The Chemical Step Is Not Rate-Limiting during the Hydrolysis by Phospholipase A2 of Mixed Micelles of Phospholipid and Detergent†

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ABSTRACT: The effect of detergents on the overall catalytic turnover by secreted phospholipase A2 (PLA2) on codispersions of the substrate phospholipid is characterized. The overall rate of interfacial catalytic turnover depends on the effective substrate "concentration" (mole fraction) that the bound enzyme "sees" at the interface. Therefore, besides the intrinsic catalytic turnover rate determined by the Michaelis–Menten cycle in the interface [Berg et al. (1991) *Biochemistry 30*, 7283], two other interfacial processes significantly alter the overall effective rate of hydrolysis: first, the fraction of the total enzyme at the interface; second, the rate of replenishment of the substrate. At low mole fractions (<0.3), bile salts promote the binding of pig pancreatic PLA2 to zwitterionic vesicles, and the rate of hydrolysis increases with the fraction of the enzyme in the interface. At higher (>0.3) mole fractions of the detergent, the bilayer is disrupted, and the rate of hydrolysis decreases by more than a factor of 10. The detergent-dependent decrease in the rate of hydrolysis of the *sn*-2-oxyphospholipids is much larger than that of *sn*-2-thiophospholipid, and therefore the element effect (O/S ratio) decreases from about 10 in bilayers to less than 2 in mixed micelles. This loss of the element effect in mixed micelles shows that the chemical step is no longer rate-limiting during the hydrolysis of mixed micelles formed by the disruption of vesicles by the detergent. Such effects were observed with phospholipase A2 from several sources acting on substrates dispersed in a variety of detergents including bile salts, 2-deoxylysophosphatidylcholine, and Triton X-100. The rationale for these effects is elaborated on the basis of the fact that the intrinsic rate of hydrolysis at the interface is fast (about 300 s^{-1} at the maximum mole fraction of the substrate, =1); therefore, a significant fraction of the substrate present in the enzyme-containing micelle (typically less than 50 phospholipid molecules per micelle) is hydrolyzed within a fraction of a second. Since the rate of intermicellar exchange of the enzyme and monomeric phospholipids is slow, the availability of the substrate to the enzyme on a micelle would be limited by the rate of replenishment of the substrate by collisional fusion–fission with excess micelles. In effect, during the hydrolysis of small particles such as mixed micelles, the steady-state condition is not satisfied microscopically on the particle to which the enzyme is bound; i.e., the overall effective rate of hydrolysis becomes limited by the local effective mole fraction of the substrate at the interface.

Kinetics of interfacial catalysis by phospholipase A2 (PLA2)[1] are described by an adaptation of Michaelis–Menten formalism (Jain & Berg, 1989; Berg et al., 1991; Jain et al., 1991a). By a combination of equilibrium and kinetic methods, it was possible to determine all the intrinsic rate and equilibrium parameters that characterize the kinetics of hydrolysis of DMPM vesicles in the highly processive scooting mode, i.e., under the conditions where the intervesicle exchange of the enzyme, substrate, and products is negligible. The intrinsic turnover rate of the enzyme in the scooting mode is about 300 s^{-1} at the maximum possible concentration of the substrate (=1 mol fraction) in the bilayer (Berg et al., 1991). Therefore, on a small vesicle with about 5000 phospholipids and 1 enzyme, the substrate concentration would change by more than 20% in less than 5 s (Jain et al., 1991c). Thus, on a microscopic level, the steady-state condition of constant substrate concentration is satisfied for less than 5 s (Berg et al., 1991), unless of course the rate of replenishment of the substrate is promoted by fusion of vesicles (Jain et al., 1991c). Such considerations illustrate a fundamental feature of interfacial catalysis; i.e., due to the local substrate depletion on the enzyme-containing aggregate, the overall catalytic turnover can become *locally* substrate-limited even if the bulk (*global*) substrate concentration in the reaction mixture remains high. Since the bound enzyme does not sample the bulk environment, on a microscopic level the local environment of the enzyme on a small aggregate changes much more rapidly than the bulk environment. Such a departure from the microscopic steady state has far-reaching kinetic consequences because it can give rise to intriguing kinetic anomalies of interfacial catalysis, which are often ascribed to the "quality of the interface" or the structure and dynamics of the aggregate (Verger & de Haas, 1977; Dennis, 1983; Jain & Berg, 1989; Jain et al., 1992).

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[1] Abbreviations: CHAPSO, 3-[(cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; cmc, critical micelle concentration; deoxy-LPC, 1-hexadecyl-2-propyl-3-phosphocholine; dithio-DMPC, 1,2-dimyristoyl-1,2-dithio-*sn*-glycero-3-phosphocholine; DOC, deoxycholate; imc, intermicellar concentration; MJ33, 1-hexadecyl-3-(trifluoroethyl)-*sn*-glycero-2-phosphomethanol (only the racemate was used for these studies); PC, *sn*-glycero-3-phosphocholine; PE, *sn*-glycero-3-phosphoethanolamine; PG, *sn*-glycero-3-phosphoglycerol; PLA2, phospholipase A2 from pig pancreas unless stated otherwise; PM, *sn*-glycero-3-phosphomethanol.

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In order to understand the kinetic consequences of the departure from the microscopic steady state, we have investigated the kinetics of hydrolysis of codispersions of phospholipids and detergents. Phospholipids "solubilized" in detergents have been used as substrates for lipolytic enzymes (Verheij et al., 1982; Dennis, 1983; Waite, 1987). Under these conditions, aggregates containing typically less than 100 phospholipid molecules are formed. This raises the possibility of significant departure from microscopic steady state if the substrate replenishment on the aggregates is not rapid during the residence time of the enzyme on the aggregate (Berg et al., 1991). Direct measurements suggest that the residence time of PLA2 on vesicles or micelles is of the order of seconds or longer (Jain et al., 1988), that the half-time for intermicellar exchange of monomeric long-chain phospholipids is of the order of 40 h (Nichols, 1988), and that the half-times for the intermicellar transfer of phospholipids by fusion–fission of micelles is of the order of several seconds (Nichols, 1988; Fullington et al., 1990). As expected on the basis of these results, in this paper we show that the "initial" rate of hydrolysis of mixed micelles by PLA2 is limited by the rate of replenishment of the substrate, and a true steady-state initial rate corresponding to the bulk substrate concentration is not observed under the conditions usually employed for the kinetic measurements on mixed micelles.

