Kinetic Study of the Hydrolysis of Lecithin Monolayers by *Crotalus adamanteus* α-Phospholipase A_2. Monomer–Dimer Equilibrium[1]

Betty W. Shen, Francis H. C. Tsao, John H. Law,* and Ferenc J. Kézdy

Contribution from the Department of Biochemistry, University of Chicago, Chicago, Illinois 60637. *Received July 17, 1974*

Abstract: The *Crotalus adamanteus* α-phospholipase A_2 catalyzed hydrolysis of 1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholine monolayers at the air–water interface requires the dimeric form of the enzyme. The dissociation constant of the dimer (K_d), its rate constant of dissociation (k_1), and the rate constant of the association of the monomer (k_2) were measured by determining the enzyme concentration dependency and the change with time of the catalytic rate. At pH 6.0 and 7×10^{-3} *M* Ca^{2+}, we found $K_d = 2.0 \times 10^{-9}$ *M*, $k_1 = 2.9 \times 10^{-3}$ sec^{-1}, and $k_2 = 1.5 \times 10^6$ M^{-1} sec^{-1}. The enzymatic reaction also requires a reversibly bound Ca^{2+} ion, with $K_{Ca^{2+}} = 1.4 \times 10^{-2}$ *M* at pH 6.0 and $K_{Ca^{2+}} = 7.4 \times 10^{-5}$ *M* at pH 8.0. The reaction is first order both with respect to the surface concentration of the substrate and the concentration of the enzyme dimer in the solution, with $k_E = 3.1 \times 10^7$ M^{-1} sec^{-1} at pH 6.0, $I = 0.1$ *M*, and saturating Ca^{2+} concentration. The value of k_E is independent of the monolayer surface pressure when measured under conditions of constant area and variable surface pressure in the 3–8 dyn/cm range, or under conditions of variable area and constant pressure at 3, 5, and 7 dyn/cm.

Interest in the physical and chemical properties of phospholipid monolayers lies in the fact that such monolayers provide the experimentally most accessible model for phospholipid bilayers, one of the essential constituents of biological membranes. Kinetic studies of the action of pancreatic lipase were proved to be sensitive probes for the structure and reactivity of triglyceride molecules in monolayers[2] and these techniques have also been applied to the study of the interaction of pancreatic phospholipase A_2 with short-chain lecithin monolayers.[3] To determine whether the kinetic behavior observed in the latter reaction is representative of the interaction of all phospholipases with lecithin monolayers, or whether at least some of the observed features are the result of the particular structure and mechanism of the pancreatic phospholipase, a comparison has to be made between the kinetic characteristics of several enzymes of similar specificities.

Various other phospholipases have been shown to react with phospholipid monolayers, but no detailed kinetic studies have yet been carried out with any of these enzymes.[4] For our comparative kinetic studies we chose α-phospholipase A_2 from the venom of the Eastern diamondback rattlesnake, *Crotalus adamanteus*. This enzyme is readily obtained in pure form and in large quantity from lyophilized venom by the procedure of Wells and Hanahan.[5] It is remarkably stable and hydrolyzes phospholipids in all physical forms, including monomolecular solutions, micelles, emulsions, and monolayers. Unlike most other phospholipases, the *C. adamanteus* enzyme exists in its native form as a dimer and the molecular weight of the subunit is comparable to that of other, monomeric phospholipases.[6] Disruption of the quarternary structure of the enzyme by extremes of pH or urea is accompanied by loss of enzymatic activity, thereby suggesting that perhaps the enzyme is active only in the dimeric form.[6] This unique requirement for a dimeric structure could then be an indication of profound structural and possibly mechanistic differences between the *C. adamanteus* enzyme and other, monomeric phospholipases.

