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Kinetic Studies on Pancreatic Lipase Activity in Micellar Systems. I.[1] Inhibition by Sodium Deoxycholate Micelles

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Pancreatic lipase activity toward vinyl laurate (VL) solubilized in sodium deoxycholate (NaDC) micelles was investigated kinetically. The inhibition by substrate-free NaDC micelles, M, was observed, and a Lineweaver–Burk plot and a plot of the reciprocal of initial rate *vs.* [M] indicated that the inhibition may be fully competitive or fully mixed inhibition, depending on the value adopted for the aggregation number of NaDC micelles. However, further consideration of the interaction of various species in the system led us to favor a fully competitive inhibition mechanism. The Michaelis constant, K_m, and the inhibition constant, K_4, which is the dissociation constant of lipase–NaDC micelle complex, were estimated. It was shown that K_m indicates the lower limit of the dissociation constant of lipase–NaDC micelle solubilizing VL, K_1. The results are discussed in relation to the results of other studies on the inhibition of the enzyme by bile salts in emulsion systems.

Keywords——pancreatic lipase activity; micellar system; vinyl laurate; inhibition by NaDC micelles; fully competitive inhibition; Michaelis constant; inhibition constant

The enzymatic hydrolysis of a lipid by pancreatic lipase, as well as by other lipolytic enzymes, is a heterogeneous reaction because the enzyme is water-soluble but the substrate is not. Therefore, the enzyme–substrate interaction must take place at the substrate–water interface. Usually pancreatic lipase activity has been measured by use of an emulsion system of plant oil, and it is known that the activity depends not on the total concentration of the emulsified substrate but on the interfacial area of emulsion droplets.[2] Since an emulsion system is thermodynamically unstable, the emulsion must be prepared carefully to have a definite interfacial area of the system, and in some instances determination of the interfacial area of the system is required. On the other hand, a micellar system is thermodynamically stable and will provide an interface for the enzyme, but little work has been done to apply a micellar system to the measurement of lipase activity.[3]

A bile salt, which is a naturally occurring surfactant and participates physiologically in lipolysis, has often been used as an accelerator for the enzyme in an emulsion system. Although it has been known that a bile salt generally acts as an accelerator at lower concentration but as an inhibitor at higher concentration,[4] quantitative studies have not been performed satisfactorily because experiments employing an emulsion system suffer the disadvantage that the extent of emulsification affects not only the effective concentration of the substrate but also the bile salt concentrations in bulk and at the interface. If a substrate solubilized in bile salt micelles is usable by the enzyme, activity measurement using the substrate may be free from this disadvantage. In addition, a quantitative analysis of the effect of bile salt on the enzyme activity may provide some information on the physiological role of a bile salt in fat digestion and absorption.

In this work, sodium deoxycholate (NaDC), which has the mildest inhibitory effect among bile salts,[4b,5] was chosen as a surfactant, and pancreatic lipase activity toward vinyl laurate (VL)[6] solubilized in NaDC micelles was kinetically investigated. The mechanism of inhibition by NaDC micelles is discussed.

Experimental

Materials——Pancreatic lipase was a product of Sigma Chemical Co., Lipase Type VI from porcine pancreas. According to the supplier, the specific activity is 340 unit/mg protein using olive oil at pH 7.7 and 37°C. VL (>99%) was obtained from Tokyo Kasei Kogyo Co. and used without further purification. NaDC (Merck Co.) was recrystallized two or three times from a mixed solution of methanol, and ethanol, and then dried *in vacuo* at 110°C. The purity was estimated to be at least 99% by pH measurement.[7] Rhodamine 6G was obtained from Katayama Chemical Co., and 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBTH) from Tokyo Kasei Kogyo Co. All other chemicals were of reagent grade. Water was passed through an ion-exchange resin and distilled twice.

Determination of the cmc of NaDC——The critical micellar concentration (cmc) was determined by the spectral shift method with Rhodamine 6G.[8] Absorption maxima were measured with a Hitachi 100-60 spectrophotometer equipped with a thermostated chamber.

