasei Kogyo Co., Ltd., respectively. All substrates and PVA were of the same lots used in our previous study.[1)]

Assay Procedure

Lipase Assay——The method using a PVA system described in the previous study[1)] was used for the lipase assay.

Measurements of Enzyme Adsorption at Oil-Water Interface

The quantities of enzyme adsorbed at oil-water interface were measured as follows: A mixture of 5 ml of olive oil emulsion, 3 ml of McIlvaine buffer solution (0.2M Na_2HPO_4 and 0.1M citric acid, pH 7.0), and 1 ml of bile salt solution was cooled to 5°, 1 ml of the enzyme solution was added, and after shaking for 30 sec, the mixture was centrifuged at $4,100 \times g$ for 5 min at 5°, 1 ml of the aqueous phase was diluted to 10 or 50 ml with cold water, and submitted to the assay. In addition, the procedure shown in Diagram 1 was used in some experiments. The amount of enzyme adsorbed at oil-water interface was calculated from the difference between the lipase activities in the absence and presence of bile salts.

Diagram 1. Measurements of Enzyme Adsorption at Oil-Water Interface by Sodium Taurocholate

Sample	Adsorption procedure[a]			
	1	2	3	4
Olive oil emulsion	5.0	5.0	5.0	5.0 ml
Phosphate buffer	3.0	3.0	3.0	3.0
Enzyme solution[b]	1.0	—	1.0	—
Water	2.25	2.25	1.25	1.25
Sod. taurocholate solution	—	—	1.0	1.0
	↓	↓	↓	↓
	Mixing for 30 sec and centrifugation for 5 min at $4100 \times g$ at 5°			
	↓	↓	↓	↓
Aqueous phase	1.0	4.5	1.0	4.5
Enzyme solution	—	0.5	—	0.5
	Dilution to 10-fold with cold water and the lipase activity was measured.			

a) All procedures were carried out at 5°.
b) The enzyme solutions were centrifuged at $12500 \times g$ for 15 min before using in this experiment.

Measurement of Diffusion by Fatty Acid from Oil-Water Interface——A mixture of 25 ml of oleic acid and 50 ml of olive oil was emulsified by 2% aqueous PVA solution previously described[1] in a homogenizer cooled with ice to below 10°, and 4 ml of the emulsion was carefully and quietly laid on a mixture of 2 ml of McIlvaine buffer (pH 7.0), pure water and bile salt solution respectively in a test tube (2.80×13.0 cm). After the test tube was incubated at 37° for 6 or 12 hr without agitation, 3 ml of the aqueous phase was carefully taken out with an injector. The fatty acid in the aqueous phase was extracted with the method of Dole,[6] and titrated with 0.01N ethanolic KOH solution, using thymol blue as an indicator.

Determination of Protein and Bile Salt Concentration——The concentration of protein and bile salts was determined from the absorbancy at 280 mμ and by the procedure of Abe,[7] respectively.

Physical Measurement and Apparatus——The apparatus described in the previous study[1] was used in this experiment. The surface tension and viscosity measurement of olive oil emulsion were performed by using a Du Nouy tensiometer and an Ostwald-type viscometer, with a flow time of 22 sec with deionized water, respectively.

Results

Effect of Bile Salts on pH-Activity

The optimum pH for lipase in the presence of VII and VIII, whose concentration was 1.5×10^{-2} and 4.0×10^{-2}M in reaction system, was determined in various kinds of buffer. This purified lipase was most active at pH 7.0, so that pH optimum of lipase was not influenced by bile salts.

Effect of Bile Salts on Heat Stability

Heat stability was measured at pH 6.0 in the presence of VII and VIII. As shown in Fig. 1, the lipase became slightly unstable by the addition of bile salts and lost most of the activity at 50° with a salt concentration of 3×10^{-2}M in the enzyme system.

6) V.P. Dole, *J. Clin. Invest.*, **35**, 150 (1956).
7) Y. Abe, *J. Biochem.* (Tokyo), **25**, 181 (1937).

