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Studies on Bile-sensitive Lipase. IX.[1] Action Pattern and Mechanism of Lipolysis by *Mucor* Lipase[2]

Taro Ogiso and Mamoru Sugiura

Gifu College of Pharmacy[3]

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To clarify the hydrolysis rate of esters at each position of triglyceride(TG), the positional specificity and mechanism of lipolysis by *Mucor* lipase, kinetic studies and action pattern of hydrolysis on glycerides were studied. It was found by thin-layer chromatography that the lipase firstly hydrolyzed the esters at outer position of TG and next the inner chain, and the hydrolysis rate of esters was in the order: TG>2,3-DG≒MG, however, in the presence of bile salts: TG>2,3-DG>MG. Although in the presence of bile salts the rate at initial stage of lipolysis was higher than that in its absence, the rate at the end became equal to or less than that in its absence. Thermodynamic values of activation state little changed by the addition of the salts. *K*m and *V*max values were found to be 7.4×10^{-3}M and 10.8 μmoles/min for trilaurin and 26.7×10^{-3} and 8.1 for 1,3-dilaurin, respectively. *k* values for TG were also higher than that for 1,3-DG. *K*m values for triolein and trilinolein were 6.3×10^{-2} and 6.5×10^{-2}M, respectively, whereas the value for trilinolenin ($\Delta^{6,7}$, $\Delta^{9,10}$ and $\Delta^{12,13}$) was 48×10^{-2}M and the result indicates that polyunsaturated fatty acids in TG molecules resist the lipolysis.

Many studies have been reported on the substrate specificity and positional specificity of pancreatic lipase.[4] It is summarized from these studies that pancreatic lipase had a high reactivity to ester links at position 1 and 3 of triglycerides; two outer chains were preferentially hydrolyzed, but the secondary ester of glycerin was little hydrolyzed,[4d,e] esters at position 1 and 3 of glyceride were hydrolyzed at the same rate,[5] and the lipase spilt triglycerides at high rate but monoglycerides at low rate.[4d,f,6] Brockerhoff also clarified the substrate specificity of pancreatic lipase as the results of detailed studies on the effect of various substitutes of both alcohol and fatty acid moities on the rate of lipolysis.[4m,n]

However, the positional specificity of lipase from microorganisms and its difference in hydrolysis rate of esters at each position were scarcely clarified; it was not yet known whether two outer and an inner chain of triglycerides were hydrolyzed at the same rate, or hydrolysis of 2-monoglyceride might be carride out, by the lipases from microbial origin.

To obtain a satisfactory answer to the questions, the positional specificity and mechanism of lipolysis by microbial lipases, especially *Mucor* lipase, were studied. In this report, action

1) Part VIII: M. Sugiura and T. Ogiso, *Chem. Pharm. Bull.* (Tokyo), **18**, 663 (1970).
2) This forms the Part LIX of "Studies on Enzymes" by M. Sugirua. This work was presented as the meeting of Tokai Branch, Pharmaceutical Society of Japan, Nagoya, February, 1971.
3) Location; *Mitahora 492—36, Gifu.*
4) *a*) F.H. Mattson, J.H. Benedict, J.B. Martin and L.W. Beck, *J. Nutr.*, **48**, 335(1950); *b*) F. Schønheyder and K. Volqvartz, *Biochim. Biophys. Acta*, **8**, 407 (1952); *c*) B. Borgström, *Acta Chem. Scand.*, **7**, 557 (1953); d) F. Schønheyder and K. Volqvartz, *Biochim. Biophys. Acta*, **15**, 288 (1954); *e*) P. Savary and P. Desnuelle, *Biochim. Biophys. Acta*, **21**, 349 (1956); *f*) F.H. Mattson and L.W. Beck, *J. Biol. Chem.*, **219**, 735 (1956); *g*) B. Entressangles, L. Paséro, P. Savary, L. Sarda and P. Desnuelle, *Bull. Soc. Chim. Biol.*, **43**, 581 (1961); *h*) G. Clement, J. Clement and J. Bezard, *Biochem. Biophys. Res. Commun.*, **8**, 238 (1962); *i*)M.H. Coleman, *J. Am. Oil Chemists' Soc.*, **40**, 568 (1963); *j*) P.J. Fritz and P. Melius, *Can. J. Biochem. Physiol.*, **41**, 719 (1963); *k*) B. Borgström, *Biochim. Biophys. Acta*, **84**, 228 (1964); *l*) R.G. Jensen, J. Sampugna and R. L. Pereira, *Biochim. Biophys. Acta*, **84**, 481 (1964); *m*) H, Brockerhoff, *Biochim. Biophys. Acta*, **159**, 296 (1968); *n*) H. Brockerhoff, *Biochim. Biophys. Acta*, **212**, 92 (1970).
5) F.H. Mattson and L.W. Beck, *J. Biol. Chem.*, **214**, 115 (1955); P. Desnuelle and P. Savary, *J. Lipid Res.*, **4**, 4 (1963).
6) B. Borgström, *Biochim. Biophys. Acta*, **13**, 491 (1954).

