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Studies on Lysophospholipases

VII. Synthesis of Acylthioester Analogs of Lysolecithin and Their Use in a Continuous Spectrophotometric Assay for Lysophospholipases, a Method with Potential Applicability to Other Lipolytic Enzymes

A. J. AARSMAN, L. L. M. VAN DEENEN, AND H. VAN DEN BOSCH

Laboratory of Biochemistry, Padualaan 8, De Uithof, Utrecht, The Netherlands

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The synthesis of acylthioester analogs of lysolecithins, i.e., 2-hexadecanoylthio-1-ethyl-phosphorylcholine and 3-hexadecanoylthio-1-propyl-phosphorylcholine is described. Both compounds were found to be hydrolysed by a homogeneous lysophospholipase from beef liver, a spectrophotometric assay for the activity of which was developed by continuous measurement of the released thiol groups in the presence of dithionitrobenzoic acid.

Phospholipase A_2 from pig pancreas effected hydrolysis of the acylthioester bond in 2-hexadecanoylthio-1-ethyl-phosphorylcholine, the enzymatic action of which could also be monitored spectrophotometrically.

Lipase from pig pancreas was found to hydrolyse acylthioester bonds in 2-hexadecanoylthio-1-ethanol. The tributyryl ester of 3-mercapto-1,2-propanediol was synthesized and used to compare the release of total acid and thiol groups during hydrolysis with lipase. A ratio of about 2 : 1 was found for these releases.

These findings clearly indicate the potential applicability of acylthioester analogs of substrates for phospholipases, lysophospholipases, and lipases in continuous spectrophotometric assays for lipolytic enzymes.

INTRODUCTION

Lipolytic enzymes are conveniently assayed by titrimetric procedures. This principle has been applied successfully for pancreatic lipase (*1*), pancreatic phospholipase A_2 (*2*), snake venom phospholipase A_2 (*3*), and *Bacillus cereus* phospholipase C (*4*). For a determination with a 100-μl burette filled with 0.01 *N* alkali, a full scale recorder deflection corresponds to 1000 nmoles of acid produced. This puts a reasonable lower limit on the detection of enzymatic activities, which should be of the order of 100 nmoles/min. Most of the intracellular lipases and phospholipases, when tested in crude systems without prior purification have activities of the order of only 1–10 nmoles/min/mg of protein, at best. For such enzymes, methods employing radioactive substrates have frequently been developed (*5*). In some instances the cumbersome and time-consuming thin-layer chromatographic separation of the radioactive reaction product has been replaced by more convenient solvent extraction procedures (*6–10*). In our research on lysophospholipases we have used up to now an assay system in which the [^{14}C]palmitate released from synthetic 1-[1-^{14}C]lysophosphatidylcholine was determined after a modified Dole-extraction procedure (*9*). However, despite the convenient extraction procedures, these radiochemical methods still suffer from the general

disadvantages inherent to fixed-time assays. We have therefore developed a continuous spectrophotometric assay for lysophospholipases by using substrate analogs in which the fatty acid is linked in thioester linkage rather than in oxyester linkage to the backbone of the substrate molecule. The release of free SH-groups during the hydrolysis is measured by its reaction with 5,5′-dithiobis(2-nitrobenzoic acid). This approach has previously been used in assaying esterases, e.g., acetylcholine esterase (*11*), and acyltransferases, e.g., lysophosphatidylcholine-acyl-CoA acyltransferase (*12*). The findings reported in this paper, indicating that not only lysophospholipases but also pancreatic phospholipase A_2 and pancreatic lipase can hydrolyse fatty acids linked in thioester linkage to their respective substrates, may prove to be applicable to other lipolytic enzymes as well. Enzymatic activities corresponding to the release of 1 nmole of fatty acid/min are easily detectable.