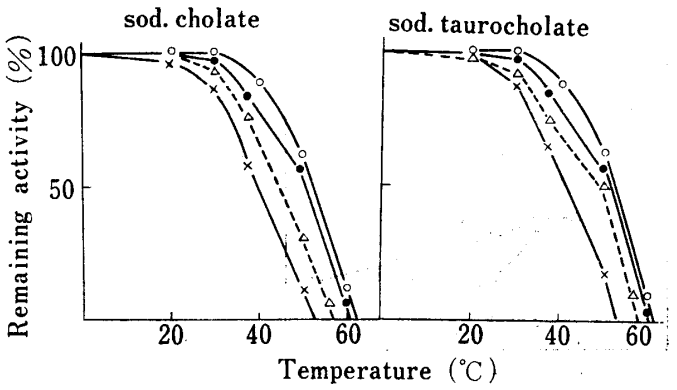

Fig. 1. Heat Stability of Lipase in Presence of Bile Salts

—○— : enzyme alone —●— : enzyme plus bile salt (0.5×10^{-2})[a]
—△— : enzyme plus bile salt (1.5×10^{-2}) —×— : enzyme plus bile salt (3.0×10^{-2})
a) mole concentration in enzyme system

Sod. cholate or sod. taurocholate solution with various concentration was added to enzyme solution (pH 6), the mixture was incubated at each temperature for 20 min, after 1 ml of each solution was diluted to 50 ml the activity was measured by method A.

Effect of Various Bile Salts and Bile Derivatives on Lipase Activity

The effect of VII and VIII on the lipase was tested in concentrations varying from 1×10^{-3} to 5×10^{-2}M at pH 5—8. This result is shown in Fig. 2. The enzyme showed the highest activity at pH 7 in all concentrations of bile salts. The effect of VIII on accelerating lipolysis at pH 7 was slightly lower than that by VII which is a conjugated salt. The inhibitory effect by bile salts was recognized at pH 5 in their low concentration.

Since bile acids occur only as the glycine or taurine conjugates in native bile, the effect of such conjugates on the lipase was tested in concentration ranging from 0.1 to 4.0% (w/v).

In Fig. 3, the relative effectiveness of bile derivatives is given. Addition of VI and VII to the substrate, which are sodium salts of 3,7,12-trihydroxycholanic acid conjugated taurin and glycine, respectively, highly increased the enzymic release of fatty acids from the substrate. Similar results were obtained by the addition of sodium salts of I and II. In contrast, the sodium salt of IV lacking 12-hydroxy group indicated an extreme inhibitory effect. The sodium salt of III and ox bile extracts, which consist primarily of V and VIII, inhibited lipolysis by the lipase in higher concentration of 2—4%, but there was a marked increase in lipolysis in lower concentration of the salts.

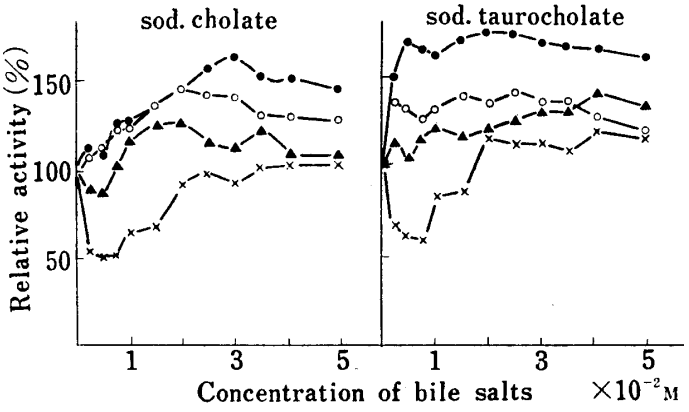

Fig. 2. Effect of Concentration of Bile Salts on Lipase Activity

—○— : activity at pH 8.0 —●— : activity at pH 7.0
—▲— : activity at pH 6.0 —×— : activity at pH 5.0
The activity was assayed by method A.

