Preparation of thioester substrates and development of continuous spectrophotometric assays for phospholipase A_1 and monoacylglycerol lipase

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Abstract Current assays of phospholipase A_1 (EC 3.1.1.32) and monoacylglycerol lipase (EC 3.1.1.23) activities in tissues are discontinuous, laborious, and expensive. Some spectrophotometric substrates were synthesized to alleviate this problem. Thioester analogs of phosphatidylcholine and phosphatidylethanolamine, rac-1,2-S,O-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol and rac-1,2-S,O-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol, were synthesized from the diacylglycerol analog, rac-1,2-S,O-didecanoyl-1-mercapto-2,3-propanediol. The latter was prepared from triacylmercaptoglycerol by lipolysis and separation by chromatography on silica gel. Monoacylglycerol thioester analogs, 1-S-hexadecanoyl-1-mercapto-2,3-propanediol and 1-S-decanoyl-1-mercapto-2,3-propanediol, were synthesized by selective acylation of mercaptoglycerol. All of the substrates were hydrolyzed by *Rhizopus delemar* lipase to release sulfhydryl groups reactive towards 4,4′-dithiobispyridine. The hydrolysis could be followed continuously in a spectrophotomer with 0.1 absorbance unit corresponding to 5 nmol product. The structure and isomeric purity of the phospholipid analogs were verified by their behavior on thin-layer chromatography, elemental analyses, infrared spectra, and by the specificity of the colorimetric reaction with lipolytic enzymes. Whereas phospholipase A_1 activity hydrolyzed both phospholipid analogs to release the theoretical amount of free thiol, neither phospolipases C nor A_2 promoted thiol release. The pH optimum, heat stability, and Ca^{2+} ion dependency were determined for the hydrolysis of each substrate by *R. delemar* lipase. The results indicate that the phospholipase A_1 and monoacylglycerol lipase activities in *R. delemar* lipase are due to separate enzymes, and that these enzyme specific assays will be of general utility for enzyme characterization and purification studies. These substrates are useful for sensitive, convenient, and specific spectrophotometric assays for phospholipase A_1 and monoacylglycerol lipase over the pH range 3 to 8.—**Cox, J. W., and L. A Horrocks.** Preparation of thioester substrates and development of continuous spectrophotometric assays for phospholipase A_1 and monoacylglycrol lipase. *J. Lipid Res.* **22:** 496–505.

Supplementary key words triglyceride lipase · *Rhizopus delemar* · phosphatidylcholine · phosphatidylethanolamine · diacylglycerol · triacylglycerol

Enzyme assays for phospholipase A_1 are generally radiochemical. Radioactive substances with labeled fatty acids at the 2-position are incubated with enzyme and the incubation products are fractionated by extraction and thin-layer chromatography. Lysophospholipid, fatty acid, and starting material are usually counted to differentiate between phospholipase A_1 and phospholipase A_2. Methods have been devised to avoid TLC separation (1, 2), but the assays are still discontinuous, time-consuming, and expensive. Even with sufficient phospholipase A_1 activity, many of these enzymes cannot be assayed continuously by titration of released fatty acids because of their neutral to acidic pH optima.

Aarsman, van Deenen, and van den Bosch (3, 4) applied the thioester substitution technique to the assay of lysophospholipase and phospholipase A_2. They prepared a glycol lecithin analog in which a thioester linkage was substituted for the oxyester. Hydrolysis by the phospholipases produced a water-soluble thiol which reacted with DTNB to produce color. We extended this concept to the assay of phospholipase C with thiophosphoester substrates (5, 6). This system was more complex than the lysophospholipase assay in that the product thiols were not water-soluble, but they were still reactive with DTNB or DTP.

Abbreviations: TLC, thin-layer chromatography; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTP, 4,4′-dithiobispyridine: PC, phosphatidylcholine; PE, phosphatidylethanolamine; MP, monopalmitin; MD, monodecanoin; GPC, glycerophosphocholine; PC (III), rac-1,2-S,O-didecanoyl-3-phosphocholine-1-mercapto-2,3 propanediol; PE (IV), rac-1,2-S,O-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol; MP (V), 1-S-hexadecanoyl-1-mercapto-2,3-propanediol; MD (VI), 1-S-decanoyl-1-mercapto-2,3-propanediol; and lyso PE (VIII), 2-O-decanoyl-3-phosphocholine-1-mercapto-2,3-propanediol.

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For the assay of phospholipase A_1, PC (III) and PE (IV) were synthesized from mercaptoglycerol. In addition, two monoglyceride lipase substrates (V and VI) were prepared. The assays were developed with the lipase from *Rhizopus delemar*, a commercially available triglyceride lipase preparation which has small amounts of phospholipase A_1 and lesser amounts of lysophospholipase activity (7). After demonstrating the enzyme specificity of these thioester substrates, the assays were used to characterize briefly the lipolytic activities of *R. delemar* lipase. The results indicate that the assays will have general utility for phospholipase A_1 and monoglyceride lipase investigations. These continuous spectrophotometric assay methods are sensitive and enzyme specific, and they can be used under acidic conditions.

