[Chem. Pharm. Bull. 22(2) 413—420 (1974)]

UDC 577.15.087 : 543.544

Analytical Studies on Esterases using Gas-Liquid Chromatography. II.[1] On the Alcoholytic and Hydrolytic Activities of Esterases from Swine Liver and *Mucor javanicus*

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(Received July 6, 1973)

Using gas–liquid chromatography in the esterase assay, the properties of esterases from swine liver and *Mucor javanicus* were studied in the presence of high concentration of alcohols, with methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl esters of *n*-butyric acid as the substrates of esterases, the relationship between the enzyme activities of alcoholysis and hydrolysis was discussed in detail.

In the previous report,[1] the authors established a new assay method of esterase activity using gas–liquid chromatography (GLC). From our subsequent work, not only the activity of hydrolysis but also the one of alcoholysis has become measurable simultaneously by using this method. When an alcohol and an acid are used as the substrates in place of corresponding ester, synthetic activity of esterase can be also determined. In the present study, esterase-catalyzed hydrolysis and alcoholysis were investigated by using our GLC method. Especially, the effects of alcohols on the reaction rates of two esterases were studied, and the suggestive information pertinent to the mechanism of action of the enzymes was obtained by using *n*-butyryl esters of aliphatic alcohols which have 1 to 5 carbon atoms, as the substrates. As the sources of animal and microbial esterases, the enzyme preparations from swine liver and *Mucor javanicus* were chosen in the present work. In the presence of high concentration (3.5 M) of methanol or ethanol, the alcoholytic activity of liver esterase was exceedingly higher than the hydrolytic one, while Mucor esterase exhibited extremely low activity of alcoholysis which amounted to a few per cent of total (hydrolytic plus alcoholytic) enzyme activity. Recently, Greenzaid and Jencks[3] have shown that in the presence of methanol the swine liver esterase catalyzes both the methanolysis and hydrolysis of acetate esters, and that the enzyme contains at least two types of active sites. In the present work, we have obtained the evidence which was different from their observation in regard to the effect of acetone on solvolysis of ethyl *n*-butyrate and phenyl acetate.

Experimental

Materials——Swine liver esterase (carboxylester hydrolase, EC 3.1.1.1), which was a purified preparation (suspension in 3.2M ammonium sulphate solution, contaminants: acid phosphatase <0.01%, cholinesterase<0.1%), was purchased from C.F. Boehringer und Soehne Gmbh. The esterase preparation had a specific activity of 30.4 by our methods (see Enzyme Assay) and a purity greater than 93% by electrophoresis of the esterase (10 μg) in 7.5% polyacrylamide gel containing Tris-glycine buffer (pH 8.6), followed by staining for protein with Coomassie blue. And the enzyme preparation was free of lipase activity determined by the method of Dole and Meinertz[4] with Ediol (a commercial emulsion of 50% coconut oil from Calbiochem)

1) Part I: H. Ikezawa, S. Asai, and H. Ishihara, *Anal. Biochem.*, **41**, 408 (1971). This work was reported at the 92nd Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April, 1972.
2) Location: *Tanabe-dori, Mizuho-ku, Nagoya.*
3) P. Greenzaid and W.P. Jencks, *Biochemistry*, **10**, 1210 (1971).
4) V.P. Dole and H. Meinertz, *J. Biol. Chem.*, **235**, 2595 (1960).

as a substrate. A commercial product of *Mucor javanicus* lipase (specific activity of esterase: 0.00111), which was kindly supplied from Amano Pharmaceutical Co., Ltd., was also used as a preparation of esterase. Swine liver esterase was diluted with 0.03M sodium phosphate buffer (pH 6.8) −2.1 mM sodium deoxycholate solution. *Mucor javanicus* esterase was dissolved in the same buffer solution and centrifuged. The protein concentration of enzyme solution was estimated by the biuret method.[5] Methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl esters of *n*-butyric acid and phenyl acetate in the reagent grade were used as substrates. All other reagents used were the purest commercial grade available.