In the present paper we will describe the determination of the kinetic parameters of the *C. adamanteus* phospholipase catalyzed hydrolysis of dioctanoyl lecithin monolayers at the air–water interface. We will show by kinetic methods that the active, dimeric form of the enzyme is in a true equilibrium with enzymatically inactive subunits and that the Ca^{2+} requirement of the surface reaction is the same as the one observed for the interaction of the enzyme with soluble substrates.[7] Finally, the kinetic behavior of the enzyme will be compared to that of pancreatic phospholipase A_2.

Experimental Section

Materials and Methods. α-Phospholipase A_2 from *C. adamanteus* was prepared according to the method of Wells and Hanahan,[5] starting with the lyophilized crude venom purchased from Miami Serpentarium Laboratories. The pure enzyme had the same electrophoretic pattern and the same specific activity toward an ether solution of egg lecithin as described.[5] The enzyme was stored as a lyophilized powder. Stock solutions of the enzyme (10^{-6} *M*, in 0.01 *M* Tris–acetate buffers, pH 6–8) were found to be stable, when refrigerated, over periods of several weeks.

Dioctanoyl-(*R*)-3-phosphatidylcholine (1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholine) was obtained from Supelco, Inc., and its purity was found to be at least 99% by tlc, using "solvent I" of the method of Skidmore and Entenman.[8] The optical purity of the compound was indicated by total hydrolysis by the *C. adamanteus* enzyme. Stock solutions of the substrate were prepared in dry benzene immediately before use.

Water was deionized by a double bed ion exchanger and distilled in an all-glass apparatus. Buffer solutions were freed from trace surfactant impurities by foaming for 5 min immediately before use.

Inorganic compounds were of analytical reagent grade and all solvents were redistilled before use.

The force–area curve of dioctanoyllecithin monolayers was measured by a du Noüy ring and a recording Cahn electrobalance after spreading of successive aliquots of a benzene solution of the substrate in a constant area trough. The same measurements were also carried out by compressing a preformed monolayer of substrate in a Langmuir-type trough. The force–area curves obtained by both methods were identical with the one described by Zografi, *et al.*[3]

The kinetics of the enzymatic hydrolysis of dioctanoyllecithin monolayers were measured at 20 ± 0.5° under conditions of variable surface pressure and constant area.[2] The reaction rates under conditions of constant surface pressure and variable area were determined by using a recording Lauda surface balance (Brinkmann Instruments).

For the determination of the dissociation constant of the enzyme dimer, the enzyme stock solution (dimer concentration, 10^{-6} *M*) was diluted to the final concentration in the appropriate buffer and placed in the Teflon trough of the surface balance. After a period of 20 min, the surface was cleaned and a benzene solution of the substrate was spread from a microsyringe. The time-dependent change of surface pressure was monitored after the evaporation of the solvent, usually not more than 30 sec.

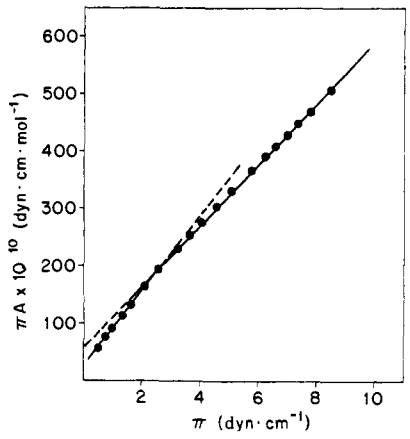

Figure 1. πA *vs.* π plot for dioctanoyl-(*R*)-3-phosphatidylcholine monolayer: 20 ± 1°; subphase, 0.01 *M* Tris-acetate, pH 6.02, $[CaCl_2]$ = 7 m*M*, [NaCl] = 0.1 *M*; initial surface = 600 cm², 1.5 × 10^{16} molecules of phospholipid.