MATERIALS AND METHODS

Materials

Rhizopus delemar lipase (glyceryl ester hydrolase, EC 3.1.1.3) in both crude (600 U/mg) and purified (6000 U/mg) forms was provided by Miles Laboratories (Elkhart, IN) (one unit of activity releases 0.05 mEq fatty acid in 150 min). Mercaptoglycerol was purchased from Evans Chemetics (Darien, CT) and fractionally distilled. Decanoyl chloride, palmitoyl chloride, bromoethanol, ethanolamine, and DTP were purchased from Aldrich Chemical Company (Milwaukee, WI). Trimethylamine (anhydrous) was purchased from Eastman Kodak Company (Rochester, NY). DTNB was purchased from Sigma Chemical Company (St. Louis, MO). Chromatography solvents were reagent grade or better. Pyridine and triethylamine were distilled from BaO, and chloroform and trichloroethylene were washed with water, dried over Na_2SO_4, and distilled from $CaSO_4$. Tetrahydrofuran was refluxed over Na chips for 2 hr prior to distillation. Bromoethylphosphorusoxydichloride was prepared from freshly distilled bromoethanol and phosphorusoxytricholoride. Bromoethanol was added to a 3 mol excess of $POCl_3$ in benzene and the solution refluxed for 2 hr in an exhaust hood. The solution was rotary evaporated at 55°C and the product was vacuum distilled in 80% yield (81°C, 0.4 mm Hg). rac-1,2-Didecanoylglycerol was synthesized as described by Jensen and Pitas (8) using boric acid to remove the dihydropyran blocking group. This was then converted into the phosphocholine derivative according to Hirt and Berchtold (9).

Analytical methods

Melting points were determined with a Thomas Hoover capillary melting point apparatus and were uncorrected. Elemental analyses were performed by Galbraith Laboratories (Knoxville, TN) after drying the samples in vacuo over P_2O_5 for 24 hr. Lipid phosphorus was determined by the method of Gottfried (10). Infrared spectra were either obtained neat or as 10% (w/v) solutions in chloroform against a blank cell containing chloroform with a Beckman Model 4230 infrared spectrophotometer. Visible and UV spectrophotometric measurements and enzyme assays were performed with a Beckman Model 25 recording spectrophotometer equipped with an automatic sample changer.

Chromatography

Analytical TLC was done with 0.5-mm thick layers of silica gel G (EM Laboratories, Elmsford, NY). Spots were visualized with either I_2 vapor, phosphate spray reagent (11), Dragendorf stain for choline (12), ninhydrin spray for primary amine (13), or DTNB (14) for sulfhydryl groups (5 mM DTNB in ethanol–water 2:1, pH 7). Unisil silicic acid was a product of Clarkson Chemical Company (Williamsport, PA). Neutral silica gel for column chromatography was prepared from silicic acid by titrating an aqueous slurry with NaOH to pH 7. The slurry was then suction filtered and dried overnight at 120°C.

Substrate suspensions

A 1-mM emulsion of PC (III) was prepared by drying 15 μmol of the lipid under N_2 in a 50-ml beaker and suspending in 15 ml of buffer by sonication for 4 min at 35% intensity with a Bronwill Biosonik III sonifier (Will Scientific, Rochester, NY) equipped with a ¾ inch probe. The beaker was immersed in ice during the sonication. The absorbance at 450 nm of 0.05 mM emulsion was 0.2. PE (IV) gave unstable crystalline dispersions under these conditions, but when small amounts of chloroform were added prior to sonication, stable emulsions with low turbidity were formed. Routine emulsions were therefore prepared by drying 15 μmol of PE (IV) from chloroform solution in a beaker under N_2, followed by heating in a 120°C oven for 1 min to completely evaporate the organic solvent. After cooling, 15 ml of buffer followed by 10 μl of chloroform was added and the mixture was sonicated as before. MP (V) and MD (VI) were suspended as semi-stable microcrystalline dispersions with the aid of didecanoyl GPC. Coevaporation of 6 μmol MG (V) of MD (VI) with 6 μmol didecanoyl GPC followed by addition of 15 ml of buffer and sonication as above gave a suspension with an absorbance at 450 nm of 0.6 for MP (V) and 0.4 for MD (VI) and no significant baseline drift. Sedimentation of MP (V) crystals occurs if the suspension

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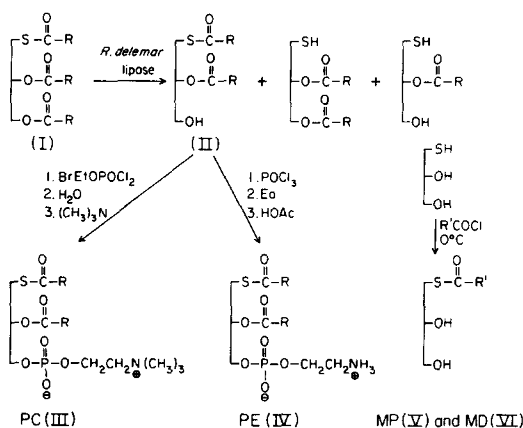

Fig. 1. Synthetic scheme for thioester substrates for phospholipase A_1 and monoglyceride lipase. R is $CH_3(CH_2)_8^-$. R' is $CH_3(CH_2)_{14}^-$ for (V) and $CH_3(CH_2)_8^-$ for (VI).

is allowed to stand for several hours, but they can be resuspended by vortexing. All suspensions were allowed to age at least 1 hr at room temperature prior to use.

Enzyme assays

Routine assay conditions with PC (III) and PE (IV) substrates included 0.5 mM substrate suspension, 0.6 mM DTP, and enzyme in 0.1 M buffer. Total volume was 1 ml. Reactions were run at room temperature and were started by adding enzyme solution. Assay conditions with MP (V) and MD (VI) substrates included 0.2 mM substrate suspension (containing 0.2 mM didecanoyl GPC), 5 mM $CaCl_2$, 0.6 mM DTP, and enzyme in 0.1 M buffer. Rates were calculated from the molar absorption coefficient for 4-thiopyridone at 324 nm of 1.98×10^4 $M^{-1}cm^{-1}$ (15). Measurements were normally made against air.

pH dependence

Substrate suspensions were prepared in 0.05 M sodium acetate–0.05 M sodium 3-(N-morpholino) propane sulfonate buffer adjusted to pH 7.5 with HCl. The suspensions were then adjusted down in 0.5 pH increments with HCl to pH 4.0. This method introduces a salt gradient, but no NaCl dependence was observed at pH 7.5.