MATERIALS AND METHODS

MJ33 and deoxy-LPC (Jain et al., 1991d), DMPM and POPM (Jain & Gelb, 1991), and dithio-DMPC (Hendrickson & Hendrickson, 1990) were synthesized as described. Other phospholipids used in this study were from Avanti. Bile salts and CHAPSO were from Sigma, and Triton X-100 was from Boehringer. PLA2 from pig pancreas and venoms were isolated as described (Jain et al., 1991b).

Stock dispersions of substrate phospholipids (typically 20 mg/mL) in water were prepared by sonication in a bath-type sonicator. The reaction progress was monitored by pH-stat titration (Upreti & Jain, 1978, 1980; Jain et al., 1986a; Berg et al., 1991). The reaction mixture contained 4 mL of vigorously stirred (at 2000 rpm without frothing) solution at pH 8.0 and 23 °C containing 1 mM NaCl and 1 mM (for anionic phospholipids) or 6 mM $CaCl_2$ (for zwitterionic phospholipids). The bulk substrate concentration was kept 1 mM (unless stated otherwise). Specific conditions and controls necessary for monitoring the hydrolysis of thioester substrates are described elsewhere (Jain et al., 1992b). The *element effect* is the O/S ratio which is defined as the ratio of the rate of hydrolysis of (dioxy)phospholipid to dithiophospholipid. Bile salt or other detergents, if present in the reaction mixture, were added as concentrated (>30 mM at pH 8.0) stock solution to the phospholipid dispersions in the reaction mixture. The reaction was typically initiated with 0.02–2 μg of PLA2 added from a stock solution of 0.05–0.1 mg/mL in water. In most cases, the order of addition of the bile salt or the enzyme did not change the initial rate of hydrolysis. The latency period or other anomalous features of the reaction progress curves observed in the absence of a bile salt (Upreti & Jain, 1980; Apitz-Castro et al., 1982) were not observed in the presence of bile salts. Essentially 100% of the substrate was accessible for the hydrolysis at >0.2 bulk mol fraction of bile salts, whereas only the outer monolayer of vesicles was hydrolyzed at the lower mole fractions of the bile salt. The steady-state initial rates of hydrolysis reported here have an uncertainty of less than 10%, and the values are expressed as catalytic turnover numbers per second (per enzyme molecule). Specific activity in units of micromole per minute per milligram of PLA2 is obtained by multiplying the turnover number by 4.1. The cmc and imc values used in this study are from Fullington et al. (1990).

The binding of PLA2 to phospholipid dispersions was monitored as the fluorescence change in the emission from Trp-3 at 333 nm with excitation at 290 nm with slitwidths of 4 nm each (Jain et al., 1982, 1986b). Other conditions are given in the figure legend or the text.

RESULTS

Long-chain diacylglycerophospholipids form bilayer vesicles in aqueous dispersions (Jain, 1988), whereas codispersions of a phospholipid and bile salt exhibit complex polymorphism depending on the relative and absolute concentrations of the components, tonicity, and pH (Lichtenberg, 1985). Thus, depending on the specific conditions, particles of yet unknown morphology and composition are formed (Carey & Small, 1981; Ollivon et al., 1988). Transitions of these organizational polymorphs exhibit considerable hysteresis, especially if concentrated codispersions of phospholipid and detergents are diluted (Lichtenberg et al., 1979, 1983; Mazer et al., 1980; Nagata et al., 1990). In order to minimize the effect of such time-dependent transitions on the kinetics, the experiments described in this paper were carried out by adding concentrated detergent solution to vigorously stirred dispersions of phospholipid. Results described below show that the isothermal polymorphic transitions that depend on the mole fraction of the detergent in the mixture, such as bilayer → disks or rods → micelles, underlie the complexity of the observed kinetics of hydrolysis of phospholipid–bile salt codispersions by PLA2.

Hydrolysis of DMPC Dispersions in the Presence of Bile Salts. The progress curves for the hydrolysis of DMPC vesicles exhibit anomalous behavior, which is appreciably simplified in the presence of anionic amphiphiles in the bilayer (Upreti & Jain, 1980; Apitz-Castro et al., 1982; Jain et al., 1982, 1989; Ghomashchi et al., 1991). Similar behavior was observed with bile salts; the latency period disappears, and the apparent rate of hydrolysis can be obtained from the initial steepest part which is typically linear for several minutes. As shown in Figure 1A, the initial rate of hydrolysis of 1 mM DMPC increased more than 10-fold in the presence of <0.2 mM deoxycholate, whereas above 0.5 mM DOC the rate of hydrolysis dropped sharply. Such a biphasic effect on the initial rate of hydrolysis was also observed in the presence of other bile salts listed in Table I. With all the anionic bile salts, the biphasic activation profiles were essentially similar to that shown in Figure 1A; however, the optimum rate of hydrolysis and the concentration of the bile salt for the maximum initial rate depended on the structure of the bile salt, as well as the structure and concentration of the phospholipid. The values of the maximum initial rate of hydrolysis at the optimum bile salt concentration and also at a concentration between the imc and cmc values obtained from such plots are summarized in Table I. These results show that the biphasic dependence of the initial rate of hydrolysis on the bile salt concentration is a general property of such anionic amphiphiles and the optimum activation is seen at concentrations well below the cmc of the bile salt. Differences between the effect of these amphiphiles arise largely from their partitioning, mixing, and phase behavior with phospholipids.

For the characterization of the effect of bile salts on the kinetics of hydrolysis of phospholipid dispersions by PLA2, we focus mainly on the effects of DOC. In some key

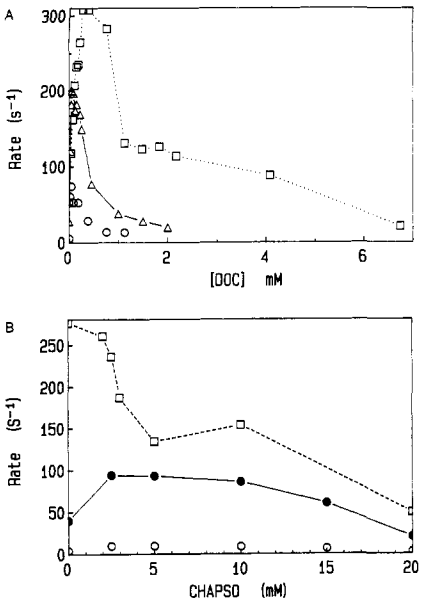

FIGURE 1: (A) Effect of [DOC] on the initial rate of hydrolysis of DMPC vesicles (10 mM, squares; 1 mM, triangles; 0.3 mM, circles) by PLA2 at pH 8.0 in 1 mM NaCl and 6 mM $CaCl_2$. (B) Effect of [CHAPSO] on the initial rate of hydrolysis of (open circles) DMPC (1 mM) or (squares) DMPM (1 mM) by PLA2 under the conditions given above; (closed circles) data points for DMPC shown on the 10× expanded scale (shown for clarity).

