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Kinetic Analysis of Phospholipase A<sub>2</sub> Activity toward Mixed Micelles and Its Implications for the Study of Lipolytic Enzymes

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A detailed kinetic scheme is proposed for the action of phospholipase A<sub>2</sub> on mixed micelles of phospholipid and surfactant:

\[
E + A \xrightleftrarrows k_1 \kern-15pt \text{EA} \xrightarrow{k_4} \kern-15pt \text{EA} + B \xleftarrow{k_2} \kern-15pt \text{EAB} \xrightarrow{k_3} \kern-15pt \text{EA} + Q
\]

where \(E\) is the enzyme, \(A\) is the mixed micelle, and \(B\) is the phospholipid substrate in the mixed micelle. This scheme takes into account quantitatively the involvement of the lipid-water interface in the action of this enzyme toward substrate in macromolecular lipid complexes. The kinetic equation for this scheme is derived and four simplifying assumptions which are necessary for its practical application are described. Kinetic data are reported for the action of cobra venom phospholipase A<sub>2</sub> (*Naja naja naja*) on 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine in mixed micelles with the nonionic surfactant Triton X-100, and these data are analyzed in terms of the kinetic equation presented. At 40°C, pH 8.0, and in the presence of 10 mM Ca<sup>2+</sup>, \(V\) was found to be about \(4 \times 10^{-5}\) \(\mu\text{mol min}^{-1}\) mg of protein<sup>-1</sup>. \(K_{\text{m}}^A\), which is the dissociation constant for the enzyme-mixed micelle complex, is about \(5 \times 10^{-5}\) M. \(K_{\text{n}}\), the Michaelis constant for the catalytic step, which is \((k_4 + k_3)/k_2\), is 1 to 2 \(\times 10^{-10}\) mol cm<sup>-2</sup>. This kinetic treatment, together with the fact that the mixed micelle system allows the concentration of the substrate in the lipid-water interface to be varied, has made possible the quantitative separation of the association of a lipolytic enzyme with the lipid-water interface (expressed as \(K_{\text{sA}}\)) and the binding to the substrate in the interface (reflected in the \(K_{\text{n}}\) term). The implications of this kinetic scheme for the analysis of phospholipase A<sub>2</sub> from other sources acting on other aggregated forms of phospholipid and for the study of other phospholipases and lipases is considered.

Phospholipase A<sub>2</sub>, as well as other lipolytic enzymes, act in vivo on substrates that are part of macromolecular aggregates. For in vitro kinetic studies, several forms of the substrate have been employed including monolayers (1), micelles of phospholipids containing short chain fatty acids (2), monomers (3), ether-water complexes (4), and mixed micelles with surfactants (5). All of the studies have suggested that the lipid-water interface of these macromolecular aggregates plays an important role in enzymatic activity, although the precise function of the interface has not been elaborated. The presence of an interface sets lipolytic enzymes apart from "normal" enzymes, and an understanding of its functioning is crucial before the detailed catalytic mechanisms of this class of enzymes can be established. The Triton X-100-phosphatidylcholine mixed micelle system allows a direct kinetic investigation of the interaction of the enzyme and the lipid-water interface, because it provides a system in which the concentration of substrate in the interface can be varied and the activity can be followed by standard kinetic techniques. We have now developed a kinetic scheme which accounts for an interfacial requirement of phospholipase A<sub>2</sub>, the theoretical and experimental kinetic analyses of this scheme are presented. We also discuss the implications of these results for other lipolytic enzymes and for analyzing phospholipase A<sub>2</sub> activity toward other types of interfaces. The studies described here were conducted with a homogeneous preparation of phospholipase A<sub>2</sub> derived from cobra venom (*Naja naja naja*) which is described in the accompanying paper (6).