pattern of lipolysis, using thin-layer chromatography, kinetics and thermodynamic studies on triglyceride (TG), 1,3-diglyceride(1,3-DG) and 1-monoglyceride(1-MG) are described.

Material and Method

Preparation of Enzyme——The lipase from *Mucor javanicus* was purified according to the method described in the previous paper,[7] and the purified lipase preparation was used throughout this work. The lipase preparations from *Candida cylindracea* and *Aspergillus* species were a gift from Meito Sangyo Co., Ltd., and Amano Seiyaku Co., Ltd., respectively.

Materials——Polyvinyl alcohol (PVA)-117, PVA-210, olive oil and Emalgen 120 (polyoxyethylene lauryl ether) were obtained from Kurashiki Rayon Co., Ltd., Iwaki Seiyaku Co., Ltd., and Kao Soap Co., Ltd., respectively. Trilaurin and 1,3-dilaurin obtained from Tokyo Kasei Kogyo Co., Ltd., were purified by repeated extraction of free fatty acid from the glycerides with MeOH at 5° and followed by drying in vaccum. Triolein ($\Delta^{9,10}$), trilinolein ($\Delta^{9,10}$ and $\Delta^{12,13}$) and trilinolenin ($\Delta^{6,7}$, $\Delta^{9,10}$, and $\Delta^{12,13}$) were gifts from Ono Yakuhin Kogyo Co., Ltd. Monoolein from Tokyo Kasei Koguo Co., Ltd., was not subjected to purification. All glycerides were checked for purity by thin-layer chromatography (TLC) on Silica gel G(E. Merck). The purity of trilaurin or monoolein was more than 90%, and 1,3-dilaurin were 95% pure by TLC. The contaminants in TG were 1,3-DG and that in 1,3-DG were 2,3-DG. TG of unsaturated acids were more than 99% pure. The other chemicals were of special or reagent grade.

Assay Procedure——Lipase Assay: Olive oil and TG emulsion were prepared by the same method as described in the previous paper.[7] Monoolein was emulsified with 3% aqueous Emalgen 120 solution in a homogenizer cooled with ice water for 10 min. Throughout the experimental work the assay of lipase activity was mainly made by Method B,[7] *i.e.*, method of Dole.[8] A part of the assay was done by the method using shaken system; in a L-shaped flask, a mixture of 0.2 ml of glyceride, 0.3 ml of *n*-heptane and 0.2 ml of McIlvaine buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid, pH 7.0) was incubated at 37° for 10 min, and then 0.5 ml of lipase solution (0.5—0.7 unit/ml) was added. The incubation was carried out at 37° with constant shaking (110 oscillations/min in 4 cm amplitude), using a Monod-type shaking machine. After 20 min the reaction was stopped by the addition of 5 ml of solution consisting of 80 ml of iso-PrOH, 20 ml of *n*-heptane and 2 ml of 2N H_2SO_4. The liberated fatty acids were extracted with 3 ml of *n*-heptane, and followed by titration with 0.01N ethanolic KOH as described previously.[7]

Thin-Layer Chromatography——TLC was performed with petroleum ether: ethyl ether: acetic acid (80: 30: 1) as the solvent, which was run on 0.25 mm thick layer of Silica gel G (Kieselgel G), and sptos were detected by spraying 10% ethanolic phosphomolybdic acid solution and heating at 130° for 20 min.

Result

The Action Pattern of Lipolysis

Hydrolysis of Olive Oil and Trilaurin——To clarify the mode of lipolytic action and specificity of *Mucor* lipase toward the position of esters, time course of hydrolysis of olive oil and trilaurin was studied by TLC. When olive oil emulsion was the substrate as shown in Fig. 1a), considerable amounts of 2,3-DG and small amounts of MG were accumlated at the initial stage of lipolysis. This suggests that the lipase firstly hydrolyzes the esters at outer position of TG and next the inner chain, and the hydrolysis rate of esters at position 3 of 2,3-DG is lower than that of position 1 of TG, namely in the order: TG>2,3-DG. Furthermore, small accumulation of MG may come to the conclusion that hydrolysis rate of outer chain of 2,3-DG is generally equal to or slightly higher than that of MG.

