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# Influence of the Structure of the Lipid–Water Interface on the Activity of Hepatic Lipase†

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*Received August 19, 1987; Revised Manuscript Received November 3, 1987*

ABSTRACT: Factors affecting the hydrolytic activity of purified rat hepatic lipase have been examined in mixed-monolayer systems. When nonsubstrate lipids [either egg sphingomyelin or β-*O*-hexadecyl-γ-*O*-(1-octadec-9-enyl)-DL-phosphatidylcholine (OPPC-ether)] were used as inert matrices, hydrolytic activity for both triolein and dioleoylphosphatidylethanolamine was shown to decrease with increasing surface pressure ($\pi$); negligible activity occurred at $\pi \geq 30$ mN/m. Examination of the effect of introduction of cholesterol into either matrix containing 2 mol % triolein indicated that the mean molecular area decreased with increasing cholesterol and that, at $\pi = 24$ mN/m, triolein was fully miscible in the sphingomyelin matrix at cholesterol concentrations ≤32.5 mol % and in the OPPC-ether matrix at cholesterol concentrations ≤49 mol %. Above these critical concentrations of cholesterol, the phase diagrams indicate transitions that suggest that triolein is forced out of the monolayer. Introduction of increasing amounts of cholesterol into either inert matrix increased the rate of hydrolysis of triolein by hepatic lipase, although by different degrees. There are at least two factors contributing to these effects: (1) condensation of the monolayer by cholesterol, thus increasing the total surface concentration of triolein at $\pi = 24$ mN/m in the constant area surface balance, and (2) some change in triolein conformation and/or accessibility since at identical surface concentrations of triolein ($8.7 \pm 0.1$ pmol/cm$^2$) and $\pi$ (24 mN/m) the rate of hydrolysis of triolein by hepatic lipase is 1.5-fold higher in the OPPC-ether matrix than in the egg sphingomyelin matrix. A number of human apolipoproteins [A-I, A-II, C-II, and C-III(1,2)] were observed to inhibit the hydrolysis by hepatic lipase of 2 mol % triolein in an OPPC-ether matrix at subphase concentrations of apolipoprotein ≥0.1 nM.

The processing of lipoproteins in the plasma compartment is mediated by two enzymes, lipoprotein lipase (LPL)[1] and hepatic triglyceride lipase (HL) (Kinnunen, 1984; Jackson, 1983). Both enzymes have the capacity to hydrolyze triglycerides as well as phospholipids; however, they have different preferences for lipoprotein substrates. LPL, which is found in extrahepatic tissues, utilizes chylomicrons and very low density lipoproteins (VLDL) as its physiologic substrates and requires the participation of apolipoprotein C-II as an activator. Although LPL can hydrolyze both phosphatidylcholine and phosphatidylethanolamine, this phospholipase $A_1$ activity is low relative to its triglyceridase activity (Jackson, 1983). As its name suggests, HL is found in the liver, although a similar if not identical activity has been found in steroidogenic tissues (Persoon et al., 1986). HL is thought to play a role in processing chylomicron remnants, intermediate-density lipoproteins, and high-density lipoproteins (Daggy & Bensadoun, 1986). Like LPL, HL is an active triglyceridase but also has phospholipase $A_1$ activity (Laboda et al., 1986).

A difficulty in characterizing and comparing substrate specificities of lipases arises in the presentation of the lipid substrate. For studies on LPL and HL, many investigators have used lipoproteins as substrates, which present a complex and often uncontrollable mixture of lipids and apolipoproteins. Even less complex substrates, such as emulsions or liposomes, are often difficult to prepare in a reproducible manner. A particularly useful system for the study of the substrate specificity of lipases has been a lipid monolayer spread at the air–water interface. In this system, the composition and concentrations of the lipids in the monolayer, as well as surface pressure, can be varied in a controlled manner. Also, the effects of the addition of individual apolipoproteins can be assessed. Previous studies on the behavior of both LPL and HL in monolayer systems indicated that the effects of monolayer composition and surface pressure on the two enzymes are quite different (Demel et al., 1982, 1984; Jackson et al., 1986; Laboda et al., 1986). In the present study, we have used the monolayer system to examine the effects of surface charge and monolayer composition on the activity of HL. In particular, we have focused on factors that might influence the activity of HL at the surface of lipoproteins, namely, the

† Supported by Research Grants HL 22633 and HL 07443 from the National Heart, Lung, and Blood Institute of the National Institutes of Health and by a grant from the W. W. Smith Charitable Trust.
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[1] Abbreviations: HL, hepatic lipase; DOPE, 1,2-dioleoyl-L-3-phosphatidylethanolamine; DOPC, 1,2-dioleoyl-L-3-phosphatidylcholine; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; OPPC-ether, β-*O*-hexadecyl-γ-*O*-(1-octadec-9-enyl)-DL-phosphatidylcholine; $\pi$, surface pressure; *A*, mean molecular area (angstroms squared per molecule); LPL, lipoprotein lipase; VLDL, very low density lipoprotein(s); apo, apolipoprotein; HDL, high-density lipoprotein(s); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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surface concentration of cholesterol and apolipoproteins. As both of these components can modulate lipid packing in monolayers, the dependence of lipolytic activity on surface pressure was also explored.