**EXPERIMENTAL PROCEDURE**

Venom from the cobra *Naja naja naja* (Pakistan), Lot no. NNPO4L, was obtained from the Miami Serpentarium in the form of a lyophilized powder, and phospholipase A<sub>2</sub> was purified to homogeneity (6). Dipalmitoyl phosphatidylcholine<sup>1</sup> (Calbiochem), Lot 300319, gave

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<sup>1</sup>The abbreviations used are: dipalmitoyl phosphatidylcholine, 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine; Triton, Triton X-100.
a single spot on thin layer chromatography and had an $[\alpha_2] = +6.4^\circ$ at 260 nm in CHCl₃. Triton X-100 (Rohm and Haas) was assumed to have a molecular weight of 624 and was used as obtained. All other chemicals were of reagent grade. Enzymatic activity was followed by the pH-stat technique (7). The assay mixture contained 10 mM CaCl₂, the indicated quantities of dipalmitoyl phosphatidylcholine and Triton X-100, and 0.1 or 0.2 µg of protein in a total volume of 2.00 ml. Assays were conducted at 40°C and pH 8.0. Plots of KOH added against time were recorded by the pH-stat apparatus, and the slopes of these plots were used to calculate the rate. One unit of activity is the amount of enzyme required to hydrolyze 1 µmol of phospholipid/min. Protein was determined by the method of Lowry et al. (8).

**Theoretical Considerations**—While the precise structure of mixed micelles is not known, a working model for the average structure of the surface of mixed micelles (9) is shown in Fig. 1. Using this model and the assumption that phospholipase $A$, associates with the lipid-water interface, a general scheme for the action of phospholipase $A$ toward phospholipids at the surface of mixed micelles is proposed as shown in Fig. 2. This scheme attempts to indicate diagrammatically the simplest possible mechanism consistent with available data.

The proposed scheme involves association of the enzyme with the mixed micelle. At the extreme, this could occur in two ways. First, association could occur primarily via interactions of the enzyme with the hydrophobic core of the mixed micelle, perhaps best considered as a hydrophobic portion penetrating into the micelle interior (1). Alternatively, association may involve primarily interactions with the hydrophilic surface. As drawn, these two extreme possibilities are represented; the actual association could include both hydrophobic and hydrophilic interactions. In order to interpret this scheme, the concentrations of $A$ and $B$ are defined more precisely. ($A$) is the molar concentration of micellar sites capable of associating with an enzyme molecule as defined in Equation 1.

$$(A) = \frac{xP + yT}{n} \tag{1.1}$$

$x =$ Average surface area of phospholipid per molecule (cm² mole⁻¹)

$y =$ Average surface area of Triton per molecule (cm² mole⁻¹)

$P =$ Concentration of phospholipid (M)

$T =$ Concentration of Triton (M)

$n =$ Micellar surface area per binding site for the enzyme (cm² mole⁻¹)

($B$) is the concentration of phospholipid in the two-dimensional surface of the micelle expressed in moles cm⁻² of surface as defined in Equation 2.

$$x_{B} = \frac{y_{B} + y_{T}}{P} \tag{1.2}$$

$x_{B} =$ Concentration of phospholipid in the two-dimensional surface of the micelle expressed in moles cm⁻² of surface

$y_{B} =$ Average surface area of phospholipid per molecule (cm² mole⁻¹)

$y_{T} =$ Average surface area of Triton per molecule (cm² mole⁻¹)

$P =$ Concentration of phospholipid (M)

$T =$ Concentration of Triton (M)

$K_{1}$ and $K_{2}$ are defined in Equations 3 and 4, respectively.

$$K_{1} = \frac{x_{B} y_{A} y_{B}}{x_{B} y_{A} y_{B}} \tag{2.1}$$

$$K_{2} = \frac{x_{B} y_{A} y_{B}}{x_{B} y_{A} y_{B}} \tag{2.2}$$

The kinetic scheme for this scheme is derived under "Appendix" and is shown in Equation 4.

$$v = \frac{V(A)(B)}{K_{m}^{2} + (A)(B) + (B)^{2}} \tag{4.}$$

It should be noted that this equation is formally the same as that considered by Cleland (10) for a soluble, bisubstrate enzyme displaying an equilibrium ordered initial velocity pattern (pp. 9–10 of Ref. 10). In our case, the micelle is kinetically analogous to a cofactor such as a metal ion which is required to bind before the substrate and is not altered by the reaction.

