Sleight, R. G., & Pagano, R. E. (1985) *J. Biol. Chem. 260*, 1146–1154.
Storch, J., & Schachter, D. (1985) *Biochim. Biophys. Acta 812*, 473–484.
Stremmel, W., Lotz, G., Strohmeyer, G., & Berk, P. D. (1985) *J. Clin. Invest. 75*, 1068–1076.
Thilo, L. (1977) *Biochim. Biophys. Acta 469*, 326–334.
Thulborn, K. R., & Sawyer, W. H. (1978) *Biochim. Biophys. Acta 511*, 125–140.
Van Deenen, L. L. M. (1975) in *Biomembranes: Structure and Function* (Gordos, G., & Szasz, E., Eds.) North-Holland Publishing Co., Amsterdam.
Wolkowicz, P. E., Pownall, H. J., Pauly, D. F., & McMillan-Wood, J. B. (1984) *Biochemistry 23*, 6426–6432.

Activation of Porcine Pancreatic Phospholipase A_2 by the Presence of Negative Charges at the Lipid–Water Interface†

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ABSTRACT: The effect of surface charge on the porcine pancreatic phospholipase A_2 catalyzed hydrolysis of organized substrates was examined through initial rate enzyme kinetic measurements. Two long-chain phospholipid substrates, phosphatidylglycerol (PG) and phosphatidylcholine (PC), were solubilized in seven detergents differing in polar head-group charge. The neutral or zwitterionic detergents selected were Triton X-100, Zwittergent 314, lauryl maltoside, hexadecylphosphocholine (C_{16}PN), and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate. The negatively and positively charged detergents used were cholate and CTAB, respectively. In general, the negatively charged phospholipid PG was hydrolyzed much more rapidly than the neutral (zwitterionic) phospholipid PC. The rate of hydrolysis of PG was rapid when solubilized in all the neutral detergents and in cholate but was essentially zero in the positively charged CTAB. Conversely, hydrolysis of PC was negligible when solubilized in neutral detergents, except C_{16}PN, and was maximal in the negatively charged detergent, cholate. The rate of hydrolysis of PC solubilized in a neutral detergent became significant only when a negative surface charge was introduced by addition of SDS. Taken together, these kinetic measurements indicate that the surface charge on the lipid aggregates is an important factor in the rate of hydrolysis of phospholipid substrates and the highest activity is observed when the net surface charge is negative. Fluorescence and electron spin resonance (ESR) spectroscopic data provide additional support for this conclusion. The fluorescence emission spectrum of the single tryptophan of phospholipase A_2 is a sensitive monitor of interfacial complex formation and shows that interaction of the protein with detergent micelles is strongly dependent on the presence of a negatively charged amphiphile. Furthermore, ESR spectra of spin-labeled detergent analogues detect a significant decrease in lipid motion in the presence of protein only when the charge on the spin-label is negative.

Pancreatic phospholipase A_2 (EC 3.1.1.4) (PLA)[1] is a small water-soluble enzyme of M_r 14 000 that catalyzes the hydrolysis of the 2-acyl ester bond of 3-*sn*-phosphoglycerides. Primary sequences of more than 30 phospholipases A_2 isolated from mammalian pancreas and various snake venoms are known (Verheij et al., 1981). Three-dimensional structures of the porcine and bovine pancreatic enzymes as well as the enzyme from *Crotalus atrox* venom have been determined by X-ray crystallography (Dijkstra et al., 1981a, 1983; Keith et al., 1981). One of the most interesting aspects of phospholipase A_2, and one that distinguishes this enzyme from other water-soluble enzymes, is that it hydrolyzes substrates at a much higher rate when they are organized into larger aggregates such as micelles (Wells, 1972; Pieterson et al., 1974). Several models have been proposed to account for the interfacial activation of PLA and other lipolytic enzymes [reviewed by Volwerk & de Haas (1982)]. In the majority of kinetic studies on pancreatic as well as snake venom phospholipases, phospholipids carrying the neutral zwitterionic phosphocholine head group have been employed as substrates either as short-chain PC's in a single component system (de Haas et al., 1971; Wells, 1972) or as long-chain PC's mixed with uncharged detergents such as Triton X-100 (Dennis, 1973).

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[1] Abbreviations: PLA, porcine pancreatic phospholipase A_2; PC, phosphatidylcholine; PG, phosphatidylglycerol; HETLC, high-efficiency thin-layer chromatography; C_{16}PN, *n*-hexadecylphosphocholine; Z314, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 314); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CHOL, sodium cholate; OG, octyl glucoside; CTAB, cetyltrimethylammonium bromide; SDS, sodium dodecyl sulfate; TX100, Triton X-100; LM, lauryl maltoside; MP*, 14-proxylstearyl methyl phosphate sodium salt; QA*, (14-proxylstearyl)trimethylammonium mesylate; cmc, critical micelle concentration; TEM, transmission electron microscopy; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Most binding studies on pancreatic phospholipase have employed nonhydrolyzable phospholipid analogues carrying the phosphocholine head group (Soares de Araujo et al., 1979; Hille et al., 1981; Donné-Op den Kelder et al., 1981). There have been some reports in the literature of phospholipase A_2 activity on different substrates and environments, but under such different conditions that the data are troublesome to correlate (Van Deenen et al., 1963; Hendrickson et al., 1981; Hoffman et al., 1983; Hille et al., 1983; Thuren et al., 1984). Our purpose here was to measure the initial rates of the PLA-catalyzed hydrolysis in a series of detergents using two different naturally occurring phospholipid substrates, one neutral (egg PC) and one with a negatively charged head group (PG derived from egg PC), to determine if there is a correlation between enzyme activity and net charge on the substrate aggregate. We find that the rate of hydrolysis is significantly higher when the aggregates are negatively charged, a fact that is likely to be functionally significant in view of the surface charge of the naturally occurring mixtures of phospholipids with bile salts.

Materials and Methods

Materials. Phospholipase A_2 from porcine pancreas was obtained as described previously (Nieuwenhuizen et al., 1974). PC was purified from egg yolks according to Singleton et al. (1965). Part of this preparation was converted into PG via transphosphatidylation with phospholipase D and purified on CM-cellulose (Whatmann, CM-52) as described by Comfurius and Zwaal (1977). Purified phospholipids were stored in chloroform/methanol (99/1 v/v) at a concentration of 24 mg/mL under Ar at −20 °C. Purity was routinely checked via HETLC (Analtech) using chloroform/methanol/water (65/25/4 v/v) as the solvent. The detergent $C_{16}PN$ was prepared as reported by Van Dam-Mieras et al. (1975). Z314, CHAPS, CHOL, and OG were from Calbiochem, CTAB and SDS were from Sigma, TX100 was from Rohm & Haas, and sodium stearate was from Matheson Coleman and Bell. LM was synthesized according to the method of Rosevear et al. (1980) and was a generous gift of Dr. S. Ferguson-Miller. The spin-labeled detergent analogues MP* and QA* were synthesized as described by Keana et al. (1982). All other reagents and materials were of the highest grade available.