Solubilization of VL by NaDC Micelles——A stock solution of VL solubilized by NaDC was prepared prior to the kinetic runs as follows. A 0.5 M solution of VL in acetonitrile was added to crystalline powder of NaDC in a volumetric flask such that the molar ratio of VL to NaDC was about 1 to 20, and then dissolved slowly by addition of such quantities of a 50 mM Tris-HCl buffer (pH 8.0, ionic strength 0.25 adjusted with NaCl) that the final concentration of buffer was 40 mM. When the powder had dissolved completely, the flask was filled to the mark with water.

Assay of Acetaldehyde——Acetaldehyde, the isomerization product of the unstable vinyl alcohol which is the hydrolysis product of VL, was determined by the MBTH method.[9] Because of pH elevation of the reaction mixture, resulting from the use of alkaline-buffered sample solution, the method was modified as described below. A 1 ml aliquot of sample solution was added to a mixture of 1 ml each of 0.4% MBTH and 0.1 N HCl in a 20 ml volumetric flask. After 30 min standing, 4 ml of 0.25% ferric chloride was added to the mixture. After a further 5 min, the mixture was diluted to 20 ml with acetone. The absorbance was measured at 663 nm against water with a Hitachi 100-60 spectrophotometer.

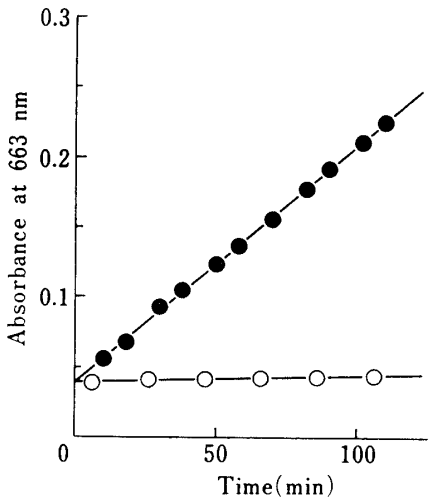

Fig. 1. Absorption-Time Curve for the Hydrolysis of Vinyl Laurate followed by the Formation of Acetaldehyde

●: in the presence of pancreatic lipase, ○: in the absence of pancreatic lipase.
Conditions: vinyl laurate 1 mM, NaDC 40 mM, Tris-HCl buffer 40 mM, NaCl 0.18 M, ionic strength 0.2, pH 8.0, temperature 25°C.

Kinetic Measurement——Various amounts of NaDC dissolved in 40 mM Tris-HCl buffer were added to definite amounts of stock solution of VL solubilized in NaDC micelles, and the mixtures were diluted to a definite volume with 40 mM Tris-HCl buffer (pH 8.0, ionic strength 0.2). Of these substrate solutions, 4 ml was pippeted into a 10 ml test tube fitted with a glass stopper, and then placed in a thermostated bath (25±0.1°C). After about 20 min, each run was started by addition of 50 μl of pancreatic lipase solution (0.005%). At appropriate intervals, 1-ml aliquots were withdrawn, and then initial rate (v) was estimated from the amount of acetaldehyde liberated. Since it was confirmed in a preliminary experiment that the absorbance increased linearly with time as shown in Fig. 1, the number of samplings was usually two or three for each run, and it was confirmed that the values extrapolated to zero time were consistent with those of the blank runs. Each solution was tested for pH constancy before and after a kinetic run, and the largest change was 0.04 pH unit. A Metrohm Model E 510 precision pH meter with an expanded scale and a combined glass electrode EA 125 was used.