It is useful to consider the expression for $v$ as a function of ($A$) when ($B$) is held constant; this expression is given in Equation 5, and the reciprocal form is given in Equation 6.

$$v = \frac{V(B)}{K_{m}^{2} + (B) + (B)^{2}} \tag{5.}$$

$$\frac{1}{v} = \frac{1}{K_{m}^{2} + (B) + (B)^{2}} \tag{6.}$$

Equation 6 shows that if initial rate studies are conducted in which the concentration of phospholipid in the surface, ($B$), is held constant while the total concentration of micellar binding sites, ($A$), is varied, then plots of $1/v$ versus $1/(A)$ at constant ($B$) should give linear plots. These lines should intersect at a point with the coordinates $-1/K_{m}^{2}$. $1/V$, $1/(B)$, and $1/(B)^{2}$.

**Figure 1** Schematic diagram of the average structure of mixed micelles of phosphatidylcholine and Triton X-100 at a stoichiometry of 2:1 Triton X-100/phospholipid.
HYDROPHOBIC BINDING:

\[ \begin{align*}
E & \rightleftharpoons A & \text{EA} \\
\text{EA} + B & \rightleftharpoons \text{EAB} & \text{EA} + Q
\end{align*} \]

HYDROPHILIC BINDING:

\[ \begin{align*}
E & \rightleftharpoons A & \text{EA} \\
\text{EA} + D & \rightleftharpoons \text{EAD} & \text{EA} + Q
\end{align*} \]

FIG. 2. Schematic diagram of the action of phospholipase A\textsubscript{2}, E, toward phospholipids, B, contained in mixed micelles, A, with surfactants such as Triton X-100 using the symbols for phosphatidylcholine and Triton X-100 employed in Fig. 1. Phospholipase A\textsubscript{2} would produce products, Q, lysophosphatidylcholine and fatty acid. The products may be released into solution or they may be retained in the mixed micelles, where under initial rate conditions, they would be diluted in the mixed micelles. For phospholipase A\textsubscript{2}, Ca\textsuperscript{2+} which is linear, and the intercept of this line on the 1/v intercept axis should be 1/V, and on the 1/(B) axis it should be \(-1/K_m\). A plot of the slope of each line in the original graph against 1/(B) should be linear and pass through the origin; the slope of the resulting line should be \(K_m/K_s V\) so that the value of \(K_s\) can be determined from this replot and the values of \(K_m^0\) and \(V\).

If data are considered at a fixed concentration of micellar binding sites, (A), while the concentration of phospholipid in the surface, (B), is varied, then Equation 7 would apply, and the reciprocal form is shown in Equation 8.

\[
v = \frac{V(B)}{k_m^0 \left( \frac{k_s}{(A)} + 1 \right) + (B)}
\]

\[
\frac{1}{v} = \frac{k_m^0}{V} \left( \frac{k_s}{(A)} + 1 \right) \frac{1}{V} + \frac{1}{V}
\]

Plots of 1/v versus 1/(B) at constant (A) would give linear plots that intersect on the 1/v axis at 1/V. Replots would allow the determination of \(K_m^0\) and \(K_s\). Specifically, a plot of the slope of each line versus 1/(A) should also be linear and should intersect the slope axis at \(K_m^0/V\) and it should intersect the 1/(A) axis at \(-1/K_s\).

Practical Considerations—In order to apply the above considerations to the action of phospholipase A\textsubscript{2} toward mixed micelles of phosphatidylcholine and Triton X-100, it is necessary to vary both (A) and (B) in a regular manner. This could be accomplished by varying P and T if the values of x, y, and n at all concentrations of P and T were known. Unfortunately, x, y, and n are not known accurately at any concentration of P and T, and they would be difficult to determine precisely. However, by applying the simplifying assumptions given below, it is possible to deal with the kinetic data.

1. The size and aggregation number of mixed micelles are constant as (A) is varied. Thus, x and y are also constant as (A) is varied at constant (B). This assumption arises from the fact that the aggregation number of pure micelles of nonionic surfactants does stay roughly constant over a large concentration range above the critical micelle concentration (11), although no data are available on mixed micelles.

2. Only 1 enzyme molecule can bind to a finite segment of a micelle and n, which defines the size of that segment, is constant under all experimental conditions.

3. The average surface area per molecule of phosphatidylcholine and Triton X-100 in mixed micelles is the same, that is x = y. There are no experimental data available for these values in mixed micelles, but the limited data available for Triton and phosphatidylcholine in separate structures are consistent with this assumption. Interfacial tension measurements of Triton X-100 in isooctane-water interfaces suggest that the average surface area is about 85 A\textsuperscript{2} (12). Monolayer studies with both natural and synthetic phosphatidylcholines suggest that phospholipase A\textsubscript{2} acts maximally when the surface pressure is such that the average surface area of the phospholipid molecules is about 80 to 90 A\textsuperscript{2} (13, 14). Although the average surface area of Triton X-100 and phosphatidylcholine molecules in mixed micelles is undoubtedly different, it is not likely that they differ greatly.