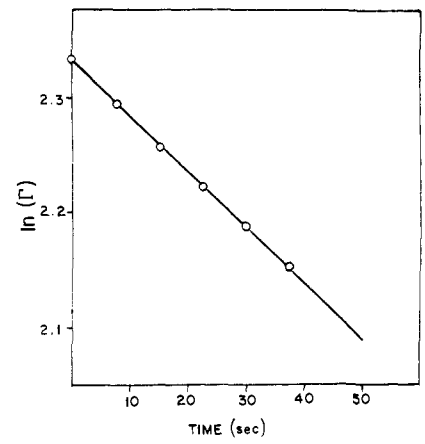

Figure 2. First-order plot for the hydrolysis of dioctanoyllecithin monolayer by α-phospholipase A_2: 20 ± 1°, pH 6.02; $[E_0]$ = 4.92 × 10^{-10} *M*; k_{exp} = 4.82 × 10^{-3} sec^{-1}.

For the measurement of the rate of dissociation of the enzyme dimer, the experimental procedure was the same, except that the substrate solution was spread at various time intervals after the dilution of the enzyme stock solution. In this way, the hydrolytic rate could be determined as early as 100 sec after dilution of the enzyme. Most of our experiments were performed at pH 6.0, an apparent pH optimum for the enzyme-catalyzed hydrolysis of the substrate monolayer. At this pH, secondary phenomena, such as enzyme denaturation and absorption of carbon dioxide in the surface layer, are negligible.

Results

The experimental force–area data for dioctanoyllecithin monolayers were analyzed by plotting πA *vs.* π according to the equation

$$\pi A = \pi A_0 + \text{cst}$$

where π is the surface pressure in dynes per centimeter, A is the molecular area in Å²/molecule, and A_0 is the limiting molecular area. In the region of π = 2.5–8 dyn/cm a linear relationship is observed between πA and π (Figure 1) indicating that in this pressure domain a single surface phase is present, characterized by A_0 = 86.6 Å²/molecule. All kinetic experiments in this paper were carried out with the substrate in this surface phase.

When a lecithin solution in benzene is spread out on a buffered enzyme solution (Ca^{2+} = 7 × 10^{-3} *M*) which has been equilibrated for 20 min at 20° in the Teflon trough of the surface balance, a rapid, time-dependent decrease in the surface pressure is observed, indicating enzymatic hydrolysis of the substrate followed by rapid dissolution of the reaction products. Under conditions of variable pressure and constant area, the enzymatic hydrolysis is first order with respect to the surface concentration of the substrate (Γ), since ln Γ *vs.* *t* plots yield a straight line. From the slope of the line an apparent first-order rate constant can be calculated (Figure 2). Identical initial reaction velocities were observed under conditions of constant pressure and variable area, at pressures of 3, 5, and 7 dyn/cm. Thus the rate constant of the *C. adamanteus* phospholipase A_2 catalyzed hydrolysis of the surface phase of dioctanoyllecithin existing between 3 and 8 dyn/cm is independent of the surface pressure.

The enzyme concentration dependency of the hydrolytic reaction was measured at 4 dyn/cm (Ca^{2+} = 7 × 10^{-3} *M*), by determining the rate of the reaction (V) in the presence of varying concentrations of the enzyme [E], after a 20-min preincubation period. The rate of the enzymatic reaction is not proportional to the enzyme concentration in the range

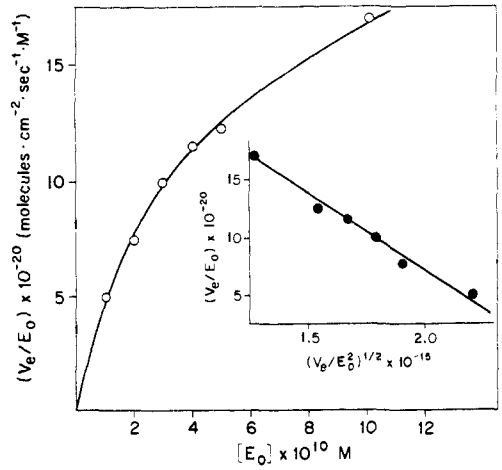

Figure 3. Rate of hydrolysis of a dioctanoyllecithin monolayer as a function of $[E_0]$: 20 ± 1°; 0.01 *M* Tris-acetate, pH 6.02; $[CaCl_2]$ = 7 m*M*.