Chemical syntheses of thioester substrates

Preparation of rac-1,2-S,O-didecanoyl-1-mercapto-2,3-propanediol (II). Mercaptoglycerol was triacylated with decanoyl chloride to form the triglyceride (I), which was then partially hydrolyzed with *R. delemar* lipase (600 U/mg) to obtain the desired diglyceride (II) (**Fig. 1**). Mercaptoglycerol (35 mmol) was dissolved in 100 ml of chloroform and 210 mmol of pyridine. Decanoyl chloride (175 mmol) was added with rapid stirring. After cooling, the mixture was allowed to stand in the dark for 2 days. It was then extracted with 500 ml of hexane and washed three times with 100-ml portions of water. Rotary evaporation of the organic phase gave 30 g of brown oil. The crude product (5 g) was purified on a 200-g column of silicic acid by elution with a hexane–diethyl ether solvent system (98:2, 95:5, and 85:15). The triglyceride (I) is obtained in 95% yield as light yellow oil that migrates just ahead of trimyristin on TLC (hexane–diethyl ether–acetic acid 90:10:1, $R_f = 0.3$). The infrared spectrum of the neat oil exhibited the following strong bands: 2970, 2940, 1460, 1375 (C-H); 1740, 1200–1150, 1110, 1050 (ester); and 1695 cm^{-1} (thioester).

The triglyceride (I) was partially hydrolyzed with *R. delemar* lipase using the assay conditions described by Fukumoto, Iwai, and Tsujisaka (16). Triglyceride (I) (10 mmol) was incubated with 5 mg of lipase in a volume of 60-ml containing 0.2 M sodium acetate buffer, pH 5.6, and 10 mM $CaCl_2$. The mixture was rapidly stirred and the progress of the reaction was followed by TLC (hexane–diethyl ether–acetic acid 60:40:1). After 1 hr of incubation, five components of the reaction mixture could be detected, and they were tentatively identified as unreacted starting material with $R_f = 0.98$; 2,3-O-didecanoyl-1-mercapto-2,3-propanediol with $R_f = 0.95$; decanoic acid with $R_f = 0.68$, 1,2-S,O-didecanoyl-1-mercapto-2,3-propanediol (II) with $R_f = 0.47$; and 2-O-decanoyl-1-mercapto-2,3-propanediol with $R_f = 0.26$. The concentration of (II) reached a maximum after about 3 hr of incubation, and the mixture was extracted with four volumes chloroform–methanol 2:1. After rotary evaporation at 30°C, the residual oil was fractionated on a 200-g column of neutral silica gel by elution with hexane–diethyl ether (90:10, 80:20, and 70:30). The desired diglyceride (II) elutes in the last fraction in 26% yield. The infrared spectrum of the neat oil was identical to (I) except for the presence of a strong hydroxyl band at 3450 cm^{-1} and weaker ester bands. A trace amount of 1,3-isomer was formed during column chromatography, but it was judged to be less than 5% of the 1,2-isomer (TLC, hexane–diethyl ether–acetic acid 60:40:1, $R_f = 0.47$ for the 1,2-isomer compared to 0.53 for the 1,3-isomer).

Preparation of rac-1,2-S,O-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol (III). The diglyceride (II) (3.45 mmol) was dissolved in 15 ml of chloroform and added dropwise to a well stirred solution of 13.8 mmol of bromoethylphosphorusoxydichloride and 28 mmol of triethylamine at 0–5°C. The mixture was stirred at room temperature for 12 hr and then

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subjected to hydrolysis, trimethylamination, and purification as described by Aarsman, van Deenen, and van den Bosch (3). Following the treatment with ion exchange resin, the crude products were taken up in chloroform and eluted from a 100-g column of silicic acid with chloroform–methanol (4:1, 1:1, 2:3, and 1:3). PC (III) elutes in the last two fractions in 40% yield. On TLC it migrated identically to didecanoyl GPC and it stained positively for both choline and phosphate groups. TLC in chloroform–methanol–water 65:35:4 showed a R_f of 0.25. The infrared spectrum of (III) is compared to didecanoyl GPC in **Fig. 2.** Assignments are as follows: 3300 (bound water); 2970, 2940, 2860, 1470, and 1380 (C-H); 1740, 1170 (ester); 1695 (thioester); 1260, 1095 (PO_2^-); 1060 ((P)OC); 970; 920 (quaternary trimethylammonium salt); 870 (PO(C)); and 820, 720–790 cm^{-1} (O-P-O). A single elemental analysis yielded the following results:

$C_{28}H_{56}O_7NSP$

requires: C 57.8, H 9.7, N 2.4, S 5.5, P 5.3%

found: C 57.6, H 9.7, N 2.4, S 5.6, P 5.4%

Preparation of rac-1,2-S,O-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol (IV). The diglyceride (II) (3.45 mmol) was converted to the phosphoethanolamine derivative as described by Eibl (17). The workup was modified, however, since the didecanoyl analog failed to precipitate after the addition to acetic acid. The solution was rotary evaporated and purified by chromatography on a 100-g column of silicic acid with a chloroform–methanol solvent system (chloroform–methanol 9:1, 6:1, 5:1, and 4:1). The desired product, PE (IV), eluted in the last fraction in 30% yield. On TLC, it migrated below bovine brain ethanolamine phosphoglyceride and stained positively for both phosphate and primary amine. TLC in chloroform–methanol–water 65:35:4 showed a R_f of 0.61. The infrared spectrum (Fig. 2) is distinguished by bands at 2400–3300, 1630, 1550 (N-H); 1230 (PO_2^-); 1070 (PO_2^-, (P)OC); 1035, 890 (C-C-N); 830, 720–800 (O-P-O). A single elemental analysis yielded the following results:

$C_{25}H_{50}O_7NSP$

requires: C 55.6, H 9.3, N 2.6, S 5.9, P 5.7%

found: C 55.5, H 9.6, N 2.5, S 5.8, P 5.6%

Preparation of 1-S-hexadecanoyl-1-mercapto-2,3-propanediol (V) and 1-S-decanoyl-1-mercapto-2,3-propanediol (VI). The sulfhydryl group of mercaptoglycerol was selectively acylated with hexadecanoyl chloride as described by Aarsman et al. (3) for the monoacylation of mercaptoethanol. Mercaptoglycerol (100 mmol) was dissolved in 100 ml of diethyl ether and 60 mmol of pyridine. To this solution was added dropwise a solution of hexadecanoyl chloride (25 mmol) in 30 ml of diethyl ether with rapid stirring at 0°C. After the addition, the mixture was allowed to stir at room temperature for 2 hr, then extracted with four 50-ml portions of water. The organic phase was dried over Na_2SO_4 and was rotary evaporated. The residue was taken up in chloroform and eluted from a 200-g column of neutral silica gel with chloroform, chloroform–methanol 98:2, and chloroform–methanol 96:4. MP (V) elutes in the last fraction, and the purified product was crystallized from benzene at 4°C in 30% yield (mp 83.5–84.5°C). It migrates slightly ahead of monopalmitin on TLC (chloroform–methanol 96:4, R_f = 0.55 for MP (V) compared to 0.50 for monopalmitin). The infrared spectrum exhibited the following strong bands: 3450, 1410 (O-H); 2960, 2930, 2860, 1460 (C-H); 1690 (thioester); 1060, 1030 (C-O). MD (VI) (mp 68.0–69.0°C) was prepared by an identical procedure from decanoyl chloride.

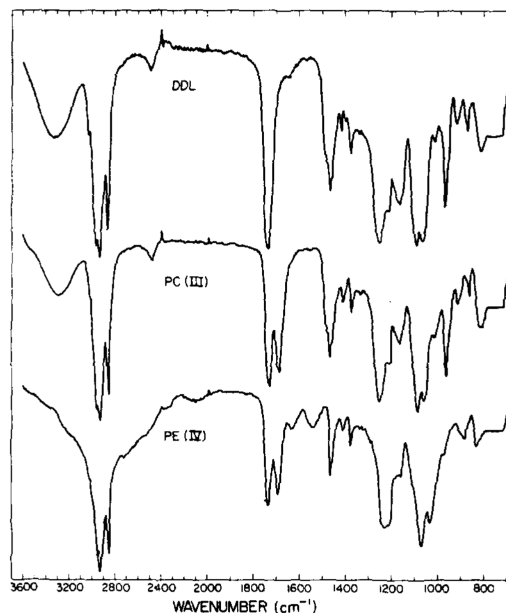

Fig. 2. Infrared spectra of rac-2,3-didecanoyl-glycero-1-phosphocholine (DDL), rac-1,2-S,O-didecanoyl-3-phosphocholine-1-mercapto-2,3-propanediol (III), and rac-1,2-S,O-didecanoyl-3-phosphoethanolamine-1-mercapto-2,3-propanediol (IV). Spectra were obtained as 10% (w/v) solutions in chloroform against a blank cell containing chloroform.

RESULTS

Thioester analogs of phosphatidylcholine (III), phosphatidylethanolamine (IV), diglyceride (II), and

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monoglyceride (V and VI) were prepared in moderate yield from mercaptoglycerol. For all of these substrates, the essential feature of any synthetic route is that it provides the final products in high isomeric purity. We have developed two synthetic routes to satisfy this criterion. The first, which is not reported in detail here, involves blocking the sulfhydryl group of mercaptoglycerol by oxidation to the disulfide. Subsequent tritylation and reduction of the disulfide with dithiothreitol gave 3-trityl-1-mercapto-2,3-propanediol. This intermediate was then converted into the diglyceride (II) by diacylation and removal of the trityl blocking group on a boric acid-silicic acid column. This five-step route to (II) was time-consuming and the yield was low.

On the other hand, partial hydrolysis of the triglyceride of racemic mercaptoglycerol with *R. delemar* lipase provides a convenient two-step route to (II) with an overall yield of 25% (Fig. 1). The success of this approach is attributable to the large difference in polarity between the various lipolysis products that allows (II) to be isolated in high purity by column chromatography. The stereo-isomeric composition of (II) depends on the stereospecificity of the lipase. This enzyme is reported to be nonstereospecific since it hydrolyzes both the 1 and 3-positions of natural triglycerides (18). We also observed the formation of the expected mixture of diglycerides and monoglycerides upon lipolysis of (I) (Fig. 1). Thus, the diglyceride (II) is probably a racemate or at least only partially resolved. Both thioester and ester bonds were hydrolyzed at comparable rates, as judged by the order of appearance of products in the reaction mixture.

The phosphocholine and phosphoethanolamine derivatives of (II) were synthesized by published procedures for diglycerides of glycerol (3, 17). Structures of PC (III) and PE (IV) were verified by TLC behavior, infrared spectra (Fig. 2) and elemental analyses. The structural isomers of PC (III) and PE (IV), 1,3-S,O-didecanoyl- and 2,3-O-didecanoyl-glycerophospholipids, could not be detected by TLC, on which both migrate slightly ahead of the 1,2-S,O-didecanoyl-isomer. These thioester substrates should therefore be specific for phospholipase A_1. The overall synthesis of both PC (III) and PE (IV) as shown in Fig. 1 requires three workups and can be easily completed in 10 days.

We also prepared two thioester analogs of monoglycerides, MP (V) and MD (VI). Neither of the purified products contained any oxyester bonds detectable by infrared spectroscopy, nor were any sulfhydryl groups detected by reaction of the dispersed substrate with DTP.