Assay Procedure——Unless otherwise stated, esterase activities were determined by the method using GLC.

1) Assay Method using GLC: Enzyme assay using GLC was carried out, according to the methods reported previously.[1] Glass-stoppered test tubes containing 1.9 ml of enzyme solutions were preincubated at 30° for 5 min and the enzyme reaction was initiated by addition of 0.5 ml of alcoholic solutions of alkyl *n*-butyrates (substrates), which contained 100 μmoles of the ester unless otherwise stated. The total rate of reaction, *i.e.*, the rate for transformation of substrate was determined from the change in amount of remaining substrate, which was corrected for nonenzymatic hydrolysis (control) under the experimental conditions. The rate of alcoholysis was obtained by measuring the amount of ester formed. Then, the rate of hydrolysis was calculated by subtracting that of alcoholysis from the total rate of reaction. One unit of esterase is expressed as that amount which will transform 1 μmole of methyl *n*-butyrate per minute in the presence of 3.5M ethanol. The specific activity is expressed in the terms of units/mg protein. Throughout the activity measurements, the enzyme activities were expressed in terms of initial reaction rates.

2) Assay Method using pH-Stat: Enzyme assay using pH-stat was carried out at pH 6.8 in a 2.4 ml reaction mixture containing 0.05M potassium chloride with a Radiometer TTT 2 titrator with magnetic stirring at 30°. A gentle stream of nitrogen was passed over the surface, and standard 0.02N sodium hydroxide was used to titrate the released protons.

3) Assay Method using Colorimetric Determination: When phenyl acetate was used as substrate, total activity was estimated colorimetrically by the method of Aldridge[6] using 4-amino antipyrine.

GLC——The conditions of GLC reported previously were slightly modified as follow: In the foregoing work, toluene was used as an internal standard. However, with ethyl *n*-butyrate as the substrate, toluene has a disadvantage that it has the same retention time as that of the substrate under the conditions of GLC. Thus, 0.2% *m*-xylene in *n*-hexane was used as the internal standard in the present work. In case that 15.8 mM ethyl *n*-butyrate was used as substrate, internal standard was further diluted (0.02%) and sensitivity of detector was raised (sensitivity=10^2 MΩ, range=0.05 V). Fig. 1 illustrates a typical pattern of chromatogram, which shows the relationship among the peaks of *n*-hexane, *m*-xylene and equal moles of alkyl *n*-butyrates and aliphatic alcohols. Calibration curves for alkyl *n*-butyrates are linear up to 120 μmoles of the esters. The actual recovery of each compound was much than 93%, with the coefficient of variation less than 3%.

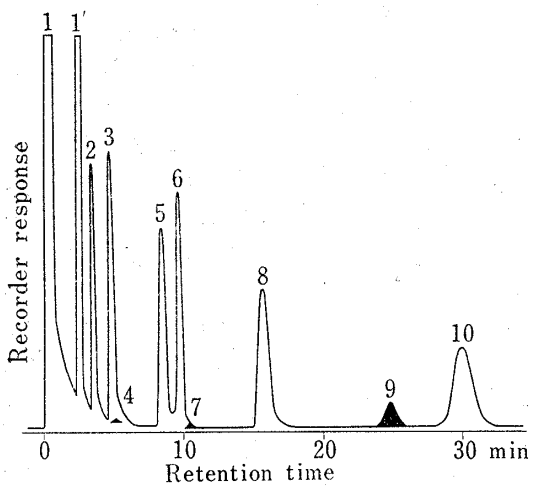

Fig. 1. Gas Chromatogram of Alkyl *n*-Butyrates, Aliphatic Alcohols and *m*-Xylene (Internal Standard)

peak 1: *n*-hexane (solvent), 1': inseparable contaminant in the solvent, 2: methyl *n*-butyrate, 3: ethyl *n*-butyrate, 4: *n*-propyl alcohol, 5: *n*-propyl *n*-butyrate, 6: *m*-xylene, 7: *n*-butyl alcohol, 8: *n*-butyl *n*-butyrate, 9: *n*-amyl alcohol, 10: *n*-amyl *n*-butyrate