Results and Discussion

Micelle Solubilization of VL by NaDC

It is well known that NaDC forms micelles, and that both the cmc and the aggregation number depend on pH, the concentration of added salts and temperature.[10] Under the present experimental conditions (pH 8.0, NaCl 0.18 M, and 25 °C), the cmc of NaDC was determined to be 1.75 mM by the spectral shift method with Rhodamine 6G. The aggregation number of NaDC under the present experimental conditions is considered to be around 17 because

it has been reported that the aggregation number of NaDC is 17 at pH 8.0, NaCl 0.15 M, and 20 °C and that it increases with increase in the concentration of added salt and with decrease in temperature.[10]

When the micelle concentration was less than the VL concentration, VL was not completely solubilized and oil droplets appeared; this solubilization behavior of VL is consistent with the previous findings[11] that the maximum amount of oleic acid solubilized by NaDC micelles is one molecule per micelle, and the aggregation numbers of simple micelles and mixed micelles of NaDC and oleic acid are the same. Therefore, it is estimated that NaDC micelles solubilizing VL each consist of about 17 molecules of NaDC and one of VL.

Inhibition by Substrate-free NaDC Micelles

For a lipolytic enzyme, the substrate of which is water-insoluble, the adsorption of the enzyme to the substrate–water interface must precede the formation of an enzyme–substrate complex.[2] Since pancreatic lipase activity was observed in NaDC micellar systems solubilizing VL, it can be considered that the enzymatic reaction in this system fundamentally follows the mechanism shown in Chart 1.[12] The symbols used are: E, pancreatic lipase; M, NaDC

$$E \overset{MS}{\rightleftarrows} EMS \rightleftarrows ESM \longrightarrow E + M + P$$

Chart 1

micelle; S, VL; MS, NaDC micelle solubilizing VL; EMS, lipase–NaDC micelle solubilizing VL complex; ESM, lipase–VL complex at micelle interface; P, products (see Nomenclature).

When the enzyme activities were measured in the above mentioned micellar systems at various concentrations of VL keeping the concentration of NaDC constant, nontypical Michaelis and Lineweaver–Burk plots were obtained (Fig. 2): the Michaelis plot is not convex but concave, and the Lineweaver–Burk plot is not linear but convex. These nontypical plots seem to be due to the adsorption of the enzyme to substrate-free NaDC micelles (M), which decrease in amount with increase in the substrate concentration. That is to say, the effective concentration of the enzyme for MS may alter because the concentration of M changes simultaneously with that of MS. Thus, in order to analyze this enzymatic reaction, it is necessary to keep the concentration of either MS or M constant.

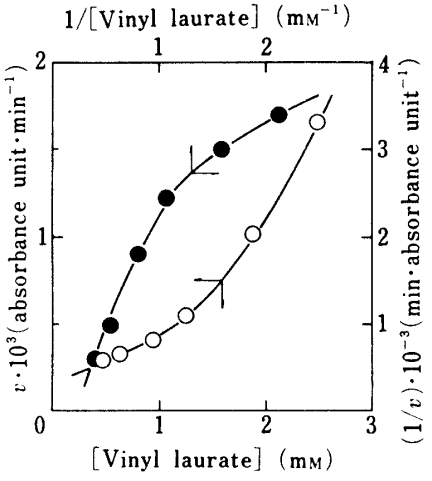

Fig. 2. Michaelis Plot and Lineweaver–Burk Plot at Constant Concentration of NaDC

○: Michaelis plot, ●: Lineweaver–Burk plot.
Conditions: NaDC 40 mM, Tris-HCl buffer 40 mM, NaCl 0.18 M, ionic strength 0.2, pH 8.0, temperature 25°C.

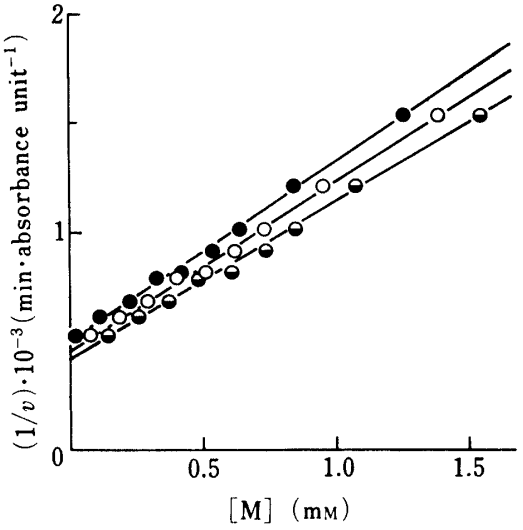

Fig. 3. Inhibition by Substrate-free NaDC Micelles

◒: $N=16$, ○: $N=17$, ●: $N=18$.
Solid lines are regression lines ($r=0.997$ in each case).