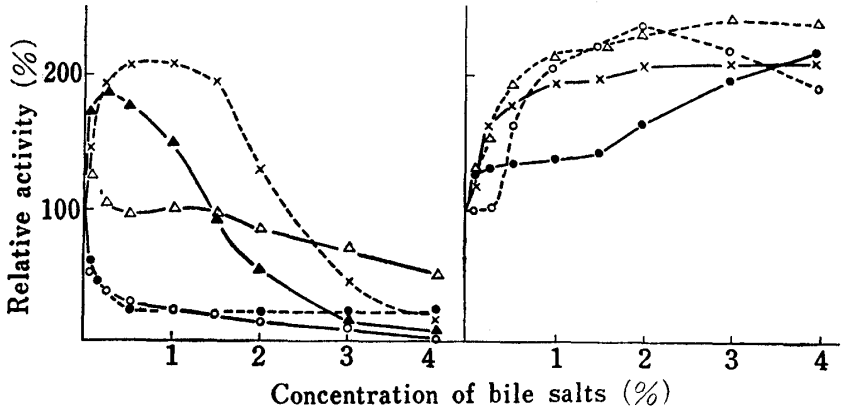

Fig. 3. Effect of Various Bile Salts on Lipase Activity

—○— : sod. ursodeoxycholate
---●--- : urso powder[a]
—△— : sod. deoxycholate
—▲— : sod. glycodeoxycholate
---×--- : ox bile extracts
---○--- : sod. glycocholate
—●— : sod. glyco-3α,12α-dihydroxy-7-oxocholanate
---△--- : sod. glyco-3α-hydroxy-12-oxocholanate
—×— : sod. taurocholate

a) This powder contains sod. ursodeoxycholate chiefly.
The activity was assayed by method B

Michaelis Constant (Km) and Maximum Velocity (V_{max}) in the Presence of Bile Salt

The values of Km and V_{max} for olive oil were determined in a concentration of 5×10^{-3}M of VII in the reaction system. As shown in Fig. 4, the value of Km was reduced from 404 to 275 (mg) and that of V_{max} increased from 1.22 to 1.68 (mg/min). Therefore, activity of the enzyme for the substrate can enhanced by the addition of bile salts.

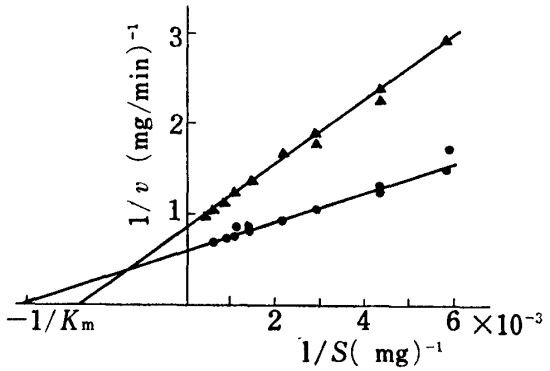

Fig. 4. Lineweaver-Burk Plots of Lipase Activity

—▲— : enzyme alone
—●— : enzyme plus sod. taurocholate[a]

a) Concentration sod. taurocholate was 5×10^{-3}M in reaction mixture.
The activity was assayed by method A

Some Experiments on Mechanism of Activation by Bile Salts

It would be very interesting to know whether the accelerating effect by bile salts in enzymic hydrolysis is due to the direct activation on the enzyme molecule or substrate, whether bile salts assist the adsorption of the enzyme at the substrate or not, if this effect is due to an emulsification of the substrate, and to see the role of bile salts to diffusion of fatty acid from the interface. In this experiment, VII and VIII were chosen because these bile salts have become something of a standard for study in this field.