MATERIALS AND METHODS

Thiourea was obtained from May & Baker Ltd., Dagenham, England. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and 3-mercapto-1,2-propanediol were purchased from Aldrich-Europe, Beerse, Belgium. Butyryl chloride and 2-mercapto-1-ethanol were products from Merck AG, Darmstadt, G.F.R., and 3-chloro-1-propanol was obtained from Fluka AG, Buchs, Switzerland. Palmitic acid was obtained from J. T. Baker Chemicals N.V., Deventer, Holland. Palmitoylchloride was prepared according to the method of Daubert *et al.* (*13*). Column chromatography was performed on Kieselgel 60 reinst (Merck, Germany), 70–230 mesh, activated at 120°C for several hours before use. The purity of the intermediates and end products was checked by thin-layer chromatography on microscope slides coated with silica gel G (Merck, Germany). The compounds on thin-layer chromatography plates were routinely visualized by charring with 20% sulfuric acid. Occasionally, a molybdate reagent (*14*) was used to identify phosphorus-containing compounds. Lipid phosphorus was determined quantitatively by the method of Chen *et al.* (*15*) on samples ashed by the procedure described by Ames and Dubin (*16*). Acid hydrolysis of thioester-containing compounds was carried out in 5% (w/v) sulfuric acid in methanol for 2 hr at 80°C in a nitrogen atmosphere. After neutralization with 1 *M* potassium hydroxide, free thiol groups were determined spectrophotometrically in a medium containing 4 m*M* DTNB and 200 m*M* potassium phosphate buffer, pH 7.5. A calibration curve was made by carrying 2-mercapto-1-ethanol through the same procedure. All spectrophotometric assays were done in a Varian spectrophotometer Model 635. When enzymatic activities had to be measured the cell compartment was thermostated at 37°C. From calibration curves with 2-mercapto-1-ethanol a molar extinction coefficient of 12,800 liters $mole^{-1}$ cm^{-1} was deduced, and this is in good agreement with literature values (*17*).

Element analyses were carried out by the Element Analytical Section of the Institute for Organic Chemistry TNO, Utrecht, The Netherlands. Nuclear magnetic resonance spectra were measured on a Varian EM-360 60 MHz spectrometer and were carried out in the Department of Instrumental Analysis, Laboratory of Organic Chemistry, State University of Utrecht. A Hitachi grating infrared spectrometer Model EPI-G3 was used to obtain infrared spectra. The measurements, with the compound between

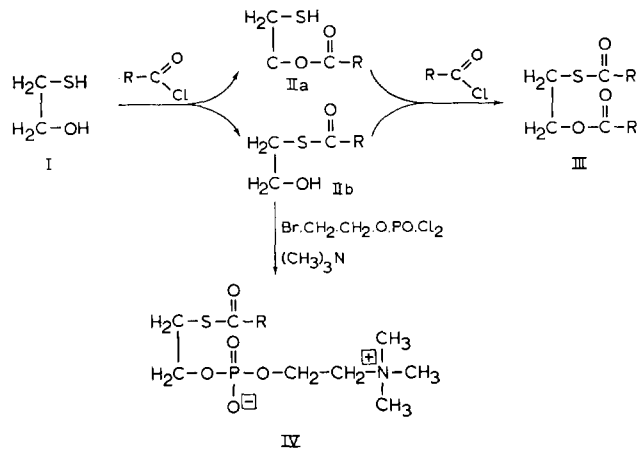
two potassium bromide plates, were carried out in the Department of General Chemistry of this University. Melting points were determined with a Leitz mikroskopheiztisch 350 and are uncorrected.

The porcine pancreatic lipase (EC 3.1.1.3) used was a generous gift from Dr. R. Verger, Marseille, France. Phospholipase A_2 (EC 3.1.1.4) from porcine pancreas was kindly donated by Dr. H. M. Verheij of this laboratory. Lysophospholipase II (EC 3.1.1.5) was purified from beef liver as described previously (*18*).

SYNTHESIS OF ANALOGS OF LYSOLECITHIN

A. 2-Hexadecanoylthio-1-ethyl-phosphorylcholine (IV)

Compound IV was synthesized by a slight modification of the methods described by Eibl *et al.* (*19*). The reaction sequence is outlined in Scheme 1. In principle, the acylation of an excess of 2-mercapto-1-ethanol with an acylchloride can give rise to the



SCHEME 1. Route of synthesis of 2-hexadecanoylthio-1-ethyl-phosphorylcholine. R stands for $-(CH_2)_{14}-CH_3$. I, 2-mercapto-1-ethanol; IIa, 1-hexadecanoyloxy-2-ethanethiol; IIb, 2-hexadecanoylthio-1-ethanol; III, 2-hexadecanoylthio-1-hexadecanoyloxy-ethane; IV, 2-hexadecanoylthio-1-ethyl-phosphorylcholine (in text, thioglycol lecithin).

formation of three products, i.e., the monoacyloxyester (IIa), the monoacylthioester (IIb), and the diacyl mixed ester (III). Under the experimental conditions described below the monoacyl product consists almost entirely of the monoacylthioester (IIb).