4. For simplicity, the average surface area per molecule of...
both phospholipid and Triton X-100 is assumed to remain constant as their molar ratio is varied. Thus, \( x \) and \( y \) are constant as \( (B) \) is varied. This assumption implies that to a first approximation, the size of the mixed micelles does not vary with changes in composition of Triton X-100 and phospholipid. Because we have found that the size and polydispersity of mixed micelles do vary with molar ratio of Triton X-100/phospholipid (15), this simplifying assumption is not completely valid and is taken into account below.

These assumptions allow Equations 1 and 2 to be simplified as shown in Equations 9 and 10, respectively.

\[
(A) = \frac{x}{n}(P + T) = \frac{x}{n}A' \quad (9.1)
\]

\[
B' = \frac{P + T}{B' + 1} \quad (10.1)
\]

\[A' \text{ is simply the sum of the molar concentrations of phospholipid and Triton X-100, and } B' \text{ is simply the mole fraction of phospholipid in the mixed micelle. Since } x \text{ and } n \text{ are constants, Equations 6 and 8 can be rewritten in terms of } A' \text{ and } B' \text{ as shown in Equations 11 and 12, respectively.}
\]

\[
\frac{1}{v} = \frac{1}{v} - \frac{\left(\frac{nK_s^A}{K_B^m}\right)A' + \left(1 + \frac{xK_s^B}{yB'}\right)1}{yB'} \quad (11.1)
\]

\[
\frac{1}{v} = \frac{1}{v} - \frac{\left(\frac{nK_s^A}{K_B^m}\right)A' + \left(1 + \frac{xK_s^B}{yB'}\right)1}{yB'} \quad (12.1)
\]

The form of the resulting equations is unchanged and experiments can be conducted by changing \( A' \) and \( B' \) in a regular manner by varying the molar concentrations of phospholipid, \( P \), and Triton X-100, \( T \). If all of the assumptions are correct, this would result in plots similar to those described for Equations 6 and 8, although values for \( x \) and \( n \) would be required to solve for \( K_s^A \) and \( K_s^B \).

These results suggest that kinetic experiments should be conducted holding either \( A' \) or \( B' \) constant. For practical purposes, it is easiest to prepare a stock mixture of Triton X-100 and phospholipid at a constant mole fraction, \( B' \), and various amounts of that mixture can be used to vary \( A' \) in each assay. This method was used to obtain the data reported here. The normal way to conduct kinetic experiments, if Triton X-100 were thought to be an activator and/or inhibitor of the enzyme, would be to hold the Triton X-100 concentration constant and vary the phospholipid concentration. However, results obtained in this manner would be difficult to interpret in terms of the kinetic equation presented above because both \( A' \) and \( B' \) would be varied simultaneously.

**RESULTS AND DISCUSSION**

**Kinetic Data**—Kinetic studies on phospholipase A\(_2\) action toward dipalmitoyl phosphatidylcholine in mixed micelles with Triton X-100 under standard assay conditions. Plot of \( 1/v \) versus \( 1/A' \) at several values of \( B' \). Data were obtained.

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Activity of phospholipase A\(_2\) toward dipalmitoyl phosphatidylcholine in mixed micelles with Triton X-100 under standard assay conditions. Plot of \( 1/v \) versus \( 1/A' \) at several values of \( B' \): ○, 0.333; ■, 0.200; △, 0.143; ●, 0.111; □, 0.091; ▲, 0.077.

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Replots of \( 1/v \) intercepts (○) and slopes (●) obtained from Fig. 3 versus \( 1/B' \).
a minimum value for it by assuming that the enzyme is a perfect sphere and that 2 enzyme molecules associated with a micelle cannot be closer than their diameters. This would imply that the micellar binding site cannot be less than the projection of the enzyme's cross-sectional area onto the micellar surface. This cross-sectional area can be calculated from the partial specific volume and the molecular weight of the enzyme as discussed elsewhere (6). This gives $n = 3.6 \times 10^{18}$ cm$^2$ mol$^{-1}$ of binding sites. Note that this corresponds to about 7 molecules of Triton X-100 and/or phospholipid. Using these values of $x$ and $n$, $K_{A}^{*}$ is about $7 \times 10^{-18}$ M and $K_{B}^{*}$ is about $1.8 \times 10^{-18}$ mol cm$^{-2}$.