of 1–10 × 10^{-10} *M*: at low enzyme concentrations the value of $V/[E]$ tends toward zero, whereas at higher enzyme concentrations $V/[E]$ tends to become independent of [E], although it does not reach a plateau even a the highest experimentally measurable rate (Figure 3). This type of behavior is compatible with a reversible dissociation of the active, dimeric enzyme into inactive subunits, provided that the dissociation constant of the dimer is of the same order of magnitude as the molarity of the enzyme. The data were analyzed according to this hypothesis. The reversible dissociation of the dimer (D) into monomers (M) is represented by the following equation

$$[D] \overset{K_d}{\rightleftharpoons} 2[M] \qquad (A)$$

with $K_d = (M)^2/(D)$ and

$$V = k_E(D)(\Gamma) = k_{exp}(D) \qquad (1)$$

If E_0 = (D) + (M)/2 then the following equation can be derived

$$V/E_0 = k_{exp} - (K_d k_{exp}/4)^{1/2}(V/E_0^2)^{1/2} \qquad (2)$$

Thus, reaction A and eq 2 predict that a plot of V/E_0 *vs.* $(V/E_0^2)^{1/2}$ should yield a straight line with a negative slope and a positive intercept on the ordinate axis. The insert in Figure 3 shows that indeed the experimental points conform to eq 2. From the intercept and slope we calculate K_d = 2.0 × 10^{-9} *M*.

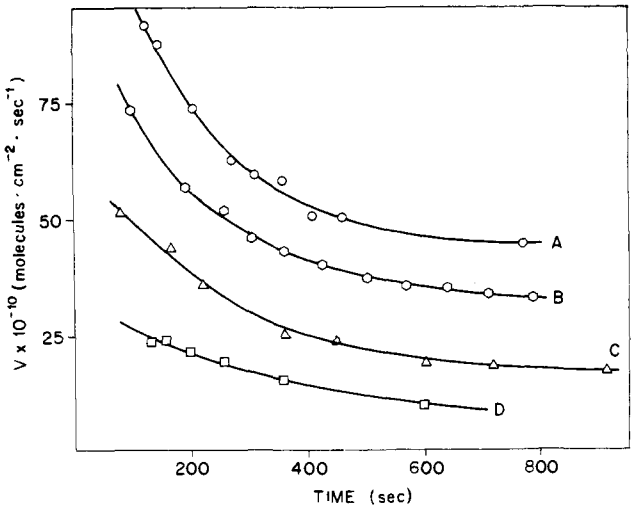

Figure 4. Rate of hydrolysis of a dioctanoyllecithin monolayer at $\pi = 4$ dyn/cm by phospholipase A_2 as a function of the time after dilution of the enzyme: pH 6.02, $[CaCl_2] = 7$ mM; A, $[E_0] = 4.08 \times 10^{-10}$ M; B, $[E_0] = 3.06 \times 10^{-10}$ M; C, $[E_0] = 2.04 \times 10^{-10}$ M; D, $[E_0] = 1.02 \times 10^{-10}$ M.