## Materials and Methods

*Lipids.* Glycerol [9,10-$^3$H]trioleate (1.0 Ci/mmol), glycerol [1-$^{14}$C]trioleate ([$^{14}$C]triolein) (55.5 mCi/mmol), 1,2-dioleoyl-L-3-phosphatidyl[2-$^{14}$C]ethanolamine (DOPE), and [1-$^{14}$C]dioleoyl-L-3-phosphatidylcholine (DOPC) (114 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Triolein, cholesterol, dicetyl phosphate, and fatty acid free bovine serum albumin (fraction V) were purchased from Sigma (St. Louis, MO). Egg sphingomyelin and β-*O*-hexadecyl-γ-*O*-(1-octadec-9-enyl)-DL-phosphatidylcholine (OPPC-ether) were purchased from Calbiochem (San Diego, CA). Dioleoylphosphatidylethanolamine was purchased from Avanti Polar Lipids (Birmingham, AL). The purities of the lipids were established by thin-layer chromatography on Anasil-G plates (Analabs, North Haven, CT) with a solvent system consisting of either petroleum ether/diethyl ether/acetic acid (60:40:1.5 v/v) or chloroform/methanol/acetic acid/water (50:30:8:4 v/v).

*Hepatic Lipase and Apolipoproteins.* HL was isolated from heparin-containing rat liver perfusates as described Jensen and Bensadoun (1981). The enzyme was subsequently concentrated on a heparin–Sepharose affinity column (bed volume 1 mL). The enzyme was eluted with 5 mM barbital buffer (pH 7.2) containing 1 M NaCl and 30% glycerol (v/v) (Laboda et al., 1986) and stored in small aliquots at −70 °C. The purified enzyme had a specific activity of 26 000 μmol of fatty acid produced $h^{-1}$ $mg^{-1}$ in an assay system of [$^3$H]triolein emulsified in 5% gum arabic (w/v) (Jensen & Bensadoun, 1981).

Human apolipoprotein A-I (apo A-I) and apolipoprotein A-II (apo A-II) were prepared from HDL. Apolipoprotein C-II (apo C-II), apolipoprotein C-III(1,2) [apo C-III(1,2)], and total apolipoprotein's C (apo C's) were prepared from VLDL. HDL and VLDL were isolated from fresh human plasma using sequential ultracentrifugation (Hatch & Lees, 1968). The total HDL was delipidated by using the procedure of Scanu (1966). Apo A-I and apo A-II were isolated as described by Reijngoud and Phillips (1984) and were stored at −20 °C. The apo A-I and apo A-II preparations both showed a single band on SDS–PAGE. The total apo C's were prepared by using a modification of the procedure of Holmquist and Carlson (1977). Apo C-II and apo C-III(1,2) were isolated from the total apo C's by chromatofocusing using a modification of the procedure described by Knipping et al. (1984). Upon analytical isoelectrofocusing, the resultant apo C-II gave a single band, and the apo C-III(1,2) gave the expected two bands. Concentrations of the apolipoproteins were routinely calculated from their absorbances at $A_{280}$. For the A apolipoproteins, standard values [apo A-I = 1.23 mL/(mg·cm) and apo A-II = 0.87 mL/(mg·cm)] were established experimentally relative to values determined by a modified Lowry assay (Markwell et al., 1978) using BSA as the standard. For the apo C preparations, published values of weight extinction coefficients [apo C-II = 1.36 mL/(mg·cm) and apo C-III = 2.24 mL/(mg·cm)] were used (Pownall & Massey, 1986).

Prior to use for the monolayer studies, all of the apoprotein preparations were dissolved at a concentration of 2 mg/mL in the trough buffer (described below) containing 4 M guanidine hydrochloride and were stored at 4 °C for at least 12 h. The apolipoproteins were then chromatographed on a Bio-Gel P-2 column (1 cm × 10 cm) to remove the guanidine hydrochloride. This treatment ensured that the apolipoproteins were in a monomeric state (Phillips & Krebs, 1986).

*Radiolabeling of Apolipoprotein A-I.* The lysine residues of apo A-I were labeled with $^{14}$C using the reductive methylation method of Jentoft and Dearborn (1983). The specific activity of the $^{14}$C-labeled apo A-I was 1.8 μCi/mg.

*Surface Pressure–Molecular Area Isotherms.* The surface pressure ($\pi$) as a function of average molecular area ($A$) of mixed lipid films at 33 °C was measured on a surface balance by a procedure described previously (Laboda et al., 1986). A movable Teflon barrier compressed the monolayer in a continuous manner at a rate of 27.4 $cm^2$/min. Surface pressures for a given molecular area (angstroms squared per molecule) were reproducible to within 1 mN/m ($n$ = 3).

*Monolayer Assay of HL Activity.* Measurements of the hydrolytic activity of HL were performed in a Teflon dish, 10.8 cm in diameter (area = 91.6 $cm^2$), as described previously (Laboda et al., 1986). The subphase contained 40 mL of buffer (100 mM Tris-HCl, pH 8.6, 155 mM NaCl, and 5 mM $CaCl_2$) at 33 °C and was stirred with small magnetic bars. The $^{14}$C-labeled substrate lipids were mixed with either OPPC-ether or egg sphingomyelin and various amounts of cholesterol in hexane/ethanol (9:1 v/v). For most of the studies, the lipids were spread as monolayers on the surface of the buffer to an initial $\pi$ of 24 ± 1 mN/m. After the surface radioactivity and $\pi$ had stabilized (4–8 min), fatty acid free BSA and HL were added to the subphase to give concentrations of 6.8 μg/mL (0.1 μM) and 1 ng/mL, respectively. The desorption of [$^{14}$C]oleic acid at a concentration of 6 mol % in a monolayer of sphingomyelin/cholesterol (2:1) followed first-order kinetics with a half-time of 5 min. This rate was at least 10 times faster than that observed for the hydrolysis of triolein. The surface radioactivity was measured by a Baird Atomic gas flow detector, and $\pi$ was measured by the Wilhelmy plate technique. To determine the effect of $\pi$ on the hydrolysis of triolein and DOPE by HL, monolayers consisting of either 2 mol % triolein or 2 mol % DOPE were spread to the desired $\pi$. BSA and HL were added to the subphase to final concentrations of 68 μg/mL (1.0 μM) and 1.5 ng/mL, respectively. The higher concentration of BSA was used to ensure the rapid desorption of the lyso-PE product of hydrolysis (Laboda et al., 1986). HL activities are expressed as micromoles of fatty acid produced per hour per milligram of injected HL unless otherwise indicated. Results are expressed as the mean ± SD of triplicate measurements, or the average of duplicates, within 10%, as indicated.