Table I: Initial Rate of Hydrolysis of DMPC (1 mM) in the Presence of Bile Salts

bile salt	imc/cmc[a]	initial rate (s^{-1}) at optimum (mM)	initial rate (s^{-1}) at cmc (mM)
deoxycholate	1.6/3.0	200 (0.15)	10 (2.0)
taurodeoxycholate	1.0/2.4	150 (0.1)	7 (3.2)
glycodeoxycholate	1.0/2.0	107 (0.125)	10 (1.6)
cholate	5.9/11.0	128 (0.3)	10 (10)
taurocholate	4.8/6.0	87 (0.2)	6 (3.1)
glycocholate	5.0/10.0	222 (0.3)	2 (7)
chapso	–/5.0	12 (0.25)	8 (2.5)

[a] All cmc/imc values, except for CHAPSO, are from Fullington et al. (1990).

experiments, zwitterionic (deoxy-LPC or CHAPSO) and nonionic (Triton X-100) detergents were also used. As shown in Figure 1B, the rate of hydrolysis of DMPC vesicles did not increase in the presence of CHAPSO; however, at higher concentrations, the rate of hydrolysis was lower.

The biphasic effect of bile salts on the rate of hydrolysis of the aqueous dispersions of zwitterionic phospholipids has been observed with virtually all secreted PLA2s (Uthe & Magee, 1971; Upreti & Jain, 1978; Nalbone et al., 1980; Hoffman et al., 1983; Gheriani-Gruszka et al., 1988). Similar behavior is observed with nonionic detergents such as Triton X-100 (Dennis, 1973). On the basis of evidence given below, the biphasic effect of anionic bile salts on the initial rate of hydrolysis of DMPC dispersions is interpreted as a result of two competing processes: (a) The initial rate of hydrolysis of zwitterionic DMPC vesicles is slow because the affinity of pig pancreatic PLA2 in the aqueous phase for zwitterionic bilayers is poor. Anionic amphiphiles such as bile salts partition into the substrate vesicles and promote binding of the enzyme to the zwitterionic bilayer. (b) It can be ruled out that the bilayer is disrupted at low mole fractions (<0.15) of the bile salt (Walde et al., 1987a,b); at higher bulk concentrations of bile salts, the disruption of the bilayer is accompanied by a marked decrease in the rate of hydrolysis.

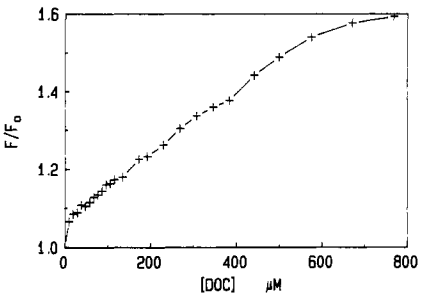

FIGURE 2: Relative fluorescence intensity of a mixture of PLA2 and DTPC (1 mM) in 40 mM Tris·HCl and 1 mM $CaCl_2$ at pH 8.0 and 23 °C as a function of DOC concentration. The excitation wavelength was 285 nm, and the emission wavelength was 333 nm with slitwidths of 4 nm.

Table II: Rate of Hydrolysis of Aqueous Dispersions of Phospholipid (1 mM) in the Presence of DOC at 23 °C

phospholipid	rate (s^{-1}) at [DOC] none	optimum (mM)	above imc (mM)
dipalmitoyl-PC	4	15 (0.25)	3.5 (1.7)
dimyristoyl-PC	8	200 (0.15)	10 (1.9)
dilauroyl-PC	12	19 (0.125)	4.4 (2.2)
dioctanoyl-PC	65	132 (0.25)	19 (1.9)
diheptanoyl-PC	10	10 (0.125)	1.3 (1.0)
dioleoyl-PC	4	42 (2.2)	11.5 (5.2)
1-palmitoyl-2-oleoyl-PC	4	28 (2.2)	1.4 (3.5)
1-oleoyl-2-palmitoyl-PC	6	32 (0.7)	7 (2.2)
1-palmitoyl-2-arachidonoyl-PC	3	11 (1.3)	0.5 (3.9)
dimyristoyl-PE	33	4 (0.37)[a]	1.9 (1)
dioleoyl-PE	73	10 (0.3)[a]	3.6 (1)
1-palmitoyl-2-oleoyl-PE	42	49 (0.3)	2.6 (1.7)
1-palmitoyl-2-oleoyl-PG	35	72 (0.54)	24 (2.4)
1-palmitoyl-2-oleoyl-PM	61	180 (1.7)	104 (6.4)
DMPM	270	240 (0.2)	33 (2.4)

[a] The phospholipid exhibits a monophasic decrease with increasing [DOC].

DOC Promotes Binding of PLA2 to Zwitterionic Bilayers. An increased rate of hydrolysis of DMPC vesicles at low concentrations of bile salts is due to an enhanced binding of the enzyme to zwitterionic bilayers as shown by direct binding studies. Such measurements are based on the fact that the binding of PLA2 to the lipid–water interface is accompanied by an increase in the fluorescence emission intensity of Trp-3 on PLA2 (Van Dam-Mieras, 1975; Jain et al., 1982) which also results in resonance energy transfer to a dansyl group localized at the interface (Jain & Vaz, 1987). Such changes are not observed on the binding of a ligand to the active site of PLA2 in the aqueous phase (Ramirez & Jain, 1991; Rogers et al., 1992). By these criteria, PLA2 does not bind to DTPC vesicles (Jain et al., 1982, 1986b); however, as shown in Figure 2, the binding of PLA2 to DTPC vesicles is enhanced in the presence of DOC. Except for a small increase above the critical micelle concentration of DOC, such a fluorescence change is not observed with DOC alone (results not shown). These observations show that the enhanced rates of hydrolysis in the presence of bile salt as summarized in Figure 1 and Table I (and also Table II and discussed below) are primarily due to enhanced binding of the enzyme to zwitterionic bilayers in the presence of anionic bile salts.

