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Studies on Bile-sensitive Lipase. VIII.[1] Product Inhibition of *Mucor* Lipase and Role of Bile Salts and Ca^{2+} to the Inhibition[2]

Mamoru Sugiura and Taro Ogiso

Gifu College of Pharmacy[3]

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In order to clarify the effect of the products formed during lipolysis by *Mucor* lipase, some experiments were done. The lipase was inhibited by soaps of long-chain fatty acids above C_{12}, while the lipase was little affected by soaps of C_4—C_8 fatty acids, glycerin and monoglycerides. Long-chain fatty acids in very low concentration, less than 5 μmoles, acted as a competitive inhibitor. Although diffusion of oleic acid from the interface was arrested by laurate and oleate because of the accumulation of the salts at the interface, by the addition of bile salt to the system diffusion of oleic acid was promoted. Bile salts reduced inhibition by long-chain fatty acids, consequently the initial rate of the reaction did not reduce for some time. Ca^{2+} also prevented the inhibition by removing the fatty acids into the oil phase after the formation of Ca-soaps.

Lipase [glycerol ester hydrolase EC 3.1.1.3.] which hydrolyzes triglycerides has special property of being capable of functioning efficiently at the oil–water interface. Therefore, any materials that can alter the nature of this oil–water interface may markedly influence the activity of lipase, and the lipolysis in heterogeneous emulsions may be controlled by other factors not normally existed in homogeneous system. It has been recognized that the hydrolysis of triglycerides by pancreatic lipase was inhibited by soaps of long-chain fatty acids.[4] The inhibition was explained to be preventing the formation of the enzyme–substrate complex[4a] and the block of the interface by the soaps,[4b] but the mechanisms of the inhibition are not yet clear.

It is supposed that the products formed from the hydrolysis of triglyceride influence the rate of lipolysis. In order to clarify the mechanism of inhibition by products, effects of sodium salts of fatty acids, free fatty acids, glycerin and monoglycerides on *Mucor* lipase were measured. At the same time, the role of bile salts and Ca^{2+} on the inhibition by the product, and the effect of them on the diffusion of oleic acid from the oil–water interface were studied.

Materials and Methods

Preparation on Enzyme——The lipase from *Mucor javanicus* was purified according to the method described in the previous paper,[5] and the purified lipase preparation was used throughout this work.

Chemicals——Sodium cholate, sodium taurocholate, sodium salts of fatty acids and triglycerides were obtained from Tokyo Kasei Kogyo Co., Ltd., olive oil and polyvinyl alcohol (PVA)[5] were obtained from Iwaki Seiyaku Co., Ltd., and Kurashiki Rayon Co., Ltd., respectively. The other chemicals were of special or reagent grade.

Assay Procedures——Lipase Assay: Olive oil emulsion and triglyceride emulsion were prepared by the same method as described in the previous paper.[5] A mixture consisting of 1 ml of the emulsion, 1 ml

1) Part VII: M. Sugiura and T. Ogiso, *Yakugaku Zasshi*, **89**, 1289 (1969).
2) This forms the Part L of "Studies on Enzymes" by M. Sugiura. This work was presented at the Meeting of Tokai Branch, Pharmaceutical Society of Japan, Nagoya, September, 1969.
3) Location: *Mitahora, Gifu.*
4) *a*) G. Benzonana and P. Desnuelle, *Biochim. Biophys. Acta*, **164**, 47 (1968); *b*) F.H. Mattson and R.A. Volpenhein, *J. Am. Oil Chemists' Soc.*, **43**, 286 (1966).
5) T. Ogiso and M. Sugiura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1025 (1969).

of McIlvaine buffer (0.2M Na_2HPO_4 and 0.1M citric acid, pH 7.0) and 0.5 ml of sodium salt solution of fatty acid was incubated at 37°, 0.5 ml of bile salt or $CaCl_2$ solution was added to the mixture if necessary. Veronal buffer (0.1M sodium barbital and 0.1N HCl, pH 7.0) was used instead of McIlvaine buffer in the presence of Ca^{2+}. Then the lipolysis was started by the addition of 0.5 ml of enzyme solution (0.4—0.5 unit/ml). After 20 min the reaction was stopped with a mixture of iso-PrOH–heptane–2N H_2SO_4 (40:20:1, by volume) and titrated with 0.01N ethanolic KOH solution by the same method as mentioned in previous study[5] (Reaction system).