Analogous results were found in *Candida* and *Aspergillus* lipase.

Since the lipase activity was enhanced by bile salts as reported previously,[9] the mode of action of lipolysis by the lipase in the presence of taurocholate was studied. The results are shown in Fig. 1b). The increase of fatty acid released at the initial stage of lipolysis may signify that the initial hydrolysis rate in the presence of bile salts is higher than that in its absence. As shown in Fig. 1, however, the rate at the end decreased rather. On the other

7) T. Ogiso and M. Sugiura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1025 (1969).
8) V.P. Dole, *J. Clin. Invest.*, **35**, 150 (1956).
9) T. Ogiso and M. Sugiura, *Chem. Charm. Bull.* (Tokyo), **17**, 1034 (1969).

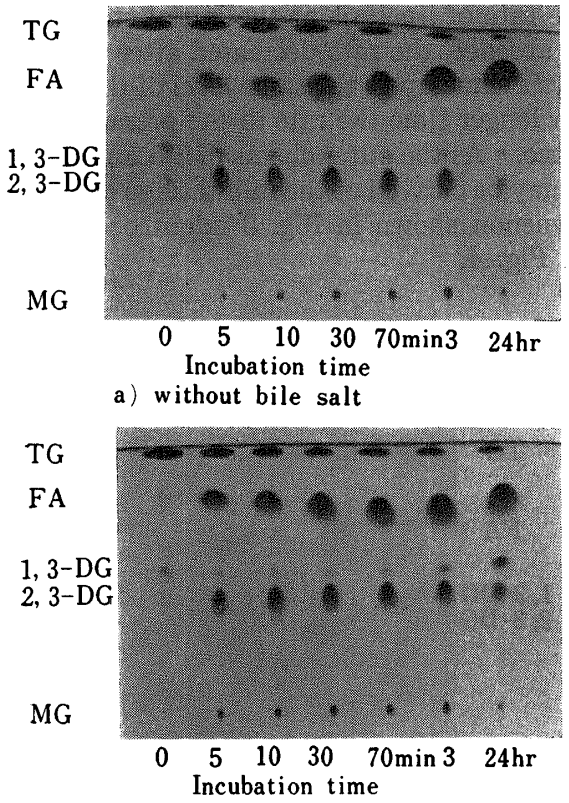

Fig. 1. Thin-Layer Chromatography of Reaction Products from Olive Oil.

The mixture which contained 0.5 ml of olive oil emulsion, 1 ml of McIlvaine buffer (pH 7.0) and 0.5 ml (44.4 units) of lipase solution was incubated for each time at 37° in the presence or absence of 0.02M taurocholate. Three μl of heptane phase was placed on the thin-layer of silica gel. solvent system: pertroleum ether (80) ethyl ether (30) acetic acid (1), developing reagent: 10% ethanolic phosphomolybdic acid solution TG: triglyceride, FA: fatty acid, DG: diglyceride, MG: monoglyceride

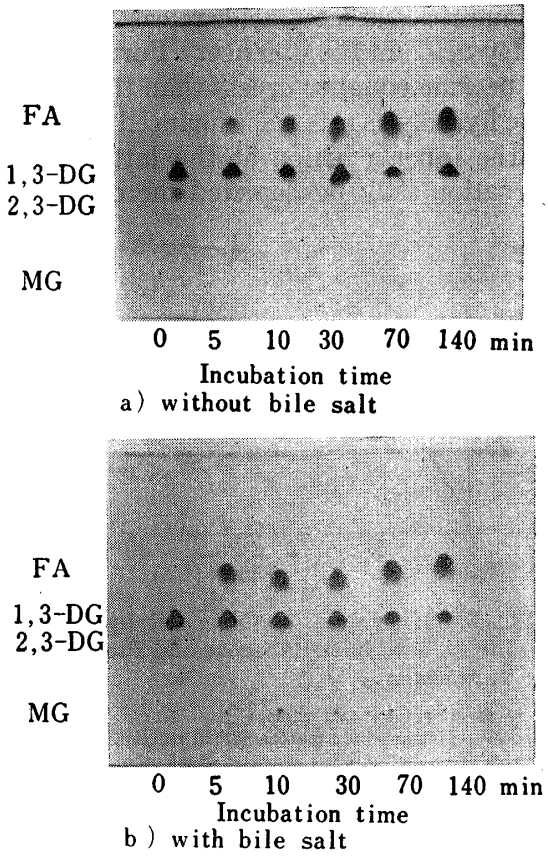

Fig. 2. Thin-Layer Chromatography of Reaction Products from Dilaurin

All procedures were caeeied out in the same way as described for Fig. 1. 46.8 units of lipase were used in the experiments.