Effect of Changing the Bulk DMPC Concentration. The effect of the concentration of DOC required for the optimum activation at three concentrations of DMPC is shown in Figure 1. The overall shape of the activation profile did not change significantly between 0.3 and 10 mM DMPC; only the maximum rate and the concentration of DOC required for

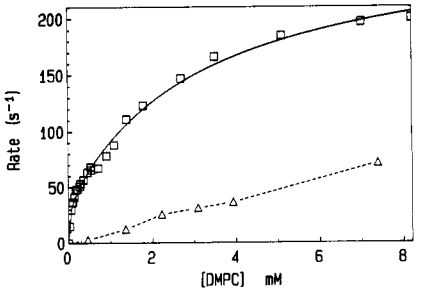

FIGURE 3: Dependence of the rate of hydrolysis of DMPC vesicles by PLA2 in the presence of 0.15 mM (squares) and 2 mM (triangles) DOC. Other conditions were as given in the legend to Figure 1.

the maximum rate increased with the bulk DMPC concentration. The effect of increasing the bulk DMPC concentration on the initial rate of hydrolysis at 0.15 and 2 mM DOC is shown in Figure 3. At 0.15 mM DOC, where the bilayer organization is retained, the maximum rate of hydrolysis was observed at lower DMPC concentrations than at 2 mM DOC, where the bilayer organization is disrupted. A comparison with the imc and cmc values in Table I suggests that the sharp drop in the initial rate of hydrolysis begins at about the imc and is substantially complete at the cmc. It may be recalled that the cmc is the concentration of the detergent above which the micelles of the detergent begin to form in the monomeric solution of the detergent, whereas the imc is the concentration at which the mixed aggregates of the detergent and phospholipids begin to separate as aggregates from the solitary monomeric detergent molecules and the bilayer of phospholipids. Taken together, results so far suggest that a sharp drop in the observed rate at higher bile salt concentration corresponds to an effectively lower rate of catalytic turnover on mixed micelles of DMPC formed at higher concentrations of DOC.

Effect of Phospholipid Structure. The effect of DOC on the initial rate of hydrolysis depends on the structure of the phospholipid. Results obtained from plots of the type shown in Figure 1 are summarized in Table II. As was the case with DMPC, the initial rate of hydrolysis of phosphatidylcholines with saturated acyl chains increased 5–15-fold in the presence of <0.3 mM DOC. Although the biphasic effect of bile salts on the rate was observed with all phosphatidylcholines, the shape of the profile changed with the structure of the phospholipid. Phosphatidylethanolamines (Figure 4A) and phosphatidylcholines (Figure 4B) showed very different dependence on DOC concentration. The complexity of the overall process is further illustrated by the fact that the dependence for the hydrolysis of isomeric phosphatidylcholines (Figure 4B) was also different. As compared in Table II, in the absence of DOC, the initial rates of hydrolysis of phospholipids with PE, PG, and PM head groups were significantly higher than for PCs. This is consistent with the conclusion that the binding is already saturated on anionic bilayers; therefore, the effect of low concentrations of DOC on the rate of hydrolysis of anionic phospholipids is small, if any. On the other hand, in all the cases that we have investigated, irrespective of the structure of the phospholipid or the detergent, above the cmc of the detergent the initial rate of hydrolysis decreases to less than 10%. As elaborated later in this paper, this effect is due to a change in the rate-limiting step. On the basis of the controls summarized below, several other possible explanations for the effect of bile salts were eliminated as these experiments showed that bile salts do not have any direct effect on PLA2 at the interface or in

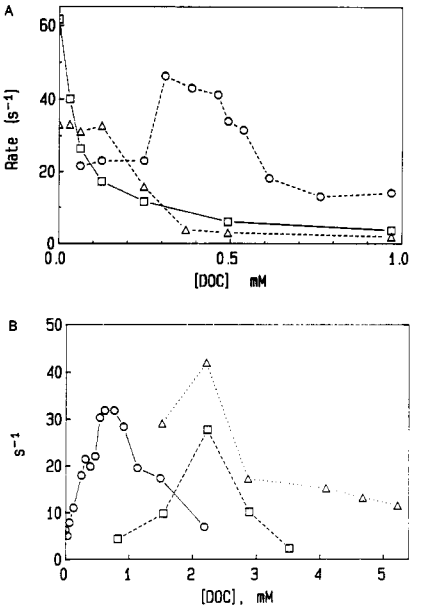

FIGURE 4: (A) Rate of hydrolysis of PEs (dioleoyl-, squares; dimyristoyl-, triangles; 1-palmitoyl-2-oleoyl-, circles) by PLA2 as a function of DOC concentration. (B) Rate of hydrolysis of PCs (1-oleoyl-2-palmitoyl-, circles; 1-palmitoyl-2-oleoyl-, squares; 1,2-dioleoyl-, triangles) by PLA2 as a function of DOC concentration. Other conditions were as given in the legend to Figure 1.

the aqueous phase, nor do they directly modulate the intrinsic catalytic activity of PLA2.

(a) The titration efficiency for fatty acids was essentially the same at all the bile salt concentrations, and the corrections were made in the reported results for small (<15%) differences that were observed.

(b) Bile salts did not inactivate or denature the enzyme as shown by a lack of any noticeable change in the progress curves obtained with PLA2 preincubated with up to 100 mM detergent.

(c) On the basis of the results of the type shown in Figure 2, it was concluded that higher concentrations of DOC did not promote desorption of PLA2 from the DTPC interface.

(d) Deoxy-LPC, DOC, and other bile salts, under monomeric as well as micellar conditions, do not protect the active site of PLA2 from alkylation (Jain et al., 1991a; de Haas et al., 1990); i.e., they do not have affinity for the active site of the enzyme in the aqueous phase or the interface.

(e) The rate of hydrolysis catalyzed by several different PLA2 and lipolytic enzymes (results not shown here) showed a biphasic effect with the detergent concentration (cf. Figure 1). This suggests that the decrease in the rate is most probably due to a nonspecific effect, such as a physical change in the state of phospholipid–bile salt codispersions. Indeed, physical studies (Carey & Small, 1970; Helenius & Simons, 1975; Ollivon et al., 1988) show that the size of the phospholipid dispersions undergoes a major decrease as the bile salt/detergent concentration approaches or exceeds the imc.

