Chem. Pharm. Bull. 24(8)1822—1828(1976)

UDC 577.153.02 : 547.564.3.09

Studies on the Mechanism of Lipase Reaction. IV.[1] Action of the Lipase from *Chromobacterium* on Monomeric *p*-Nitrophenyl Acetate

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(Received November 14, 1975)

The hydrolytic activity of the lipase (EC 3.1.1.3) from *Chromobacterium* for the monomeric esters was found. The hydrolysis of *p*-nitrophenyl acetate by the lipase proceeded with three step reaction in which acylation and deacylation processes of the enzyme were involved. The rate limiting step was found to be the acylation.

The enzymic hydrolysis was accelerated about 2-fold by the additions of hydrophobic glass beads. Water soluble organic solvents did not activate but inhibit the enzymic hydrolysis and they did not affect significantly on the activating effect by hydrophobic glass beads. It was found that the lipase was adsorbed on the glass beads and once adsorbed enzyme was difficult to desorb. These results were discussed relation to the reaction mechanism of lipolysis by lipase and an assumption was made.

Lipases (glycerol ester hydrolase; EC 3.1.1.3) have been differentiated from esterase (carboxylic ester hydrolase; EC 3.1.1.1) in their substrate specificity. Lipases hydrolyze the emulsion[3] or aggregates (micelle,[4,5] monolayer[6]) of insoluble long chain fatty acid esters of glycerol and other alcohols at a very high rate but their action on the fully dispersed substrate molecules in an aqueous system is comparatively weak. On the other hand, esterase hydrolyzes the water soluble esters and not the aggregated ester molecules.[3] Recently, it was found that various lipases had a wide substrate specificity and hydrolyzed tripropionin[7,8] or *p*-nitrophenyl acetate[9–12] which was dispersed in water. Brockman, *et al.*[7] found that the pancreatic lipase hydrolyzed the water soluble tripropionin and the hydrolysis was substantially stimulated in the presence of hydrophobic surface. They ascribed the activation to an increased local concentration of the substrate at the liquid-solid interface. Desnuelle, *et al.*[12] reported that the hydrolysis of *p*-nitrophenyl acetate by the pancreatic lipase involved an acylation and deacylation step like that by ordinary esterase and the rate limiting step was the deacylation. The enzymic reaction was also accelerated by various interfaces. The hydrolysis of monomeric tripropionin by pancreatic lipase[8] was substantially activated by the addition of low concentrations of water miscible organic compounds to an aqueous system. From the fact, an assumption was made the organic compounds modify the structure of liquid water and causes a transconformation of the enzyme. Also the mechanism of action of pancreatic lipase was discussed in the light of current concepts.

1) This forms Part CXVII of "Studies on Enzyme" by M. Sugiura, preceeding paper Part CXVI: M. Sugiura, M. Isobe, T. Oikawa, and H. Oono, *Chem. Pharm. Bull.* (Tokyo), **24**, 1202 (1976).
2) Location: *1432-1, Horinouchi, Hachioji-shi, Tokyo.*
3) L. Sarda and P. Desnuelle, *Biochim. Biophys. Acta*, **30**, 513 (1958).
4) B. Entressangles and P. Desnuelle, *Biochim. Biophys. Acta*, **159**, 285 (1968).
5) P.W. Albro and A.D. Latimer, *Biochemistry*, **13**, 1431 (1974).
6) J.W. Lagocki, J.H. Law, and F.J. Kézdy, *J. Biol. Chem.*, **248**, 580 (1973).
7) H.L. Brockman, J.H. Law, and F.J. Kézdy, *J. Biol. Chem.*, **248**, 4965 (1973).
8) B. Entressangles and P. Desnuelle, *Biochim. Biophys. Acta*, **341**, 437 (1974).
9) T. Egelrud and T. Olivecrona, *Biochim. Biophys. Acta*, **306**, 115 (1973).
10) O. Hernell and T. Olivecrona, *Biochim. Biophys. Acta*, **369**, 234 (1974).
11) J.E. Fulton, N.L. Noble, S. Bradley, and W.M. Awad, *Biochemistry*, **13**, 2320 (1974).
12) M. Sémériva, C. Chapus, C. Bovier-Lapierre, and P. Desnuelle, *Biochem. Biophys. Res. Commun.*, **58**, 808 (1974).