Incubation of *R. delemar* lipase with substrate suspensions in the presence of DTP resulted in a linear increase in absorbance at 324 nm. The contribution of a turbidity change to the total absorbance change was evaluated at 450 nm, where neither DTP nor thiopyridone absorb. Independent incubations conducted in the absence of DTP had verified that the absorbance change at 450 nm was not significantly different from that measured at 324 nm. With the phospholipid substrates, however, this change was opposite to that measured in the presence of DTP, presumably because DTP modifies the products and influences their water solubility. In the absence of DTP, there was a slight increase in turbidity, whereas in the presence of DTP, there was a slight decrease. This negative absorbance change measured at 450 nm for DTP containing incubations was less than 10% of the total absorbance change at 324 nm. The change at 450 nm developed slowly with time, so that it had no effect on initial rate measurements. For evaluation of absorbance change at 324 nm for complete substrate hydrolysis, the negative ΔA_{450} was substracted from ΔA_{324} to give the net ΔA_{324}. Because the turbidity change was dependent on DTP, it is invalid to blank the complete incubation system at 324 nm against a cell lacking DTP.

When 50-μM substrate concentrations were used (50 nmol of substrate in 1.0 ml volume), complete hydrolysis of all thioester bonds corresponded to a net ΔA_{324} of 0.99. The value actually recorded for PC (III), PE (IV), and MP (V) corresponded to 104.7, 99.6, and 99.7% of the theoretical value, respectively. No discontinuities were detected in any of the progress curves to suggest enzymic stereoselectivity for the racemic substrates. Treatment of PC (III) and PE (IV) with either phospholipase A_2 (*Crotalus adamanteus*) or phospholipase C (*Bacillus cereus*) resulted in no release of thiol.

Suspensions of substrates PE (IV) and MP (V) required stabilization to prevent sedimentation of very fine crystals. If chloroform was not completely removed from PE (IV) prior to addition of buffer and sonication, stable emulsions with low turbidity were produced. A series of emulsions containing PE (IV) and various amounts of chloroform were therefore prepared to examine the solvent effect more closely. Concentrations of chloroform as low as 0.8 mM for a 0.5 mM emulsion of PE (IV) reduced the absorbance at 450 nm by 72% and increased the rate of hydrolysis by 800% (**Fig. 3**). There was a good inverse correlation between the absorbance at 450 nm and hydrolysis rate curves ($P > 95\%$). Both the duration of the linear phase of the progress curve and the enzyme concentration range over which linear enzyme dependencies

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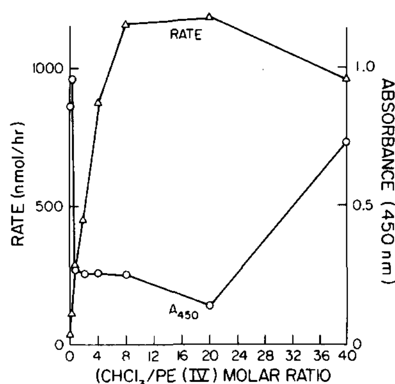

Fig. 3. Effect of chloroform on the A_{450} of PE (IV) emulsions (○---○) and on *R. delemar* lipase activity (△---△). Incubations were conducted at pH 7.0 with 1 mg powder (600 U/mg).

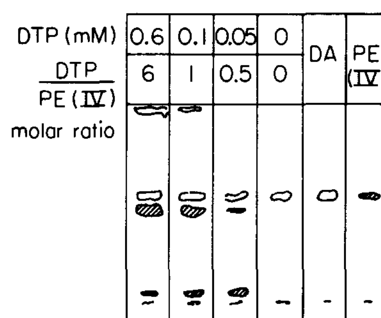

Fig. 4. TLC of reaction products from incubations of PE (IV) with *R. delemar* lipase at different concentrations of DTP. Solvent system: chloroform–methanol–ammonia 65:35:4. Standards are decanoic acid (DA) and PE (IV). Hatch marks represent a positive stain for phosphorus. Incubations contained 0.1 mM PE (IV) at pH 6.0.

could be obtained were increased by increasing the concentration of chloroform. At the same concentration, neither diethyl ether nor isopropanol were as effective as chloroform at producing stable emulsions of PE (IV). However, the chloroform did not facilitate the dispersion of MP (V). Sodium taurocholate was only satisfactory above pH 6, due to precipitation at lower pH values. Didecanoyl GPC produced semistable suspensions in a 1:1 molar ratio with MP (V), and was unaffected by pH. It was therefore selected as the dispersing agent for both MP (V) and MD (VI) for further studies.

Reaction product composition as a function of DTP concentration from the incubation of *R. delemar* lipase with PE (IV) emulsions was examined by TLC (**Fig. 4**). The three DTP-containing incubations had identical progress curves even though the DTP in one of the incubations was insufficient to react with the lyso PE (VII) product (**Fig. 5**). The other hydrolysis product, decanoic acid, co-migrates with PE (IV) in the chloroform–methanol–NH_4OH 65:35:4 solvent system. After the incubation, this area no longer stained positively for phosphorus. The lysoPE (VII) product formed in the absence of DTP was not extracted into the organic phase, but two new phosphorus-containing spots appeared in the extracts from DTP-containing incubations. The less polar of the pair was the major product in the presence of high DTP concentrations and this material is presumably the mixed disulfide (VIII). The more polar spot predominates as the DTP concentration decreases, and it is probably the symmetrical disulfide of lysoPE (IX). Unreacted DTP migrates to the solvent front.

Linear enzyme dependencies were obtained for each substrate over a minimum 10-fold range of protein concentration (**Fig. 6**). Reduction of the DTP concentration did not affect the slopes of these plots, but did reduce the protein concentration range over which a first order dependency was obtained.