(f) Partitioning of the bile salt decreases the mole fraction of the substrate that the bound enzyme "sees" at the interface. This possibility is inconsistent with the results shown in Figure 1, where a 30-fold change in the bulk DMPC concentration had only a minor effect on the shape of the profile. Also as discussed in the next section, this effect due to dilution of the substrate in the interface could be dissected on the basis of the interfacial K_M^* (=0.35 mol fraction on DMPM vesicles) values (Berg et al., 1991; Jain et al., 1991a); up to about 1

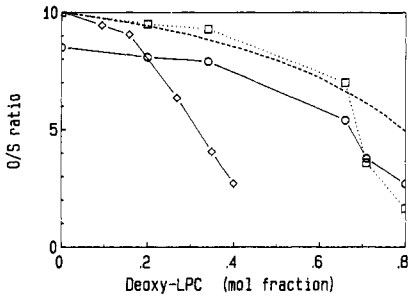

FIGURE 5: Normalized initial rate of hydrolysis of (squares) 1 mM DMPM and (circles) the O/S ratio for DMPM/dithio-DMPM as a function of the mole fraction of deoxy-LPC ($=X_{ND} = 1 - X_S$) in 1 mM $CaCl_2$, 1 mM NaCl, and 5 mg/mL polymyxin B/mL. (Diamonds) Normalized initial rate of hydrolysis of large DMPM (1 mM) vesicles in 0.6 mM $CaCl_2$ and 1 mM NaCl; the O/S ratio obtained under these conditions paralleled the rate profile (data not shown). The rates are given on an arbitrary scale; however, the maximum turnover number is 300 s^{-1}. The critical micelle concentration of deoxy-LPC is 0.01 mM, and it is assumed that it is completely partitioned into substrate dispersions. The dashed curve is the theoretical curve for the dependence of the initial rate calculated (eq 2) with $K_M^* = 0.35$ mol fraction.

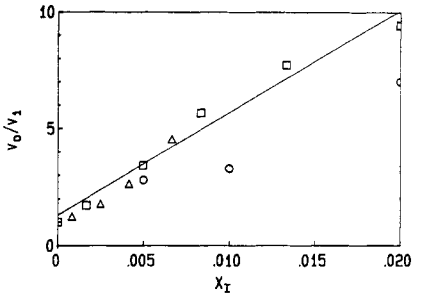

FIGURE 6: Plot of the relative rate of hydrolysis of codispersions of DMPC (1 mM) with DOC (0.065 mM, triangles; 0.45 mM, squares; 3 mM, circles) by PLA2 as a function of the mole fraction of rac-MJ33 [1-hexadecyl-3-(trifluoroethyl)-*sn*-glycero-2-phosphomethanol]. Other conditions were as in the legend to Figure 1. The mole fraction values are corrected for the surface dilution.

mM DOC, the predicted effect of "surface dilution" is considerably smaller than the overall observed effect.

Detergent as a Neutral Diluent. By definition, a neutral diluent molecule does not have an affinity for the active site of the enzyme. When incorporated into bilayers, it changes only the mole fraction of amphiphiles in the interface without changing the factors that determine the catalytic turnover (Jain et al., 1991a). The relative rate of interfacial catalysis in the absence and in the presence of an inhibitor (v_0/v_I) in the interface at mole fraction X_I is given by (Berg et al., 1991):

$$\frac{v_0}{v_I} = 1 + \left(\frac{1 + 1/K_I^*}{1 + 1/K_M^*}\right)\left(\frac{X_I}{1 - X_I}\right) \quad (1)$$

To account for the surface dilution effects of the neutral diluent, the relevant equation would be

$$\frac{v_0}{v_I} = 1 + \left(\frac{1 + X_S^0/K_I^*}{1 + X_S^0/K_M^*}\right)\left(\frac{X_I}{X_S^0 - X_I}\right) \quad (1a)$$

Here K_M^* is the interfacial Michaelis constant for the substrate, and X_S^0 is the mole fraction of substrate in the mixed micelle before the addition of an inhibitor. The effect of the presence of the neutral diluent is to reduce the value of $X_I(50)$ roughly in proportion to the dilution, so that the inhibitor would appear to become more effective when substrate is diluted.

For a neutral diluent, $K_I^* \gg 1$ mol fraction. Since the mole fraction of the substrate that the enzyme "sees" at the interface containing only two amphiphiles is $X_S = 1 - X_I$, the relative rate of hydrolysis at X_S is given by

$$v_0 = \frac{k_{cat} X_S}{X_S + K_M^*} \quad (2)$$

If a neutral diluent partitions completely into the bilayer, $X_I = X_{ND}$ would change in proportion to its bulk concentration with the bulk concentration of the substrate. As shown elsewhere, ideal and complete mixing is seen with neutral diluents like deoxy-LPC (Jain et al., 1991d) or 2-hexadecyl-*sn*-glycero-3-phosphocholine (Jain et al., 1991a), both of which have a very low cmc (about 0.01 mM) and $K_I^* > 2$.

As summarized in Figure 5 (diamonds), in the presence of deoxy-LPC, the rate of hydrolysis of DMPM vesicles decreases abruptly at $X_{ND} > 0.2$ mol fraction. Similar behavior was observed with 2-hexadecyl-*sn*-glycero-3-phosphocholine as a neutral diluent (Jain et al., 1991a). Under the assumption that the intrinsic catalytic rate and equilibrium parameters for the enzyme are the same in the micelle and the bilayer, with experimentally determined $k_{cat} = 400$ s^{-1} and $K_M^* = 0.35$ mol fraction for DMPM (Berg et al., 1991), the calculated dependence of v_0 on X_S is shown in Figure 5 (dashed line); i.e., a 50% decrease in the rate is expected at $X_{ND} > 0.7$ mol fraction, which is considerably more than was observed (diamonds in Figure 5). In other words, at $X_{ND} = 0.7$ mol fraction, the expected initial rate is 150 s^{-1} compared to the observed rate of about 10 s^{-1}.

A similar argument can also be developed for DMPC vesicles for interpretation of the results shown in Figure 1. The K_M^* for DMPC in bilayers is approximately 0.6 mol fraction (Jain et al., 1991a; Ghomashchi et al., 1991); therefore, a change in X_{ND} from 0 to 0.4 mol fraction would decrease the observed rate only by a factor of 2. Here again, the sharp decrease in the rate is observed at $X_{ND} = 0.2$ even if it is assumed that all the bile salt is incorporated into vesicles.

The possibility that the K_M^* for the mixed micelle is significantly different than that for bilayers was also discounted. As shown in Figure 5 (dotted line connecting the square data points), in the presence of polymyxin B the rate of hydrolysis of DMPM vesicles in the presence of increasing mole fractions of the neutral diluent decreases less sharply than it does in its absence (diamonds). Here the correspondence with the surface dilution effects (dashed curve) is seen over a wider range of $X_{ND} = 0$–0.65. If the sharp decrease at higher X_S is due to "local" substrate depletion on the enzyme-containing particle, the effect can be accounted for on the basis of the observation that polymyxin B promotes exchange of anionic phospholipids between vesicles (Jain et al., 1991c). Therefore, even at $X_{ND} = 0.65$, the "effective" particle size remains sufficiently large because the substrate replenishment is rapid enough to keep the effective X_S the same as under the initial conditions.