The experiments in enzyme system was done in the same procedure as described in Fig. 1.

One unit of lipase was defined as the amount of enzyme which was able to liberated 1 μmole of free fatty acid per min under the conditions as described in previous paper.[5]

Measurement of Diffusion of Oleic Acid from Oil-Water Interface——An emulsion was obtained by homogenizing 25 ml of oleic acid, 50 ml of olive oil and 225 ml of 2% solution of a mixture of 9-volume of PVA 117 and one-volume of PVA 210 as described previously.[6] Four ml of this emulsion was carefully and quietly laid on a mixture consisted of 2 ml of McIlvaine buffer (or 0.1M acetic acid, pH 6.8), 2 ml of sodium salt of fatty acid and 2 ml of bile salt (or $CaCl_2$) solution in a test tube (2.8×11.0 cm) with a stopper. After incubation for 6 or 12 hr at 37° without any agitation, about 3 ml from the aqueous phase (total volume 6 ml) was carefully transferred to another test tube with a syringe. The fatty acid in the sample was extracted with the method of Dole,[7] and titrated with 0.01N ethanolic KOH solution, using thymol blue as an indicator.

Results

Effect of Sodium Salts of Fatty Acids on Lipase Activity

The effect of varying concentration of sodium salts of fatty acids added to the standard incubation mixture was tested. As shown in Table I, the inhibition by any concentration of sodium salts of C_4—C_8 fatty acids was not observed, while sodium salts of fatty acids above C_{12} inhibited the lipase activity in proportion to the amount of soaps added. Strong inhibition of lipolysis by oleate was found at concentration more than 0.2% (w/v). The rate of inhibition by sodium salts of fatty acids was generally enhanced with the increase of carbon number of fatty acid; lipolysis was markedly inhibited by stearate, oleate and linolate.

As shown in Fig. 1, moreover, the powerful inhibition by sodium salts of long-chain fatty acids was found in enzyme system.

Table I. Effect of Sodium Salts of Fatty Acids on Lipase Activity

Concentration[a] of sod. salts of fatty acid (%, w/v)	Remaining activity Sodium salt of fatty acid									
	C_4	C_6	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	C_{18-1}	C_{18-2}
None	100[b]	100	100	100	100	100	100	100	100	100
0.001	98	102	99	98	97	99	103	97	99	98
0.0025	97	97	102	102	99	97	102	96	99	79
0.005	98	98	97	100	100	98	95	84	90	69
0.01	101	101	100	100	101	91	87	74	88	52
0.025	98	99	96	100	97	75	76	73	84	27
0.05	104	100	98	93	86	67	71	61	62	21
0.1	99	100	97	95	66	47	46	38	40	22
0.2	100	106	95	89	55	—	—	—	0	15
0.5	104	110	101	95	32	—	—	—	0	7
1.0	116	111	92	87	6	—	—	—	0	0

a) concentration in reaction mixture b) Figures are the mean values of each 4 incubations.

Since 80% of the added soap is found as free fatty acid and only 20% is soap at pH 7,[4b] free fatty acids appear to be a cause of the inhibition mentioned above. To clarify the expec-

6) T. Ogiso and M. Sugiura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1034 (1969).
7) V.P. Dole, *J. Clin. Invest.*, **35**, 150 (1956).