We reported that lipases from various origins were adsorbed on various interfaces[13–15] and made an assumption that the affinity for water insoluble esters, which was most characteristic property of lipases, will result from the accumulation of the enzyme at the interface. The present report will show that the hydrolytic activity of the lipase from *Chromobacterium* for the monomeric esters and the effect of hydrophobic glass beads for the hydrolysis of *p*-nitrophenyl acetate. The results were discussed in relation to the reaction mechanism of lipolysis by the lipase.

Material and Method

Enzyme——The lipase from *Chromobacterium* was purified as reported previously.[16] The homogeneity of the purified lipase was confirmed by disc electrophoresis at pH 9.4.

Material——Phenyl acetate, *o*-nitrophenyl acetate and *p*-nitrophenyl acetate were obtained from Tokyo Kasei Co., Ltd. Various organic solvents and the other reagents used were of the reagent grade. Silicone oil DC QF-1 was product of Applied Science Lab. Inc.

Coating of Hydrophobic Materials on Glass Beads——Glass beads (size: 100—200 mesh) were rinsed with detergent solution, rinsed with water, soaked in chromic acid cleaning solution for a day, rinsed to neutrality in distilled water, and dried under vacuum. One ml of 2% acetone solution of liquid paraffin or silicone oil DC QF-1 was added to 1 g of glass beads (surface area: 1700 cm^2/g) and the solvent was removed with evaporation.

Assay of Enzymic Activities and Protein——The activity of lipase toward olive oil emulsion was assayed according to the method of previous paper.[16] The rate of hydrolysis of *p*-nitrophenyl acetate by the lipase was assayed by following the release of *p*-nitrophenol spectrophotometrically at 400 nm or the release of acetate ion titrimetrically at 30° and pH 7.0. The protein content was determined according to the method of Lowry, *et al.*[17]

Disc Electrophoresis——Disc electrophoresis was carried out with use of the 7.5% polyacrylamide gel at pH 9.4, glycine-Tris buffer[18] and electrophoresed at a constant current of 3 mA/tube for 90 min. After the gel was sliced, the enzymic activities were assayed. Staining of protein was carried out using amido black 10B.

Results

Hydrolysis of Water Soluble Ester

The enzymic activity of the lipase on the hydrolysis of water soluble ester was shown by disc electrophoresis. The lipase of *Chromobacterium* was electrophoresed at pH 9.4 and 3 mA/tube for 90 min. After the gel was sliced and extracted with water, the activity for the hydrolysis of olive oil and *p*-nitrophenyl acetate was assayed and protein was stained by amido black 10B (Fig. 1). Both of the enzymic activities were electrophoresed in the same position as that of protein band and well proportional each other. The results suggested that the hydrolysis of *p*-nitrophenyl acetate is an action of the lipase. The presence of hydrolytic activity against *p*-nitrophenyl acetate in the lipase was also supported by heat inactivation (Fig. 2).

The lipase dissolved in 0.1M phosphate buffer (pH 7.0) was incubated at 50° for various periods of time and residual activities against *p*-nitrophenyl acetate and olive oil emulsion were assayed. Both of the activities decreased with the incubation time and at the same rate. It was provided further evidence that only one enzyme not contaminant was involved in the hydrolysis of these substrates. There is more evidence for the concept that the hydrolysis of both substrates were carried out by the same enzyme protein. The enzymic hydrolysis of *p*-nitrophenyl acetate was strongly inhibited by olive oil emulsion added in the reaction

13) M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), **23**, 1221 (1975).
14) M. Sugiura and M. Isobe, *Biochim. Biophys. Acta*, **397**, 413 (1975).
15) M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), **24**, 72 (1976).
16) M. Sugiura and M. Isobe, *Biochim. Biophys. Acta*, **341**, 195 (1974).
17) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Rundall, *J. Biol. Chem.*, **193**, 265 (1951).
18) B.J. Davis, *Ann. N. Y. Acad. Sci.*, **2**, 404 (1964).