Validity of Assumptions—When the data were considered as though $B'$ was varied at constant values of $A'$, the results shown in Fig. 5 were obtained. It was not possible to draw straight lines through the data because the experimental points clearly curve upwards. We discussed above the fact that assumption 4 was not valid since the average size of mixed micelles (and thus presumably the area per molecule) does vary with the mole fraction of phospholipid in the mixed micelle. We have found that mixed micelles at a mole fraction of about 0.09 are about the same size as pure Triton X-100 micelles (15). The precise structure of Triton X-100 micelles is not known but Kushner and Hubbard (16) have suggested that they are comprised of about 150 monomers and are spherical. Since it appears unlikely that this number of hydrophobic groups could pack in a purely spherical manner, the micelles are probably at least somewhat ellipsoidal, but it is clear that the much larger structures formed at larger mole fractions must be on the average quite ellipsoidal. Similar changes in shape of mixed micelles of Triton X-100 and sphingomyelin have been suggested on the basis of experimental measurements (17). Furthermore, we have found on gel chromatography that two main populations of micelles occur at lower molar ratios (termed mixed micelles and quasi-mixed micelles) (15). It may be that the proportion of larger micelles is merely decreasing as the molar ratio is increased, and the enzyme may have different activities toward each type of micelle. If this is the case, however, it would express itself in a change in the average structure of the mixed micelle with molar ratio. Thus, if the average shape of the mixed micelles changes from a spherical or somewhat ellipsoidal to a progressively more ellipsoidal shape as the mole fraction increases, then the surface area per molecule would decrease due to the decreasing average curvature of each molecule.

Because the precise quantitative effect on $x$ and $y$ is not known, assumption 4 was employed in the evaluation of the results. Since it appears that the lack of linearity in the plots shown in Fig. 5 is most likely due to the crudeness of assumption 4, the following considerations were applied to the data. If it is assumed that $x$ and $y$ decrease by a factor of 2 as the mole fraction, $B'$, goes from 0.077 to 0.33, reasonable straight lines intersecting on the l/B axis could be drawn as shown in Fig. 6a. Conversely, if it is assumed that $x$ and $y$ increase by the same amount as $B'$ changes, the results shown in Fig. 6b are obtained. It is clear that the curvature is much more pronounced in Fig. 6b than in Fig. 5, and that the values used in Fig. 6a give rise to a more linear plot and are probably more correct. This is the same as assuming that the average surface area per molecule at a mole fraction of 0.077 is about 85 A$^2$, and it decreases proportionally to 42.5 A$^2$ at a mole fraction of 0.33. This is a reasonable range for the surface area of these molecules. It should be noted that the values of $x$ used in obtaining Fig. 6a resulted from simply varying $y$ by a factor of 2 in a regular manner; no attempt was made to determine precise values of $x$ that would give the best fit to straight lines.

A linear replot of the slopes in Fig. 6a is obtained as shown in Fig. 7. Values of $V$ (from the intersecting velocity pattern in Fig. 6a) and $K_{A}^{*}$ and $K_{B}^{*}$ (from Fig. 7) are generally similar to those obtained from replots of Fig. 3. They are $V = 4 \times 10^8$ mol min$^{-1}$ mg of protein$^{-1}$, $K_{A}^{*} = 1.1 \times 10^{-18}$ mol cm$^{-3}$, and $K_{B}^{*} = 5 \times 10^{-17}$ M. The data in Fig. 3 were replotted using the same assumption for the values of $x$ and $y$ used in Fig. 6a as shown in Fig. 8, and the replots are shown in Fig. 9. The value of $V$, $K_{A}^{*}$, and $K_{B}^{*}$ differ by less than a factor of 2 from those obtained in Fig. 4 and agree closely with those obtained from Figs. 6a and 7. Since the values obtained after invoking the correction to assumption 4 are probably more correct, we will consider these latter data as the kinetic constants, although similar values can be obtained by using assumption 4 directly.