As a test of the general utility of these spectrophotometric assays, they were used to characterize briefly the lipolytic activities in *R. delemar* lipase with respect to Ca^{2+} ion dependence, pH optima, and heat stability. As shown in **Fig. 7**, although Ca^{2+} moderately stimulated the initial rate of hydrolysis of sodium taurocholate-MP (V) dispersions and had little effect on the hydrolysis of didecanoyl GPC-MP (V) dispersions, it inhibited the hydrolysis of both PC (III) and PE (IV). The inhibitory effect with PE (IV) is at least

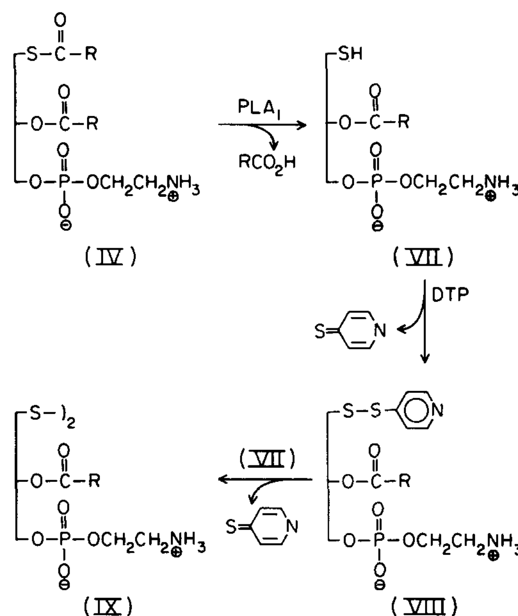

Fig. 5. Reaction scheme for the hydrolysis of PE (IV) by *R. delemar* lipase. In the presence of excess DTP, (VIII) is the major product. Decreasing the DTP concentrations causes the formation of (IX).

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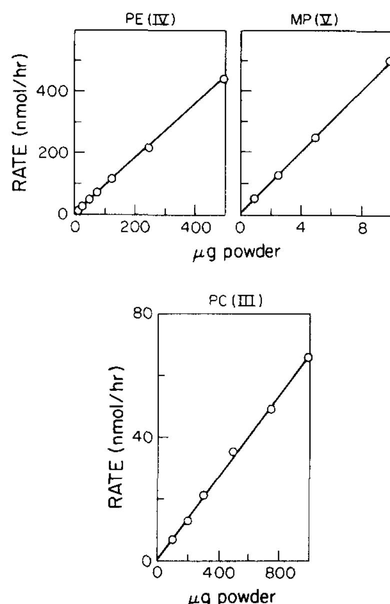

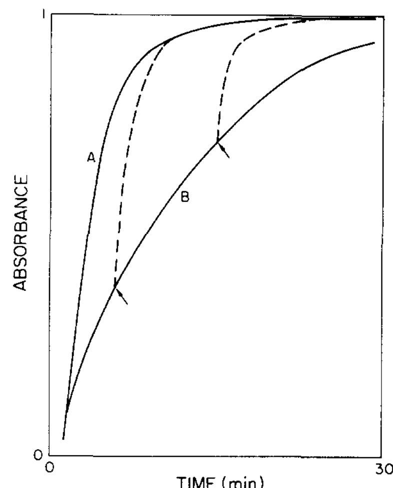

Fig. 8. Ca^{2+} stimulation of MP (V) hydrolysis by *R. delemar* lipase. Curve (A) is with 5 mM $CaCl_2$, and curve (B) is with 1 mM EDTA. Arrows indicate the addition of 5 mM $CaCl_2$ to curve (B).

Fig. 6. Enzyme dependency curves for the reaction of *R. delemar* lipase with PC (III), PE (IV), and MP (V) at pH 7.0. Incubations contained 0.6 mM DTP.

partly due to the destabilization of the PE emulsion and the precipitation of the substrate. However, inhibition of PC (III) hydrolysis was not associated with a visible (450 nm) substrate perturbation. The modulatory effect of Ca^{2+} on the initial rates of hydrolysis may be due to either specific enzyme interactions or to modification of the substrate structure and/or surface charge. The latter possibility seems to be a plausible explanation, especially since the stimulatory effect of Ca^{2+} on MP (V) hydrolysis was dependent on the dispersing agent (taurocholate versus lecithin).

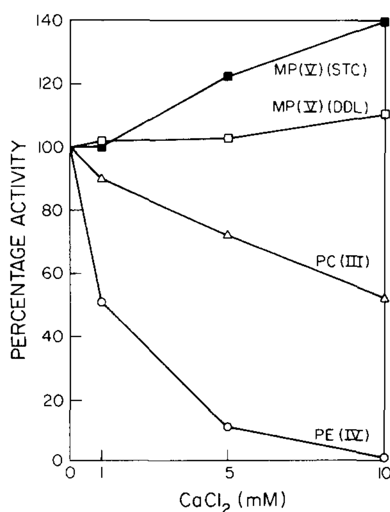

Fig. 7. Effect of Ca^{2+} on the activity of *R. delemar* lipase with thioester substrates. MP (V) was dispersed with 1:1 molar ratios of sodium taurocholate (STC) or didecanoyl lecithin (DDL). Incubations contained 0.5 mM PC (III) or PE (IV), or 0.2 mM MP (V). PE (IV) emulsions containing $CaCl_2$ were unstable.

Although Ca^{2+} had no influence on the initial rate of hydrolysis for MP (V)/lecithin dispersions, it did prevent enzyme inhibition during the later stages of hydrolysis (**Fig. 8**). In the absence of Ca^{2+}, the rate begins to slow almost immediately. If Ca^{2+} is added any time during the incubation, however, the hydrolysis accelerates to reach the control value obtained with incubations containing Ca^{2+}. A similar study with PC (III) failed to demonstrate any ability of Ca^{2+} to stimulate hydrolysis. Ca^{2+} stimulation was also observed with MD (VI) so that the opposite results with PC (III) and MP (V) are not attributable to the difference in fatty acid chain length between the substrates.