It is possible that the interface that the enzyme "sees" and on which hydrolysis occurs has a different composition than the bulk composition of the amphiphiles present in the reaction mixture. This possibility is ruled out by the following considerations. Results in Figure 6 show that the $X_I(50)$ value for MJ33, a specific competitive inhibitor of PLA2 (Jain et al., 1991d), at low concentrations of DOC (<0.5 mM) was either the same or somewhat lower than for vesicles without any DOC. According to eq 1a, the $X_I(50)$ value is related to K_M^* and K_I^* (Jain et al., 1991a,d). The K_I^* value for rac-MJ33 measured in micelles of a neutral diluent like deoxy-LPC was 0.0016, and it does not seem to change in the presence

of low mole fractions of DOC. Therefore, it may be concluded that the K_M^* value for DMPC or DMPM either remains the same or decreases in the presence of DOC.

The $X_I(50)$ values at higher DOC concentration (3 mM) were significantly higher (Figure 6). This can be ascribed to an apparently lower K_M^* in eq 1a. As quantitatively developed elsewhere (Berg et al., 1991), for interfacial catalysis under limiting substrate concentration, the K_M^* (=0.35) term in eq 1 or eq 1a is replaced by K_P^* = 0.025 mol fraction (Jain et al., 1991a). Therefore, higher $X_I(50)$ values are predicted in mixed micelles under substrate-limited rate conditions. While these results are in accord with eq 1a and its counterpart under the substrate-limited conditions, a quantitative analysis of this trend is not possible for the mixed-micelle system unless the mole fraction of the detergent in the enzyme-containing micelles can be established.

The Chemical Step Is Not Rate-Limiting for the Hydrolysis of Mixed Micelles. A 10-fold difference in the rates of hydrolysis of *sn*-2-oxy- versus thioester substrate (O/S ratio) during interfacial catalysis by PLA2 in the scooting mode suggested that the chemical step is rate-limiting for several PLA2s (Jain et al., 1992b). Under the conditions where the chemical step is not rate-limiting, the O/S ratio is expected to approach 1 because the oxygen versus sulfur element effect for the physical processes leading to catalytic turnover or those involved in catalytic turnover are not expected to be significantly different for the oxy and thio analogs of the substrates. This line of reasoning was applied to mixed micelles under three different sets of conditions.

(a) As shown in Figure 5, the O/S ratio for the hydrolysis of DMPM dispersions in the presence of polymyxin B by pig pancreatic PLA2 decreases with increasing mole fraction of deoxy-LPC, and this decrease essentially parallels the decrease in the rate of hydrolysis. In the absence of polymyxin B, a sharp decrease in the O/S ratio was observed at lower mole fractions of deoxy-LPC (results not shown) which paralleled the decrease in the rate of hydrolysis under comparable conditions (diamonds in Figure 5). These results showed that at higher X_{ND}, the chemical step is not rate-limiting. The drop in O/S ratio not only parallels the decrease in the rate of hydrolysis of the oxy substrate but the sharpest decrease also followed the breakdown of vesicles by the neutral diluents (Jain et al., 1991a). Since polymyxin B increases the rate of exchange of the anionic substrate and products (Jain et al., 1991c), the increase in the rate of hydrolysis and the O/S ratio at intermediate mole fractions of deoxy-LPC in the presence of polymyxin B suggested that a lower rate in the presence of the neutral diluent was due to the formation of smaller particles where the substrate replenishment was rate-limiting.

(b) As shown in Figure 7A, the rate of hydrolysis of DMPC dispersions by pig pancreatic PLA2 in the presence of taurodeoxycholate followed a more complex profile. However, the O/S ratio for the PC analogs also parallels the profile, and the ratio decreased from about 10 to 2.

(c) The O/S ratio for the hydrolysis of DMPC vesicles by the basic PLA2 from *Agkistrodon halys Blomhoffii* was about 25, which is the same as seen with DMPM vesicles (Jain et al., 1992b). As shown in Figure 7B, the rate of hydrolysis and the O/S ratio paralleled the changes observed with DMPC in the presence of Triton X-100.

(d) If the fusion–fission of micelles is the main (collisional) mechanism for replenishment of the substrate on the enzyme-containing micelle, it can be argued that at very high bulk substrate concentrations the effective rate of substrate replenishment in micelles may exceed the intrinsic rate of catalytic turnover. If so, the effective substrate concentration in the interface that the bound enzyme "sees" would remain constant. In some preliminary experiments, we found that at least up to 5 mM DMPC + tauro-DOC (1/9 mole ratio) the initial rate did not saturate. Such experiments suggested that at least in this concentration range it is not possible to arrive at the fast replenishment limit. It may also be recalled that the phase behavior of bile salt and phospholipid dispersions exhibits pronounced concentration dependence (Carey & Small, 1981; Ollivon et al., 1988; Lichtenberg, 1985); therefore, it cannot be assumed that only the micelle concentration, but not the composition and properties of the micelles, changes with the bulk concentration.

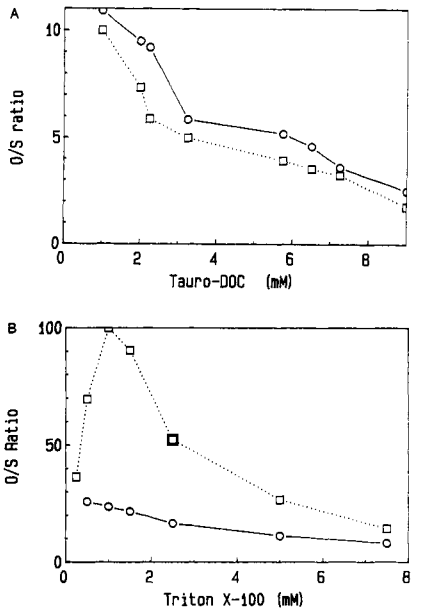

FIGURE 7: (Circles) O/S ratio and (squares) normalized initial rate of hydrolysis of 1 mM DMPC dispersions (A) by pig pancreatic PLA2 as a function of taurodeoxycholate concentration and (B) by PLA2 (basic) from the venom of *Agkistrodon halys blomhoffii* as a function of Triton X-100 concentration. The ordinate scale for the O/S ratio is shown; the ordinate for the rate is given in an arbitrary scale that can be judged from the maximum initial rate of hydrolysis (v_0) with pig pancreatic PLA2 as 180 s^{-1}, or v_0 with the *Agkistrodon* enzyme as 165 s^{-1}.