Result

Activities of Esterases from Swine Liver and *Mucor javanicus* on Alkyl *n*-Butyrates

With alkyl *n*-butyrates having 1 to 5 carbon atoms in alcohol moieties as substrates, the esterase activities of swine liver and *Mucor javanicus* were measured in the presence of 3.5 M methanol or ethanol. The relative activity with each ester was calculated by taking the total activity with *n*-propyl *n*-butyrate in 3.5 M methanol as 100, since the highest activity was obtained with this substrate. The results with liver and Mucor esterases are collected

5) A.L. Gornall, C.J. Bardawill, and M.M. David, *J. Biol. Chem.*, 177, 751 (1949).
6) W.N. Aldridge, *Biochem. J.*, 57, 692 (1954).

in Tables I and II, respectively. As shown in Table I, the relative activities of liver esterase with *n*-propyl, *n*-butyl, and *n*-amyl *n*-butyrates in 3.5 M methanol were 2.5—2.9 times as much as those in 3.5 M ethanol. On the other hand, the ratios far from this range were obtained with methyl and ethyl *n*-butyrates as the substrates. The result with Mucor esterase in Table II shows a different characteristic, from that with liver esterase in Table I. In 3.5 M methanol, ethyl *n*-butyrate was transformed by liver esterase in a comparable rate to *n*-propyl *n*-butyrate. However, with Mucor esterase, the relative activity with ethyl *n*-butyrate was extremely lower than that with *n*-propyl ester in the presence of 3.5 M methanol. So far as esterases from swine liver and *Mucor javanicus* were concerned, *n*-propyl *n*-butyrate was shown to be the best substrate of five esters tested, from Tables I and II.

Table I. Relative Activity[a] of Liver Esterase on Five Alkyl *n*-Butyrates

n-Butyrate esters	Relative activity		Activity ratio[b]
	in 3.5 M CH_3OH	in 3.5 M C_2H_5OH	
Methyl	9.21	9.53	0.97
Ethyl	94.3	7.53	12.5
n-Propyl	100(340)[c]	39.8	2.5
n-Butyl	88.6	33.1	2.7
n-Amyl	57.3	19.7	2.9

a) Calculated as (the total activity with each ester/the total activity with *n*-propyl ester in 3.5M CH_3OH)×100
b) Calculated as the relative activity in CH_3OH/the relative activity in C_2H_5OH
c) The value in parenthesis means the total activity with *n*-propyl *n*-butyrate, in terms of μmoles of substrate transformed per min per mg enzyme protein.

Table II. Relative Activity[a] of Mucor Esterase on Five Alkyl *n*-Butyrates

n-Butyrate esters	Relative activity		Activity ratio[b]
	in 3.5 M CH_3OH	in 3.5 M C_2H_5OH	
Methyl	1.64	0.180	9.1
Ethyl	2.52	0.291	8.7
n-Propyl	100(0.603)[c]	52.7	1.9
n-Butyl	94.5	44.8	2.1
n-Amyl	32.0	16.4	2.0

a), *b*) and *c*) are the same as shown in Table I.

Formation of Methyl and Ethyl *n*-Butyrates as the Products of Alcoholysis by Esterases

During the study on liver esterase, it was found by using GLC that a significant amount of methyl or ethyl *n*-butyrate was formed from other alkyl *n*-butyrate by liver esterase in the presence of 3.5 M methanol or ethanol. On the other hand, the formation of methyl or ethyl ester by Mucor esterase was only a few per cent of transformed substrate under the same conditions. The results are summarized in Tables III and IV. Under the assay conditions employed, approximately 70—80 per cent of transformed substrate was converted into methyl or ethyl *n*-butyrate, by the action of liver esterase preparation.