The pH dependencies for the hydrolysis of MP (V), PE (IV), and PC (III) by the lipase are given in **Fig. 9**. PE (IV) emulsions were unstable below pH 4.3. However, there were no significant changes in the absorbance at 450 nm for the pH 4.3 to 7.5 emulsions of all three substrates. Linear enzyme dependencies were obtained with PE (IV) at each pH, indicating that the thiol detection system is still responsive under mildly acidic conditions. The pH optima for the hydrolysis of MP (V) and PE (IV) were similar at pH 6.0 and 5.7, respectively, but the pH optimum for PC (III) was less than 4.5. At pH 6.0, the PE (IV) hydrolyzing activity was only 3% and that for PC (III) was 0.3% of the activity with MP (V). MD (VI) was hydrolyzed 75% as fast as MP (V). The lipase and phospholipase activities were also different in heat stability. Although both showed acti-

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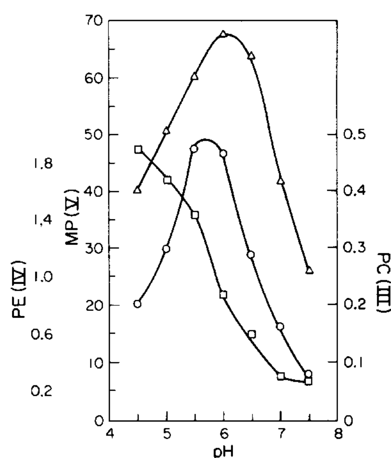

Fig. 9. pH Dependency curves for the hydrolysis of MP (V) (△——△), PE (IV) (○——○), and PC (III) (□——□) by *R. delemar* lipase (600 U/mg). Rates are expressed on the vertical axis as μmol/h/mg powder.

vation by heating up to 48°C, the monoglyceride lipase activity continued to increase up to 56°C while the phospholipase activity declined over the same period. A ratio analysis of the enzyme activities at pH 6.0 and 4.5, after preincubation of the enzyme at various temperatures, revealed a difference in heat stability between lipase and the phospholipase activities (**Table 1**). Whereas the ratio of the MP (V) hydrolyzing activities at pH 6.0 and 4.5 is unchanged by heating, the ratios for both PC (III) and PE (IV) are altered. In general, the PE (IV) hydrolyzing activity is less stable than either the PC (III) or MP (V) activities. Both the PC (III) and PE (IV) activities at pH 4.5 are less stable than the same activities at pH 6; this is especially true of PE (IV).

These experiments indicate that the phospholipase and lipase activities of *R. delemar* lipase may be separable. Consequently, a higher purity grade of *R. delemar* lipase was examined for the relative amounts of each activity at pH 4.5 and 6 (**Table 2**). The purified preparation is seven to eight times as active as the crude preparation for each substrate at both pH values with the exception of a 10-fold increase for PC (III) hydrolysis at pH 6.

The pH optima for the hydrolysis of PC (III) and PE (IV) by a commercial *Rhizopus arrhizus* preparation (Boehringer Mannheim, New York, NY) was also examined. It closely resembled the pH dependency observed with *R. delemar* lipase. PC (III) hydrolysis had two optima: a major peak from pH 4 to 5 and a minor peak coinciding with PE (IV) hydrolysis at pH 6.0. The triglyceride lipase optimum is 7.0 (19).

DISCUSSION

Synthetic procedures for thioester analogs of glycerolipids must provide the products as a single structural isomer. Contaminating isomers, in which the thioester bond is present but attached to a different glycerol carbon, can serve as colorimetric substrates for other lipolytic enzymes and reduce the enzyme specificity of the assay. The synthesis of isomerically pure phospholipase A_1 substrates depended on a method to protect the 3-hydroxy group of mercaptoglycerol. The best results were obtained when mercaptoglycerol was triacylated and then subjected to partial lipolysis (Fig. 1). This synthetic route presumably yields a racemic mixture, since the starting material is racemic mercaptoglycerol and *R. delemar* lipase is not stereospecific with triglycerides (18). Optically active products can be prepared either by enzymic resolution of PC (III) and PE (IV) racemates, or by beginning the synthesis with optically active mercaptoglycerol (20).

In order to test for the presence of structural isomers of PC (III) and PE (IV), the substrates were treated with *Bacillus cereus* phospholipase C, *Crotalus adamanteus* phospholipase A_2, and *Rhizopus delemar* lipase which has phospholipase A_1 activity (7). Although both phospholipases C (5, 6) and A_2 (3) have been shown to hydrolyze thioester substrates in the presence of thiol reagents, only *R. delemar* lipase

TABLE 1. Effect of heat pretreatment on the ratio of lipase activities as assayed with thioester substrates at pH 4.5 and 6.0. Ratios are calculated as the ratio of enzyme specific activities.

Temperature (°C)	Ratio								
	$\frac{PE_{6.0}}{PE_{4.5}}$	$\frac{PC_{6.0}}{PC_{4.5}}$	$\frac{MP_{6.0}}{MP_{4.5}}$	$\frac{PE_{6.0}}{PC_{6.0}}$	$\frac{PE_{4.5}}{PC_{4.5}}$	$\frac{MP_{6.0}}{PE_{6.0}}$	$\frac{MP_{4.5}}{PE_{4.5}}$	$\frac{MP_{6.0}}{PC_{6.0}}$	$\frac{MP_{4.5}}{PC_{4.5}}$
30	2.96	0.31	2.57	5.68	0.60	26.7	30.8	152	18.6
40	3.33	0.33	2.69	6.20	0.63	27.1	33.6	168	20.8
48	3.79	0.32	2.74	6.82	0.58	22.9	31.8	156	18.3
56	4.58	0.35	2.78	6.04	0.46	30.0	49.2	182	22.8
63	8.65	0.80	2.70	1.47	0.14	79.0	254	117	34.5

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TABLE 2. Comparisons of lipolytic activities of crude (600 U/mg) and purified (6000 U/mg) *R. delemar* lipase as assayed with thioester substrates at pH 4.5 and 6.0.[a]

$Substrate_{pH}$	Purified/Crude Activity Ratio[b]
$PE_{6.0}$	7.5
$PE_{4.5}$	7.4
$PC_{6.0}$	10.4
$PC_{4.5}$	7.1
$MP_{6.0}$	7.8
$MP_{4.5}$	7.2

[a] Specific activities and activity unit definition are as described by Miles Laboratories (Elkhart, IN).