DISCUSSION

The kinetic implications of a sharp decrease in the rate of hydrolysis of bilayer vesicles at higher concentrations of amphiphilic additives relate to yet unappreciated but fundamental features of interfacial catalysis (Verger & de Haas, 1977; Dennis, 1983; Jain & Berg, 1989). On the basis of the weight of evidence summarized in the preceding section, it may be concluded that the decrease in the rate of hydrolysis of DMPC or DMPM vesicles at higher concentrations of a detergent is due to a change in the rate-limiting step from the chemical step to a physical process leading to or as a part of the catalytic turnover cycle. This change was observed with all the substrate and detergent combinations that we have tested, and it occurred at the mole fractions of detergent that disrupt bilayer organization.

The conclusion that the rate-limiting step in mixed micelles is dominated by a physical process clearly raises serious doubts about the usefulness of detergent-dispersed micelles to characterize the kinetics and related features of interfacial catalysis. This is because the steady-state condition is not satisfied at the microscopic level; that is, the "local" substrate concen-

tration that the bound enzyme "sees" is different than that expected from the averaged bulk composition of the reaction mixture.

Since the constraints and boundary conditions for the expression of the intrinsic rate parameters have been developed in detail for interfacial catalysis in the scooting mode on bilayers (Berg et al., 1991), it is now possible to gain a qualitative appreciation of the complexity of the kinetics on mixed micelles. The following discussion is an attempt to formulate some useful generalizations that could serve as guide for the design of further studies on interfacial catalysis.

Besides the factors that control the binding of the enzyme to the interface, the key variable in the interface is not only the concentration but also the number of substrate molecules in the interface. Obviously, in a system where the lipid is dispersed in relatively small aggregates, the effective turnover cannot be faster than the effective rate of bringing the substrate to the bound enzyme, even if one chooses to ignore the steady-state concentration of the products and terms related to "local" surface dilution. As a first approximation, the effective rate of replenishment of the substrate molecules becomes rate-limiting if the residence time of the enzyme on the target interface is long compared to the replenishment time. This limit can be estimated as follows. The intrinsic rate of catalytic turnover by PLA2 is of the order of 300 s^{-1} at X_S = 1 mol fraction (Berg et al., 1991; Jain et al., 1991c). Thus, 20% of the substrate molecules in a micelle would be hydrolyzed in about 30 ms. In order to maintain a steady-state rate of hydrolysis, the underlying replenishment process must be faster than the observed rate of hydrolysis, which is approximately 10 s^{-1} in DMPC codispersions containing DOC at its cmc. As a general case, this situation should be modeled in a true steady state where the substrate mole fraction in the enzyme-containing micelle is reduced to a level where the catalysis exactly matches the rate of replenishment. This is not possible yet; however, a limit-estimate of the process underlying the substrate replenishment in micelles can be obtained from the following considerations:

(a) The effective steady-state rate of hydrolysis under conditions limited by the exchange of monomer substrate is given by

$$\text{rate} = k_{exch} N_T (1 - X_S) = \frac{k_{cat} X_S}{K_M^* + X_S} \qquad (3)$$

where k_{exch} is the rate of replenishment and N_T is the total number of exchangeable molecules in the micelle. The product inhibition term is not included; however, in effect, the X_S term will adjust to the level where the rate of catalysis (on the right-hand side) exactly matches that of replenishment (on the left-hand side). The half-time for the spontaneous transfer of monomeric long-chain diacylphospholipids between micelles as monomers through the aqueous phase is several hours (Arvinte & Hildenbrand, 1984; Fullington et al., 1990). Therefore, the observed rate of about 10 s^{-1} is much too fast to be accounted for in terms of monomer exchange.

(b) The rate of replenishment of the substrate by fusion–fission of micelles is a likely mechanism for the exchange of phospholipids between mixed micelles. This is suggested by the fact that the rate of hydrolysis increases with the bulk concentration of mixed micelles; however, the underlying mechanism is not understood yet. According to the simplest scenario, the replenishment is slow but takes place in bursts as a fusion–fission event brings a fresh load of substrate to the enzyme-containing micelle. If N substrate molecules are replenished after an average time T, the effective turnover would be N/T, if T is slow on the time scale with which the enzyme can hydrolyze the substrates. For N = 50 and an observed rate of hydrolysis of 10 s^{-1}, T would be around 5 s. This is consistent with the estimate that it would not take more than a second to hydrolyze a large fraction of the 50 substrate molecules initially present in the micelle. The half-time for the fusion and fission of mixed micelles of bile salts is also of the order of seconds (Fullington et al., 1990). As a general case where the rate of hydrolyzing the whole micelle is similar to the rate of replenishment, one would have to take the proper time average of the reaction progress in each individual micelle [for a formalism, see Berg et al., (1991)].

(c) The formalism for the exchange of the enzyme between micelles would also be essentially the same as for case b. In this case, replenishment occurs in bursts as the enzyme is released and finds a fresh micelle. The residence time of PLA2 on micelles is at least of the order of several seconds if not slower (Jain et al., 1988).

These considerations limit the choice of the rate processes that control the rate of replenishment of the substrate in mixed micelles to fusion–fission. Even if the relative contribution of the replenishment processes is fully characterized, one may ask if the kinetics on micelles as such can ever provide a way to obtain the primary kinetic and equilibrium parameters. The following considerations would be necessary to adequately address the key issue of the steady-state condition at the microscopic level. Codispersions of phospholipids and bile salts exhibit pronounced polymorphism; therefore, a rigorous attempt to model the kinetics of hydrolysis of mixed micelles in terms of the primary rate and equilibrium parameters would require an understanding of the partitioning behavior of monomeric bile salts, as well as a detailed understanding of the morphology, stoichiometry, dispersity in size and composition, and dynamics of fusion and fission of the particles where hydrolysis occurs.

Finally, in order to understand the detergent concentration dependence of the kinetics, one may also have to surmount yet another level of complexity. The decrease in the rate of hydrolysis at higher detergent concentrations is due to the effect of detergents on the stability and solubility of phospholipid vesicles (Kratohvil et al., 1983; O'Connor & Wallace, 1985). The dynamics of lamellar to micellar phase transformation are influenced not only by the nature and the concentration of the detergent and phospholipid (Carey & Small, 1970; Helenius & Simons, 1975; Schurtenberger et al., 1984; Lichtenberg et al., 1979; Lichtenberg, 1985; Paternostre et al., 1987; Almog et al., 1986; Ollivon et al., 1988) but also by the presence of calcium (Almog & Lichtenberg, 1988). The overall process occurs in three stages. Disruption of the bilayer begins at about >0.2 mol fraction of the bile salt; however, formation of classical spherical micelles occurs only at high bile salt concentrations. What happens at intermediate concentrations is not clear yet.

REFERENCES

Almog, S., & Lichtenberg, D. (1988) *Biochemistry 27*, 873–880.

Almog, S., Kushnir, T., Nir, S., & Lichtenberg, D. (1986) *Biochemistry 25*, 2597–2605.