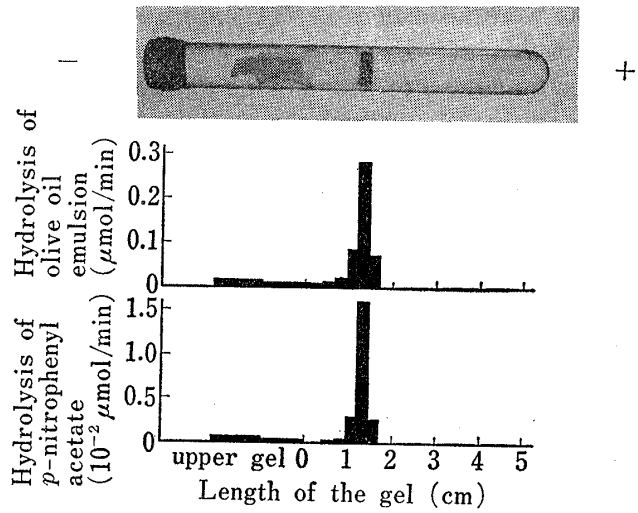


Fig. 1. Disc Electrophoresis of *Chromobacterium* Lipase and Enzymic Activities in the Gel

Disc electrophoresis was carried out at pH 9.4. After the electrophoresis, gel was sliced and extracted with water and enzymic activities were assayed by the standard method. Protein was stained by amido black 10B.

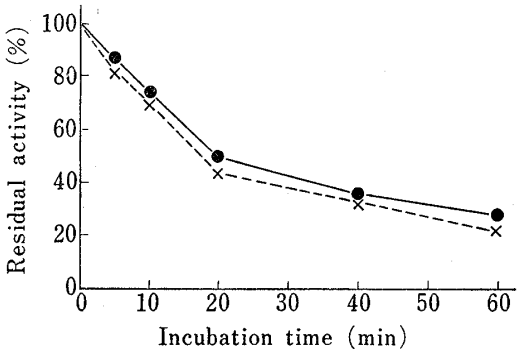


Fig. 2. Thermal Inactivation of *Chromobacterium* Lipase as Monitored by Two Substrates

The enzyme was dissolved in 0.1M phosphate buffer (pH 7.0) and incubated at 50° for various periods of time. Thereafter, the solution was plunged into an ice bath, residual activity for the hydrolysis of olive oil emulsion (×) and *p*-nitrophenyl acetate (●) was assayed.

mixture with its amount (Table I). The hydrolytic action of the lipase against several substrates were assayed (Table II). The rate of hydrolysis of *p*-nitrophenyl acetate was estimated about 1/20 compared to that of olive oil emulsion. On the other hand, *o*-nitrophenyl acetate and phenyl acetate were more inferior substrates to *p*-nitrophenyl acetate. In the subsequent experiments on the hydrolytic action of the lipase against water soluble ester, *p*-nitrophenyl acetate was used as the substrate.

TABLE I. Inhibition of Enzymic Hydrolysis of *p*-Nitrophenyl Acetate by Olive Oil Emulsion

Concentration of olive oil emulsion (%)	Relative rate of hydrolysis (%)	Concentration of olive oil emulsion (%)	Relative rate of hydrolysis (%)
None	100	1.0	23
0.1	74	2.5	8
0.5	38		

Reaction mixture containing 4 ml of *p*-nitrophenyl acetate solution (1 mM), 4 ml of 50 mM phosphate buffer (pH 7.0), 1 ml of olive oil emulsion, and 1 ml of enzyme solution was incubated at 30° for 20 min. After centrifugation at 4°, the absorbance of the aqueous phase at 400 nm was assayed.

TABLE II. Action of *Chromobacterium* Lipase on Various Substrates

Substrate	Concentration	Initial rate of hydrolysis (mole/min mg protein)
Olive oil emulsion	10%	6.64×10^{-3}
p-Nitrophenyl acetate	1 mM	3.52×10^{-4}
o-Nitrophenyl acetate	1 mM	7.38×10^{-7}
Phenyl acetate	1 mM	1.51×10^{-7}

The hydrolytic action on olive oil emulsion was assayed titrimetrically according to the method of Dole. The initial rate of hydrolysis of *p*-nitrophenyl acetate, *o*-nitrophenyl acetate and phenyl acetate were measured in a cuvette by spectrophotometer at 30° and pH 7.0.

Table III. Effects of Acetate Ion, *p*-Nitrophenol and Phenol on the Hydrolysis of *p*-Nitrophenyl Acetate by *Chromobacterium* Lipase

Concentration (mM)	Relative rate of hydrolysis (%)		
	Acetate ion	*p*-Nitrophenol	Phenol
None	100	100	100
5	97	43	98
15	96	32	80
30	95	26	67
60	98	—	41
120	102	—	2

Hydrolysis of *p*-nitrophenyl acetate was carried out at pH 7.0 and 30°. The rate of hydrolysis was assayed spectrophtometrically and titrimetrically.