A question arises as to whether this conversion was due to alcoholysis or synthesis by esterases. When these enzymes were incubated with 100 μmoles of *n*-butyric acid in the presence of 3.5 M methanol or ethanol, the amount of methyl or ethyl *n*-butyrate synthesized was less than 1 μmole for 30 min, and only 1 to 3 μmoles for prolonged incubation. Accordingly, the synthetic activity of esterases had little, if any, contribution to the formation of methyl or ethyl *n*-butyrate. Therefore, the results in Tables III and IV are explained to be due to alcoholysis.

Table III. Formation of Methyl *n*-Butyrate by Esterases in the Presence of Methanol

n-Butyrate esters	Per cent alcoholysis[a]	
	Liver esterase	Mucor esterase
Ethyl	80	4
n-Propyl	84	4
n-Butyl	77	2
n-Amyl	81	5

a) Calculated as (methyl *n*-butyrate formed/transformed substrate) × 100

Table IV. Formation of Ethyl *n*-Butyrate by Esterases in the Presence of Ethanol

n-Butyrate esters	Per cent alcoholysis[a]	
	Liver esterase	Mucor esterase
Methyl	74	1
n-Propyl	82	1
n-Butyl	76	2
n-Amyl	84	5

a) Calculated as (ethyl *n*-butyrate formed/transformed substrate) × 100

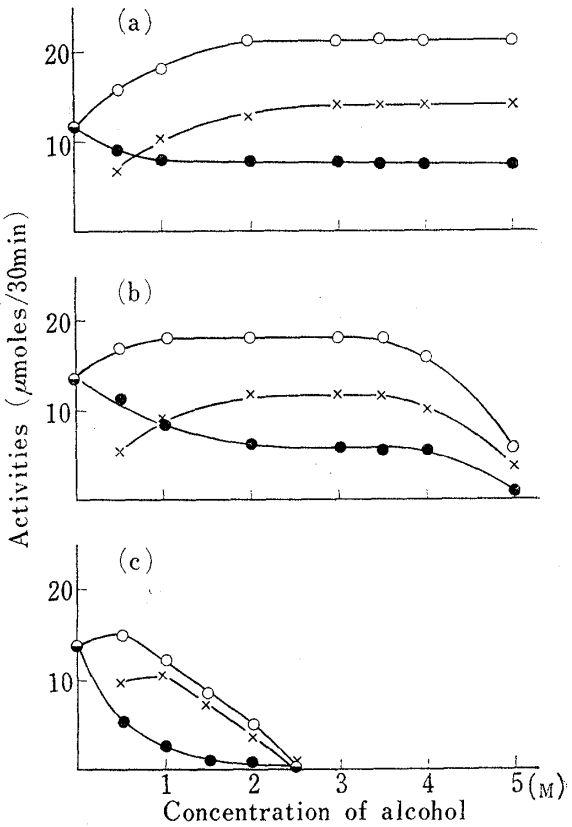

Fig. 2. Effect of Methanol, Ethanol and *n*-Propanol on the Liver Esterase-Catalyzed Hydrolysis and Alcoholysis of *n*-Butyl *n*-Butyrate

Ten microliter (=64 μmoles) of *n*-butyl *n*-butyrate was added to 1.4 ml of 0.03M sodium phosphate buffer (pH 6.8)–2.1 mM sodium deoxycholate solution containing various concentrations of alcohols. The enzyme reaction was started by addition of 1 ml of enzyme solution.
(a) in the presence of methanol (enzyme 1.9 μg)
(b) in the presence of ethanol (enzyme 3.8 μg)
(c) in the presence of *n*-propanol (enzyme 3.8μg)
—○—: total reaction (alcoholysis plus hydrolysis)
—×—: alcoholysis
—●—: hydrolysis