To determine the effect of the apolipoproteins on the hydrolysis of triolein by HL, a monolayer consisting of 2 mol % triolein in OPPC-ether/cholesterol (2:1 mol/mol) was spread. The apolipoproteins were added to the subphase and were mixed for 30 min before the addition of BSA and HL. The surface concentration of apo A-I was determined by injecting $^{14}$C-labeled apo A-I into the subphase of a monolayer consisting of 2 mol % triolein in OPPC-ether/cholesterol (2:1 mol/mol). The amount of apo A-I adsorbed to the surface was calculated from the increase of radioactivity after 30 min.

*Analytical Procedures.* The protein concentration of HL was measured by the method of Bradford (1976), using BSA as the standard, or by the absorbance at 280 nm as described above. Phospholipid phosphorus was determined according to the procedure of Sokoloff and Rothblat (1974).

## Results

*Effects of Surface Pressure, Surface Charge, and Enzyme Concentration.* A comparison of the effect of increasing

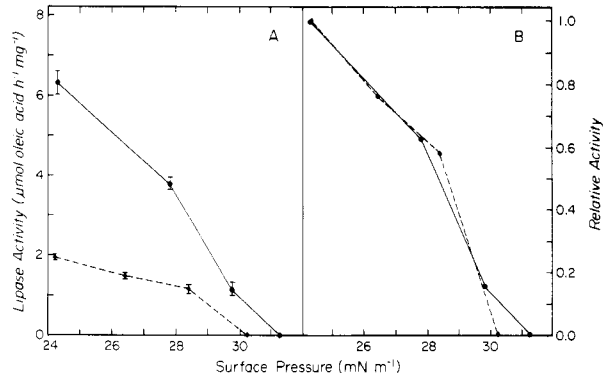


FIGURE 1: Effect of surface pressure on the hydrolysis of [$^{14}$C]triolein and $^{14}$C-labeled DOPE by HL. The monolayers were composed of either 3 mol % triolein (●—●) or 3 mol % DOPE (♦---♦) in an OPPC-ether matrix. (A) Rate of oleic acid production from triolein or DOPE by HL as a function of surface pressure ($n$ = 3). (B) Relative activity as a function of surface pressure: hepatic lipase activity at 24 mN/m = 1. The details for the hydrolysis reaction are described under Materials and Methods. The subphase consisted of 100 mM Tris-HCl (pH 8.6), 155 mM NaCl, and 5 mM $CaCl_2$, $T$ = 33 °C. The concentrations of fatty acid free bovine serum albumin and HL in the subphase were 68 μg/mL and 1.5 ng/mL, respectively.

surface pressure on the hydrolysis of triolein or DOPE in an OPPC-ether matrix is shown in Figure 1 (panels A and B). In each case, the substrate lipid was present at 3 mol %, a concentration previously shown to be completely miscible in phospholipid matrices (Laboda et al., 1986; Cullis et al., 1985). It is apparent from Figure 1B that an increase of surface pressure decreased the activity of HL in a similar manner when either triolein or DOPE was used as substrate. At $\pi$ = 28 mN/m, the enzyme activity was 50% of maximum, and at 30–31 mN/m, very little activity could be measured. The absolute rates of oleic acid production from triolein and from DOPE in the expanded OPPC-ether monolayer are shown in Figure 1A. At $\pi$ = 24 mN/m under similar experimental conditions, the activity of the enzyme with DOPE as a substrate was one-third of that with triolein. We reported earlier (Laboda et al., 1986) that HL produced oleic acid from 1 mol % triolein and DOPE in a condensed monolayer of sphingomyelin/cholesterol (2:1 mol/mol) at equal rates. Comparison of these results suggests that, when $\pi$ = 24 mN/m, HL hydrolyzes triolein and DOPE equally well when they are present in a condensed monolayer but triolein is a better substrate when the monolayer is expanded. The reasons for this difference in relative substrate specificity are not obvious but presumably involve some change in conformation of either the triolein or the DOPE molecules.

The effect of a negative charge in the lipid monolayer was explored by using a monolayer containing dicetyl phosphate in a sphingomyelin/cholesterol matrix. To optimize the capacity for detecting changes in hydrolytic rate, a sphingomyelin:cholesterol ratio of 3:1 (mol/mol) was chosen. The hydrolysis of 2 mol % triolein in the monolayer of sphingomyelin/cholesterol (3:1) was approximately twice that observed in a similar monolayer wherein 5 mol % dicetyl phosphate was substituted for 5 mol % of the sphingomyelin: 17.6 ± 1.6 versus 9.3 ± 0.4 μmol of oleic acid produced h$^{-1}$ (mg of HL)$^{-1}$ ($n$ = 3).