Effects of *p*-Nitrophenol, Phenol and Acetate Ion

Acetate ion and phenols which are the reaction products and product analogues, were added in the reaction mixture and the initial velocity of the enzymic reaction was assayed (Table III). The enzymic hydrolysis of *p*-nitrophenyl acetate was strongly inhibited by *p*-nitrophenol or phenol but not inhibited by acetate ion. Further, the inhibition by *p*-nitrophenol and phenol was examined and the inhibition was judged to noncompetitive type in the double reciprocal plots (Fig. 3). From these results, it was suggested that the enzymic hydrolysis proceeded with three step reaction in which acyl-enzyme intermediate should be involved simillar to those of various proteases, esterase and pancreatic lipase.

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} ES \xrightarrow{k_{+2}} ES + P_1 \xrightarrow{k_{+3}} E + P_2$$

Where S, P_1 and P_2 is *p*-nitrophenyl acetate, *p*-nitrophenol and acetate ion respectively. The other experiment, in which high concentration of the enzyme solution was used and time course of the hydrolysis of *p*-nitrophenyl acetate was followed, showed that the burst of *p*-nitrophenol in the initial stage of the hydrolysis was not observed and the release of *p*-nitro-

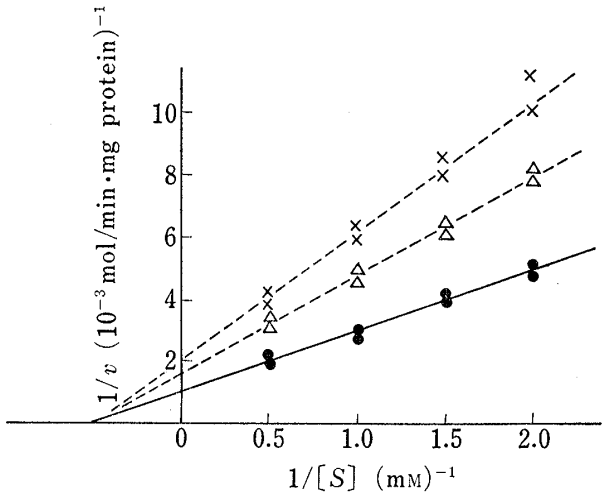


Fig. 3. Effects ot *p*-Nitrophenol and Phenol on the Hydrolysis of *p*-Nitrophenyl Acetate by *Chromobacterium* Lipase

Enzymic hydrolysis of *p*-nitrophenyl acetate was carried out in the presence of 1 mM *p*-nitrophenol (△), 20 mM phenol (×), or in the absence (●). The initial velocity of hydrolysis was assayed spectrophotometrically at 400 nm and titrimetrically.

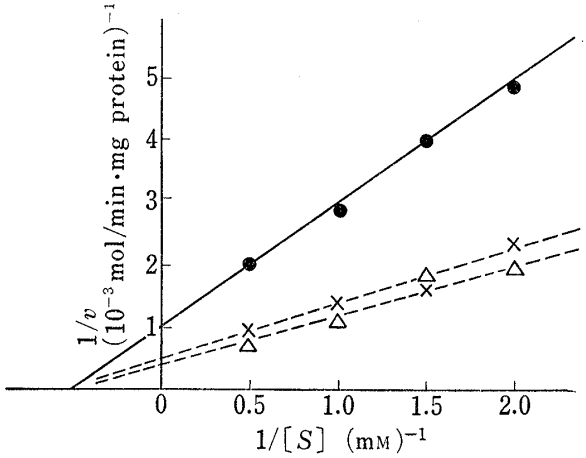


Fig. 4. Effects of Hydrophobic Glass Beads on the Hydrolysis of *p*-Nitrophenyl Acetate by *Chromobacterium* Lipase

Reaction mixture containing 3 ml of *p*-nitrophenyl acetate dissolved in 50 mM phosphate buffer (pH 7.0), and 0.5 ml of enzyme solution was incubated at 30°. The initial velocity of hydrolysis in the presence of 100 mg of glass beads which coated with silicone oil (△) or liquid paraffin (×) and in the absence (●) were assayed spectrophotometrically at 400 nm.

phenol was directly proportional to the incubation time. Since the rate-limiting step in the enzymic hydrolysis of *p*-nitrophenyl acetate appears to be the acylation of the lipase, and deacylation was more rapid ($k_{+2}<k_{+3}$).