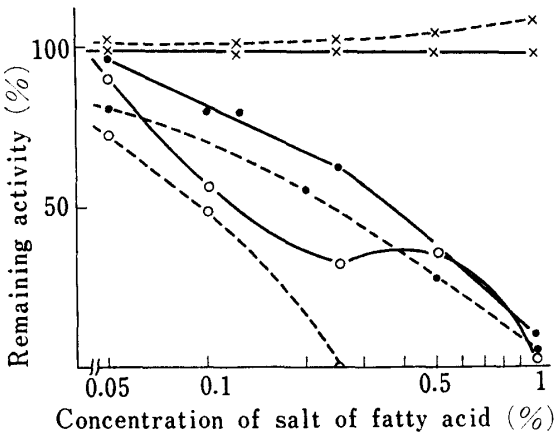

Fig. 1. Comparison between Effect of Sodium Salts of Fatty Acids in Enzyme System and Reaction System on Lipase Activity

The effect in enzyme system was done as follows; a mixture consisting of 1 ml of enzyme solution (50 units/ml) 0.5 ml of sodium salt of fatty acid and McIlvaine buffer (pH 7.0) was incubated at 37° for 20 min. After 50-fold dilution with water the activity was assayed.

——: in enzyme system ------: in reaction system
●: with sodium laurate ○: with sodium oleate
×: with sodium butyrate

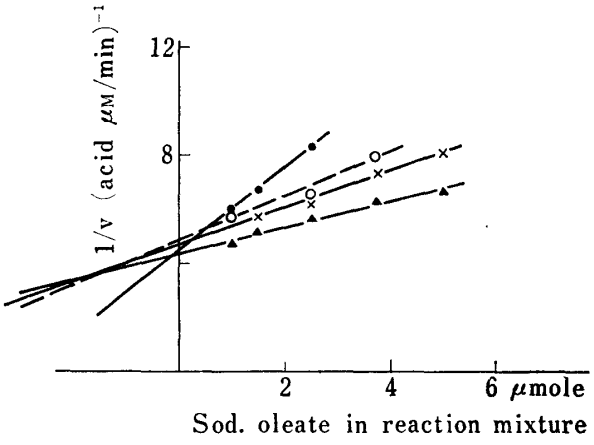

Fig. 2. Inhibition of Lipase Activity by Sodium Oleate

The reaction mixture contained each 1 ml of olive oil emulsion and McIlvaine buffer (pH 7.0), 0.5 ml of sodium oleate and 0.5 ml (0.25 unit) of enzyme solution. The reaction mixture was incubated at 37° for 20 min.

●—●: 5%(v/v) olive oil emulsion
○—○: 7.5%
×—×: 10%
▲—▲: 12.5%

tation, olive oil emulsion with oleic acid added was prepared, and the activity on the emulsion was compared with on a normal emulsion, The results were shown in Table II, the inhibition of lipolysis was enhanced with an increase of oleic acid. Consequently, it is suggested that a part of inhibition of the activity by the addition of sodium salts of fatty acids at pH 7 may be due to free fatty acids.

Table II. Inhibition of Lipase Activity by Oleic Acid in Emulsion and Protecting Effect of Bile Salt

Concentration of oleic acid in reaction mixture (%, v/v)	Activity (%)		
	Without bile salt	With taurocholate	
		0.0125M[a)]	0.025M[a)]
none	100	100	100
0.1	58	94	104
0.2	41	69	90
0.4	38	60	87
0.5	26	40	61

a) concentration in reaction mixture
Emulsions with oleic acid added into 0.5—2.5%(v/v) were prepared, respectively, and the activities on the emulsions were assayed.

Mode of Inhibition by Sodium Satls of Long-Chaine Fatty Acid in Low Concentration

It is clear from the above experiments that lipolysis by the lipase is inhibited by sodium salts of long-chain fatty acids, but it is necessary to clarify whether the salts inhibited the liapse irreversibly or competitively as substrate analogue.

The mode of inhibition by oleate in dilute soludion, less than 10 μmoles, was studied and the results were shown in Fig. 2. It was observed by the Dixon's plot[8)] that low concentration (less than 5 μmoles) of the salt inhibited competitively.

8) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

Similar results were also obtained by laurate.