hand, since quantities of MG produced during lipolysis in the presence of bile salts increased in comparison with that in its absence as presented in Fig. 1, the hydrolysis rate of MG in the presence of the salts may be lower than that of 2,3-DG. The mode of lipolysis, however, was not changed in the presence or absence of bile salts. Similar results were also obtained in the experiments on trilaurin substrate.

Hydrolysis of 1,3-Dilaurin——The results obtained by hydrolysis of 1,3-dilaurin are shown in Fig. 2 by TLC technique. It was found that one of ester linkages of 1,3-dilaurin was split off and MG produced during hydrolysis was converted into glycerin and fatty acid, and the mode of hydrolysis in the presence of bile salts was similar to that in its absence. However, quantities of fatty acids released from 1,3-DG and of MG accumulated at the initial stage of lipolysis were more than those in the absence of bile salts. The results also indicate that in the presence of bile salts hydrolysis of an outer chain of 1,3-DG occurs at a higher rate than that of MG.

Hydrolysis Curve of Olive Oil——As a result of the above experiments, it was found that lipolytic action of *Mucor* lipase was accelerated by bile salts at the initial stage of hydrolysis. However, the effect of bile salts on the overall reaction was not unclear. Therefore, a long-lipolysis on olive oil substrate was studied, using the lipase with very high units, 745 units.

The result is shown in Fig. 3. Although the rate of lipolysis at the initial stage in the presence of bile salts was higher than that in its absence, the rate decreased after few hours and quantities of fatty acids released during lipolysis came up to the same level of the amount of fatty acids in the absence. Furthermore, after longer time the rate in the presence of the salts became equal to or less than that in its absence. As the outer chains of TG and 2,3-DG, whose hydrolysis rate was enhanced by bile salts, may be cleaved at the initial stage of lipolysis and the inner position at the latter, the phenomenon shown in Fig. 3 might occur. The observation may be depicted more clearly from Fig. 4.

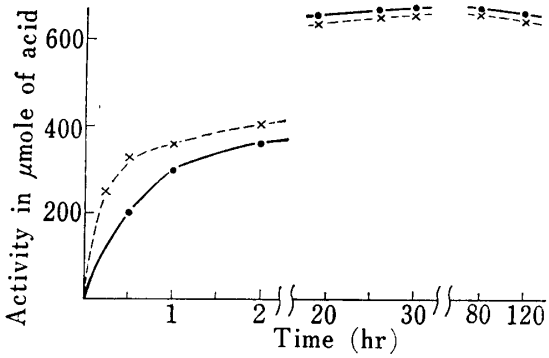

Fig. 3. Hydrolysis of Olive Oil by *Mucor* Lipase both in the Absence and Presence of Taurocholate

—●—: without taurocholate
—×—: with 0.015M taurocholate in reaction mixture
The reaction mixture consisted of 0.5 ml of olive oil emulsion, 1 ml of McIlvaine buffer (pH 7.0), 1 ml (745 units) of enzyme solution and 0.5 ml of taurocholate or water.

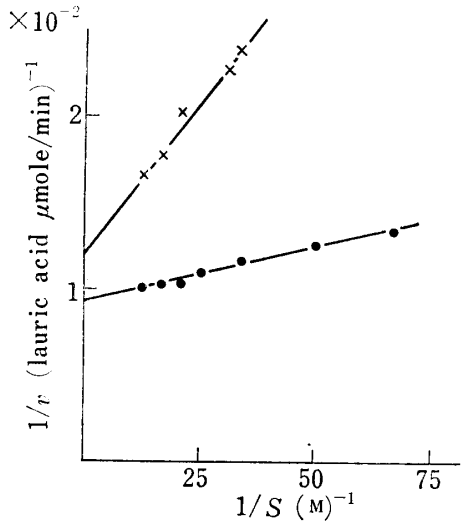

Fig. 5. Lineweaver–Burk Plots of Lipase Activity

—●—: on trilaurin emulsion
—×—: on 1,3-dilaurin emulsion
All procedures were carried out in the same way as described for assay procedure