The rate of hydrolysis of triolein by HL as a function of the amount of enzyme is shown in Figure 2. With 2 mol % triolein in a monolayer of sphingomyelin/cholesterol (2:1 mol/mol), the hydrolysis of triolein was dependent upon the amount of enzyme added up to 1 ng/mL. Within the concentration range tested (0.25–2.0 ng/mL), no change in surface pressure was observed upon addition of the enzyme.

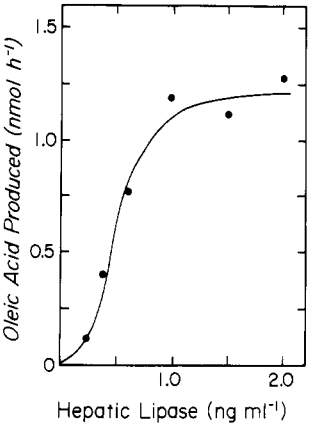


FIGURE 2: Effect of HL concentration on the production of oleic acid from triolein. The monolayer was composed of 2 mol % [$^{14}$C]triolein in an egg sphingomyelin/cholesterol (2:1 mol/mol) matrix spread at $\pi$ = 24 mN/m. The subphase consisted of 100 mM Tris-HCl (pH 8.6), 155 mM NaCl, and 5 mM $CaCl_2$, $T$ = 33 °C. The concentration of fatty acid free bovine serum albumin in the subphase was 6.8 μg/mL, and hepatic lipase was added at the indicated concentrations. The details of the hydrolysis reactions are described under Materials and Methods. The hydrolysis rates are an average of two analyses, which agreed to within 10%.

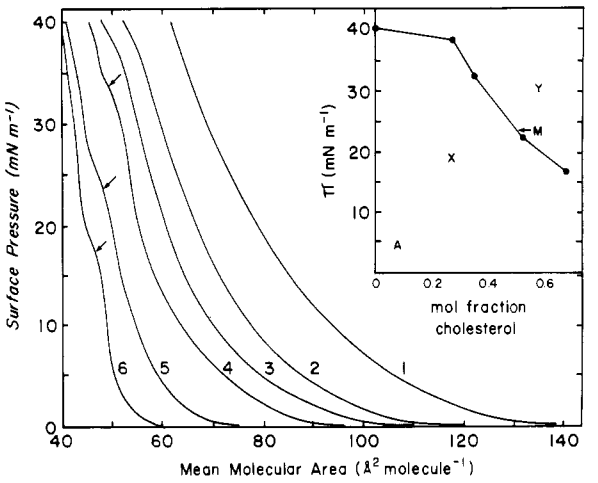


FIGURE 3: Surface pressure–molecular area isotherms for monolayers of 2 mol % triolein in an OPPC-ether matrix with increasing concentrations of cholesterol: curve 1, 0 mol %; curve 2, 14 mol %; curve 3, 24.5 mol %; curve 4, 32.8 mol %; curve 5, 49 mol %; curve 6, 65.3 mol %. The subphase contained 100 mM Tris-HCl (pH 8.6), 155 mM NaCl, and 5 mM $CaCl_2$ at a temperature of 33 °C. Transitions occurring in the isotherms below 40 mN/m are marked with an arrow. The inset shows the phase diagram for the triolein/OPPC-ether monolayer. The transition pressures were identified from the surface pressure–molecular area isotherms. Below the transition pressure, one phase (X) existed in the mixed monolayers, indicating that the three lipids were miscible. Above the transition pressure, two phases (Y) existed in the mixed monolayer: miscible lipids and triolein-rich lenses. At a surface pressure of 24 mN/m, triolein is completely miscible at concentrations of cholesterol <49 mol % (M).

*Effects of Cholesterol. (A) Lipid Packing.* To determine the effect that cholesterol has on the matrix lipids and to establish the degree to which triolein is miscible in monolayers containing cholesterol, $\pi$-A isotherms were determined for monolayers consisting of 2 mol % triolein in either OPPC-ether or sphingomyelin with different concentrations of cholesterol. The $\pi$-A isotherms of the monolayers used in this study reflect the well-known condensing effect that cholesterol has on phospholipids in the liquid-crystal state (Phillips, 1972) and the incomplete miscibility of triolein at several cholesterol concentrations. The triolein/OPPC-ether monolayer at 33 °C was expanded, and triolein was completely miscible (Figure

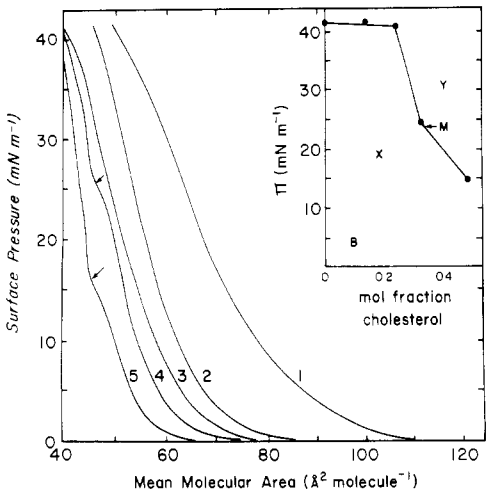


FIGURE 4: Surface pressure–molecular area isotherms for monolayer consisting of 2 mol % triolein in an egg sphingomyelin matrix with increasing concentrations of cholesterol: curve 1, 0 mol %; curve 2, 14 mol %; curve 3, 24.5 mol %; curve 4, 32.5 mol %; curve 5, 49 mol %. The subphase is the same as that described in Figure 3. Transitions occurring in the isotherms below 40 mN/m are marked with an arrow. The inset shows the phase diagram for triolein/egg sphingomyelin/cholesterol monolayers. At $\pi$ = 24 mN/m, triolein is completely miscible in the monolayer at cholesterol concentrations ≤32.5 mol % (M).