From the binding constants of fatty acid *p*-nitrophenyl esters to NaDC micelles,[13] it is presumed that VL binds nearly quantitatively to NaDC micelles. Thus, the concentration of substrate solubilized in NaDC micelles, [MS], and substrate-free NaDC micelles, [M], can be approximated by Eqs. (1) and (2), respectively, provided that the solubilization of VL does not modify the cmc and the aggregation number of NaDC micelles, as is usually assumed for a simple solubilization system.

$$[MS] = [S]_t \tag{1}$$

$$[M] = [M]_t - [MS] = \frac{[D]_t - cmc}{N} - [MS] \tag{2}$$

where $[S]_t$, $[M]_t$, and $[D]_t$ represent the total concentrations of VL, NaDC micelles, and NaDC, respectively, and N is the aggregation number. Thus, kinetic measurements at constant [MS] are made possible by varying the concentration of NaDC at a constant concentration of VL with $[M]_t > [S]_t$. When the reciprocal of initial rate ($1/v$) was plotted against the micelle concentration, [M], for three values of N (16, 17, and 18), a linear relationship was obtained in each case (Fig. 3). This demonstrates that substrate-free NaDC micelles inhibit the enzymatic reaction.

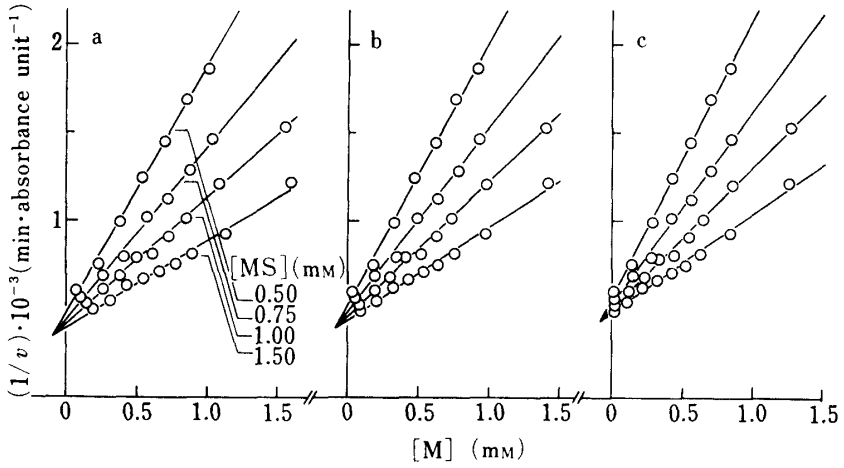

Fig. 4. Plots of $1/v$ *vs.* [M] for Three Values of Aggregation Number

a: $N=16$, b: $N=17$, c: $N=18$.
Solid lines are regression lines ($r=0.995$—0.999).

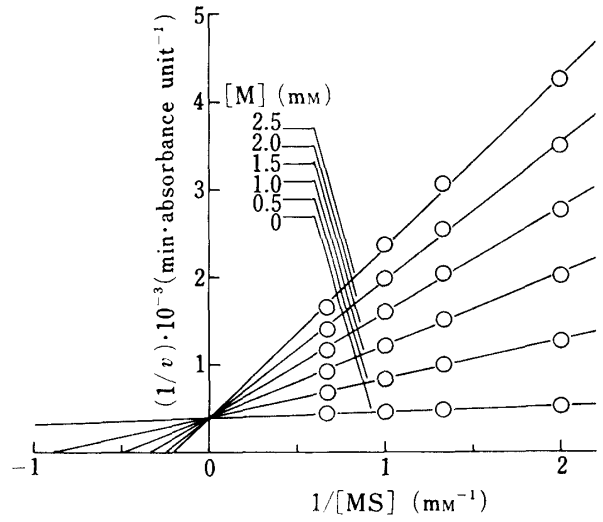

Fig. 5. Lineweaver–Burk Plots for $N=17$

Solid lines are regression lines. The correlation coefficients are 0.999 for all values of [M] except for [M]=0 ($r=0.989$).