Preparation of Mixed Micelles. Stock solutions of PC or PG in chloroform and the various detergents in methanol were mixed to give the desired detergent/lipid ratios and dried with nitrogen or by rotary evaporation followed by at least 2 h under high vacuum. The dry lipid/detergent films were then taken up in 0.1 M NaCl by vortexing with the appropriate amount of solvent. In most cases the final concentration of phospholipid was 5 mM, but further dilution was sometimes necessary because of the high viscosity of solutions containing PC and neutral detergent at high detergent/lipid ratios. At molar ratios of 2 and higher, optically clear solutions were obtained, which indicated complete solubilization of the phospholipids. In these systems there are no ambiguities involving phospholipid vesicles or multilayered phospholipid aggregates [see Lichtenberg et al. (1983)]. The absence of phospholipid vesicles in these mixed micellar preparations was confirmed in specific cases by transmission electron microscopy (TEM). TEM of negatively stained preparations of PC and PG dispersed in aqueous solution exhibited the characteristic multilamellar phospholipid structures in the absence of detergents (Bangham & Horne, 1964). After solubilization of the phospholipids in Triton X-100 or cholate (3/1 detergent/phospholipid molar ratio), no phospholipid vesicles were observed.

For the optically clear solutions consisting of mixed micelles the initial rate of the PLA-catalyzed hydrolysis was identical when measured immediately after solubilization or after incubation of the mixtures for several hours at room temperature. At detergent/lipid ratios below 2, however, the mixtures showed varying degrees of turbidity even after prolonged vortexing and bath sonication, indicating incomplete solubilization of the lipids. For these mixtures reproducible initial rate measurements could not be obtained.

In our hands only substrate mixtures prepared as described above at detergent/lipid molar ratios of 2 or higher showed acceptable kinetics; i.e., the initial rates were linear for at least 3 min and reproducible to within 10%, and there was good proportionality between the initial rate and the amount of enzyme added. The alternative procedure of mixing separately prepared aqueous solutions of detergent and phospholipids was not satisfactory even when the lipid was dispersed in the form of unilamellar vesicles. Under these mixing conditions reproducibility was poor, and the measured initial rate was strongly dependent on the time elapsed between mixing of lipid and detergent and the addition of enzyme. Furthermore, it took up to several hours for some combinations of lipid and detergent to produce optically clear solutions. This result was somewhat surprising because Jackson et al. (1982) reported that, contrary to multilamellar vesicles, unilamellar vesicles were solubilized rather rapidly by several detergents including octyl glucoside. We therefore also examined the solubilization of PC and PG by octyl glucoside and some of the detergents used in our study via turbidity measurements as described by Jackson et al. (1982). In agreement with the observations of these authors we found that solubilization of both PC and PG in the form of large unilamellar vesicles by octyl glucoside was essentially complete within a few minutes. However, solubilization of PC was much slower with cholate, Triton X-100, and $C_{16}PN$. To become optically clear required several hours with the first two mixtures and days for mixtures of PC with the detergent $C_{16}PN$. Solubilization of PG was generally faster than that of PC with the same detergents. Some tentative conclusions that can be drawn from these solubilization experiments are the following: (1) The rate of solubilization of phospholipids dispersed as bilayers by detergents is related to the cmc values of the detergents and is fast for detergents with a high cmc (octyl glucoside, cmc = 22 mM) and slow for detergents with a low cmc ($C_{16}PN$, cmc = 10 μM). (2) The solubilization rate for neutral detergents is dependent on the lipid head group and is faster for the negatively charged PG than for the net neutral PC.

Vesicle Preparation. Large unilamellar vesicles containing PG and PC in various proportions were prepared by three different methods: (1) reverse-phase evaporation (Szoka & Papahadjopoulos, 1978); (2) ethanol injection (Kremer et al., 1977); (3) dialysis from octyl glucoside (Mimms et al., 1981). The kinetics of the PLA-catalyzed hydrolysis was similar for all three vesicle preparations.

Initial Rate Measurements. Initial rates of the PLA-catalyzed hydrolysis of PC and PG were determined by automatic titration of the fatty acid products in a pH state (Nieuwenhuizen et al., 1974). Kinetic measurements were performed at 25 °C and pH 8.0 by using a jacketed titration cell connected to a circulating water bath. The total volume in the titration cell was 3 mL. The fatty acids liberated as a function of time were titrated with 0.01 N NaOH prepared fresh weekly from a 1 N stock solution via dilution with degassed (boiled) glass-distilled water. The machine was calibrated immediately before a series of measurements via titration of

a 0.01 N HCl solution prepared by dilution of a standardized 2 N HCl solution (Sigma). A nitrogen screen was used to reduce the background due to CO_2 uptake, and the titrant was protected from CO_2 uptake by a KOH trap. The final assay mixture contained 0.1 M NaCl and 10 mM $CaCl_2$ unless otherwise stated. No albumin was added because these are initial rate measurements and no significant amounts of lysolecithin or fatty acid products are present. In one experiment involving the hydrolysis of PG solubilized in lauryl maltoside, 1 mg of albumin/mL of assay mixture was added, with no observable effect on the initial rate measurements [see also Pluckthun & Dennis (1985)]. PLA was added usually as 10–50-μL aliquots from a 50-fold dilution of a stock solution containing 1 mg/mL protein in distilled water. The solution containing 1 mg/mL protein was stable for several days when kept on ice and stored in the refrigerator. There was a fairly rapid loss of activity, however, in the 50-fold diluted sample probably because of irreversible adsorption of the protein to the walls of the glass container. Therefore, a fresh dilution of protein was prepared every hour. Measurements were in general reproducible within 10%. During a series of measurements the blank values showed a tendency to increase, probably because traces of enzyme accumulated in the titration cell in spite of thorough rinsing with distilled water. When necessary this was circumvented by rinsing the cell quickly with concentrated bleach followed by dilute acetic acid and multiple rinses with distilled water.