Reactions of Liver Esterase in the Presence of Various Amounts of Alcohols

Fig. 2 illustrates the effects of concentrations of methanol, ethanol and *n*-propanol on the hydrolytic and the alcoholytic activities of liver esterase. The substrate used was *n*-butyl *n*-butyrate. Within lower range of alcohol concentration, the increase in molarity of alcohol resulted in a marked decrease of hydrolytic activity of the enzyme. However, this activity was kept constant at concentrations of methanol above 1 M, gradually reduced at concentrations of ethanol higher than 1 M, and considerably inactivated at concentrations of *n*-propanol above the same molarity. On the other hand, the total activity of liver esterase reached to maximum at concentrations above 2 M with methanol, 1 M with ethanol and 0.5 M with *n*-propanol. These optima were slightly lower than those for alcoholytic activity of the enzyme, which were 3 M with methanol, 2 M with ethanol and 1 M with *n*-propanol. The difference between the optima of the total and alcoholytic activities was due to the fact that the alcoholytic activity became dominant over the hydrolytic one with increase in alcohol concentration. As the number of carbon atoms of alcohol increased, the esterase became more sensitive to alcohol. Especially, *n*-propanol completely inactivated total activity of the enzyme at concentrations around 2.5 M.

Inactivation of Liver Esterase by Heating and by Treatment with Bis-[*p*-nitrophenyl] phosphate (DNPP)

For the purpose of getting exact information on the relationship between hydrolytic and alcoholytic activities of liver esterase preparation, the changes in two activities were compared after inactivation of the enzyme by heat treatment and irreversible inhibitor, DNPP.

Swine liver esterase was stable at 50° for 1 hr, while it completely lost its activity at 60° for the same period. Then, the hydrolytic and alcoholytic activities of the enzyme preparation were measured with four substrates in the presence of 3.5 M methanol or ethanol, after heat treatment at 60° for 15 min. Table V illustrates per cent inactivation of these activities after heating. The extent of inactivation of hydrolytic activity was almost the same as that of alcoholytic activity, irrespective of the substrates used.

Recently, Heymann, *et al.*[7] showed that DNPP reacts specifically with a highly purified preparation of carboxylesterase from swine liver in a stoichiometrical manner, and that as the result of this reaction irreversible inactivation of enzyme rapidly takes place. In the present study, the activities of liver esterase were measured after preincubation of the enzyme at 30° for 10 min in the absence (control) or presence of 10^{-5} M DNPP. Table VI shows per cent inactivation of hydrolytic and alcoholytic activities, with four substrates in two alcohols. Per cent inactivation of hydrolytic activity with DNPP was in good agreement with that of alcoholytic activity, irrespective of the substrates used. From the results shown in Tables V and VI, it is concluded that the same enzyme is responsible for the hydrolysis and alcoholysis of alkyl *n*-butyrates.

Table V. Inactivation of Liver Esterase by Heating

n-Butyrate esters	Assay in 3.5 M CH_3OH			Assay in 3.5 M C_2H_5OH		
	Enzyme (μg)	% inactivation[a]		Enzyme (μg)	% inactivation[a]	
		Alcoholysis	Hydrolysis		Alcoholysis	Hydrolysis
Methyl	—	—	—	22.8	59	59
Ethyl	1.9	46	48	—	—	—
n-Propyl	1.9	43	36	3.8	67	61
n-Butyl	1.9	48	44	3.8	68	66
n-Amyl	3.8	23	24	7.6	64	56

a) Calculated as (A–B/A)×100, where A=the activity of unheated enzyme (control) and B=the activity of heated (at 60° for 15 min) enzyme

Table VI. Inactivation of Liver Esterase by DNPP

n-Butyrate esters	Assay in 3.5 M CH_3OH			Assay in 3.5 M C_2H_5OH		
	Enzyme (μg)	% inactivation[a]		Enzyme (μg)	% inactivation[a]	
		Alcoholysis	Hydrolysis		Alcoholysis	Hydrolysis
Methyl	—	—	—	21.6	54	59
Ethyl	1.8	49	38	—	—	—
n-Propyl	1.8	50	42	3.6	60	65
n-Butyl	1.8	46	35	3.6	51	59
n-Amyl	3.6	54	51	7.2	59	64

a) Calculated as (C–D/C)×100, where C=the activity of untreated enzyme (control) and D=the activity of 10^{-5} M DNPP-treated enzyme

7) E. Heymann and K. Krisch, *Z. Physiol. Chem.*, **348**, 609 (1967).