Effect of Bile Salts on Enzyme

The enzyme solution was incubated with various concentrations of bile salts at 37° and pH 6, for 10 min, and after dilution to 25-fold with cold water the activity was assayed. As shown in Table I, the activation of the enzyme by bile salts was not recognized in any concentrations of them, while the effect of bile salts on the enzyme was examined by variation of preincubation time at 20° and pH 6. As shown in Table II, the effect of preincubation time was very small or none. As a result, the accelerating effect of bile salts on the enzyme molecule seems to be very small.

Table I. Effect of Concentration of Bile Salt on Lipase

Concentration[a] of bile salt ($\times 10^{-2}$M)	Relative activity (%)	
	Sod. cholate	Sod. taurocholate
none	100	100
0.5	91	99
0.75	88	93
1.0	90	100
1.5	67	95
2.0	59	94
2.5	81	93
3.0	78	88
4.0	98	98

a) concentration of bile salt in enzyme system
One ml of enzyme solution (pH 6) and 1 ml of bile salt solution with various concentration were mixed, after preheating at 37° for 10 min, 1 ml of the mixture was diluted to 25 ml with cold water and then the activity was measured (method A).

Table II. Effect of Preincubation Time with Bile Salts on Lipase Activity

Concentration[a] of sod. taurocholate (M)	Relative activity				
	Time 0	15	30	60	90 (min)
1.5×10^{-2}	100	97	98	95	93
3.0×10^{-2}	100	100	97	94	90

a) concentration of sod. taurocholate in enzyme system
4.5 ml of enzyme solution and 0.5 ml of sod. taurocholate solution were mixed, after preheating at 20° for each time 1 ml of the mixture was diluted to 25 ml with cold water and then the activity was measured (method B).

Effect of Bile Salts on Substrate

1) Using Ion Exchanger——The procedure and results are given in Diagram 2. A mixture consisting of 20 ml of olive oil emulsion and 2 ml of 0.2M of VII, and one consisting of the identical amounts of the emulsion and 2 ml of water were incubated at 37° for 10 min. These mixtures were separately passed through Amberlite IRA-900 column (1.7×40 cm), which had been treated with 1M acetic acid, followed by washing with water. Additionally, the same mixtures described above were prepared as a control. The lipase activity was assayed on these four substrates. As shown in Diagram 2, the activity on the substrate from which VII was removed by the ion exchanger was entirely the same as that on the substrate without VII, while the activity on the substrate containing VII reached 158%.

Diagram 2. Effect of Sodium Taurocholate on Substrate

Sample	1	2	3	4	5
Sod. taurocholate solution (0.2M)	—	—	2	2	2 ml
Olive oil emulsion	20	20	20	20	—
Water	2	2	—	—	15
Preheating for 10 min at 37°	↓	↓	↓	↓	↓
		Amberlite IRA −900 column (1.7×40 cm)			
	↓	↓	↓	↓	↓
Lipase activity (%)	100	99	98	158	0
Cholic acid[a] (OD_{475} mμ/ml)	0	0	0	0.380	0.385

a) Mixture was centrifuged at $5500\times g$ for 10 min and cholic acid in aqueous phase was assayed by the procedure of Abe.[7]

2) Variation of preincubation Time with Bile Salt——A mixture consisting of 0.1M solution of VII and several substrates were incubated for various periods at 37°, and then assayed with the addition of enzyme. As shown in Table III, the relative activity after various preincubation periods gave almost the same value. Thus, direct effect of VII on substrates, olive oil, tricaprylin, and triolein, was not found.

Table III. Effect of Preincubation Time with Sodium Taurocholate[a] on Substrates

Preheating time (min)	Relative activity (%)		
	Olive oil	Tricaprylin	Triolein
0	100	100	100
15	99	105	98
30	104	97	97
60	96	105	94
90	100	106	97
120	101	106	101
150	102	104	100

a) Concentration of sod. taurocholate in substrate was 2.5×10^{-2}M.
One ml of emulsion, 0.5 ml of McIlvain buffer (pH 7) and 0.5 ml of sod. taurocholate (0.1M) solution were mixed, after preheating at 37° for each time 0.5 ml of enzyme solution was added to the mixture, and the activity was measured (method B).