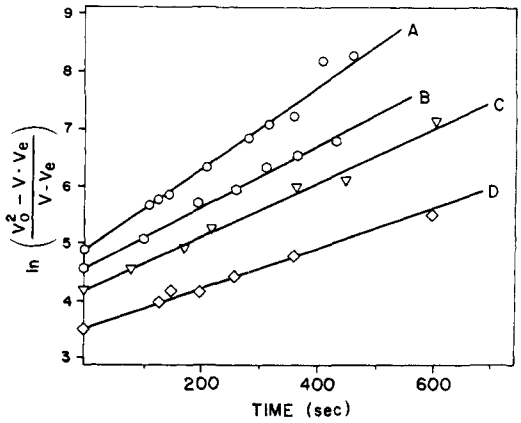

Figure 5. Time dependency of the dissociation of α-phospholipase A_2 plotted according to eq 3: pH 6.02, $[CaCl_2] = 7$ mM; line A, $[E_0] = 4.08 \times 10^{-10}$ M; line B, $[E_0] = 3.06 \times 10^{-10}$ M; line C, $[E_0] = 2.04 \times 10^{-10}$ M; line D, $[E_0] = 1.02 \times 10^{-10}$ M.

If the dissociation of the dimer is an equilibrium process, then the rate of approach to equilibrium should be slow enough to be observed at low enzyme concentrations, even if the association of the monomers is diffusion controlled. Thus, an initial, time-dependent loss of enzyme activity should occur when the reaction is initiated shortly after dilution of a concentrated enzyme stock solution. Figure 4 shows that this is in fact the case. We also found, that the value of V was the same, whether the enzyme solution was covered with substrate or not during the incubation period. The data of this initial period after dilution were analyzed assuming first-order dissociation (k_1), and second-order reassociation (k_2). The kinetic equations pertaining to such a system have been solved[9] and if D_0, D_e, and D represent the concentration of the dimer at $t = 0$, at equilibrium and at time $= t$, respectively, then the integration of the rate equation gives

$$\ln\frac{D_0 - \frac{D_e}{D_0}D}{D - D_e} = \frac{D_0 + D_e}{D_0 - D_e}k_1 t = \ln\frac{D_0^2 - D_e D}{D - D_e} - \ln D_0 \qquad (3)$$

with the initial conditions $M_0 = 0$ and $(D)_{t=0} = D_0$.

Since, according to eq 1, V is proportional to D at constant Γ, the rates measured at $\pi = 4$ dyn/cm were plotted as $\ln[(V_0^2 - V_e V)/(V - V_e)]$ *vs.* t, the time elapsed since the dilution of the initially dimeric enzyme. As shown in Figure 5, such plots yielded straight lines for several different initial enzyme concentrations, thereby supporting the validity of eq 3 and the assumptions behind it. From the slope of these lines a value for k_1 can be calculated, if V_0 and V_e are known. V_e is readily determined experimentally and for V_0 the value used was obtained by extrapolation from the insert in Figure 3. The value obtained this way for the rate constant of the dissociation of the dimer, $k_1 = 2.9$ (± 0.2) $\times 10^{-3}$ sec^{-1}, was found to be independent of the initial dimer concentration over a fourfold enzyme concentration range. The value of k_2 was calculated from k_1 and K_d, and we found $k_2 = 1.5 \times 10^6$ M^{-1} sec^{-1}, a reasonable order of magnitude for the diffusion controlled association of two protein molecules[10] and comparable to values found in other protein-protein associations.[11]

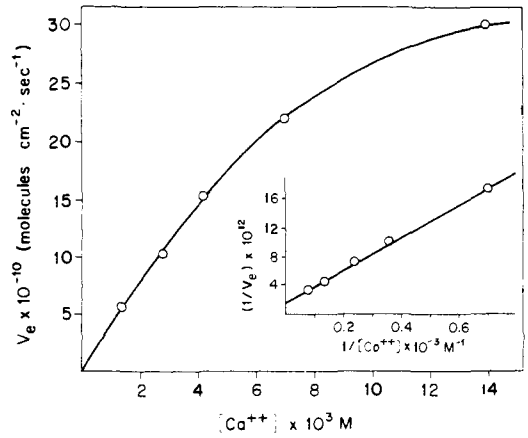

Figure 6. $[Ca^{2+}]$ dependence of the α-phospholipase A_2 catalyzed lecithin monolayer hydrolysis: 0.01 M Tris-acetate, pH 6.02, [NaCl] = 0.1 M, $[E_0] = 4.94 \times 10^{10}$ M.