Effect of Hydrophobic Surface

Effect of hydrophobic surface on the hydrolysis of *p*-nitrophenyl acetate by the lipase was investigated. The rate of hydrolysis was accelerated by the addition of glass beads which were coated with silicone oil or liquid paraffin and the activation was about 2-fold independently on the coating materials. The value of *Km* in the presence of hydrophobic surface was found to be identical as that of the enzymic hydrolysis in the absence of hydrophobic surface but the value of V_{max} was increased. The results were shown as a double reciprocal plots (Fig. 4).

Various water soluble organic solvents which change the dielectric constant of the water phase and the property of the hydrated water at the surface were added to the reaction mixture and the rate of hydrolysis by the lipase was assayed. In this experiments, acetonitrile, formamide, *tert*-butyl alcohol and methyl alcohol were used as the water soluble organic solvents and the data of acetonitrile was shown (Fig. 5).

Acetonitrile did not activate but inhibit the enzymic hydrolysis and the activating effect of hydrophobic glass beads was observed in the any concentration of the acetonitrile. The similar results were also observed by the other organic solvents.

Further the effect of the hydrophobic glass beads on the enzymic action was investigated in relation to the adsorption of the lipase at the surface. The enzymic hydrolysis was carried out in the presence of siliconized glass beads and, in the result, the rate of hydrolysis increased to 165% of the original rate. After the reaction mixture was centrifuged, the supernatant and the precipitate were separated to assay the enzymic activity. Therefore the supernatant contained 54% of the enzymic activity and if the denaturation of the enzyme protein did not occur, it was estimated that the amount of the enzyme protein adsorbed on the glass beads was 46% of the added enzyme and the adsorbed enzyme had an activity about 2-fold compared to the lipase in the aqueous solution. After the precipitate and substrate were incubated for 20 min at 30°, the mixture was centrifuged once and the enzymic activity in the supernatant and precipitate was assayed. As the result, most of the enzymic activity was found in the precipitate and it was concluded that the lipase which was once adsorbed on the siliconized

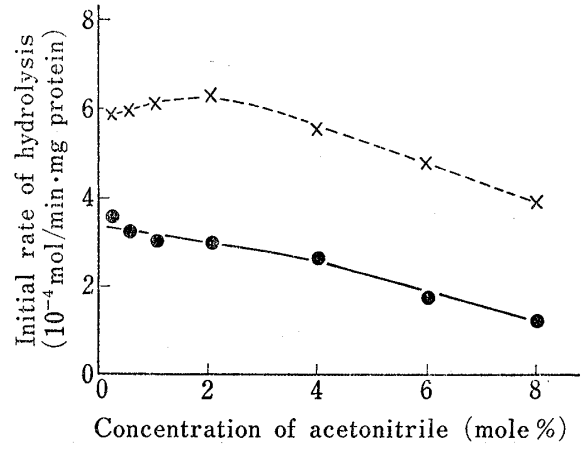


Fig. 5. Effect of Acetonitrile on the Hydrolysis of *p*-Nitrophenyl Acetate by *Chromobacterium* Lipase

Reaction mixture containing 3 ml of *p*-nitrophenyl acetate solution (0.8 mM) dissolved in various concentration of acetonitrile and 0.5 ml of enzyme solution. Enzymic reaction was carried out at pH 7.0 and 30° in the presence of siliconized glass beads (×) or in the absence (●)

Table IV. Effect of Siliconized Glass Beads on the Hydrolysis of *p*-Nitrophenyl Acetate by *Chromobacterium* Lipase

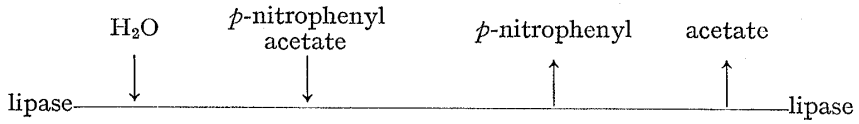
Reaction system	Relative rate of hydrolysis (%)
Lipase+substrate	100
Lipase+substrate+glass beads after centrifugation	165
Precipitate+substrate after centrifugation	95
Precipitate+Substrate	90
Supernatant	1
Supernatant	54

As the substrate, 1 mM *p*-nitrophenyl acetate dissolved in 50 mM phosphate buffer (pH 7.0) was used. The enzymic reaction was carried out at 30° in a cuvette and the rate of hydrolysis was assayed spectrophotometrically at 400 nm.

glass beads was difficult to desorb from the surface and the lipase was active in the form of adsorbed. These data were summarized (Table IV).