Fluorescence Measurements. Tryptophan emission spectra were recorded on a Spex Fluorolog fluorometer essentially as described by Volwerk et al. (1985). The buffer used was 0.1 M Tris-HCl (pH 8.0) containing 10 mM $CaCl_2$ and 0.1 M NaCl, and the protein concentration was 0.2 mg/mL. Fluorescence emission spectra were recorded with excitation at 295 nm after addition of small aliquots of a stock solution containing 75 mM total detergent to the protein solution. Some background fluorescence was observed for the detergent LM. When PLA was titrated with micelles containing LM, the emission intensity was corrected for the contribution by this detergent assuming a linear proportionality between the emission intensity of LM and its concentration.

ESR Measurements. Electron spin resonance spectra were collected on a Varian E-line 9.5-GHz spectrometer interfaced with a 32K Varian 620/L100 computer. During the ESR run the sample temperature of 25 °C was monitored by a thermocouple inserted into the sample holder just above the cavity; temperature was controlled to ±0.1 °C. Spectra were typically collected at a power setting of 5 mW with scan times of 8–30 min, filter time constants of 0.1–0.3 s, modulation amplitudes of 0.8–1.25 G (depending on line width), and a scan range of 100 G. Samples were prepared by adding buffer or buffer containing $C_{16}PN$ micelles and/or PLA to a dried (nitrogen) film of the spin-labeled component followed by bath sonication for 5 min. The buffer used was 0.1 M sodium acetate/acetic acid (pH 6.0) containing 0.1 M NaCl. The concentration of the spin-labeled molecule was about 0.1 mM, $C_{16}PN$ was 10 mM, and protein was 7 mg/mL (0.5 mM). A total sample volume of about 50 μL was placed in the microwave cavity by using flame-sealed glass capillary tubes.

RESULTS

Enzyme Kinetics. The effect of the head-group charge of the phospholipid substrate on the PLA-catalyzed hydrolysis was examined by measuring the enzyme activity on egg PC and PG solubilized in various neutral and charged detergents. In these and all the enzyme kinetics experiments, the reaction was initiated by the addition of the enzyme and progress was

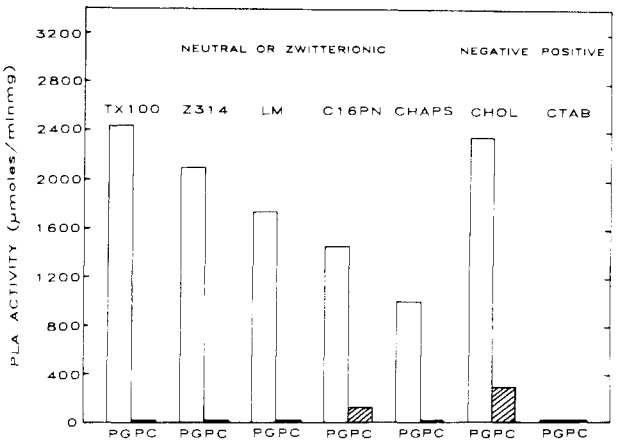


FIGURE 1: Comparison of the maximum initial rates of the PLA-catalyzed hydrolysis of PG and PC solubilized in seven different detergents. For all detergents, except cholate (CHOL), the highest activity was observed at a detergent/lipid molar ratio of 2. The very low activity observed for the combinations PC/Triton X-100 (TX100), PC/Z314, PC/lauryl maltoside, PC/CHAPS, PC/CTAB, and PG/CTAB was in the range between 0 and 5 μmol min^{-1} mg^{-1}.

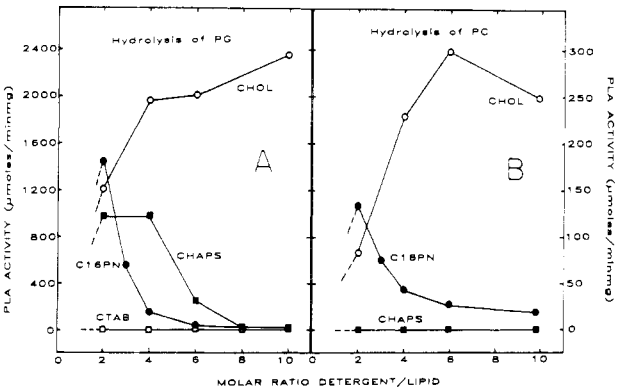


FIGURE 2: Initial rates of the phospholipase A_2 catalyzed hydrolysis of PG (A) and PC (B) solubilized in detergent as a function of the detergent/lipid molar ratio. Enzyme activities were measured at 25 °C and pH 8.0 in the presence of 10 mM $CaCl_2$ and 0.1 M NaCl. The substrate (PG or PC) concentration was 1 mM. Initial rate measurements were reproducible within 10%.

then followed with time for a few minutes. The initial rate is the slope of the progress curve at $t = 0$. Progress curves were linear in this region, and the slopes were proportional to the amount of enzyme added. This was found to be true for all combinations of the phospholipids and detergents of this study provided that the detergent/phospholipid molar ratio was two or higher. Initial rate measurements were used throughout this study because the substrate concentration is very nearly constant during this short time interval, and there is no significant accumulation of products that could influence the reaction rates.

The optimal initial rates observed for phospholipid hydrolysis in the series of detergents are summarized in Figure 1. These hydrolysis rates are much higher with the negatively charged PG than with the zwitterionic PC (except for the positively charged detergent CTAB, where the activity is zero with both substrates). Significant activity with PC was observed only with $C_{16}PN$, which carries the zwitterionic phosphocholine head group, and the negatively charged detergent cholate.

The range of initial rates observed as a function of the detergent/phospholipid molar ratio is shown in Figure 2. For the combination PG with each of the detergents Triton X-100, Z314, and lauryl maltoside, the enzyme activity as a function of the detergent/substrate ratio followed a pattern similar to the one shown for the PG/$C_{16}PN$ mixture (Figure 2A). At