3, curve 1); at $\pi$ = 24 mN/m, the mean molecular area for the monolayer was 74 Å$^2$. With increasing concentrations of cholesterol up to 65.3 mol %, the mean molecular area at this surface pressure decreased to 44 Å$^2$ (Figure 3, curve 6). With concentrations of cholesterol of 32.8, 49.0, and 65.3 mol %, transitions are apparent in the $\pi$-A isotherms, indicating that the component forming the least stable monolayer is preferentially forced out of the surface (see arrows in curves 4, 5, and 6 of Figure 3). Since among the lipids used in this study triolein forms the least stable monolayer with a low collapse pressure of approximately 10 mN/m (Merker & Daubert, 1964; Smaby & Brockman, 1978), a triolein-rich mixture is presumably being forced out of the surface. A plot of the transition pressure versus the mole fraction of cholesterol in the triolein/OPPC-ether monolayers gives a phase diagram for the surface phase (Figure 3, inset). At the $\pi$ at which most of the hydrolytic studies are conducted, 24 mN/m, triolein is miscible with the other lipids at concentrations of cholesterol ≤49 mol % (see point M in Figure 3, inset).

The triolein/egg sphingomyelin monolayer was more condensed than the triolein/OPPC-ether monolayer (Figure 4, curve 1). At 24 mN/m, the mean molecular area was 65 Å$^2$; with increasing percentages of cholesterol up to 49 mol %, the mean molecular area decreased to 43 Å$^2$. At cholesterol concentrations of 32.8 and 49 mol %, there were transitions in the $\pi$-A isotherms (see arrows in curves 4 and 5 in Figure 4), and the phase diagram (Figure 4, inset) shows that at 24 mN/m triolein was miscible in the monolayers at concentrations of cholesterol <32.5 mol % (see point M in Figure 4, inset).

(*B*) *Enzymatic Activity.* The effect of the cholesterol content of the monolayer on the hydrolysis of triolein by HL was investigated by using OPPC-ether or egg sphingomyelin matrices at 24 mN/m. When either type of matrix contained 2 mol % triolein, there was an increase in the rate of hydrolysis with increasing percentages of cholesterol up to the miscibility limits for each matrix [49 and 33 mol % for the OPPC-ether and sphingomyelin matrices, respectively (Figure 5A)]. Although the mole percent of triolein was maintained at 2 mol %, the condensing effect of cholesterol (cf. Figures 3 and 4)

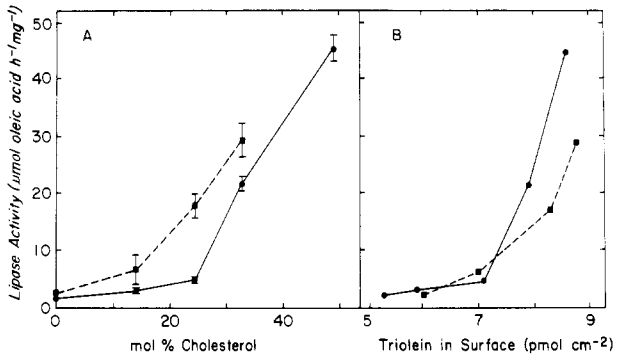


FIGURE 5: Effect of the monolayer cholesterol content (A) and the consequent triolein surface concentration (B) on HL activity. Panel A shows the hydrolytic activity of HL as a function of increasing cholesterol concentration between 0 and 49 mol % in a monolayer consisting of 2 mol % [$^{14}$C]triolein in an OPPC-ether matrix (●—●) and from 0 to 32.5 mol % in a monolayer consisting of 2 mol % [$^{14}$C]triolein in an egg sphingomyelin matrix (■- - -■) ($n$ = 3). Panel B shows the hydrolytic activity of HL as a function of the surface concentration of triolein in an OPPC-ether matrix (●—●) and an egg sphingomyelin matrix (■- - -■). The concentrations of triolein in the OPPC-ether matrix were 5.3, 5.9, 7.1, 7.9, and 8.6 pmol/cm$^2$, respectively, at cholesterol concentrations of 0, 14, 24.5, 32.8, and 49 mol % (cf. Figure 3), and the concentrations of triolein in the egg sphingomyelin matrix were 6.0, 7.0, and 8.3, and 8.8 pmol/cm$^2$, respectively, at cholesterol concentrations of 0, 14, 24.5, and 32.5 mol % (cf. Figure 4). The surface pressure was 24 mN/m, and the other experimental conditions were the same as those described in the legend to Figure 2.

allows a greater number of lipid molecules to occupy a given surface area. The hydrolytic activity of HL at each cholesterol concentration plotted as a function of the surface concentration of triolein is shown in Figure 5B. The maximum activity of HL was obtained when triolein was present at a concentration of 8.7 ± 0.1 pmol/cm$^2$ in both the OPPC-ether and the sphingomyelin matrices, which corresponds to monolayer compositions of cholesterol of 49 and 33 mol %, respectively. When the mole fraction of cholesterol was increased to 65 mol % in the OPPC-ether matrix, the surface concentration of triolein increased to 9.8 pmol/cm$^2$; when cholesterol was increased to 49 mol % in the sphingomyelin matrix, the surface concentration of triolein increased to 9.2 pmol/cm$^2$. However, there were 65% and 29% reductions of the hydrolytic activity of HL in the OPPC-ether and sphingomyelin matrices, respectively, probably reflecting the incomplete miscibility of triolein at these cholesterol concentrations and the breakdown of the homogeneous monolayer system.