Apitz-Castro, R. J., Jain, M. K., & de Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 349–356.

Arvinte, T., & Hildenbrand, K. (1984) *Biochim. Biophys. Acta 775*, 86–94.

Berg, O. G., Yu, B.-Z., Rogers, J., & Jain, M. K. (1991) *Biochemistry 30*, 7283–7297.

Carey, M. C., & Small, D. M. (1970) *Am. J. Med. 49*, 590–608.

Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5394.
Dennis, E. A. (1973) *J. Lipid Res. 14*, 152–159.
Dennis, E. A. (1983) *Enzymes (3rd Ed.) 16*, 307–353.
Fullington, D. A., Shoemaker, D. G., & Nichols, J. W. (1990) *Biochemistry 29*, 879–886.
Gheriani-Gruszka, N., Almog, S., Biltonen, R. L., & Lichtenberg, D. (1988) *J. Biol. Chem. 263*, 11808–11813.
Ghomashchi, F., Yu, B.-Z., Berg, O., Jain, M. K., & Gelb, M. H. (1991) *Biochemistry 30*, 7318–7329.
Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.
Hendrickson, H. S., & Hendrickson, E. K. (1990) *Chem. Phys. Lipids 53*, 115–120.
Hoffman, W. J., Vahey, M., & Hajdu, J. (1983) *Arch. Biochem. Biophys. 221*, 361–370.
Jain, M. K. (1988) *Introduction to Biomembranes*, p 423, Wiley, New York.
Jain, M. K., & Vaz, W. L. C. (1987) *Biochim. Biophys. Acta 906*, 1–8.
Jain, M. K., & Berg, O. G. (1989) *Biochim. Biophys. Acta 1002*, 127–156.
Jain, M. K., & Rogers, J. (1989) *Biochim. Biophys. Acta 1003*, 91–97.
Jain, M. K., & Gelb, M. H. (1991) *Methods Enzymol. 197*, 112–125.
Jain, M. K., Egmond, M. R., Verheij, H. M., Apitz-Castro, R. J., Dijkman, R., & de Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 341–348.
Jain, M. K., Rogers, J., Jahagirdar, D. V., Marecek, J. F., & Ramirez, F. (1986a) *Biochim. Biophys. Acta 860*, 435–447.
Jain, M. K., Rogers, J., Marecek, J. F., Ramirez, F., & Eibl, H. (1986b) *Biochim. Biophys. Acta 860*, 462–474.
Jain, M. K., Rogers, J., & de Haas, G. H. (1988) *Biochim. Biophys. Acta 940*, 51–62.
Jain, M. K., Yu, B.-Z., & Kozubek, A. (1989) *Biochim. Biophys. Acta 980*, 23–32.
Jain, M. K., Yu, B.-Z., Rogers, J., Ranadive, G. N., & Berg, O. G. (1991a) *Biochemistry 30*, 7306–7317.
Jain, M. K., Ranadive, G. N., Yu, B.-Z., & Verheij, H. M. (1991b) *Biochemistry 30*, 7330–7340.
Jain, M. K., Rogers, J., Berg, O. G., & Gelb, M. H. (1991c) *Biochemistry 30*, 7340–7348.
Jain, M. K., Tao, W., Rogers, J., Arenson, C., Eibl, H., & Yu, B.-Z. (1991d) *Biochemistry 30*, 10256–10268.
Jain, M. K., Yu, B.-Z., Rogers, J., Gelb, M. H., Tsai, M. D., Hendrickson, E. K., & Hendrickson, H. S. (1992a) *Biochemistry 31*, 7841–7847.
Jain, M. K., Yu, B.-Z., Gelb, M. H., & Berg, O. G. (1992b) *Mediators Inflammation 1*, 85–100.
Kratohvil, J. P., Hsu, W. P., Jacobs, M. A., Aminabhavi, T. M., & Mukunoki, Y. (1983) *Colloid Polym. Sci. 261*, 781–785.
Lichtenberg, D. (1985) *Biochim. Biophys. Acta 821*, 470–478.
Lichtenberg, D., Zilberman, Y., Greenzaid, P., & Zamir, S. (1979) *Biochemistry 18*, 3517–3525.
Lichtenberg, D., Robson, R. J., & Dennis, E. A. (1983) *Biochim. Biophys. Acta 737*, 285–304.
Mazer, N. A., Benedek, G. B., & Carey, M. C. (1980) *Biochemistry 19*, 601–615.
Nagata, M., Yoesuyanagi, T., & Ikeda, K. (1990) *Chem. Pharm. Bull. 38*, 1341–1344.
Nalbone, G., Lairon, D., Augeire, M. C., Vigne, J., Leonardi, J., Chabert, C., Hauton, J. C., & Verger, R. (1980) *Biochim. Biophys. Acta 620*, 612–625.
Nichols, J. W. (1988) *Biochemistry 27*, 3925–3931.
O'Connor, C. J., & Wallace, R. G. (1985) *Adv. Colloid Interface Sci. 22*, 1–111.
Ollivon, M., Eidelman, O., Blumenthal, R., & Walter, A. (1988) *Biochemistry 27*, 1695–1703.
Paternostre, M., Roux, M., & Rigaud, J. T. (1988) *Biochemistry 27*, 2668–2677.
Ramirez, F., & Jain, M. K. (1991) *Proteins: Struct., Funct., Genet. 9*, 229–239.
Roger, J., Yu, B.-Z., & Jain, M. K. (1992) *Biochemistry 31*, 6056–6062.
Schurtenberger, P., Mazer, N., Waldvogel, S., & Kanzig, W. (1984) *Biochim. Biophys. Acta 775*, 111–114.
Upreti, G. C., & Jain, M. K. (1978) *Arch. Biochem. Biophys. 188*, 364–375.
Upreti, G. C., & Jain, M. K. (1980) *J. Membr. Biol. 55*, 113–121.
Uthe, J. F., & Magee, W. L. (1971) *Can. J. Biochem. 49*, 776–784.
Van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5394.
Verger, R., & de Haas, G. H. (1977) *Annu. Rev. Biophys. Bioengg. 5*, 77–117.
Verheij, H. M., Slotboom, A. J., & de Haas, G. H. (1981) *Rev. Physiol., Biochem. Pharmacol. 91*, 91–137.
Waite, M. (1987) *The Phospholipase*, Plenum, New York.
Walde, P., Sunamoto, J., & O'Connor, C. J. (1987a) *Biochim. Biophys. Acta 905*, 30–38.
Walde, P., Sunamoto, J., & O'Connor, C. J. (1987b) *Biochim. Biophys. Acta 905*, 39–47.