All preceding experiments were performed at Ca^{2+} concentrations of 7×10^{-3} M, since Ca^{2+} was reported to be required for enzymatic activity.[5] The dependency of the surface reaction on the Ca^{2+} concentration was studied at pH 6.0 and ionic strength = 0.1 M, by measuring again the rate of the reaction (V) at $\pi = 4$ dyn/cm as a function of the Ca^{2+} concentration, after 20 min of preincubation of the enzyme solution. Figure 6 shows that under these conditions increase of the Ca^{2+} concentration produces a typical saturation curve. Indeed, a plot of $1/V$ *vs.* $1/(Ca^{2+})$ is linear, yielding a positive slope and a positive intercept on the ordinate axis (see insert, Figure 6). From these, a value of $K_{Ca^{+2}} = 1.4 \times 10^{-2}$ M was calculated. Table I shows the ionic strength dependency and the pH dependency of this equilibrium constant.

Finally, the Ca^{2+} dependency of K_d, the dissociation constant of the dimer, is summarized in Table II.

Discussion

The linearity of the πA *vs.* π plot between 3 and 8 dyn/cm (Figure 1) clearly establishes the existence in this pressure range of a single surface phase of the dioctanoyllecithin monolayer. Within the domain of existence of this phase, the rate of the enzyme-catalyzed reaction is truly independent of the surface pressure, regardless of the method of measurement, whether under constant surface area or under constant surface pressure. This contrasts sharply with the results reported for pancreatic phospholipase A_2.[3] The apparent pressure dependency observed in the latter case is thus certainly not a general characteristic of all interfacial

Table I. Binding of Ca^{2+} to α-Phospholipase A_2[a]

pH	Ionic strength, M	$K_{Ca^{2+}} \times 10^3$, M
6.0	0.01	5.0
6.0	0.1	14
8.0	0.1	0.074

[a] 20°, 0.01 M Tris–acetate buffer, π = 4.0 dyn/cm.

Table II. Dimerization Equilibrium of α-Phospholipase A_2[a]

$[Ca^{2+}] \times 10^3$, M	Buffer ionic strength, M	$K_d \times 10^9$, M
0.7	0.01	2.4
3.5	0.01	1.4
7	0.01	2.0
7	0.1	0.37

[a] 20°, 0.01 M Tris–acetate buffer, pH 6.02, π = 4.0 dyn/cm.

phospholipase reactions and the cause for it has then to be sought in terms of the particular structural features of the pancreatic enzyme.

In agreement with the indirect evidence presented by Wells,[6] we found that the dimeric form of the enzyme is the sole active species. The dissociation of the dimer is a simple thermodynamic equilibrium with $K_d = 2.0 \times 10^{-9}$ M at pH 6.0. This represents a free energy of association of 11.6 kcal/mol, thus reflecting an unusually strong and specific interaction between the subunits. The results in Table II show that the dissociation is independent of Ca^{2+} in the ragne of 10^{-3}–10^{-2} M; *i.e.*, the dimerization site is independent from the site involved in the interaction of the enzyme with the loosely bound metal ion.

The requirement for a dimeric form for enzymatic activity is rather surprising, since phospholipases A_2 from a variety of other sources were shown to exist in the monomeric state and are apparently active as such.[6] For alkaline phosphatase from *Escherichia coli* and a number of other enzymes, only the dimeric form is active, since the reaction mechanism involves the cooperative interaction of two active sites.[12] It is possible that the existence of monomeric and dimeric phospholipases reflects a mechanistic diversity of these enzyme-catalyzed reactions.