Inhibition Mechanism

Plots of $1/v$ *vs.* [M] at several constant concentrations of VL are shown in Fig. 4, in which the straight lines clearly intersect in the left upper quadrant in all cases.

Dixon *et al.* stated that inhibitions of single substrate reaction can be classified into four categories, *i.e.*, competitive,

non-competitive, mixed, and uncompetitive, and they are subdivided individually into two types, *i.e.*, full and partial type.[14] Of these eight types of inhibition mechanisms, fully competitive and fully mixed inhibition would produce the results in Fig. 4. These two inhibition mechanisms can be distinguished by means of a Lineweaver–Burk plot. Using estimates for $(1/v)_{[M]=const}$ obtained by regression analysis of the data of Fig. 4, Lineweaver–Burk plots for the three values of N were prepared. In the case of $N=17$, straight lines intersecting on the $1/v$ axis could be drawn (Fig. 5), indicating that the mechanism must be competitive inhibition. For other values of N, the intersection of lines in the left upper quadrant suggests the mixed inhibition mechanism (Fig. 6).

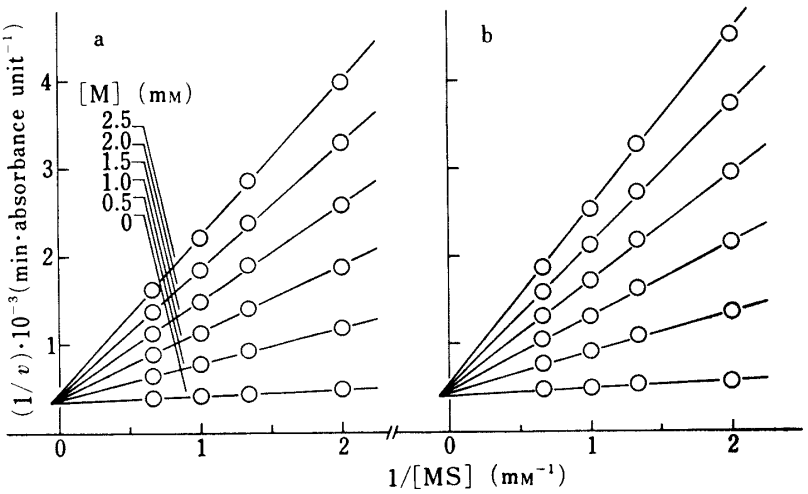

Fig. 6. Lineweaver–Burk Plots for $N=16$ and $N=18$

a: $N=16$, b: $N=18$.
Solid lines are regression lines. The correlation coefficients are 0.999 for all values of [M] except for [M]=0 ($r=0.992$ for $N=16$, 0.987 for $N=18$).

The above two possible mechanisms may be described as shown in Charts 2 and 3.[15] Although, in general, the step depicted by broken arrows in Chart 2 does not exist in a fully competitive inhibition mechanism, it may need to be taken into account in this study. For

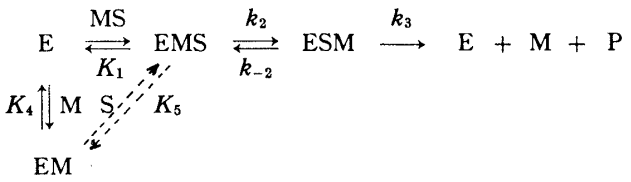

Chart 2. Fully Competitive Inhibition

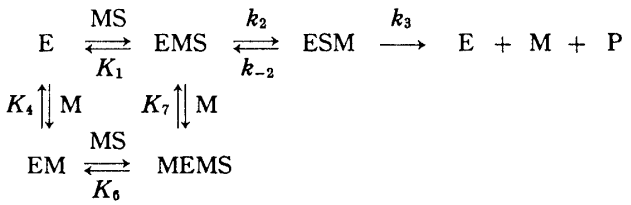

Chart 3. Fully Mixed Inhibition

the fully mixed inhibition mechanism, species involving E and M, other than EM, must be present. Chart 3, where MEMS is assumed as such a species, may be representative of fully mixed inhibition mechanisms.[16] However, it is unlikely that MEMS, generated by the binding of M to EMS, does not break down to give products (not *via* EMS) at all, whereas EMS does; if MEMS gives products directly, contrary to Chart 3, the inhibition mechanism becomes