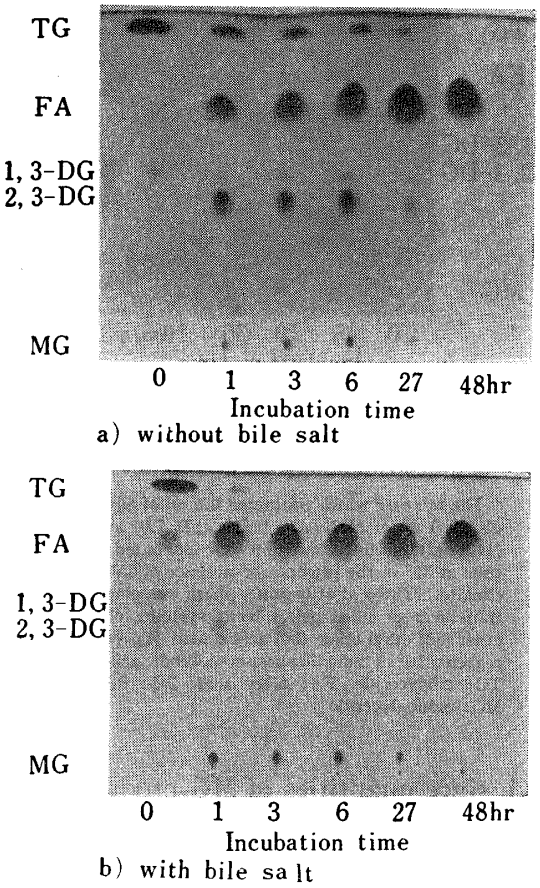

Fig. 4. Thin-Layer Chromatography of Reaction Products from Olive Oil in the Presence or Absence of High Concentration of Bile Salt

All procedures were carried out in the same way as described for Fig. 1. 75.8 units of lipase and 0.167M taurocholate were used in the experiments.

Kinetics for Lipolysis of TG, DG and MG

As a result of those experiments, a difference in the hydrolysis rate between TG, 1,3-DG and MG was postulated. In order to certify the postulation kinetics parameters for trilaurin, 1,3-dilaurin and triolein were measured. Lineweaver–Burk plots of the lipase activity on

trilaurin and 1,3-dilaurin and the parameter, Km and Vmax, obtained from the plots are shown in Fig. 5 and Table I, respectively. Km values were found to be 7.4×10^{-3}M for trilaurin and 26.7×10^{-3}M for 1,3-dilaurin, and Vmax values was 10.8 and 8.1(μmole/min), respectively. Therefore, the formation of the E·S complex between the lipase and TG seems to be much easier than that of the lipase and 1,3-DG, but the rate of breakdown of E·TG may be slightly higher than that of E·DG. While, the lipase activity (as μmoles of fatty acid released for 20 min under the experimental condition) on 1,3-dilaurin was a 77% hydrolysis of trilaurin. The velocity constants (k) for trilaurin and 1,3-dilaurin in the presence or absence of taurocholate is shown in Table II.

Table I. Kinetics Parameters for Hydrolysis of Olive Oil and Trilaurin

Kinetic parameter	K_m ($\times10^{-3}$M)	V_{max} (μmole/min)
Trilaurin	7.4	10.8
1.3-Dilaurin	26.7	8.1

Table II. Velocity Constants (k) for Hydrolysis of Trilaurin and 1,3-Dilaurin

	Taurocholate[a]	k(min^{-1})	
		30°	37°
Trilaurin[b]	none	5.5×10^{-3}	6.5×10^{-3}
	0.015M	9.1×10^{-3}	11.7×10^{-3}
1,3-Dilaurin	none	4.8×10^{-3}	6.1×10^{-3}
	0.015M	6.8×10^{-3}	8.4×10^{-3}

a) concentration of taurocholate in substrate-enzyme mixture
b) To compare value of k for trilaurin with that for 1,3-dilaurin, the value was calculated removing one lauric acid residue from trilaurin molecule.

k values for trilaurin in the presence of 0.015M taurocholate were 1.7—1.8 times as many as those in its absence, while, the values for 1,3-dilaurin in the same condition were 1.3—1.4 times. Thus, it was clarified that the hydrolysis rate of ester at position 1 of TG was higher than that of the chain at position 1 of 1,3-DG, so that bile salts greatly contribute to increasing the rate of hydrolysis of an outer chain of TG, but slightly to that at position 1 of 1,3-DG. The hydrolysis rate of triolein and 1-monoolein in the presence of n-heptane which was added to equalize the physical state of the substrates was measured in a shaking system. The rate of 1-monoolein was a 70% hydrolysis of triolein. While, the hydrolysis rate of 1-monoolein in the presence of 0.005—0.025M taurocholate or cholate was equal to or slightly lower than that in those absence. Therefore, bile salts will not be able to accelerate the hydrolysis rate of 1-monoolein by the lipase.