The binding constant of Ca^{2+} to the enzyme was determined previously by using dibutyryllecithin, a water-soluble substrate.[7] At pH 8.0 the reported value for $K_{Ca^{2+}}$ is 4.0×10^{-5} M with the latter substrate, although the ionic strength was not strictly controlled in these experiments. Nonetheless, at pH 8 the value of $K_{Ca^{2+}}$ is in excellent agreement with the one reported here for the monolayer reaction. This identity of the Ca^{2+} binding further supports the great mechanistic similarity of the homogeneous and the heterogeneous reaction. At pH 6 the binding of Ca^{2+} is considerably weaker than at pH 8, presumably because of the accumulation of positive charges on the enzyme as the pH is lowered. The same reasoning can be applied to the ionic strength dependency of K_d (Table II).

The fact that the hydrolytic reaction is strictly first order with respect to the enzyme dimer shows that the dissolution of the reaction products cannot be rate limiting even at the highest enzyme concentrations used, a conclusion reached independently by direct measurement of dissolution rates.[13] We cannot assess the exact enzyme concentration near the interface, which is undoubtedly different from that in the bulk phase. However, the proportionality of the rate to the enzyme concentration and the calcium ion concentration in the bulk phase points to the existence of a rapid equilibrium between molecules close to the surface with those in the subphase. This is also supported by the fact that stirring of the subphase does not change the velocity of the monolayer reaction at all stages during the establishment of the monomer–dimer equilibrium. None of our experimental results suggest the occurrence of enzyme penetration into the lipid phase of the insoluble monolayer. In particular the pressure independency of the rate of the enzymatic reaction and the strict first-order dependency on both substrate and enzyme dimer strongly argue against penetration.

These experiments demonstrate the utility of substrate monolayer techniques in probing mechanistic details of enzyme reactions. Because of the possibilities for manipulating and monitoring parameters such as surface concentration and orientation of substrate molecules, one can ensure that all reacting molecules are equivalent (*i.e.*, in the same phase). Furthermore, because contact between subphase components with monolayer molecules can take place freely, the system offers a conceptually simple and experimentally very accessible method for probing lipid–protein ineractions.

References and Notes

(1) The research presented in this paper was supported by Grants GM 13863 and GM 13885 from the National Institute of General Medical Sciences and Grant 1 P17 HL 15062 from the National Heart and Lung Institute, U.S. Public Health Service.
(2) J. W. Lagocki, N. D. Boyd, H. H. Law, and F. J. Kézdy, *J. Amer. Chem. Soc.*, **92**, 2923 (1970); J. W. Lagocki, J. H. Law, and F. J. Kézdy, *J. Biol. Chem.*, **248**, 580 (1973).
(3) G. Zografi, R. Verger, and G. H. de Haas, *Chem. Phys. Lipids*, **7**, 185 (1971); R. Verger, M. C. E. Mieras, and G. H. de Haas, *J. Biol. Chem.*, **248**, 4023 (1973).
(4) For a review, see B. A. Pethica in "Structural and Functional Aspects of Lipoproteins in Living Systems," E. Tria and A. Scanu, Ed., Academic press, New York, N.Y., 1969, Chapter A2, p 65.
(5) M. A. Wells and D. J. Hanahan, *Biochemistry*, **8**, 414 (1969).
(6) M. A. Wells, *Biochemistry*, **10**, 4074 (1971).
(7) M. A. Wells, *Biochemistry*, **11**, 1030 (1972).
(8) W. D. Skidmore and C. Entenman, *J. Lipid Res.*, **3**, 471 (1962).
(9) S. W. Benson, "The Foundations of Chemical Kinetics," McGraw Hill, New York, N.Y., 1960, p 30.
(10) M. Eigen and G. G. Hammes, *Advan. Enzymol.*, **25**, 1 (1963).
(11) J.-P. Vincent and M. Lazdunski, *Biochemistry*, **11**, 2967 (1972).
(12) M. Lazdunski, C. Petitclerc, D. Chappelet, and C. Lazdunski, *Eur. J. Biochem.*, **20**, 124 (1971).
(13) W. R. Snyder, Ph.D. Thesis, University of Chicago, 1972.