Although sodium salts of long-chain fatty acids in very dilute solution will be competitive with the substrate, the salts at higher concentration may be combined with the enzyme rather than with the substrate, making the lipase inactive as shown in Fig. 1.

Effect of Glycerin and Monoglycerides on Lipase Activity

Glycerin and monoglyceride, which are end product and intermediate of the lipolysis, respectively, were added to the reaction mixture in order to know the possible effect on the enzyme. As shown in Table III, glycerin, monostearin and monoolein did not inhibit the activity, on the contrary monoolein was stimulated the hydrolysis to a small extent only. It is clear from the above result that a small amount of glycerin and monoglycerides formed during initial lipolysis did not influence the lipase action at all.

Table III. Effect of Glycerin and Monoglycerides on Lipase Activity

Concentration (%, w/v)	Activity (%)		
	On olive oil emulsion		On trilaurin emulsion
	Glycerin	Monostearin[a)]	Monoolein[a)]
None	100	100	100
0.005	103	96	111
0.01	100	94	106
0.025	97	98	113
0.05	97	95	110
0.1	101	96	106
0.25	97	—	105
0.5	99	—	95
1.0	101	—	—

a) The values were corrected for lipase activity on monoglyceride.

Effect of Bile Salts on Inhibition by Sodium Salts of Fatty Acids

It is very interesting to know protecting action of bile salts against inhibition by sodium salts of long-chain fatty acids, and to know whether one of accelerating effect on lipolysis by bile salts is due to protection against the inhibition, thus some experiments on the effect of bile salts were done.

Effect of sodium salts of fatty acids and bile salt on diffusion of oleic acid: Diffusion of oleic acid from oil-water interface was measured in the presence of sodium salts of fatty acids or bile salt. As shown in Fig. 3a and 3b, diffusion of oleic acid was arrested by the addition of laurate and oleate. Only 2 mg of laurate or oleate blocked the interface (minus values in Fig. 3 indicate the accumulation of sodium salts of fatty acids added) and hindered diffusion of oleic acid from the interface. On the contrary butyrate and caprylate slightly accelerated the diffusion. Therefore, sodium salts of long-chain fatty acids appear to hinder the diffusion of the products from the interface and cause the inhibition apparently.

In the presence of bile salts, *i.e.* taurocholate, the accumulation of soaps of long-chain fatty acids reduced and oleic acid diffused into the aqueous phase. The rate at which oleic acid was diffused from the interface was accelerated in proportion to the increasing concentration of taurocholate added. However, when sodium salts of long-chain fatty acids relatively increased as compared with the amount of taurocholate, the acccumulation of the salts at the interface was observed. This suggests that bile salts have a limitary function in accelerating diffusion of fatty acids.

Protecting effect of bile salts on inhibition by sodium salts of long-chain fatty acids: In Fig. 4, inhibition of lipolysis by soaps of long-chain fatty acids, such as myristate, palmitate