A comparison of the absolute activity of HL at 8.7 pmol of triolein/cm$^2$ shows a 1.5-fold higher rate of hydrolysis of triolein in the OPPC-ether matrix as compared to the sphingomyelin matrix: 46 ± 2 versus 29 ± 3 μmol of oleic acid h$^{-1}$ (mg of HL)$^{-1}$, respectively. In addition, another difference between the two types of matrix can be seen. The graph for the hydrolysis of triolein by HL as a function of cholesterol concentration in the OPPC-ether monolayers (Figure 5A) shows two relatively linear regions: (1) from 0 to 24.5 mol % cholesterol, where low activity of HL is observed (≤5 μmol of oleic acid h$^{-1}$ mg$^{-1}$); (2) from 24.5 to 49 mol % cholesterol, which corresponds to much higher activity of HL (up to 46 μmol of oleic acid h$^{-1}$ mg$^{-1}$). A similar relationship is seen with the activity of HL as function of the triolein concentration in the OPPC-ether monolayers (Figure 5B). In contrast, the activity of HL as a function of cholesterol (Figure 5A) or triolein concentration (Figure 5B) in the sphingomyelin monolayers shows a progressive increase.

*Effects of Apolipoproteins.* The effects of apolipoproteins on the hydrolytic activity of HL were studied by using a

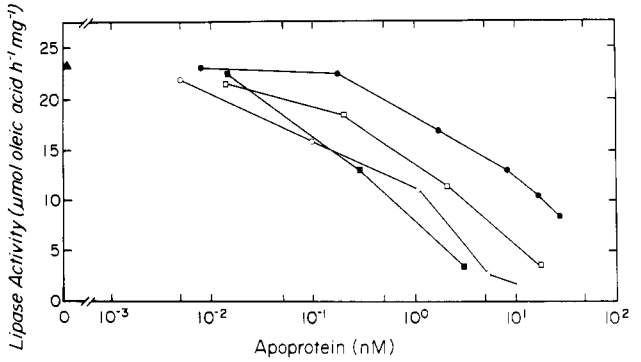


FIGURE 6: Effect of apolipoproteins on the hydrolytic activity of HL. The monolayer conisted of 2 mol % [$^{14}$C]triolein in OPPC-ether/cholesterol (2:1 mol/mol). After the monolayer was stabilized at a surface pressure of 24 mN/m, the individual apoprotein preparations were injected into the subphase and were mixed for 30 min. Fatty acid free bovine serum albumin and HL were subsequently added to the subphase to concentrations of 6.8 μg/mL and 1.0 ng/mL, respectively, and the hepatic lipase activity was determined: apo A-I (○); apo A-II (●); apo C-II (■); apo C-III(1,2) (□); no apoprotein (▲). The other experimental conditions are the same as those in Figure 2.

monolayer containing 2 mol % triolein in an OPPC-ether/cholesterol (2:1) matrix. The hydrolytic activity of HL decreased as the concentration of apoproteins that were injected into the subphase increase from $5 \times 10^{-3}$ to $2.9 \times 10^{1}$ nM (Figure 6). Overall, apo C-II had the greatest inhibiting effect followed by apo A-I > apo C-III(1,2) > apo A-II. A mixture of total apo C's also had an inhibiting effect on the hydrolytic activity when 0.12 and 1.2 μg were injected into the subphase: 17.0 and 11.4 μmol of oleic acid $h^{-1}$ (mg of HL)$^{-1}$, respectively, versus 22.8 μmol of oleic acid $h^{-1}$ (mg of HL)$^{-1}$ in the absence of apolipoproteins. There was no increase in $\pi$ associated with the addition to the subphase of any of the apo C preparations or with the addition of apo A-I or apo A-II at concentrations below $1 \times 10^{0}$ and $1.8 \times 10^{-1}$ nM, respectively. With higher concentrations of apo A-I and apo A-II, there was an increase in $\pi$ to a maximum value of about 28 mN/m. The HL activity with an apo A-I concentration of $1.1 \times 10^{1}$ nM at $\pi$ = 28 mN/m was 1.8 μmol of oleic acid $h^{-1}$ (mg of HL)$^{-1}$ while the activity with an apo A-II concentration of $2.9 \times 10^{1}$ nM at $\pi$ = 27 mN/m was 8.4 μmol of oleic acid $h^{-1}$ (mg of HL)$^{-1}$. These were 8% and 37% of the control hydrolytic activity of HL in the absence of apolipoproteins at $\pi$ = 24 mN/m. When the hydrolytic activity of HL was measured in the absence of apolipoproteins but at $\pi$ = 28 mN/m, the rate was 56% of the rate measured at $\pi$ = 24 mN/m: 12.7 μmol of oleic acid $h^{-1}$ $mg^{-1}$ versus 22.8 μmol of oleic acid $h^{-1}$ (mg of HL)$^{-1}$.

To quantitate the amount of apo A-I that adsorbed to the triolein/OPPC-ether/cholesterol monolayer, 12 μg (428 pmol) of $^{14}$C-labeled apo A-I was injected into the subphase which is equivalent to a subphase concentration of $1.1 \times 10^{1}$ nM. The increase of radioactivity in the surface was measured after 30 min, and the amount of apo A-I was calculated. Approximately 7 pmol or 1.6% of the apo A-I was adsorbed to the surface. Adsorption of smaller amounts of $^{14}$C-labeled apo A-I could not be monitored because of the low sensitivity of the gas flow detector.