Assumption 3 stated that $x = y$. The linearity of the plot in Fig. 3 is also rather insensitive to this assumption as shown in the plots in Fig. 10, where it was assumed that the average surface area of Triton X-100 is either twice or one-half that of the phospholipid. Lines were drawn as in Fig. 3. Replots are not obviously less linear than the original plots, and the kinetic parameters do not vary greatly. Also, plots of $1/v$ versus $1/(B)$ at constant $A$ do not significantly alter the apparent curvature in Fig. 5. Thus, even if assumption 3 is not completely valid, it does not affect our results significantly.

In summary, the data presented here are consistent with the kinetic scheme presented in Fig. 2 and Equation 4 considering all of the simplifying assumptions required; however, the

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig5.png}
\caption{Plot of the kinetic data in Fig. 3 in the form of $1/v$ versus $1/B'$ at several values of $A'$: ○, 40 mM; □, 20 mM; Δ, 10 mM; ●, 5 mM; ▼, 4 mM; ▲, 2.5 mM.}
\end{figure}

$^4$It should be noted that $K_{A}^{*}$ and $K_{B}^{*}$ in Equation 4 are constants, but in the mixed micelle system, it is possible that variations of $x$ and $y$ with $B'$ cause changes in the physical state of the interface and consequently affects these constants and this is responsible for the curvature in the plots. When $B'$ is less than 0.09, $x$ and $y$ should be constant (15) and this possibility would then be a moot point. Experimental limitations prevented conducting these experiments at very low $B'$. However, experiments with phosphatidylserine decarboxylase were conducted at low $B'$, and the application of Equation 4 did not require an assumption about the variations of $x$ and $y$ with $B'$ (18).
FIG. 6. Plot of the kinetic data in Fig. 5 as $1/v$ versus $1/(B)$ at several values of (A), with the assumption that $x$ varies with $B'$ as a test of assumption 4 in the text. (A) and (B) were calculated for the data points from the value of $n$ estimated in the text and the appropriate value of $x$ based on the assumption that the average surface area per molecule at various values of $B'$ is as shown: $\circ$, when $B' = 0.077$, the average surface area is 85 Å$^2$; $\circ$, $B' = 0.091$, (0.9) 85 Å$^2$; $\circ$, $B' = 0.11$, (0.8) 85 Å$^2$; $\circ$, $B' = 0.14$, (0.7) 85 Å$^2$; $\circ$, $B' = 0.20$, (0.6) 85 Å$^2$; $\circ$, $B' = 0.33$, (0.5) 85 Å$^2$, $b$, when $B' = 0.077$, the average surface area is (0.5) 85 Å$^2$; $\circ$, $B' = 0.091$, (0.6) 85 Å$^2$; $\circ$, $B' = 0.11$, (0.7) 85 Å$^2$; $\circ$, $B' = 0.14$, (0.8) 85 Å$^2$; $\circ$, $B' = 0.20$, (0.9) 85 Å$^2$; $\circ$, $B' = 0.33$, (1.0) 85 Å$^2$. In both $a$ and $b$, (A) has the values: $\circ$, 1.4 mM; $\square$, 0.71 mM; $\bigcirc$, 0.43 mM; $\bullet$, 0.36 mM; $\bigtriangleup$, 0.29 mM; $\bigtriangleup$, 0.24 mM.

Kinetic results do not necessarily rule out other schemes for this system such as classical competitive inhibition by Triton X-100. It does appear that the kinetic constants derived from plots of $1/v$ versus $1/A'$ at constant $B'$ and the appropriate replots are not greatly affected by the two most questionable assumptions (3 and 4). However, it is important to note that with the assumptions required and the method of drawing lines employed, the values of $V$ and $K_s^A$ are clearly only meaningful within an order of magnitude. Furthermore, the results show that the $K_m^B$ is 0.5 to 1 (in mole fraction units) which is so
on a substrate localized in a large lipid aggregate, the enzyme probably first associates with the lipid-water interface. There-complex formation. In order for a water-soluble enzyme to act interfacial binding of the enzyme or the subsequent Michaelis determined. \(K_{aA}\) and \(K_{s}\) were not separated out of the apparent \(K_{a}\) and thus little can be concluded about the yields a rate equation in which only an apparent \(K_{a}\) could be containing two intermediate complexes (19). This treatment results obtained by others, the phospholipase \(A_{2}\) phospholipid by Brockerhoff and Jensen (19). In summarizing the kinetic system was treated as if it was a single substrate reaction presented for other forms of the substrate as recently reviewed based is not radically different from schemes that others have.