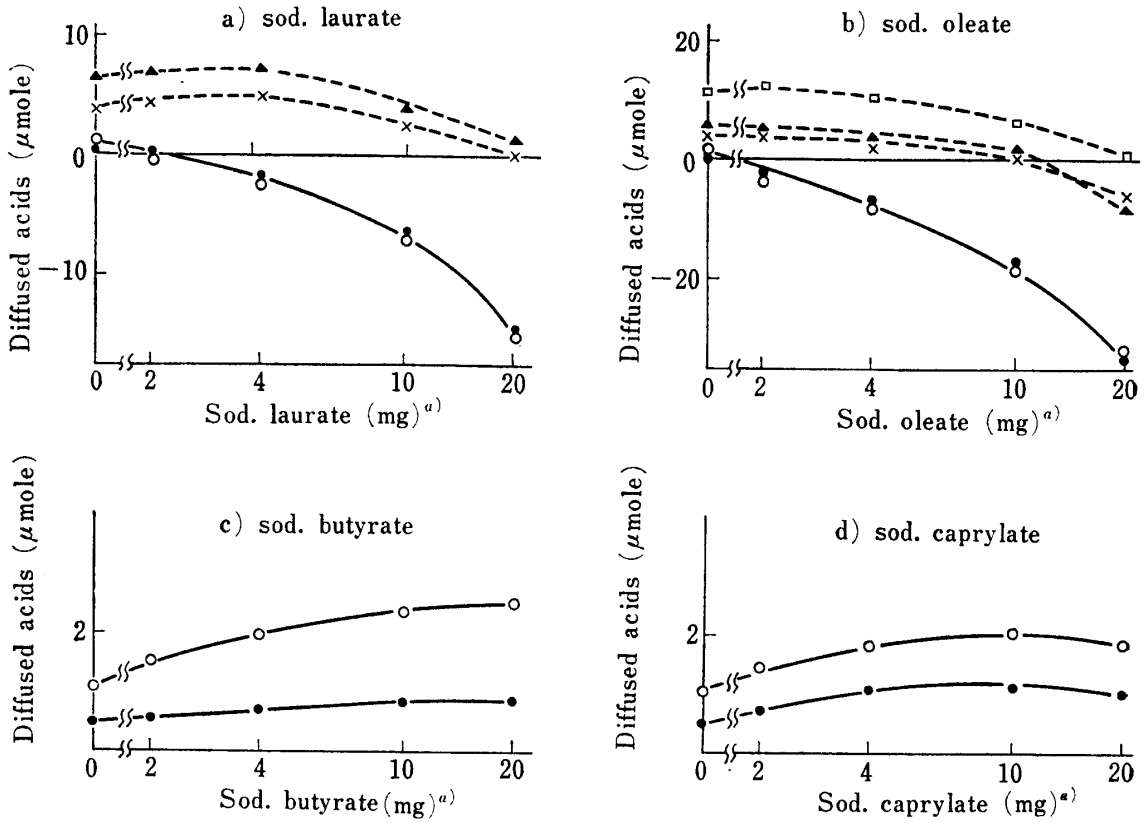

Fig. 3. Effect of Sodium Salts of Fatty Acids on Diffusion of Oleic Acid from Oil-Water Interface in the Presence or Absence of Bile Salt at pH 7.0

—●—: amount of acid (μmole) in the aqueous phase after 6 hr in the absence of taurocholate
—○—: amount of acid (μmole) after 12 hr in the absence of taurocholate
---×---, ---▲---: amount of acid (μmole) after 6 and 12 hr in the presence of 100 μmole of taurocholate, respectively
---□---: amount of acid (μmole) after 12 hr in the presence of 300 μmoles of taurocholate
a) amount of sodium salt of fatty acid added to the aqueous phase

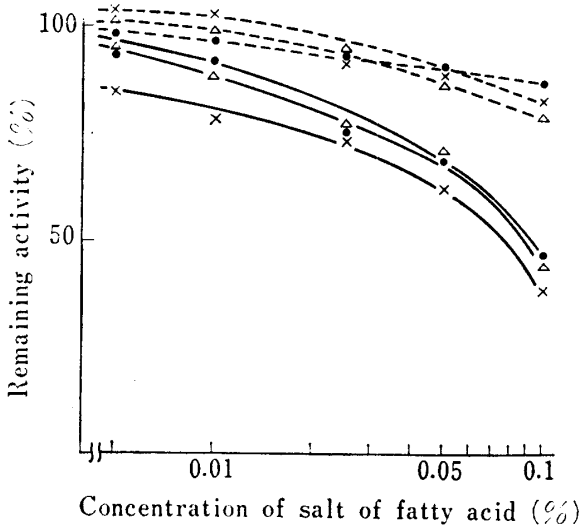

Fig. 4. Protecting Effect of Bile Salt on Inhibition by Sodium Salt of Fatty Acid

——: without bile salt
——: with 0.0125M taurocholate in reaction mixture
●: C_{14} △: C_{16} ×: C_{18}