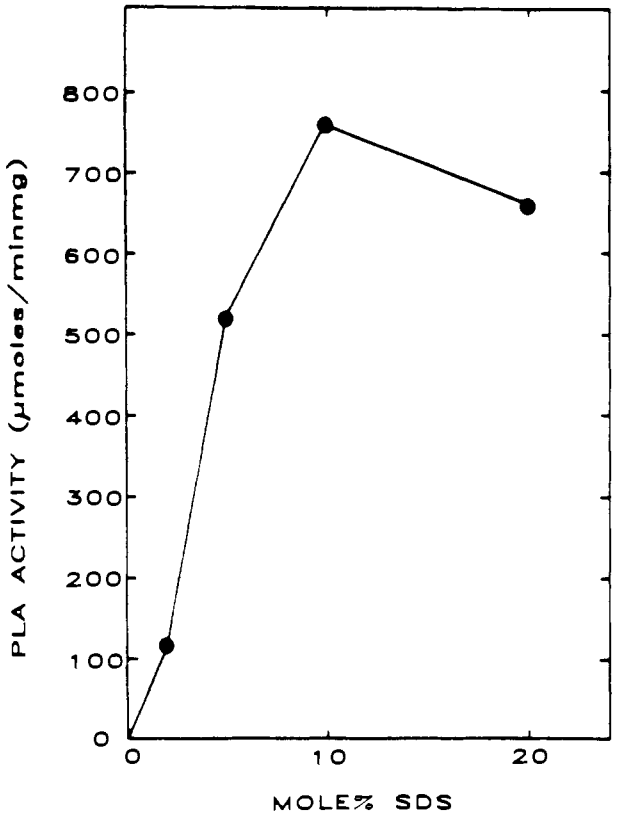


FIGURE 3: Effect of adding a negatively charged detergent (SDS) on the PLA-catalyzed hydrolysis of PC solubilized in the neutral detergent, lauryl maltoside. PC/detergent mixtures were prepared via cosolubilization with a constant molar ratio total detergent/PC of 3; conditions were the same as for Figure 2. The reproducibility of these measurements was about 15% due to the high viscosity of the SDS-containing mixed micelles in the presence of Ca^{2+}.

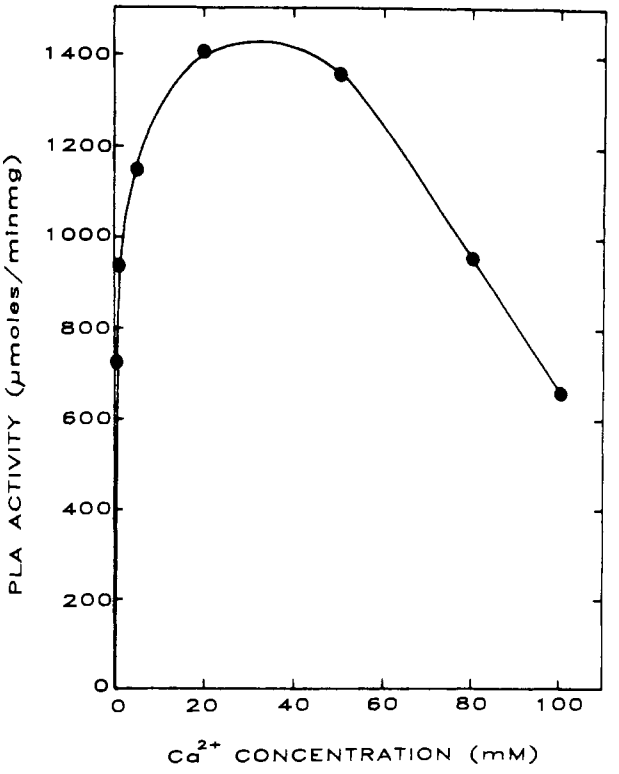


FIGURE 4: Initial rate of the PLA-catalyzed hydrolysis of PG solubilized in lauryl maltoside as a function of the Ca^{2+} concentration. Enzyme activities were measured at 25 °C and pH 8.0 with 1 mM PG and 3 mM lauryl maltoside in the presence of 0.1 M NaCl and varying Ca^{2+} and were reproducible to within 10%.

low detergent/lipid molar ratios (below 2) the high turbidity of the mixtures indicated incomplete solubilization of the phospholipid. In this range of detergent/lipid ratios the kinetics behaved erratically, and reliable quantitative measurements could not be obtained, but there was a general tendency toward higher activities with increasing detergent/lipid ratios. For PG solubilized in any of the detergents Triton X-100, Z314, lauryl maltoside, or $C_{16}PN$, the initial rate of hydrolysis showed a sharp optimum at a detergent/lipid ratio of 2 with a fairly rapid decrease of activity at higher detergent/lipid ratios. At lipid/detergent ratios of 2 and higher, PG was completely solubilized and formed optically clear mixed micellar solutions. A sharp optimum in the activity was not observed with the PG/cholate mixture. Instead, the activity continued to increase above a detergent/lipid ratio of 2, but appeared to plateau at higher ratios. With CHAPS there was a decrease at higher detergent/lipid ratios, although the region of optimal activity appeared to be somewhat broader (Figure 2A).

A low activity of zero, or very nearly zero, was observed when the substrate was PC solubilized in any one of the detergents lauryl maltoside, Triton X-100, Z314, CTAB, and CHAPS (Figure 2B). All of these mixed micelles are characterized by either a net neutral or a net positive (CTAB) surface charge and are clearly not good substrates for PLA. The only exception was the PC/$C_{16}PN$ mixture, which showed an activity pattern similar to that observed for PG/$C_{16}PN$, although the optimum level of activity was much lower (compare the *y*-axis scales in parts A and B of Figure 2). The PC/cholate mixture also behaved similarly to the PG/cholate mixed micelles, but again the level of activity was only about 10% of that found with the latter.

PC hydrolysis could be stimulated by adding a negatively charged detergent to a mixture of PC and a neutral detergent (Figure 3). Incorporation of a negatively charged molecule (SDS) into mixed micelles of PC and the uncharged detergent lauryl maltoside leads to a pronounced increase in the initial rate of PC hydrolysis. This experiment was carried out at a total detergent/PC molar ratio of 3 to ensure complete solubilization of the lipid. A similar stimulation of hydrolysis was observed when PC was solubilized in the neutral detergent TX-100 and the negative charge was introduced by addition of SDS or when PC was solubilized in lauryl maltoside and sodium stearate provided the negative charge. The stimulation of PLA activity was not nearly as pronounced when $C_{16}PN$ was the neutral detergent. Addition of SDS to the $C_{16}PN$/PC mixed micelles produced only a marginal increase of the activity from about 80 μmol min^{-1} mg^{-1} measured in the absence of added negatively charged detergent to about 100 μmol min^{-1} mg^{-1} in the presence of 20 mol % SDS.