Conclusions and Implications for Study of Lipolytic Enzymes—We have developed a kinetic equation to analyze the activity of phospholipase \(A_{2}\) toward phospholipid-Triton X-100 mixed micelles. The scheme upon which this equation is based is not radically different from schemes that others have presented for other forms of the substrate as recently reviewed by Brockerhoff and Jensen (19). In summarizing the kinetic results obtained by others, the phospholipase \(A_{2}\) phospholipid system was treated as if it was a single substrate reaction containing two intermediate complexes (19). This treatment yields a rate equation in which only an apparent \(K_{a}\) could be determined. \(K_{aA}\) and \(K_{s}\) were not separated out of the apparent \(K_{s}\) and thus little can be concluded about the interfacial binding of the enzyme or the subsequent Michaelis complex formation. In order for a water soluble enzyme to act on a substrate localized in a large lipid aggregate, the enzyme probably first associates with the lipid-water interface. There-

The rate equation now has the same form as a simple monosubstrate Michaelis-Menten reaction. Pancreatic phospholipase \(A_{2}\) does demonstrate such kinetics when acting on these micelles. This illustrates the problem mentioned above, in that \(K_{aA}\) and \(K_{s}\) cannot be separated without additional independent data. With the introduction of Triton X-100 into the micelles, \((B)\) becomes less than \(1/x\) and is no longer constant. This dilution of the phospholipid in the interface has been described as a "surface dilution phenomenon" (5). This in turn forces the use of the complete kinetic equation, Equation 4, and allows the determination of \(K_{s}\) and \(K_{s}\). Verger et al. (1) have also suggested a scheme similar to the one presented in Fig. 2 to explain the action of pancreatic phospholipase \(A_{2}\) toward monolayers and micelles of phospholipid, and their analysis provides information about the enzyme-interface interaction. However, their experiments did not lead to a determination of the kinetic constants equivalent to \(K_{aA}\) and \(K_{s}\) defined here.

We have found in preliminary experiments with phospholipase \(C^{2}\) and a solubilized form of the membrane-bound enzyme phosphatidylserine decarboxylase (18) acting on mixed micelles of Triton X-100 and phospholipid that the kinetic results are also analyzable with the scheme and equation presented here. It is also possible that triglyceride lipases act in a fashion similar to phospholipase \(A_{2}\). However, analyzing the triglyceride substrate in this manner would be difficult since as generally studied, triglyceride lipases act on lipid emulsions in which only a fraction of the triglyceride substrate is at the lipid-water interface where it is exposed to the enzyme. Thus, the dependence of total surface and the
concentration of substrate in the surface on lipid concentration would be more difficult to quantitate than in the case of phospholipids. Yet, the scheme we have presented and the results obtained from applying it to the phospholipase A_2 mixed micelle system does aid in the investigation of other phospholipases and may with suitable manipulation of the substrate aid in studies on triglyceride lipase.

Acknowledgment—We wish to thank Mr. Thomas G. Warner for critical and helpful discussions of this work.

APPENDIX

\[
\begin{align*}
E + A & \xrightarrow{k_1} EA \\
EA + B & \xrightarrow{k_2} EAB \xrightarrow{k_3} EA + Q
\end{align*}
\]

Steady-state assumption, initial rate conditions:

\[
\frac{d(EA)}{dt} = \frac{d(EAB)}{dt} = 0
\]

Conservation of mass:

\[
E_T = (E) + (EA) + (EAB)
\]

Solving for (EAB) using Cramers Rule:

\[
(EAB) = \frac{K_1(A)(B)}{K_1/k_2 + K_2/k_3 + K_3/k_4}
\]

Since \( v = k_3(EAB) \) and \( V = k_3(E) \):

\[
V = \frac{V(A)(B)}{k_4(E) + k_3(EA) + (A)(B)}
\]

where \( \frac{k_i}{k_j} \) and \( \frac{k_m}{k_n} \)

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