Effect of Temperature on Rate of Lipolysis

The effect of temperature on the velocity of the lipase reaction was studied. In Fig. 6, logarithm of rate constant was plotted against the inverse of the absolute temperature. The relation was linear, so that activation energy is obtained from the slope according to Arrhenius equation, $\log k=-E/2.303RT+$constant, where k is rate constant, E for the activation energy, R for the gas constant and T for the absolute temperature. Thermodynamic values of activation state, *i.e.* enthalpy ΔH, the free energy ΔG, and entoropy ΔS of activation are calculated by the follwing equations derived from absolute reaction rate theory.[10]

10) S. Glastone, K.J. Laidler and H. Eyring,"The Theory of Rate Processes," McGraw-Hill, New York, 1941; M. Dixon and E.C. Webb, "Enzymes," Longmans, London, 1964, pp. 145—166.

$$\Delta H = E - RT$$
$$\Delta G = 4.58T(10.318 + \log T - \log K)$$
$$\Delta S = (\Delta H - \Delta G)/T$$

The caluculated values of ΔH, ΔG and ΔS are also listed in Table III. The fact that thermodynamic values of activation little changed by the addition of bile salts, although the value of E decreased to a small extent, suggests that conformation of the enzyme does not change by the addition of the salts.

Table III. Some Thermodynamic Data of *Mucor* Lipase

Substrate	Taurocholate[a)]	E (kcal)	ΔH (kcal)	ΔG (kcal)	ΔS (*e.u.*)
Olive oil	none	7.94	7.33	22.92	−50.3
	0.015M	6.84	6.22	22.67	−53.1
Trilaurin	none	8.55	7.94	22.87	−48.2
	0.015M	7.58	6.97	22.62	−50.5

condition: at pH 7.0, 37°
a) concentration of taurocholate in substrate-enzyme mixture

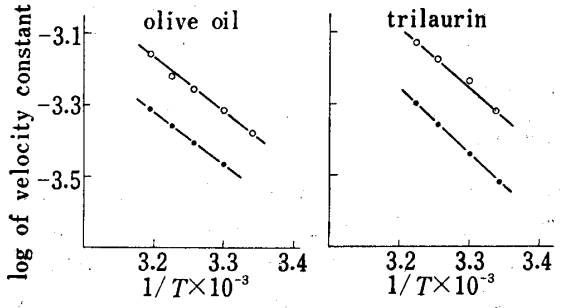

Fig 6. Temperature Dependence of Velocity Constants
—○—: in the presence of 0.015M taurocholate
—●—: in the absence of taurocholate

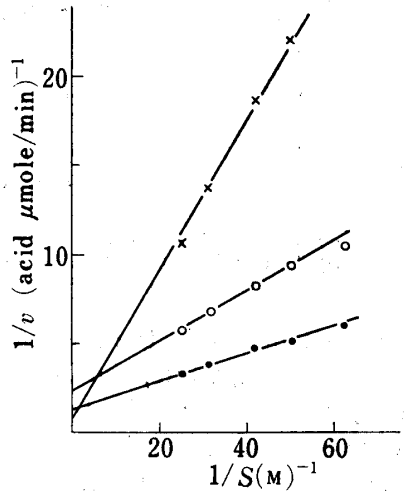

Fig. 7. Lineweaver–Burk Plots of Lipase Activity on Triglycerides of Unsaturated Acids

All procedures were carried out in the same way as described for assay procedure. 0.35 unit of lipase was used for hydrolysis of triolein (—○—), 0.67 unit for trilinolein (—●—), and trilinolenin (—×—), respectively

Lipolysis of Triolein, Trilinolein and Trilinolenin

It was found that unsaturation on the chain length of 2—5 carbons[4n)] and polyunsaturated fatty acids in TG molecules[11)] led to a relative resistance of the esters against pancreatic lipase. When the lipase activity to TG of unsaturated C_{18}-fatty acid was measured, the following rate values were obtained: triolein 100, trilinolein 92 and trilinolenin 25. Lineweaver–Burk plot of the lipase activity on these substrates are shown in Fig. 7. *Km* values for triolein and trilinolein were 6.3×10^{-2} and 6.5×10^{-2}M, respectively. However, the value for trilinolenin was very high, 48×10^{-2}M. The result indicates that polyunsaturated fatty acids in

11) N.R. Bottino, G.A. Vanderburg and R. Reiser, *Lipids*, 2, 489 (1967).