The enzyme activity was measured as a function of the Ca^{2+} concentration because of the possible interaction of this positively charged ion with the negatively charged substrates and detergents. Previous work has shown that the activity of PLA on neutral short-chain lecithins as a function of the Ca^{2+} concentration varies according to the Michaelis–Menten theory; i.e., at high concentrations the initial velocity approaches asymptotically a limiting value corresponding to complete saturation of the enzyme with Ca^{2+} ions (de Haas et al., 1971). With PG/lauryl maltoside as substrate, however, there was a *decrease* in activity at Ca^{2+} concentrations above 40 mM (Figure 4). This observation is most readily interpreted as a competition of the Ca^{2+} ions with the enzyme for the negatively charged groups on the micellar surface and suggests that electrostatic interactions between these negatively charged groups and protein residues contribute to the for-

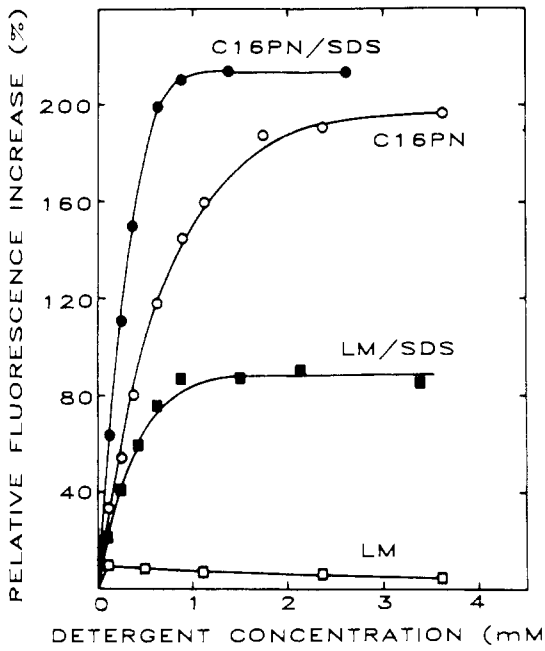


FIGURE 5: Comparison of the effect of the presence of neutral detergent micelles and negatively charged mixed detergent micelles on the tryptophan fluorescence of PLA. The fluorescence emission intensity at 346 nm is plotted as the relative fluorescence increase $100(F - F_0)/F_0$, where F and F_0 are the emission intensities in the presence and absence of detergent, respectively. The open symbols correspond to the increase in the fluorescence emission of the protein in the presence of neutral micelles ($C_{16}PN$ or lauryl maltoside), and the solid symbols correspond to the fluorescence increase in the presence of the same detergents to which 10 mol % SDS had been added in order to produce a net negative surface charge on the micelles.

mation of the lipid/protein complex. Even at these high Ca^{2+} concentrations no visible precipitation occurred, indicating that any Ca^{2+}/PG complex formed was fully solubilized by the excess detergent.

Kinetic parameters for the PLA-catalyzed hydrolysis of PG and PC solubilized in a 3-fold molar excess of $C_{16}PN$ were determined at 25 °C and pH 8.0 in the presence of 10 mM $CaCl_2$ and 0.1 M NaCl from double-reciprocal plots of initial velocity vs. substrate concentration. The apparent V_{MAX} values were 83 ± 11 and 550 ± 50 μmol min^{-1} mg^{-1} for PC and PG, respectively, and the corresponding values for the apparent K_M were 0.23 ± 0.03 mM for PC and ≤0.01 mM for PG. The rates of hydrolysis for PG were essentially independent of the phospholipid substrate concentration at concentrations above 0.01 mM. These data show that there is a large effect of the head-group charge of the lipid substrate on the PLA activity and that both parameters, the apparent V_{MAX} and the apparent K_M, are affected.

Although the focus of this work and all the data presented here involve mixed micelles, it was also of interest to examine the effect of phospholipid head-group charge on the hydrolysis of egg PC and PG in the form of bilayers. Pure PC vesicles were not a substrate for the pancreatic PLA. When pure PG vesicles were the substrate, a fairly slow hydrolysis rate of about 50 μmol min^{-1} mg^{-1} was observed. In this case, however, addition of $CaCl_2$ to the vesicle suspension led to massive vesicle aggregation and formation of a precipitate. With PC/PG mixed vesicles the kinetics showed multiphasic behavior with poor reproducibility. A rapid initial burst was followed by a slow phase sometimes decreasing to almost zero activity. In a qualitative sense these experiments on bilayers confirmed the stimulating effect of negative charge on the phospholipase A_2 catalyzed hydrolysis, in that hydrolysis was more rapid in the presence of PG.

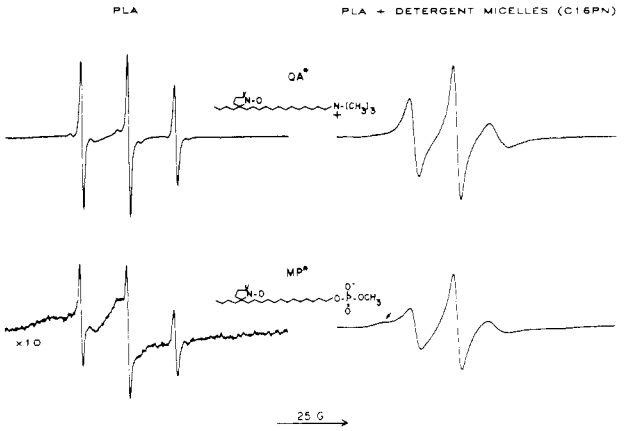


FIGURE 6: Comparison of the effect of phospholipase A_2 on the ESR line shapes of a positively charged (QA*) and a negatively charged (MP*) spin-labeled detergent analogue in the presence (right) and absence (left) of neutral detergent micelles ($C_{16}PN$). Spectra were recorded at 25 °C in 0.1 M sodium acetate/acetic acid, 0.1 M NaCl (pH 6.0) containing 7 mg/mL (0.5 mM) PLA, 10 mM $C_{16}PN$, and 0.1 mM of the spin-labeled molecule (QA* or MP*).

Fluorescence Spectroscopy. Figure 5 shows the increase in the fluorescence emission intensity of the single tryptophan of PLA as a function of the detergent concentration comparing neutral and negatively charged micelles. PLA binds fairly strongly to the $C_{16}PN$ micelles, and the interaction is accompanied by about a 200% increase in the fluorescence emission intensity. The affinity of the enzyme for the lipid–water interface was clearly improved, however, when a negative surface charge was introduced via formation of $C_{16}PN$/SDS mixed micelles. Compared to the uncharged $C_{16}PN$ micelles, saturation was reached at a lower total detergent concentration, although the limiting value of the relative fluorescence increase was similar to that observed with $C_{16}PN$ alone. Both for the $C_{16}PN$ and for the $C_{16}PN$/SDS charged micelle, binding of PLA to the lipid–water interface was accompanied by a blue shift of about 8 nm in the fluorescence emission maximum. Thus, the indole side chain of the tryptophan residue of PLA is probably in a similar environment when a complex with either micelle is formed.