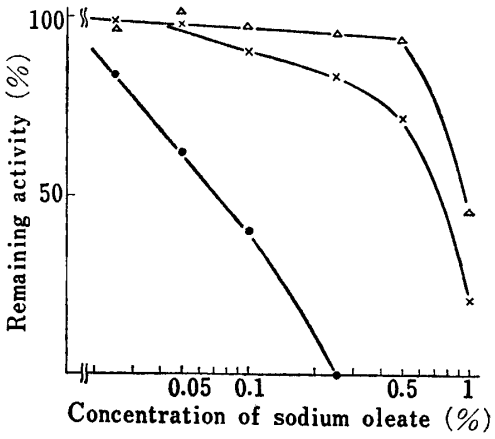

Fig. 5. Protecting Effect of Sodium Taurocholate on Inhibition by Sodium Oleate

—●—: without bile salt
—×—: with 0.0125M taurocholate in the reaction mixture
—△—: with 0.025M taurocholate in the reaction mixture

and stearate, and protecting effect of taurocholate on the inhibition were shown. Inhibition by the soaps reduced in the presence of 0.0125M taurocholate. Inhibition by laurate also diminished to some extent by the addition of bile salt. As shown in Fig. 5, taurocholate was also found to have excellent protecting effect on inhibition by oleate. Although 0.25% oleate in reaction mixture gave 100% inhibition of the lipolysis, 0.025M taurocholate produced a 95% reduction of the inhibition. A similar result was shown in the case of inhibition by linolate and free oleic acid (Table II). The protecting effect of cholate was slightly lower than that of taurocholate which is a conjugated salt in all cases. As a result of these experiments, bile salts appear to be able to reduce inhibition by soaps of long-chain fatty acids.

Effect of sodium oleate and bile salt on the rate of lipolysis: It is suggested from above results that long-chain fatty acids formed during lipolysis may affect hydrolysis by the enzyme at the interface to some extent, and the inhibition by the acids may be prevented, if bile salt is in the system. Therefore, the rate of lipolysis was measured in the presence of oleate and bile salt. The results were shown in Fig. 6. By the addition of 10 μmoles of sodium oleate the rate was decreased soon. On the other hand, when fatty acid formed during lipolysis was over a certain amount a decrease of the rate was also observed. In the presence of 0.015M taurocholate the rate did not decrease and even by the addition of 10 μmoles of sodium oleate a decrease of the rate was not found till 50 min. Therefore, it is supposed that bile salts may be able to prevent inhibition by long-chain fatty acids, consequently the initial rate of the reaction will not decrease for some time.

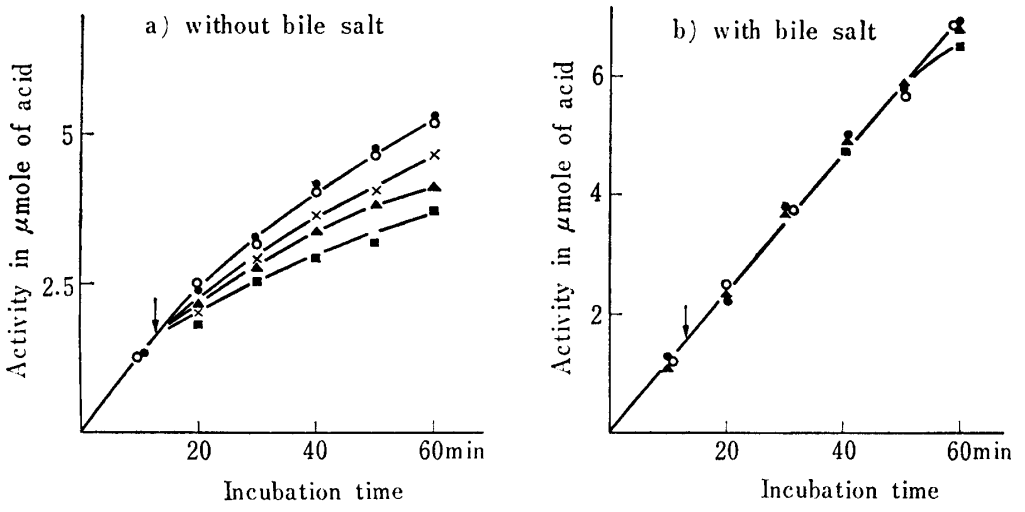

Fig. 6. Effect of Sodium Oleate and Bile Salt on Rate of Lipolysis of Olive Oil Emulsion