Discussion

It has been considered that lipases hydrolyze the water insoluble esters but not the water soluble esters, in this substrate specificity, lipases were distinguished from esterases which appeared to act readily on monomers.[3] But recently it was found[7-12] that lipase hydrolyzes the dispersed ester molecule in the aqueous system though the rate of hydrolysis was comparatively slow to those of the hydrolysis of triglyceride emulsions. Also we found the activity for the hydrolysis of *p*-nitrophenyl acetate in the purified lipase from *Chromobacterium* and the enzymic properties were studied in relation to the reaction mechanism of lipolysis. The electrophoresis and denaturation studies with either *p*-nitrophenyl acetate or olive oil emulsion as substrate indicated that the lipase, not trace amount of contaminant, hydrolyzed both substrates. Further, it was assumed by the competition study with *p*-nitrophenyl acetate and olive oil. The hydrolysis of *p*-nitrophenyl acetate was not inhibited by acetate ion but inhibited noncompetitively by *p*-nitrophenol and phenol which are the reaction product and product analogue respectively. From the pattern of product inhibition in the various reaction mechanism,[19] the reaction by the lipase was considered to proceed by the Ordered Bi Bi mechanism.



The first step which was incorporation of water in the enzyme was actually negligible for the reaction in water solution and the reaction can be treated by three step reaction. The rate limiting step was found to be the acylation and it was considered to be reasonable for the enzymic reaction. The lipase hydrolyzes preferentially the long chain fatty acid esters[20] and the released free fatty acid did not solve in water phase but remain at the substrate-water interface in which enzymic reaction proceed. If the rate limiting step was the deacylation, the enzymic hydrolysis will be influenced by product inhibition strongly. Sémériva, *et al.* reported[12] that the enzymic hydrolysis of *p*-nitrophenyl acetate by pancreatic lipase involves, like that catalyzed by ordinary esterase and some proteases, an acylation and a deacylation step and also that the deacylation step was the rate limiting step. The result was differed from that of *Chromobacterium* lipase and it should be related to the substrate specificity of pancreatic lipase which act preferentially on the ester of short chain fatty acids.[21]

The activation of lipase reaction by various surface and organic solvents had been reported and the characteristic property of the affinity for the insoluble substrates were discussed.[7,8,12] Brockman, *et al.*[7] reported that the reaction of pancreatic lipase with the soluble triglyceride, tripropionin, was stimulated substantially in the presence of hydrophobic surfaces and the enhancement of the hydrolysis velocity on the surface was ascribed to an increased local concentration of the substrate at the liquid-solid interface. On the other hand, Sémériva, *et al.* found[12] that the hydrolysis of monomeric *p*-nitrophenyl acetate by pancreatic lipase in the presence of acetonitrile was accelerated about 500-fold by various interfaces. Also Entressangles and Desnuelle found[8] the similar increasing effect of low concentration of water soluble organic compounds on the activity of pancreatic lipase towards monomeric tripropionin. They considered that low concentration of organic solvents and surfaces modify

19) W.W. Cleland, *Biochim. Biophys. Acta*, **67**, 104, 173, 188 (1963).
20) M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), **23**, 1226 (1975).
21) B. Entressangles, L. Pasero, P. Savary, L. Sarada, and P. Desnuelle, *Bull. Soc. Chim. Biol.*, **43**, 581 (1961).

the structure of liquid water and the modification was favorable for the action of lipase through a transconformation of the enzyme molecule or any other process. We also have studied the effect of hydrophobic interfaces and organic solvents on the hydrolysis of monomeric *p*-nitrophenyl acetate by *Chromobacterium* lipase. As the results, it was found that the hydrophobic interfaces accelerated the enzymic hydrolysis about 2-fold but not activate so much as that reported by pancreatic lipase in any conditions, and the water soluble organic solvents did not activated the enzymic action but inhibited. Therefore the *Chromobacterium* lipase was not so much activated that could account for the specific action on the insoluble substrates, an concept that inactive enzyme is activate at the interface of substrate and the lipolysis was carried out was not reasonable. We found[13-14] a general property among various lipases but not of the other enzymes that adsorbed on various interfeces. From the results of this and previous experiments. the assumption of the reaction mechanism of lipolysis is made. The difference between lipase and esterase should be attributable to the affinity of the enzyme molecule at the interface. The lipase is accumulate in the interface by the similar interaction to those of surface active agents and the enzyme molecule may be placed in a position which is better for the orientation of the substrate molecules with respect to the geometry of its active site.