3) Effect on Physical Properties of Substrate——A mixture of 5 ml of olive oil emulsion, 4 ml of McIlvaine buffer (pH 7.0) and 1 ml of bile salt solution was submitted to viscosity, surface tension, and particle size measurement. As indicated in Table IV and Fig. 5, viscosity value of the mixture containing a low concentration of bile salts was lower than that in their absence, while that at a high concentration of bile salts was higher. Surface tension of the mixture decreased with the concentration of the salts. However, particle size of olive oil was not changed by the addition of salts, as shown in Fig.5, which was observed under a microscope. The emulsion with bile salts added became unstable and separated in a short time. This fact suggests that oil particles having a weak negative charge will be subjected to the electrostatic repulsion by bile salts which are anionic and negatively charged.

Table IV. Viscosity and Surface Tension of Substrate with Bile Salts Added

Concentration of bile salt ($\times10^{-2}$M)[a]	Sod. cholate		Sod. taurocholate	
	Viscosity (cps)	Surface tension (dyn/cm)	Viscosity (cps)	Surface tension (dyn/cm)
none	2.79±0.09[b]	58.3±0.7[b]	2.79±0.09[b]	58.3±0.7[b]
0.1	2.81±0.05	54.8±0.3	2.89±0.07	54.0±1.2
0.25	2.78±0.03	51.3±0.5	2.79±0.03	50.6±0.5
0.5	2.69±0.06	48.8±0.2	2.62±0.09	48.5±0.6
1.0	2.64±0.04	47.3±0.2	2.63±0.09	48.0±0.6
2.0	2.73±0.07	46.8±0.3	2.77±0.03	47.4±0.5
3.0	2.82±0.08	46.4±0.4	2.90±0.04	47.3±0.8
4.0	2.87±0.05	46.6±0.4	3.01±0.06	47.2±0.9
5.0	2.95±0.04	46.4±0.7	3.11±0.09	47.2±0.8

a) concentration of bile salts in substrate b) mean±S.E of each 5 samples
Substrates consisted of 5 ml of olive oil emulsion, 4 ml of McIlvaine buffer (pH 7.0) and 1 ml of bile salt solution. The measurements were carried at 25°.

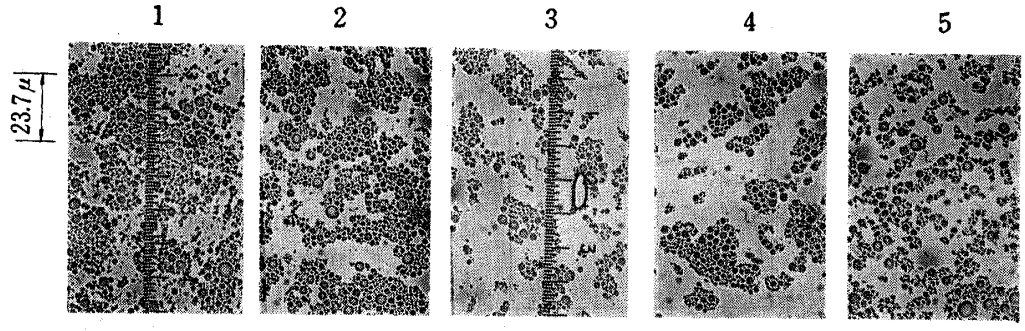

Fig. 5. Microscopic Photograph of Oilve Oil Emulsion

One division of a microscopic eyepiece micrometer is equivalent to 2.37 μ. The emulsion consisted of 5 ml of olive oil emulsion, 4 ml of McIlvaine buffer (pH 7.0) and 1 m lof solution of bile salts.