From the fluorescence measurements, the difference in the affinity of PLA for the detergent micelles in the presence and absence of SDS was much greater in the case of lauryl maltoside than for $C_{16}PN$ (Figure 5). The affinity of PLA for the neutral lauryl maltoside micelle was negligible. The small initial increase in the fluorescence intensity is probably due to binding of a lauryl maltoside monomer in the active site of PLA, saturating already at low lauryl maltoside concentrations. The absence of any further increase in the fluorescence upon addition of more lauryl maltoside micelles suggests that PLA does not bind to the micellar interface. The slight decrease observed instead is probably because of a small correction in the relative fluorescence increase due to fluorescence of the detergent itself. A fairly large increase in the emission intensity of the tryptophan, accompanied by a blue shift in the emission maximum of about 6 nm, was obtained when PLA was titrated with mixed micelles of lauryl maltoside and SDS in a 9/1 molar ratio (Figure 4). Similar results were obtained with Triton X-100/SDS and Z314/SDS (molar ratio 9/1), whereas the positively charged detergent CTAB did not produce changes in the tryptophan emission spectrum indicative of interfacial binding.

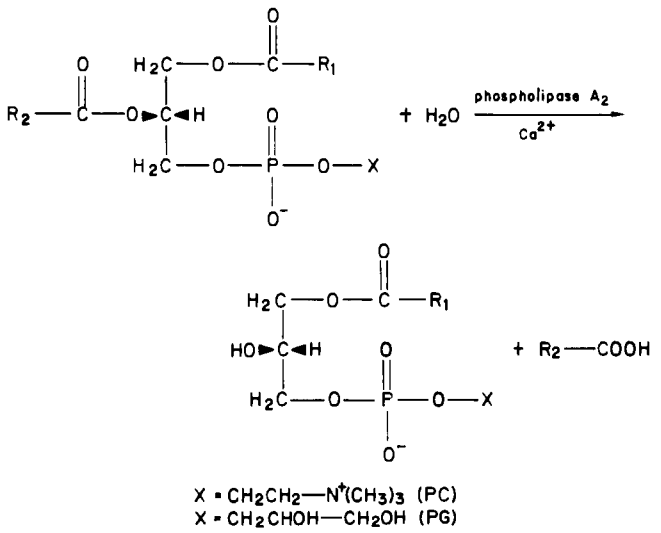
Electron Spin Resonance Spectroscopy (ESR). Both spin-labeled detergent analogues (MP* and QA*) in buffer

at 0.1 mM produced a sharp three-line spectrum characteristic of a rapidly tumbling spin-label (similar to Figure 6, top left), indicating that this concentration is below the cmc of these compounds. When excess PLA was added to buffer containing the spin-label molecules, the heights of the first derivative ESR lines of both spin-labels decreased, indicating line broadening caused by interaction of the spin-labels with the enzyme (Figure 6, left column). However, a much larger change in the line shape of the negatively charged spin-label MP* was observed. This line shape is characteristic of spin exchange and results from a high local concentration of spin-labels, in this case a clustering of spin-labels on the protein surface. This is consistent with protein-induced formation of a micelle-like structure as described by Hille et al. (1983).

In the absence of protein, addition of 10 mM $C_{16}PN$ changed the sharp three-line spectrum to a more motion-restricted line shape similar to Figure 6 (top right). Under these conditions both MP* and QA* are apparently completely taken up in the micelles and produce identical spectra. Addition of excess protein to 10 mM $C_{16}PN$ micelles containing 0.1 mM of spin probes MP* or QA* (1 mol %) produced the spectra shown in the right column of Figure 6. In these experiments the protein concentration (0.5 mM) represents about a 2-fold excess of protein over the available number of binding sites on the micellar surface assuming that the stoichiometry of the lipid–protein complex is not changed significantly by the presence of the spin-label (Soares de Araujo et al., 1979). This excess was used to ensure that the effect of the protein on the spectral line shape would be optimal. The appearance in the bottom right spectrum of Figure 6 of a broader component (see arrow) underlying the micellar line shape indicated that there was a greater degree of association with the protein for the negatively charged spin-label (MP*) than for the positively charged spin-label (QA*).

Discussion

Our purpose in carrying out this study was to examine the effects of charge interactions on the kinetics of the reaction:



One reason that neutral phospholipids have been employed almost exclusively in kinetic and binding studies of PLA is probably the fact that this enzyme requires a Ca^{2+} ion as an essential and highly specific cofactor. Since it is well-known that Ca^{2+} and similar divalent metal ions interact strongly with anionic phospholipids and anionic detergents, complexes formed between these compounds and Ca^{2+} may produce inhomogeneities or precipitates in the substrate mixture and complicate interpretation of the data. Although this may hamper a detailed analysis of the pathways leading to formation of the Michealis complex and product release, our results show that studies of negatively charged substrates and detergents, even in the presence of Ca^{2+}, can provide useful information about the role of surface charge in PLA function.

One source of surface charge is the phospholipid substrate itself. We selected two naturally occurring substrates with the same composition of fatty acyl chains but differing in the structure of the polar head groups. The egg PC is neutral (zwitterionic), and the PG prepared from it is negatively charged. The most significant observation from this comparison is that in every case examined the rate of the PLA-catalyzed hydrolysis of the negatively charged PG is much greater. This suggests that the negative surface charge contributed by PG is a significant factor in the observed increase in hydrolysis rate. There are of course other differences between PC and PG, notably the geometry and size of the head groups, that may affect the hydrolysis rates.

Another source of surface charge is the detergent solubilizing the phospholipid substrate. We therefore also examined each phospholipid substrate in a series of seven detergents with different head-group charges. All combinations of phospholipid and detergent, with the exception of cholate, exhibit a rather sharp optimum at detergent/lipid ratios around 2, with decreasing activity both at higher and at lower ratios. This decrease in enzyme activity at high detergent has been observed earlier for cobra venom PLA-catalyzed hydrolysis of PC in one detergent, Triton X-100, and was explained by surface dilution of the substrate at higher detergent/lipid ratios (Dennis, 1973) but could also result from competitive inhibition by the detergent. Interestingly, we do not observe a drop in activity at higher detergent/lipid ratios when cholate is the detergent, so the chemical nature of the detergent can influence the rate of phospholipid hydrolysis.

The comparison of the series of detergents shows that the rate of enzyme-catalyzed hydrolysis depends markedly on the charge of the detergent. For both substrates, the rate of hydrolysis is maximal in the negatively charged cholate and minimal (essentially zero) in the positively charged detergent, CTAB. Furthermore, we observe a marked stimulation of the PLA activity when negatively charged amphiphiles (e.g., SDS) are added to PC in uncharged detergents, where the activity is practically zero in the absence of the negatively charged molecule (Figure 3). With each detergent there will of course be some variation in the state of the substrate (i.e., conformation, organization, state of hydration, and dynamics). However, the effects on enzyme activity in this series of detergents with widely differing structures were clearly correlated with the surface charge. We conclude that the surface charge on the substrate aggregate is an important factor in the observed rate of hydrolysis by phospholipase A_2, and the maximum rates occur when the surface charge is negative.