TG molecules resist the lipolysis by the lipase, and the resistance of unsaturated acids will be due to steric hindrance during the formation of the activated complex.

Discussion

Action pattern and mechanism of lipolysis by lipases from microorganisms were not yet clear, whereas the substrate specificity and positional specificity of pancreatic lipase have been clarified. In order to clearify the positional specificity, the mode of action and mechanism of lipolysis by *Mucor* lipase, the hydrolysis rate of TG, 1,3-DG and 1-MG were studied. To determine the true rate of hydrolysis of individual glyceride, each glyceride, *e.g.* 2,3-DG and 2-MG, must be isolated in pure state. However, it is impossible for us because of isomerization of acyl groups at position 2. Therefore, 1,3-DG and 1-MG were mainly used for the studies.

Lipolysis was mentioned to start with a nucleophilic group of the enzyme attacking the carbonyl carbon of the substrate,[4m] therefore, if the carbon is made electrophilic the reaction will become faster. As a result of the studies on lipolysis of TG and 1,3-DG TLC technique (Fig. 1,2 and 4), determination of the rate constants of lipolysis (Table II) and *K*m values (Table I), it was clarified that the rate of hydrolysis of ester at position 1 of TG was faster than that at position 1 of 1,3-DG. Since *K*m is the equilibrium constant of reaction as, E $+S \rightleftharpoons ES$, and *V*max is a measure of the velocity constant of breakdown of the complex,[12] the many difference in *K*m values and the minor one in *V*max values (10.8 and 8.1 μmole/min) for trilaurin and 1,3-dilaurin suggest that the formation of E·S complex between TG and the lipase is much easier than that between 1,3-DG and the enzyme, and that there is only a little difference in the velocity of breakdown of these complex. The reason why the lipolysis rate at position 1 of TG is faster can be interpreted as the result of activation by a neighbouring electrophilic ester group,[4m] consequently ester groups of TG accelerate lipolysis, while, hydroxyl group which is electron-donating, relative to hydrogen[4m] may prevent the formation of E·S complex between the enzyme and 1,3-DG to some extent. Therefore, we will find the diffrence in the hydrolysis rate as shown in Fig. 1 and 4; accumulation of 2,3-DG. Furthermore, simultaneous disapperance of 2,3-DG and MG (Fig. 4) suggests that the hydrolysis rate of ester at position 3 of 2,3-DG is generally equal to that of MG, perhaps 2-MG. To compare the lipolysis rate of triolein and 1-monoolein, the activities in the presence of *n*-heptane were determined. Although physical property of 1-monoolein (suspension) differed from that of triolein (emulsion) to some extent, a difference in both the hydrolysis rate indicates that the rate of 1-MG is less than that of TG.

The chief mechanism of lipolysis by pancreatic lipase can be formulated as follows[4a-c, 4e,5,6,13]: TG→2.3-DG→2-MG. *In vivo*, an isomerization of 2,3-DG and 2-MG, followed by lipolysis, and a little direct action of the other enzyme in pancreatic juice on position 2[14] convert TG into glycerin and fatty acids. The reason why pancreatic lipase can not act the group at inner position is interpreted as the result of steric hindrance besides deactivation by neighbouring hydroxyl group as mentioned above.[4m] On the other hand, *Mucor*, *Candida* and *Aspergillus* lipase hydrolyzed the inner chain of glycerides more rapidly and perfectly than pancreatic lipase. Thus, the microbial lipases may be able to hydrolyze the ester at position 2(2-MG) directly. This suggests that the positional specificities of lipase from microbials are less than that of pancreatic lipase. However, the fact that lipolysis by microbial lipase does not start at the 2-ester of TG whose carbonyl must be even more electrophilic than those at position 1 and 3 since it is flanked by two activating groups may be based on

12) M. Dixon and E.C. Webb, "Enzymes," Longmans, London, 1964, pp. 63—67.
13) *a*) B. Borgström, *J. Lipid Res.*, **5**, 522 (1954); *b*) B. Entressangles, H. Sari and P. Desnuelle, *Biochim. Biophys. Acta*, **125**, 597 (1966).
14) F.H. Mattson and R.A. Volpenhein, *J. Lipid Res.*, **7**, 536 (1966) ; **9**, 79 (1968).

effect of steric hindrance by ester group at outer positions of TG and 2,3-DG as same as in pancreatic lipase.[4m]

The lipase activities on TG of $C_{18:1}$ and $C_{18:2}$-fatty acid were very high, however, that on trilinolenin which contains three double bonds decreased extremly, 25% activity of that of triolein. The explanation for this phenomenon may lie in differences in their molecular structure, because of the terminal methyl groups of linolenic acids lie close to their carboxyl groups as shown in Fig. 8. This proximity may cause a steric hindrance to the formation of the activated complex (E·S complex), consequently decrease of hydrolysis by the lipase. Since the steric hindrance of polyunsaturated fatty acids in TG to lipolysis is also found in pancreatic lipase,[4n,11] the phenomenan may be common to lipases.