The arrow indicates the addition of sodium oleate.
●—●: enzyme alone ○—○: with 2.5 μmole of sodium oleate ×—×: with 5 μmole of sodium oleate
▲—▲: with 7.5 μmole of sodium oleate ■—■: with 10 μmole of sodium oleate
0.12 and 0.06 unit of lipase were used in Fig. 6a) and Fig. 6b) respectively. Concentration of taurocholate was 0.015M in reaction mixture.

Effect of Ca^{2+} on Inhibition by Sodium Salts of Fatty Acids

Effect of Ca^{2+} on diffusion of oleic acid: Although it has been known that lipolysis was enhanced by Ca^{2+},[9] a role of Ca^{2+} on the removal of fatty acids formed during lipolysis was unclear. To clarify the action of Ca^{2+} on the diffusion of the acids from the interface, diffusion of oleic acid was measured in the presence of 0.008—0.17M Ca^{2+}: Diffusion of oleic acid into the aqueous phase was not accelerated by Ca^{2+}, and the difference between the rate of diffusion in the absence or presence of Ca^{2+} was not found.

9) F. Schønheyder and K. Volqvartz, *Acta, Physiol. Scand.*, **10**, 62 (1945); B. Borgström, *Biochim. Biophys. Acta*, **13**, 491 (1954); Y. Ota and K. Yamada, *Agr. Biol. Chem.* (Tokyo), **30**, 1030 (1966); S. Oi, A. Sawada and Y. Satomura, *Agr. Biol. Chem.* (Tokyo), **31**, 1357 (1967).

Effect of Ca^{2+} on inhibition by sodium oleate: As shown in Fig. 7, marked protection against the inhibition was obtained in the presence of 0.01 M Ca^{2+}. As a result of this experiment, it was found that 0.01 M Ca^{2+} prevented inhibition by oleate (oleic acid) below 0.4—0.5% (0.01 M Ca^{2+} is equivalent to 0.61% sodium oleate), while 0.001 M Ca^{2+} was not able to reduce the inhibition.

By the addition of Ca^{2+} to a mixture consisting of sodium oleate solution and veronal buffer (pH 7.0), insoluble precipitate of calcium oleate was formed, which was identified with a flame reaction and a thin-layer chromatography. When olive oil was laid on the mixture with Ca^{2+} added, the precipitate rose to the interface and dissolved into the olil phase. In this way Ca^{2+} was able to remove long-chain fatty acids from the interface. Therefore it is clearly suggested that Ca^{2+} is possible to protect the enzyme from the inhibition by long-chain fatty acids, forming Ca-soaps.

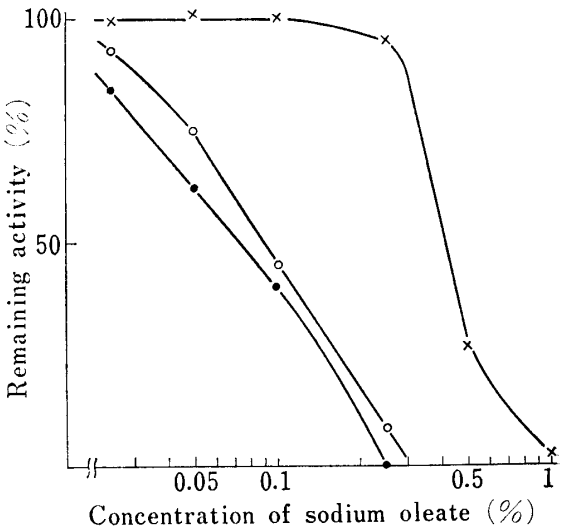

Fig. 7. Protecting Effect of Ca^{2+} on Inhibition by Sodium Oleate

—●—: without Ca^{2+}
—○—: with 1×10^{-3} M Ca^{2+} in reaction mixture
—×—: with 1×10^{-2} M Ca^{2+} in reaction mixture