1: emulsion without bile salts
2: emulsion adding sod. cholate (VIII) into the concentration of 0.005M in the mixture
3: emulsion adding VIII into 0.03M
4: emulsion adding sod. taurocholate (VII) into 0.005M
5: emulsion adding VII into 0.03M

Adsorption of Enzyme at Oil-Water Interface

The quantities of enzyme adsorbed at oil–water interface are shown in Table V. When the solution of VII was added to the substrate, the lipase activity in the aqueous phase, which was obtained by centrifugation at 4,100×***g***, markedly decreased according to increasing concentration of bile salts. The difference between the activity in aqueous phase in the absence and presence of bile salts would be in the number of enzyme molecules adsorbed at oil–water interface. Therefore, the number of enzyme molecules adsorbed at the interface would be increased by the addition of bile salts to the reaction mixture, and the enzyme activity would be enhanced. Similar results were also obtained in the substrates of triglycerides such as tricaplyrin, trilaurin, and tricaprin.

Table V. Relation between Lipase Activity in Aqueous Phase and Quantities of Enzyme adsorbed at Oil-Water Interfase by Bile Salts

Concentration[a] of bile salt (×10^{-2}M)	Sod. cholate			Sod. taurocholate		
	Lipase[b] activity	%	Adsorbed[c] enzyme (%)	Lipase activity	%	Adsorbed enzyme (%)
none	55.0	100	0	55.0	100	0
0.1	55.2	102	0	55.1	101	0
0.5	45.9	84	16	29.5	54	46
1.0	37.4	68	32	29.4	54	46
1.5	30.7	56	44	25.5	46	54
2.0	30.6	56	44	24.9	45	55
3.0	26.9	49	51	26.5	48	52
4.0	29.8	54	46	26.9	49	51
5.0	30.5	56	44	27.4	50	50

a) mole concentration of bile salt in substrate–enzyme mixture
b) lipase activity in μmole of acid/ml by method A
c) Quantities of enzyme adsorbed at oil–water interface were calculated from the difference between the lipase activities in the absence and presence of bile salts.

Correlation among the concentration of bile salts, the quantities of enzyme adsorbed at oil–water interface, and the relative activity is indicated in Fig. 6. Since there was a very well proportional relation between the increased relative activity and the quantities of enzyme adsorbed at the interface, it is concluded that the acceleration of lipolysis by bile salts will depend on the increase in the enzyme molecules adsorbed at oil–water interface.

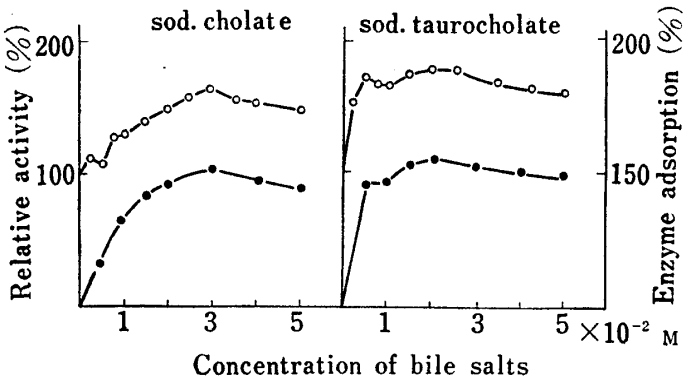

Fig. 6. Correlation between Lipase Activity and Quantities of Enzyme Adsorbed at Oil-water Interface

—○— : lipase activity (method A)
—●— : quantities of enzyme adsorbed to oil-water interface

Adsorption of various enzymes at the interface was therefore measured, using the procedure described in Diagram 1. As shown in Table VI, quantities of the enzyme adsorbed at the interface also increased in proportion to the increasing concentration of bile salts. Analogous results were found in pancreatic lipase whose activation by bile salts had already been recognized,[4] though the quantities of the adsorbed enzyme were comparatively less than that in *Mucor* lipase. However, adsorption of *Candida* lipase, slightly inhibited by bile salts, decreased by the salts, and some of