Some insight into the factors contributing to the activation of phospholipase A_2 can be obtained by considering the kinetic mechanism proposed by Verger et al. (1973):

$$E \underset{1}{\rightleftharpoons} E^* \overset{+S}{\underset{2}{\rightleftharpoons}} E^*S \xrightarrow[3]{} E + P$$

Three steps are required: (1) reversible association of the enzyme with the lipid–water interface, which may be accompanied by an activation of the enzyme (indicated by E*); (2) binding of a single substrate molecule in the active site forming the Michealis complex E*S; (3) product formation and product release. With the usual caveats inherent in interpreting the complex interfacial kinetics of lipolysis (Verger, 1980; Volwerk

& de Haas, 1982), the significantly reduced apparent K_M value for PG suggests that incorporation of anionic groups in the micellar interface increases the affinity of PLA for the mixed micelle (step 1). At the same time there is an increase in the apparent V_{MAX} as well, which indicates that step 2 and/or step 3 is also affected by the substrate head-group charge. Thus, when the negative charge is on the substrate itself, there appear to be two effects. One is improved binding to the interface, and the other is acceleration of a subsequent step in the mechanism (e.g., better binding of anionic phospholipids in the active site, or an increase in the rate of breakdown of the Michealis complex induced by lipid–protein charge interactions). However, when the negative charge is on the detergent rather than on the substrate, the first effect appears to be predominant. Evidence for this arises out of the fluorescence binding data combined with the kinetic data involving the addition of the negatively charged SDS to PC solubilized in the neutral detergents C_{16}PN and lauryl maltoside. These combined data suggest that improved affinity of the protein for the lipid–water interface is the primary reason for the observed increase in the PLA-catalyzed hydrolysis of PC when a negatively charged detergent is introduced.

From the data presented here together with earlier findings (Soares de Araujo et al., 1979; Hille et al., 1981; Donné-Op den Kelder et al., 1981), a picture of those elements that lead to an optimally functional, active complex between PLA and its organized substrate is emerging. Both hydrophobic and electrostatic interactions contribute to formation of the interfacial complex. In general, a negative surface charge is required either in the form of an anionic lipid substrate or in the form of an anionic detergent added as a third component. Some of our data suggest that there are faily strict constraints with respect to the geometrical relationship between the charged groups in the lipid–water interface and the apolar core of the lipid aggregate. For example, hydrolysis is observed in the case of micelles composed of lipids and detergents both carrying the naturally occurring phosphocholine head group. If the zwitterionic dipole of the solubilizing detergent is reversed (e.g., Z314 and CHAPS), there is no activity, unless a negative charge is provided by the substrate.

Most likely the negatively charged groups of the lipids at the interface must be situated so that they pair up with positively charged amino acid side chains of the enzyme and, at the same time, allow some interdigitation of apolar groups on the lipid binding surface of the enzyme into the lipid aggregate. This is supported by the ESR spin-labeling data which show that significant motion restriction due to interaction with the protein occurs with the negatively charged spin-labeled detergent analogue, MP*, but not with the positively charged analogue, QA*. X-ray crystallography data on phospholipase A_2 are also consistent with this picture (Dijkstra et al., 1981b). Positively charged lysine and arginine residues (e.g., Arg-6 and Lys-10, -56, -116, -121, and -122 in the porcine enzyme) are present on the enzyme surface, located around a region containing exposed hydrophobic side chains including the single tryptophan (Trp-3). The active site is located near the center of this lipid binding surface (interface recognition region). This geometry provides easy access of the active site for the substrate molecules after the enzyme binds to the lipid aggregate. Calcium ions bind to the enzyme active site, whether or not phospholipids or detergents are present. With negatively charged substrates or detergents there will also be some binding of calcium ions at the lipid–water interface. This, however, is most likely not the primary cause of the interfacial activation by negatively charged phospholipids and detergents for the following reasons: (1) It is inconsistent with the crystal structure data (only positively charged lysine and arginine groups are suitably located at the lipid binding surface of the enzyme). (2) The data of Figure 4 show that there is an inhibition at high calcium concentrations. The positively charged calcium ions compete with the positively charged lysine and arginine residues of the enzyme for the available negatively charged lipids and detergents. (3) The spin-labeling data show an interaction of the enzyme with the negatively charged detergent analogue in the absence of calcium ions (Figure 6). (4) Some snake venom phospholipases, which also require calcium as an essential cofactor, show the opposite effect. These enzymes are not activated and are even inhibited by negatively charged lipids (de Haas et al., 1966; Pluckthun & Dennis, 1985).

It is interesting to relate these results with what is known about the physiological states of the enzyme and substrates. Phospholipids and other fat molecules present in the chyme passing from the stomach into the small intestine are solubilized by bile salts secreted into the duodenum. In contrast to most water-soluble enzymes that act on substrates in monomeric form, the phospholipase must be able to recognize and bind to these phospholipid/bile salt aggregates in a complex mixture in order to achieve effective hydrolysis of its substrates. The bile salts are naturally occurring negatively charged detergents composed of cholate and related molecules conjugated to either glycine or taurine via amide bonds (Small et al., 1966). Thus, the conclusion from the present study of phospholipid/detergent mixtures that a net negative charge promotes phospholipase activity has physiological implications. The net negative surface charge on the mixed micelle helps the pancreatic phospholipase A_2 to recognize and bind to these structures, after which the enzyme is in a position to hydrolyze a number of phospholipid molecules in the micelle.

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Registry No. C_{16}PN, 93597-88-7; CHAPS, 75621-03-3; CHOL, 361-09-1; LM, 69227-93-6; OG, 29836-26-8; PLA, 9001-84-7; SDS, 151-21-3; TX100, 9002-93-1; Z314, 14933-09-6; Ca, 7440-70-2.

REFERENCES

Bangham, A. D., & Horne, R. W. (1964) *J. Mol. Biol. 8*, 660–668.

Comfurius, P., & Zwaal, R. F. A. (1977) *Biochim. Biophys. Acta 488*, 36–42.

de Haas, G. H., Bonsen, P. P. M., & van Deenen, L. L. M. (1966) *Biochim. Biophys. Acta 116*, 114–124.

de Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., & Van Deenen, L. L. M. (1971) *Biochim. Biophys. Acta 239*, 252–266.

Dennis, E. A. (1973) *Arch. Biochem. Biophys. 158*, 485–493.