Kinetics Parameters for Esterase-Catalyzed Hydrolysis of *n*-Butyrate Esters

Methyl, ethyl and *n*-propyl *n*-butyrate were chosen as substrates. *K*m and V_{max} for esterase-catalyzed hydrolysis of these *n*-butyrate esters in the presence and absence of alcohols were calculated from Lineweaver–Burk's plot. Under the assay conditions employed, the linear plots were obtained. The parameters obtained (*K*m and V_{max}) are listed in Table VII. In the presence of 3.5 M ethanol, the *K*m values were higher than those in the presence of 3.5 M methanol; especially the difference was the most marked with methyl *n*-butyrate as the substrate. On the other hand, V_{max} values in the presence of 3.5 M ethanol were almost the same as those in the presence of 3.5 M methanol.

Effect of Acetone on Solvolysis of Ethyl *n*-Butyrate and Phenyl Acetate

In order to clarify the effect of acetone on solvolysis of esters, ethyl *n*-butyrate and phenyl acetate were chosen as the aliphatic and the aromatic ester substrates, respectively. The results are listed in Table VIII. The esterase-catalyzed methanolysis of 3.95 mM phenyl acetate was inhibited by the presence of 0.25 M acetone. However, the same concentration of acetone had little effect on the rate of methanolysis of 15.8 mM ethyl *n*-butyrate. On the other hand, acetone served as a modifier increasing the rates of hydrolysis of both esters, either in the presence or absence of 0.05 M methanol.

Table VII. Kinetic Parameters for Esterase-Catalyzed Hydrolysis of *n*-Butyrate Esters

n-Butyrate esters	*K*m (mM)	V_{max} (μmoles/mg·min)	Concn. range (mM)
Methyl	1.9	146	2.8—14
in 3.5 M CH_3OH	2.4	19	2.8—14
in 3.5 M C_2H_5OH	33	19	2.8—14
Ethyl	3.1	185	2.4—12
in 3.5 M CH_3OH	3.1	23	2.4—12
in 3.5 M C_2H_5OH	10	20	2.4—12
n-Propyl	3.9	167	1.3—6.4
in 3.5 M CH_3OH	3.9	26	1.3—6.4
in 3.5 M C_2H_5OH	7.1	26	1.3—6.4

The rate of esterase-catalyzed hydrolysis was determined at 30° on pH-stat at pH 6.8 in the presence of 0.05M KCl.

Table VIII. Effect of Acetone on Solvolysis of Ethyl *n*-Butyrate and Phenyl acetate

CH_3OH	Relative rate[a]	Ethyl *n*-butyrate	Phenyl acetate
0	hydrolysis[b]	131	123
0.05 M	hydrolysis[b]	128	126
0.05 M	methanolysis	100[c]	23[d]

a) In each series, the initial rates in the presence of acetone were compared with that measured in the absence of acetone, which was taken as 100.
[phenyl acetate]=3.95 mM
[ethyl *n*-butyrate]=15.8 mM
[acetone]=250 mM
b) method using pH-stat
c) method using GLC
d) method using colorimetric determination and pH-stat

Discussion

By use of GLC in the esterase assay, the five esters of *n*-butyric acid, containing 1 to 5 carbon atoms in the straightchain alkyl moieties, were made available as the substrates of esterases. Among these esters, *n*-propyl *n*-butyrate was the best substrate for both esterases from swine liver and *Mucor javanicus*. This high reactivity for "medium-chain length"

ester is consistent with the results on liver esterase obtained by Webb.[8] In the text, relative activities of methyl *n*-butyrate in the presence of 3.5 M methanol and ethyl *n*-butyrate in the presence of 3.5 M ethanol are measured only as the apparent hydrolytic activities because, in the presence of the component alcohol, the remaining substrates are not distinguished from the ester which might reversibly be formed by alcoholysis. On the contrary, the low reaction rate with methyl *n*-butyrate in the presence of 3.5 M ethanol is due to the highest *K*m for this ester among the substrates, regardless of its V_{max} value.