## Discussion

The use of monolayer systems to study the activity of lipolytic enzymes has an advantage over bulk assay systems in that the composition and the packing of the lipid molecules in the monolayer can be varied and controlled (Verger & Pattus, 1982). Such control of the physical state of the substrate facilitates studies of the influence of substrate molecular structure on the rate of lipolysis. HL is a serine esterase, a property it has in common with pancreatic lipase and lipoprotein lipase (Komaromy & Schotz, 1987). These lipases first adsorb to the lipid–water interface through a lipid binding site(s), and then the catalytic step ensues (Brockman, 1984). A determinant of the rate of hydrolysis is the interfacial concentration of active lipase molecules. In order to obtain reliable kinetic measurements for hydrolysis by HL, it is necessary to control the interfacial concentration of HL molecules. This condition can be achieved either by saturating the lipid–water interface with HL molecules (cf. Figure 2) or by maintaining a constant interfacial concentration of HL. When a given phospholipid monolayer at a fixed $\pi$ is employed as the inert matrix for a few mole percent of substrate molecules, a given concentration of HL in the well-mixed subphase provides an essentially constant interfacial concentration of enzyme molecules. This latter condition is employed in the present investigation of the influence of the "quality of the interface" (Verger & Pattus, 1982) on the activity of HL. In particular, the ways that variations in surface pressure, cholesterol content and apolipoprotein content influence the enzyme–substrate interaction are explored as these parameters are likely to affect the behavior of a lipoprotein particle as a substrate for HL in vivo.

With increasing surface pressure, both the relative triolein hydrolase and phospholipase $A_1$ activities of HL decreased in a similar manner as the molecules in the triolein/OPPC-ether monolayer became more tightly packed. At surface pressures >30 mN/m, HL activity is negligible (Figure 1B). This is consistent with the results reported by Jackson et al. (1986) for the hydrolysis of triolein in an egg PC monolayer by either LPL or HL. This phenomenon has been demonstrated by Verger et al. (1976) for pancreatic lipase $A_2$ and is thought to be due to the inability of enzyme molecules to penetrate the tightly packed surface to interact with the lipid substrate. Generally, when $\pi \geq 30$ mN/m, protein molecules cannot penetrate into a phospholipid monolayer [for a review, see Verger and Pattus (1982)].

The enhancement in HL activity with increasing cholesterol content in either OPPC-ether or egg sphingomyelin monolayers (Figure 5A) is probably due to the condensation of phospholipid molecules by cholesterol. This effect increases the total surface concentration of substrate triolein molecules packed in a given surface area. When the lipolytic activities are normalized with respect to the total triolein surface concentration, the activity of HL is essentially the same in both egg sphingomyelin and OPPC-ether monolayers at triolein concentrations below 7 pmol/cm$^2$ (equivalent to 14 and 25 mol % cholesterol for the sphingomyelin and OPPC-ether monolayers, respectively). At higher triolein surface concentrations, the OPPC-ether matrix give greater HL activity than the sphingomyelin/cholesterol matrix (Figure 5B). At 8.7 ± 0.1 pmol of triolein/ cm$^2$, the mean molecular area in either matrix is 48 Å$^2$/molecule at $\pi$ = 24 mN/m (cf. curve 5 in Figure 3 and curve 4 in Figure 4), so the difference in HL activity is not simply attributable to a difference in average lipid packing density. Other phenomena that might account for the observed difference in activity are a change in the conformation/accessibility of the triolein substrate molecules, a change in the conformation of HL at the interface, or a change in the surface concentration of HL. To clarify the role of this last variable, an independent measure of the concentration of HL adsorbed to the lipid monolayer is required; this measurement is not technically feasible at present. Nonetheless, the data in Figure

5 suggest that the relatively close molecular packing induced by high levels of cholesterol tends to promote the rate of hydrolysis by HL of any triolein molecules in the monolayer.

It is well-known that apolipoproteins can adsorb to air–water interfaces (Phillips & Sparks, 1980) and to phospholipid–water interfaces (Jackson et al., 1980; Krebs et al., 1983; Phillips & Krebs, 1986), thereby altering the surface pressure and molecular packing density. There is some controversy in the literature about the influence of apolipoproteins on the activity of hepatic lipase. Using bulk assay systems, Jahn et al. (1981, 1983) have reported that apo A-II serves as an activator for HL, yet a number of other investigators (Kinnunen & Ehnholm, 1976; Kubo et al., 1982; Shinomiya et al., 1982) have reported inhibition of HL by apolipoproteins. Although primary data were not presented, Jackson et al. (1986) reported inhibition of HL by apolipoproteins in a monolayer system similar to that used for the present studies and suggested that the inhibition of HL was due to an increase in the packing density of the lipid molecules in the monolayer induced by adsorption of apolipoprotein molecules. The data in Figure 6 showing that various apolipoproteins at subphase concentrations of $\geq 1 \times 10^{-1}$ nM can inhibit HL are consistent with the results reported by Jackson et al. (1986). However, in our experiments, the only increases in $\pi$ occurred with apo A-I and apo A-II at subphase conentrations $\geq$ 5.3 nM. At lower concentrations of these apolipoproteins and with all of the apo C fractions at all concentrations, there was no increase of $\pi$, although the hydrolytic activity of HL was decreased. The increase in surface pressure and lateral packing density caused by the apo A molecules may contribute to inhibition of HL, but it is probably not a major factor. When an experiment was performed at $\pi$ = 28 mN/m in the absence of apolipoproteins, the rate of lipolysis by HL was 12 times faster than that in the presence of apo A-I or apo A-II at the same $\pi$ value. It is possible that there is an interaction between HL and the apoproteins in the subphase that reduces adsorption of HL to the interface, thereby inhibiting lipolytic activity. This is consistent with the suggestion by Kubo et al. (1982) that there is competition between apo A-I and apo A-II and HL for binding to the surface of lipid substrate particles. The inhibitory effect of the apolipoproteins might also be related to their charge. At pH 8.6, the apolipoproteins we have studied have a negative charge so that they confer a negative charge on the zwitterionic PC or sphingomyelin monolayers. Since HL itself has a net negative charge at pH 8.6, the inhibition of the lipolytic activity could be due to a repulsion of the enzyme from the lipid monolayer. Consistent with this concept, when a negative charge was incorporated into the lipid monolayer by the addition of dicetyl phosphate, the hydrolysis of triolein by HL was reduced approximately 50%.