Table VI. Adsorption of Enzyme at Oil–Water Interface by Sodium Taurocholate

Concentration[a] of sod. taurocholate (M)	Sample[b]	Enzyme[c] Mucor lipase Activity[d] μmole of acid	Mucor lipase Activity[d] (%)	Mucor lipase Adsorbed Enzyme (%)	Pancreatic lipase Activity[d] μmole of acid	Pancreatic lipase Activity[d] (%)	Pancreatic lipase Adsorbed Enzyme (%)	*Candida* lipase Activity[d] μmole of acid	*Candida* lipase Activity[d] (%)	*Candida* lipase Adsorbed Enzyme (%)
1×10^{-3}	1	13.7	72	28	10.3	56	44	3.9	10	90
	2	19.0	100	0	18.3	100	0	37.1	100	0
	3	12.6	66	34	9.8	54	46	12.5	35	65
	4	19.0	100	0	18.3	100	0	37.2	100	0
1×10^{-2}	1	9.2	71	29	7.2	55	45	1.5	11	89
	2	12.9	100	0	13.1	100	0	13.5	100	0
	3	7.5	58	42	6.0	46	54	8.5	63	37
	4	13.0	101	0	13.2	101	0	13.6	101	0

a) concentration of sod. taurocholate in substrate–enzyme mixture
b) The samples were prepared by the procedure described in Diagram 1.
c) These enzyme solutions were centrifuged at 12500×*g* for 15 min before using in this experiment.
d) activity in aqueous phase
The activity was measured by method A.

the inhibition of *Candida* lipase by bile salts may be based on the decreased adsorption of the enzyme at oil–water interface.

Effect of Bile Salts on Diffusion of Fatty Acid from Oil-Water Interface

It has been known that lipolysis was inhibited by amphipathic soaps as a product arising during the enzyme reaction,[8] therefore, to remove the products from oil–water interface is an important factor for progress of the reaction. In order to

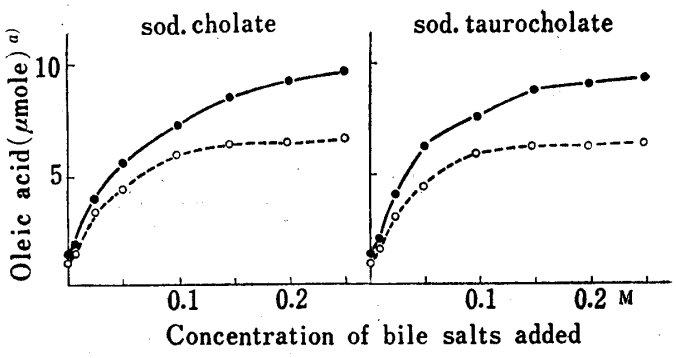

Fig. 7. Effect of Bile Salts on Diffusion of Oleic Acid from Oil-Water Interface

a) μmoles of oleic acid diffused from the interface of 6.15 cm²
---○--- : after 6 hr —●— : after 12 hr

clarify the role of bile salts to diffusion of fatty acid from the interface, the effect of the salts on diffusion of oleic acid in the emulsion was examined. As shown in Fig. 6, the rate at which oleic acid was diffused from the interface was very slow unless bile salts were added. However, the rate was markedly increased by the addition of the salts and enhanced in proportion to the increasing concetration of bile salts. Therefore, bile salts are able to accelerate diffusion of fatty acid arising during the reaction at oil–water interface.

Discussion

In order to clear the mechanism of potentiation of *Mucor* lipase activity by bile salts, their effect on the enzyme and substrate was studied.