Discussion

It is important to know the effect of products formed during lipolysis on lipase action in order to clarify the mechanism of lipolysis. It has been known that lipolysis by pancreatic lipase was inhibited by long-chain soaps and the inhibition was prevented by Ca^{2+} and bile salts to some extent.[4a] However, the mechanism of which lipolysis is inhibited by long-chain soaps and of which Ca^{2+} and bile salts prevent inhibition has remained unclear.

As a results of the present experiments, *Mucor* lipase action was inhibited by soaps of long-chain fatty acids above C_{12}. Formation of the aggregate of long-chain fatty acids at pH 7 was observed under a microscope. The aggregate may be associated with the enzyme molecules and cause a steric hinderance to the active centers in enzyme molecules. Moreover, poor diffusion and accumulation of soaps of long-chain fatty acids formed during lipolysis will induce early inhibition of tle reaction at the interface, where lipolysis by lipase is suggested to take place.[4a,10] The accumulated soaps will influence by either a steric or a charge effect the access of lipase to the triglyceride molecules situated at the interface. Thus, long-chain fatty acids and the soaps formed during the reaction may cause inhibition on the lipase action. On the other hand, lipolysis was not inhibited by soaps of short-chain fatty acids. This is readily explained by the free diffusion of soaps of shortchain fatty acids. Even if the soaps combine with enzyme, the complex will readily split by the free diffusion. This is also inferred from the results shown in Fig. 3.

As shown by kinetic studies (Fig. 2), long-chain fatty acids in very low concentration are considered to act as a competitive inhibitor in presence of excess amount of triglycerides. However, powerful inhibition by possible combination of soaps of long-chain fatty acids with enzyme molecules was observed, when the amount of long-chain fatty acid was increased. Since lipolysis by the lipase was not inhibited by glycerin and monoglycerides which are products of the reaction, product inhibition may be mainly caused by soaps of long-chain fatty

10) P. Desnuelle and P. Savary, *J. Lipid Res.*, **4**, 369 (1963).

acids above C_{12}.

A role of bile salts on the inhibition by soaps of long-chain fatty acids was suggested as follows: separation of the complex between the enzyme and the soaps, inhibition of the formation of the complex, and protection of the enzyme from the inhibition by the soaps. The protecting action is strengthened with increasing concentration of bile salts. This fact is suggested by the results in Fig. 6, where initial velocity of the reaction did not decrease in the presence of taurocholate, whereas in the absence of the salt the rate decreased rapidly. Moreover, bile salts were able to accelerate diffusion of the products, *e.g.* long-chain fatty acids, from the interface and so enhance lipolysis.

It is reported that a role of Ca^{2+} is not to activate pancreatic lipase, but rather to prevent inhibition by soaps.[4a] With the inhibition of *Mucor* lipase by long-chain fatty acids, similar results were given. The mechanism appears to prevent the formation of the complex between the soaps and enzyme, forming insoluble Ca-soaps, and to enhance lipolysis by removing fatty acids as Ca-soaps into the oil phase. One mole of Ca^{2+} will probably combine with two moles of fatty acid. Fukumoto, *et al.* mentioned that activation of lipolysis by Ca^{2+} was found after a certain amount of fatty acids was formed.[11] This observation is consistent with our view that Ca^{2+} prevents the enzyme from the inhibition by long-chain fatty acids formed during lipolysis and removes the acids into the oil phase. Thus, the mechanism by which Ca^{2+} prevents the enzyme from the inhibition is not the same as that by bile salts.

11) J. Fukumoto, M. Iwai and Y. Tsujisaka, *Symposia on Enzyme Chemistry*, **18**, 53 (1962).