One of the main characteristics of GLC method is its ability to measure the extent of alcoholysis of the substrate as well as the total amount of the substrate transformed. In the foregoing work, we used 0.2% toluene in *n*-hexane as the internal standard of GLC, and failed to detect ethyl *n*-butyrate, which was formed from methyl ester and ethanol by alcoholytic action of liver esterase. The reason why we overlooked alcoholysis is due to the fact that ethyl *n*-butyrate have approximately the same retention time as that of toluene under the conditions of GLC. In the present study, an improvement was made on the procedures of GLC by using 0.2% *m*-xylene in *n*-hexane as the internal standard, instead of 0.2% toluene. In Fig. 1, there is no significant difference between the retention times of ethyl *n*-butyrate and *n*-propyl alcohol, under the GLC conditions employed in this study. Thus some difficulty was encountered when *n*-propyl *n*-butyrate in ethanol was used as the substrate of liver esterase. In this case, ethyl *n*-butyrate was formed as the product of ethanolysis of the substrate, with concomitant release of *n*-propyl alcohol (the product of hydrolysis and ethanolysis). Consequently, it was probable that the amount of ethyl *n*-butyrate was overestimated in the presence of *n*-propyl alcohol. In fact, the correction of the amount of ethyl *n*-butyrate should be inevitable in the case of Mucor esterase, which showed little activity of alcoholysis. However, the contribution of *n*-propyl alcohol to the apparent amount of the ester was actually negligible in the study of liver esterase, which had high activity of alcoholysis.

It is interesting that both total and alcoholytic activities of liver esterase were maximally exhibited at concentrations above 0.5—3 M with three alcohols. Presumably, with respect to the acceptor function for acyl groups, these alcohols are more effective than water. However, the esterase inactivation became greater with increasing number of carbon atoms in the alcohol molecule. Recently, Simonianová, *et al.*[9] has reported the same relationship in the study of inhibition of rat serum N-acetyl-L-tyrosine ethyl esterase (chymotrypsin-like enzyme) by aliphatic alcohols.

Since we had used a commercial preparation of liver esterase, it was necessary to confirm that the same enzyme was responsible for both hydrolytic and alcoholytic activities. Fortunately, treatment of enzyme solution by heating or with DNPP resulted in parallel decrease of these two activities. On the other hand, we observed the difference between the effects of acetone on methanolysis and hydrolysis. This observation shows that the enzyme contains at least two different types of active sites. Thus, the result obtained is different from the conclusion proposed by Greenzaid, *et al.*[3] Consequently, the identical enzyme, not the identical site of a enzyme, must catalyze both hydrolysis and alcoholysis of the esters.

The data for *n*-propyl *n*-butyrate in Fig. 2, when replotted of H/A (hydrolysis/alcoholysis ratio) *vs* reciprocal of alcohol concentration according to Wynne, *et al.*,[10] resulted in straight line with an intercept on the ordinate, indicating that a scheme for partitioning of an acyl enzyme between water and alcohol is most probably represented by the Scheme 2, which was proposed by Wynne, *et al.*[10]

The percent alcoholysis of liver esterase was constant for the various leaving alkyl groups, then the reaction most probably occurrs *via* an acyl enzyme mechanism.[3,10] Mucor esterase

8) M. Dixon and E.C. Webb, "Enzymes," 2nd ed, Longmans, Green and Co. Ltd., London, 1964, p. 218.
9) E. Simonianová, M. Petaková, and M. Rybak, *Collection Czechoslov. Chem. Commun.*, **35**, 2650 (1970).
10) D. Wynne and Y. Shalitin, *Eur. J. Biochem.*, **31**, 554 (1972).

had little activity of alcoholysis, so it is not clear whether the mechanism of reaction catalyzed by Mucor esterase occurrs *via* an acyl enzyme or not.

Acknowledgement We wish to express our thanks to Amano Pharmaceutical Co., Ltd. for their gift of esterase preparation.