a partially mixed one, and plots of $1/v$ *vs.* [M] would be nonlinear in contrast to the results in Figs. 3 and 4. Furthermore, if MEMS is formed, MEM, which results from the binding of two NaDC micelles to one enzyme, would also be present and plots of $1/v$ *vs.* [M] would no longer be linear because of the appearance of a squared term in [M] in the rate equation. For these two reasons, the existence of MEMS can be ruled out practically, and thus the fully mixed inhibition mechanism is ruled out. Consequently, it can be concluded that the inhibition mechanism in this micellar system is a fully competitive one.

Estimation of Kinetic Parameters

By making reasonable assumptions, Eq. (3) can be derived from the inhibition mechanism shown in Chart 2 (see Appendix).

$$v = \frac{V[\mathrm{MS}]}{K_\mathrm{m}(1+[\mathrm{M}]/K_4)+[\mathrm{MS}]} \tag{3}$$

As shown in Table I, the meanings of V and K_m depend on the assumptions used. It should be noted that K_m implies the lower limit of K_1.

Eq. (3) can be rewritten in the forms of Eq. (4) or (5).

$$\frac{1}{v} = \frac{K_\mathrm{m}}{K_4 V[\mathrm{MS}]} \cdot [\mathrm{M}] + \frac{1}{V}(1 + K_\mathrm{m}/[\mathrm{MS}]) \tag{4}$$

$$\frac{1}{v} = \frac{K_\mathrm{m}(K_4+[\mathrm{M}])}{K_4 V} \cdot \frac{1}{[\mathrm{MS}]} + \frac{1}{V} \tag{5}$$

Table I. Meanings of V and K_m on Various Assumptions

Assumption: Rapid equilibrium	Assumption: Steady state	Assumption: Rate-determining step	V	K_m
Steps 1, 4, 5	[ESM]	Step 3	$\frac{k_2 k_3[\mathrm{E}]_0}{k_2+k_{-2}+k_3}$	$K_1 \cdot \frac{k_{-2}+k_3}{k_2+k_{-2}+k_3}$
Steps 1, 2, 4, 5	—	Step 3	$\frac{k_2 k_3[\mathrm{E}]_0}{k_2+k_{-2}}$	$K_1 \cdot \frac{k_{-2}}{k_2+k_{-2}}$
Steps 1, 4, 5	—	Step 2	$k_2[\mathrm{E}]_0$	K_1

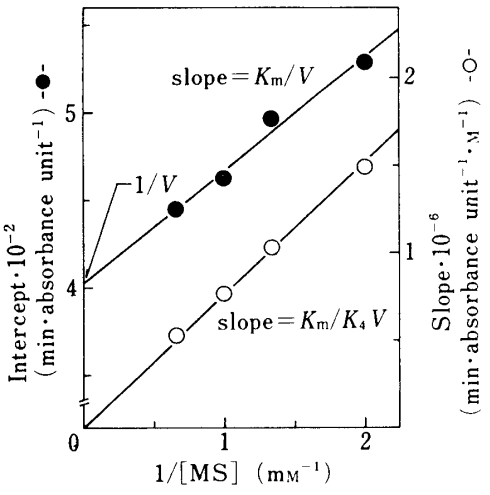

Fig. 7. Replots of Slopes and Intercepts obtained from Fig. 4b against 1/[MS]

○: slope, ●: intercept.