Effect of pH on enzyme activity in the presence of bile salts was the same as that in their absence. Therefore, optimum pH of the lipase was found not to be influenced by the salts. Borgström had concluded that the concentration of VII influenced the optimum pH of the lipolytic reaction of pancreatic lipase.[9]

As shown in Fig. 2, even at the same concentration of bile salts, various rates of hydrolysis occurred at different pH, a higher lipolysis at pH 6—8 and a slight inhibition at pH 5. This may be due to the decreased action as the anionic surfactant at pH 5, and adsorption of enzyme molecules at oil–water interface will decrease. This fact is demonstrated by the result in Table VII, in which the enzyme and substrate were mixed, and the lipase activity in the aqueous phase was measured after centrifugation. The activity increased with decreasing pH of the mixture.

Table VII. Adsorption of Enzyme at Oil–Water Interface at Various pH

Concentration of sod. taurocholate ($\times 10^{-2}$M)	Relative activity of aqueous phase (%)			
	pH 5	6	7	8
0.75	136	125	100	90
1.0	136	119	100	92

All procedures were carried out in the same way as described for assay procedure.

A smaller *K*m value of the enzyme in the presence of bile salt, as shown in Fig. 4, will be due to the increased numbers of enzyme molecules adsorbed at oil–water interface by the salts.

With the potentiation of pancreatic lipase by various bile acid, it was concluded that the lipolytic action was highly accelerated by 3,12-dihydroxybile acid, *i.e.*V. Lipolysis of *Mucor* lipase was accelerated by 3,7,12-trihydroxybile acids, such as VI and VII, but V, lacking 7-hydroxy group, and IV, lacking 12-hydroxy group showed inhibitory effect. Inhibitory effect of the latter, which caused gelation of the emulsion, is an undesirable action against substrate. However, IV and V will be expected to exert a pronounced activating effect mediated by bile salts *in vivo*.

There are numerous reports on the mechanism of lipolytic potentiation of pancreatic lipase by bile salts.[4c,10] However, the mechanism of the activation by bile salts remains obscure.

As a result of the present experiments, the enzyme and substrate were not activated by bile salts directly. Although physical properties of the substrate, such as viscosity and sur-

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face tension, changed to some extent, a change in the properties was not proportional to the lipolytic potentiation of the enzyme by bile salts. Olive oil emulsion, well emulsified by PVA, was not subjected to further emulsification and rather, the emulsion with bile salts added became unstable and separated. However, quantities of the enzyme adsorbed at the interface were increased by the salts. Therefore, one of the essential roles of bile salts to this lipase will be to increase the adsorption of the enzyme molecules at oil–water interface rather than to act as a true lipase activator or to emulsify the substrate. The reason why *Mucor* lipase was adsorbed at the interface more than other microbial lipase, *e.g.*, *Candida* lipase,[5b] is considered as follows. The lipase is an acidic protein, whose isoelectric point measured with a Tiselius–type apparatus was found to be about pH 3.8, so that the enzyme would have a negative charge at pH 7.0 and would be subjected to electrostatic repulsion by the negative charge of bile salts in the aqueous phase. Consequently, the enzyme might be crowded out near the interface. It has often been emphasized that electrostatic forces played an important role for the binding of lipase[8] and phospholipase[11] at the substrate–water interface, so that many enzyme molecules crowded out near the interface would be fixed at the interface by Coulomb's force.

In both of *Mucor* and pancreatic lipase, lipolytic action is accelerated by bile salts. However, the mechanism of activation of the former seems not to be identical with that of the latter because of difference in the qualities of these enzymes. To cite one example, the isoelectric point of the former is about pH 3.8, while that of the latter (hog pancreatic lipase) is about 5.2.[12]

In order to reproduce as closely as possible the conditions existing during lipolysis, a mixture of oleic acid and olive oil was emulsified by 2% aqueous PVA solution., and diffusion of oleic acid from oil–water interface was measured. Since the rate of diffusion was markedly increased by the addition of bile salts, another important role of bile salts is to enhance lipolysis by the continuous removal of fatty acid and soaps from the interface. The salts seems to remove fatty acid and soaps from the interface through formation of water–soluble molecular or micellar associations.[13]

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