Dijkstra, B. W., Kalk, K. H., Hol, W. G., & Drenth, J. (1981a) *J. Mol. Biol. 147*, 97–123.

Dijkstra, B. W., Drenth, J., & Kalk, K. H. (1981b) *Nature (London) 289*, 604–606.

Dijkstra, B. W., Renetseder, R., Kalk, K. H., Hol, W. G., & Drenth, J. (1983) *J. Mol. Biol. 168*, 163–179.

Donné-Op den Kelder, G. M., Hille, J. D. R., Dijkman, R., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4074–4078.

Hendrickson, H. S., Trygstad, W. M., Loftness, T. L., & Sailer, S. L. (1981) *Arch. Biochem. Biophys. 212*, 508–514.

Hille, J. D. R., Donnê-Op den Kelder, G. M., Sauve, P., de Haas, G. H., & Egmond, M. R. (1981) *Biochemistry 20*, 4068–4073.
Hille, J. D. R., Egmond, M. R., Dijkman, R., Van Oort, M. G., Sauve, P., & de Haas, G. H. (1983) *Biochemistry 22*, 5353–5358.
Hoffman, W. J., Vahey, M., & Hajdu, J. (1983) *Arch. Biochem. Biophys. 221*, 361–370.
Jackson, M. L., Schmidt, C. F., Lichtenberg, D., Litman, B. J., & Albert, A. D. (1982) *Biochemistry 21*, 4576–4582.
Keana, J. F. W., Boyd, S. A., McMillen, D. A., Bernard, E. M., & Jost, P. C. (1982) *Chem. Phys. Lipids 31*, 339–349.
Keith, C., Feldman, D. S., Deganello, C., Glick, J., Ward, K. B., Jones, E. O., & Sigler, P. B. (1981) *J. Biol. Chem. 256*, 8602–8607.
Kremer, J. M. H., Van den Esker, M. W. J., Pathmamanoharan, C., & Wiersema, P. H. (1977) *Biochemistry 16*, 3932–3935.
Lichtenberg, D., Robson, R. J., & Dennis, E. A. (1983) *Biochim. Biophys. Acta 737*, 285–304.
Mimms, L. T., Zampighi, G., Nozaki, Y., Tanford, C., & Reynolds, J. A. (1981) *Biochemistry 20*, 833–840.
Nieuwenhuizen, W., Kunze, H., & de Haas, G. H. (1974) *Methods Enzymol. 32B*, 147–154.
Pieterson, W. A., Vidal, J. C., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13*, 1455–1460.
Pluckthun, A., & Dennis, E. A. (1985) *J. Biol. Chem. 260*, 11099–11106.
Rosevear, P., Van Aken, T., Baxter, J., & Ferguson-Miller, S. (1980) *Biochemistry 19*, 4108–4115.
Schmidt, D., Gahwiller, Ch., & Von Planta, C. (1981) *J. Colloid Interface Sci. 83*, 191–198.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.
Small, D. M., Bourgès, M. C., & Dervichian, D. G. (1966) *Biochim. Biophys. Acta 125*, 563–580.
Soares de Araujo, P., Rosseneu, M. Y., Kremer, J. M. H., Van Zoelen, E. J. J., & de Haas, G. H. (1979) *Biochemistry 18*, 580–586.
Szoka, F., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.
Thuren, T., Vainio, P., Virtanen, J. A., Somerharju, P., Blomqvist, K., & Kinnunen, P. K. J. (1984) *Biochemistry 23*, 5129–5134.
Van Dam-Mieras, M. C. E., Slotboom, A. J., Pieterson, W. A., & de Haas, G. H. (1975) *Biochemistry 14*, 5387–5394.
Van Deenen, L. L. M., de Haas, G. H., & Heemskerk, C. H. Th. (1963) *Biochim. Biophys. Acta 67*, 295–304.
Verger, R. (1980) *Methods Enzymol. 64B*, 340–392.
Verger, R., Mieras, M. C. E., & de Haas, G. H. (1973) *J. Biol. Chem. 248*, 4023–4034.
Verheij, H. M., Slotboom, A. J., & de Haas, G. H. (1981) *Rev. Physiol. Biochem. Pharmacol. 91*, 91–203.
Volwerk, J. J., & de Haas, G. H. (1982) in *Lipid-Protein Interactions* (Jost, P. C., & Griffith, O. H., Eds.) Vol. I, pp 69–149, Wiley-Interscience, New York.
Volwerk, J. J., Jost, P. C., de Haas, G. H., & Griffith, O. H. (1984) *Chem. Phys. Lipids 36*, 101–110.
Wells, M. A. (1972) *Biochemistry 11*, 1030–1041.

Inhibition of Lipases by Proteins: A Binding Study Using Dicaprin Monolayers†

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ABSTRACT: We previously reported that the inhibition of pancreatic and *Rhizopus delemar* lipases by proteins is due to the protein associated with lipid and is not caused by direct protein–enzyme interaction in the aqueous phase [Gargouri, Y., Piéroni, G., Rivière, C., Sugihara, A., Sarda, L., & Verger, R. (1985) *J. Biol. Chem. 260*, 2268–2273]. In this study, using radiolabeled lipases, serum albumin, and β-lactoglobulin A, we investigated their respective binding with respect to lipolysis of dicaprin monolayers. Lipase inhibition was found to be correlated with a lack of lipase binding to mixed protein–dicaprin films or to a desorption of lipase from the interface when inhibitory protein was added later. Since a large proportion of the lipid film remained potentially accessible to the enzyme in the presence of inhibitory protein, it was concluded that the observed decrease in lipase binding to the interface was due to a variation of the physicochemical properties of the lipid–water interface following binding of inhibitory protein. On the basis of the results presented here, it is proposed that mixed protein–glyceride films could be used to characterize the interaction of various lipases with lipid substrates and to classify these enzymes according to their penetration power.

Hydrolysis of triacylglycerol by lipase occurs at the oil–water interface where the enzyme binds. As shown by several authors, pancreatic and microbial lipase activities are inhibited by bile salts at concentrations in the millimolar range (Canioni et al., 1977; Borgström et al., 1979; Sémériva & Desnuelle, 1979). Several research groups (Borgström, 1975; Chapus et al., 1975; Vandermeers et al., 1975, 1976; Momsen & Brockman, 1976a) have presented evidence that inactivation of pancreatic lipase by bile salts results from enzyme desorption from the interface. A hypothesis was initially put forward according to which the lipase might be prevented from reaching its substrate by a layer of bile salt molecules formed at the surface of the emulsified substrate (Morgan et al., 1969;

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