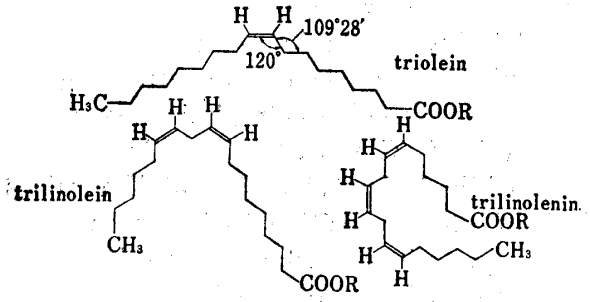

Fig. 8. Chemical Constitution of Triglycerides

As a result of the present studies, the marked increase in lipolysis by addition of bile salts was found in early stage of lipolysis, whereas at last stage the rate of lipolysis decreased rather than that in the absence of the salts (Fig. 3 and 4). As reported previously,[9] the accelerating effect of bile salts on the initial rate was due to the increase of adsorbed enzyme at the interface. The adsorbed enzyme may be followed by the formation of E·S complex: $[E+S]_{adsorbed} \rightleftharpoons E \cdot S$, consequently, hydrolysis of esters at position 1 and 3 of glycerides may be accelerated. On the other hand, a decreased rate of lipolysis at last stage in the presence of bile salts may be attributed to the salts forming mixed micelles with MG and fatty acids,[13a] and preventing the enzyme from approach to the substrate.

Since enthalpy ΔH, the free energy ΔG and entropy ΔS of activation in the presence of bile salt were almost equal to those in its absence as shown in some thermodynamic data (Table III), bile salts may not change conformation of the enzyme to activate.[15] The result strengthens more the previous conclusion that bile salts did not activate the enzyme directly.[9] While, a few decrease of activation energy in the presence of bile salts may be due to the salts accelerating the rate of diffusion of long chain fatty acids from the intereface[9] and preventing inhibition by the acids.[1]

From the above results, we will summerize the hypothetical course of lipolysis and the factors that determine the rate:

In the absence of bile salts

$$E + TG \overset{\text{(slow)}}{\longleftrightarrow} [E+TG]_{adsorbed} \longleftrightarrow E \cdot TG \xrightarrow{\text{(fast)}} E \cdot DG \xrightarrow{\text{(slow)}} E \cdot MG \xrightarrow{\text{(slow)}} E + G$$
$$E \cdot TG \rightarrow FA, \quad E \cdot DG \rightarrow FA, \quad E \cdot MG \rightarrow FA$$

In the presence of bile salts

$$E + TG \overset{\text{(fast)}}{\longleftrightarrow} [E+TG]_{adsorbed} \longleftrightarrow E \cdot TG \xrightarrow{\text{(fast)}} E \cdot DG \xrightarrow{\text{(slightly fast)}} E \cdot MG \xrightarrow{\text{(slow)}} E + G$$
$$E \cdot TG \rightarrow FA, \quad E \cdot DG \rightarrow FA, \quad E \cdot MG \rightarrow FA$$

where E is enzyme, FA for fatty acid and G for glycerin. Since quantities of the lipase adsorbed at the oil-water interface were parallel with the activity,[9] an equilibrium may be between the adsorbed enzyme, $[E+S]_{adsorbed}$, and E·S complex, thus increase of $[E+S]_{adsorbed}$ causes

15) H.G. Bray and K. White, "Kinetics and Thermodynamics in Biochemistry," J. and A. Churchill Ltd., 1957, Chap. 5 and 6.

acceleration of the enzyme activity. The overall hydrolysis of TG by the lipase under the condition of these experiments must consequently go to completion in spite of the fact that simultaneously with the hydrolysis TG and DG are synthesized from DG and MG, respectively,[4k,6] because of the last step in the reaction sequence, splitting of 2-MG, is irreversible.[4k,6]

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