[b] The average deviation from the mean for two separate analyses (different substrate preparations) was ±0.5.

hydrolyzed PC (III) and PE (IV) to cause a release of free thiol groups. The absorbance change corresponded to the theoretical amount of thioester bonds present (assuming a thioester:phosphorus ratio of 1.0). These substrates are therefore isomerically pure and specific for phospholipase A_1. The enzyme catalyzed hydrolysis of PC (III) and PE (IV) by *R. delemar* lipase was followed continously on a spectrophotometer at two wave lengths: 324 nm for the release of thiopyridone from the reaction of free thiol with DTP, and 450 nm for changes in turbidity. At 450 nm, there was a clearing effect during the hydrolysis, i.e., a decrease in turbidity resulting from conversion of the substrate into a more water soluble form. The clearing slowly developed with time and was not a factor in initial rate measurements. When the slight decrease in absorbance was taken into account at 324 nm, the net absorbance increase corresponded to a 100% hydrolysis of thioester bonds. The progress curves did not display the characteristic shape expected for the hydrolysis of a racemate by a stereospecific enzyme. Some investigators have suggested that the presence or absence of stereospecificity be used as a criterion to distinguish true phospholipase A_1 from triglyceride lipase (1, 23). However, at least one example of a stereospecific triglyceride lipase has now been discovered (21), and *Clostridium perfringens* phospholipase C is only partially stereospecific (5, 6), so that it may be premature to adopt this classification system.

The hydrolysis products were analyzed by TLC to verify the formation of fatty acid and to ascertain if the product identity was dependent on DTP. For water-insoluble thiols, disulfide exchange to form symmetrical disulfides at the micelle surface predominates regardless of the DTP concentration (5). The results from our experiments with phospholipase A_1 substrates were those expected of a homogenous system; there did not appear to be any surface effect. A phosphorus-containing material with the expected R_f of the disulfide (VIII) formed from lysoPE (VII) and DTP was the predominant product at high DTP concentrations (Figs. 4 and 5). Low concentrations of DTP resulted in the formation of a phosphorus-containing material with the polarity expected of the symmetrical lysoPE disulfide (IX). The shape of the progress curve was not influenced by changing the DTP/PE ratio from 6 to 0.5.

Linear enzyme dependencies were obtained for each substrate, and for PE (IV) over the pH range 3–8 (15), thus allowing assays to be performed in acidic media. Identical rates were obtained at pH 7 using either DTP or DTNB, and altering the concentration of the thiol reagent had no effect on the slope of the enzyme dependency curve.

Since *R. delemar* lipase is primarily a neutral glyceride lipase (18, 22), two monoglyceride lipase substrates (V and VI) were synthesized by the selective acylation of mercaptoglycerol for comparison of lipase and phospholipase activities. With didecanoyl GPC as a dispersing agent at pH 6, MP (V) was hydrolyzed 30 and 300 times faster than PE (IV) and PC (III), respectively. The relative inertness of didecanoyl GPC allows it to be used as a neutral dispersing agent for MP (V).

These phospholipase and lipase assays were tested for their general utility by attempting to characterize briefly the activities in *R. delemar* lipase. Three experiments were performed with each substrate: Ca^{2+} ion dependence, pH optimum, and heat stability. All three experiments demonstrated the ability of these enzyme specific substrates to discriminate between enzyme activities in a crude lipase preparation.

As measured with MP (V), the monoglyceride lipase activity is similar to the triglyceride lipase activity, and the two probably represent the same enzyme. The pH optimum for MP (V) hydrolysis is 6.0, compared to the literature value of 5.6 for triglycerides (22), and the Ca^{2+} stimulation pattern for MP (V) hydrolysis measured spectrophotometrically is identical to the stimulation of triglyceride hydrolysis measured with a fatty acid titration procedure (18).

In contrast, the PC (III) hydrolyzing activity appeared to be distinct from the monoglyceride lipase activity. The pH optimum was less than 4.5, and it was more heat stable than the MP (V) hydrolyzing activity. Furthermore, there were two distinct differences in Ca^{2+} effects on the activities. Ca^{2+} decreased the initial rate of hydrolysis of PC (III) whereas it had no effect on the hydrolysis of MP (V)/lecithin dispersions. It also prevented enzyme inhibition during MP (V) hydrolysis, as witnessed by dramatic changes in the incubation progress curve

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(Fig. 8), but failed to elicit similar changes in the progress curve for PC (III) hydrolysis.

These results are rather indirect, but are suggestive that the PC phospholipase A_1 and triglyceride lipase activities are physically separable. Even so, the PC (III) phospholipase A_1 activity is only 1% of the MP (V) monoglyceride lipase activity at pH 4.5, and probably less of the triglyceride lipase activity. A higher purity grade of *R. delemar* lipase was therefore assayed for these activities, but there was no significant difference between the enrichment of PC (III) activity at pH 4.5 and MP (V) or PE (IV) activity at either pH 4.5 or 6.0. However, there was a small but significant relative enrichment of PC (III) activity at pH 6.0. For comparison, the lipase from another species, *R. arrhizus*, was similarly tested and found to hydrolyze both PC (III) and PE (IV). The PE (IV) pH optimum was identical for both species, but the PC (III) activity had an optimum below pH 4.5 for *R. delemar*, and an optimum at pH 5.0 and a definite shoulder at pH 6.0 for *R. arrhizus*.

These results indicate that the thioester substrates can be of general utility in discerning phospholipase and monoglyceride lipase activities in enzyme mixtures, and should be valuable for enzyme purification studies. The assays are sensitive (10 nmol/h corresponds to 0.2 A/h with DTP), enzyme specific, and useful over the pH range 3–8 with DTP and 7–8.5 with DTNB.

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