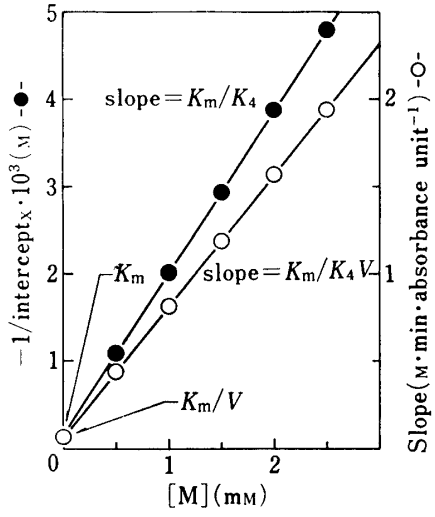

Fig. 8. Replots of Slopes and Reciprocals of Abscissa Intercepts obtained from Fig. 5 against [M]

○: slope, ●: reciprocal of abscissa intercept.

Eq. (4) predicts that the intersection of the lines in Fig. 4b is $(-K_4, 1/V)$ and replots of both the slopes and the intercepts in Fig. 4b against 1/[MS] should yield straight lines. In fact, these replots gave satisfactorily linear relationships as shown in Fig. 7. On the other hand, Eq. (5) predicts that the intersection on the ordinate in Fig. 5 is $1/V$ and replots of both the slopes and the reciprocals of abscissa intercepts in Fig. 5 against [M] should give straight lines. Again these replots gave good linearity as shown in Fig. 8.

From the slopes and the intercepts of straight lines in Figs. 7 and 8, the kinetic parameters V, K_m, and K_4 can be calculated individually; the results are summarized in Table II. The values for kinetic parameters obtained by the two different methods were in fair agreement with each other. This supports the conclusion that the inhibition mechanism by NaDC micelles is a fully competitive one along the lines shown in Chart 2.

TABLE II. Kinetic Parameters obtained by Two Methods

Kinetic parameter	Method	
	$1/v$ *vs.* [M] plot	$1/v$ *vs.* 1/[MS] plot
V (absorbance unit·min^{-1})	2.48×10^{-3}	2.53×10^{-3}
K_m (M)	1.60×10^{-4}	1.64×10^{-4}
K_4 (M)	8.50×10^{-5}	8.66×10^{-5}
K_m/K_4[a]	1.88	1.89

a) K_m/K_4 represents the lower limit of K_1/K_4.

Conclusions and Comparison with Other Studies regarding Inhibition by Bile Salts

The inhibition of pancreatic lipase by bile salts in an emulsion system has been correlated to the decrease in the amount of the enzyme adsorbed at the substrate–water interface.[17] Although it is generally accepted that monomeric bile salt competes with the enzyme at the interface,[17b,18] another mechanism, in which lipase–bile salt micelle complex with a weak affinity for the interface can be formed, has also been proposed.[19] The present findings that pancreatic lipase binds to NaDC micelles may support the latter mechanism.

Vandermeers *et al.* reported that the inhibition of lipase by taurodeoxycholate micelles in an emulsion system is competitive.[20] However, the concentration of bile salt was not described in terms of the micelle concentration but the total concentration, and a detailed analysis was not carried out. Experiments employing an emulsion system cannot avoid the unfavorable fact that the extent of emulsification affects not only the effective concentration of substrate but also bile salt concentrations in bulk and at the interface, because the amount of bile salt binding to the interface of an emulsion droplet depends on the interfacial area. Therefore, it is difficult to analyze quantitatively the interaction between lipase and bile salt, and equilibrium constants such as the dissociation constant of lipase–bile salt micelle complex have hardly been estimated. Although Myrick *et al.* performed the measurement of lipase activity in a mixed micellar system,[3] no effort was made to clarify the composition of the mixed micelles, the amount of solubilized substrate per micelle, or the inhibition me mechanism. Our results indicate that the inhibition is fully competitive inhibition by substrate-free NaDC micelles and that the dissociation constant of lipase–NaDC micelle complex, K_4, is about 9×10^{-5} M, while that of lipase–NaDC–VL complex, K_1, is more than 1.9 times K_4.