The results presented in this paper, together with those published previously (Laboda et al., 1986), indicate that the hydrolytic activity of HL is affected by a number of factors that influence the enzyme–substrate interaction in the lipid–water interface. In addition to variables such as lipid class and acyl chain composition, the rate of hydrolysis of a given lipid such as triolein is altered by (1) the packing density of the surface molecules as influenced by the surface pressure, (2) the miscibility of the substrate with the phospholipid matrix, (3) the surface charge, and (4) the cholesterol content. These parameters are all a consequence of the size, shape, and composition of a circulating lipoprotein particle. On the basis of the monolayer data (Figure 6), HL does not appear to have a specific requirement for an apolipoprotein cofactor in order to be active against lipoprotein particles. Since, unlike other lipases such as lipoprotein lipase, the rat HL molecule apparently contains two lipid binding domains, it may not require an apoprotein cofactor to mediate adsorption to the lipid–water interface (Komaromy & Schotz, 1987).

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# A New Kinetic Approach for Studying Phospholipase C (*Clostridium perfringens* $\alpha$ Toxin) Activity on Phospholipid Monolayers†

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*Received July 15, 1987; Revised Manuscript Received October 22, 1987*

ABSTRACT: The enzymatic activity of purified phospholipase C ($\alpha$ toxin) from *Clostridium perfringens* was investigated with various phospholipid monolayers. A two-step reaction was used. Enzymatic hydrolysis of insoluble lecithin films by phospholipase C, generating 1,2-diacylglycerol and water-soluble phosphocholine, was coupled with the action of pancreatic lipase in order to give rise to fatty acid and 2-monoacylglycerol, which are rapidly desorbed from the interface. With this new procedure, it is possible to obtain continuous and accurate kinetic measurements of the phospholipase C catalyzed reaction with phospholipid monolayers as the substrate. It is thus possible to avoid the use of radiolabeled substrates as necessary in previous studies, and the difficulties caused by diacylglycerol accumulation in the lipid film are minimized. No hydrolysis was detected when either phosphatidylethanolamine, phosphatidylserine, or phosphatidylglycerol films were used as substrates. By means of a film transfer technique, $Ca^{2+}$ and $Zn^{2+}$ ions were found to play a specific and critical role. The present study demonstrates clearly for the first time that $Ca^{2+}$ is essential for enzyme binding to lipid films, whereas $Zn^{2+}$ is specifically involved in the catalytic hydrolysis of the substrate.

*Clostridium perfringens* $\alpha$ toxin is generally described as being lethal, dermonecrotic, and hemolytic (McDonel, 1980). It is known to be the main toxin of *Clostridium perfringens* and to constitute a major pathogenic factor in the development of gas gangrene and septicemia (McDonel, 1980).

Since the enzymatic nature of $\alpha$ toxin (phospholipase C) was discovered by McFarlane and Knight (1941), most studies dealing with its mode of action have used assays involving either detergent-solubilized phospholipid substrates (Yamakawa & Ohsaka, 1977) or erythrocyte lysis (Taguchi & Ikezawa, 1976).

Phospholipid hydrolysis by phospholipase C leads to the production of water-insoluble diacylglycerols and water-soluble phosphocholine. In previous investigations, kinetic studies were performed with phospholipase C on monomolecular film with $^{32}P$-labeled phospholipids (Bangham & Dawson, 1962; Hirasawa et al., 1981) or [$^{3}H$]choline- or [$^{14}C$]choline-labeled phospholipids (Miller & Ruysschaert, 1971; Demel et al., 1975). During hydrolysis by phospholipase C, the solubilization of radiolabeled phosphocholine in the water subphase leads to a decrease in the surface film radioactivity. However, diacylglycerol accumulation with time results in a decrease in the phospholipid surface density. Furthermore, the lipid composition of the monolayer varies during the experiment, making it difficult to interpret the kinetics. In order to minimize the problems associated with product accumulation, we have developed a new procedure where the diacylglycerols formed during phospholipase C action are hydrolyzed by the action of a pancreatic lipase into fatty acid and 2-monoacylglycerol, which are then desorbed from the interface. It has been shown previously that pancreatic lipase does not

† This is paper 10 in a series on enzyme reactions in a membrane model. For paper 9, see Gargouri et al. (1986). This study is part of the doctoral thesis obtained by H.M. at Paris VII University, June 25, 1985. The key concepts were presented at the second European Workshop on Bacterial Protein Toxins in Wepion, Belgium, June 30, 1985, to July 4, 1985.

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0006-2960/88/0427-2319$01.50/0 