It has been pointed out that colipase, which is a protein with a molecular weight of *ca.* 10000, participates in the adsorption of lipase to the interface in such a way that it binds strongly to the lipid–water interface covered with bile salts and functions as an anchor for lipase.[17a,b,18,20] It was also reported that colipase and taurodeoxycholate micelles form a 1 : 1 complex.[18a,20,22] Because pancreatic lipase used in this work was not separated from colipase, the species, EM, EMS, and ESM, may involve colipase. Recently, it was found that pancreatic lipase binds strongly to colipase at the surface of triacylglycerol-covered glass

beads in the presence of taurodeoxycholate[17c] and at the interface of taurodeoxycholate micelles:[23] the dissociation constants were estimated to be about 1×10^{-9} M. Therefore, the values obtained here for K_m and K_4 may be related to the dissociation of colipase from those complexes (EM, EMS, and ESM), and the K_4 value may be comparable with the estimated value for the dissociation constant of colipase–taurodeoxycholate micelle complex, 1×10^{-4} M.[22]

The micellar system used in this work may be applicable for the measurement of pancreatic lipase activity, subject to further development, and may be useful for studies on the interactions of lipase and/or colipase with bile salt micelles or the substrate solubilized in the micelles.

Appendix

In deriving the rate equation for Chart 2, three kinds of assumptions seem reasonable:
1) rapid equilibrium in steps 1, 4, and 5; steady state in [ESM]; rate-determination at step 3
2) rapid equilibrium in steps 1, 2, 4, and 5; rate-determination at step 3
3) rapid equilibrium in steps 1, 4, and 5; rate-determination at step 2.

In case 1), Eqs. (6)—(9) are obtained.

$$\frac{[E][MS]}{[EMS]} = K_1 \tag{6}$$

$$\frac{[E][M]}{[EM]} = K_4 \tag{7}$$

$$\frac{d[ESM]}{dt} = k_2[EMS] - (k_{-2}+k_3)[ESM] \tag{8}$$

$$[E]_0 = [E] + [EM] + [EMS] + [ESM] \tag{9}$$

Solving for [ESM],

$$[ESM] = \frac{\dfrac{k_2}{k_2+k_{-2}+k_3}\cdot[E]_0[MS]}{K_1\cdot\dfrac{k_{-2}+k_3}{k_2+k_{-2}+k_3}\cdot\left(1+\dfrac{[M]}{K_4}\right)+[MS]} \tag{10}$$

Since $v=k_3[ESM]$,

$$v = \frac{V[MS]}{K_m\cdot\left(1+\dfrac{[M]}{K_4}\right)+[MS]} \tag{3}$$

where $V=k_2k_3[E]_0/(k_2+k_{-2}+k_3)$, $K_m=K_1(k^{-2}+k_3)/(k_2+k_{-2}+k_3)$.

In cases 2) and 3), Eq. (3) is obtained in a similar manner, though the meanings of V and K_m are different. These are listed in Table I in the text.

Nomenclature

E	pancreatic lipase.
M	sodium deoxycholate (NaDC) micelle.
S	vinyl laurate (VL).
P	products.
MS	NaDC micelle solubilizing VL.
EM	lipase–NaDC micelle complex.
EMS	lipase–NaDC micelle solubilizing VL complex.
ESM	lipase–VL complex at micelle interface.
MEMS	NaDC micelle–lipase–NaDC micelle solubilizing VL complex.
k_j	rate constant for forward reaction of step j.
k_{-j}	rate constant for backward reaction of step j.
K_j	dissociation constant of step j (K_4: inhibition constant).
K_m	Michaelis constant.
V	maximum velocity.

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References and Notes

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15) We assumed that, for a fully mixed inhibition mechanism, the enzyme might interact with the micelle in two ways, binding near the active site and binding at some other part of the molecule.
16) Other charts may be possible, *e.g.*, one includes MESM instead of MEMS, and another includes both of them. For these charts, however, the discussion is analogous to that in the text.
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