Procedure. 2-Mercapto-1-ethanol (I), 11.5 g (147 mmoles) was mixed with 10 ml of dry ether and 5 ml of anhydrous pyridine. To this mixture, 8.4 g (31 mmoles) of palmitoylchloride in 10 ml of ether was added dropwise, at 0°C. After the addition the reaction mixture was kept at room temperature for 1 hr and then analyzed by thin-layer chromatography. The result is reproduced in Fig. 1. It can be seen from this figure that the monoacyl product of 2-mercapto-1-ethanol behaves chromatographically identically to a diacylglycerol, whereas the diacyl product of 2-mercapto-1-ethanol shows the same *Rf*-value as a triacylglycerol referent. It is clear from Fig. 1 that the reaction

mixture contained very little free fatty acid, but a considerable amount of diacyl product had been formed. The reaction mixture was diluted with 100 ml of ether and then extracted five times with 50 ml of water to remove the excess of 2-mercapto-1-ethanol. The remaining ether layer was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in warm *n*-hexane and left at −20°C for 18 hr, after which the precipitate was collected on a G2 glass filter. Both filtrate and precipitate were investigated by thin-layer chromatography. It is clear from Fig. 1

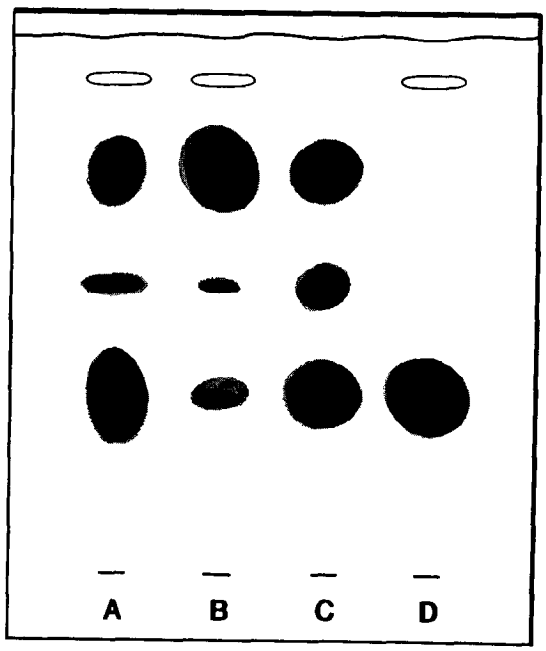


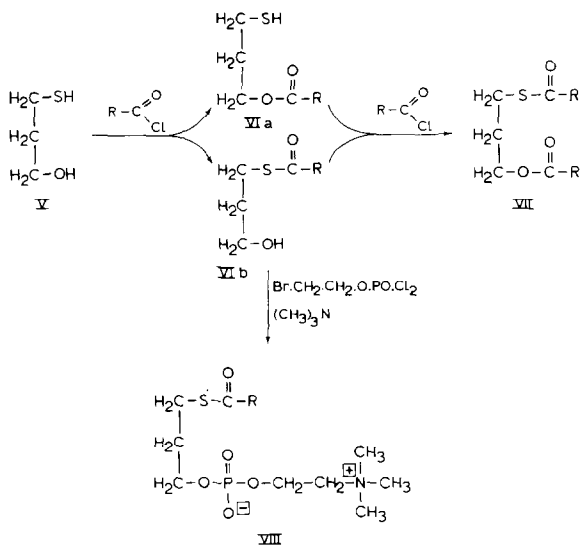
FIG. 1. Thin-layer chromatogram of products obtained during acylation of 2-mercapto-1-ethanol with palmitoylchloride. Developing solvent mixture: petroleum ether (bp 40–60°C)–diethyl ether–formic acid (60 : 40 : 1.5, v/v/v). A, total reaction mixture; B, filtrate; C, mixture of reference compounds containing (from bottom to top) diglyceride (prepared by phospholipase C hydrolysis of egg phosphatidylcholine), oleic acid, and triglyceride (triolin); D, precipitate.

that the precipitate (7.0 g; 71%) consisted of almost pure monoacyl product. Upon analysis with DTNB it was found to contain less than 0.1% of free thiol groups, whereas after acidic hydrolysis in methanol 93% of the thiol groups, on a weight basis, could be recovered. We therefore concluded that the isolated product consisted of 2-hexadecanoylthio-1-ethanol. In a slight modification of the method of Hirt and Berchtold (*20*) 1.58 g (5 mmoles) of this compound (IIb) in dry chloroform was added dropwise to 4.9 g (20 mmoles) of 2-bromoethylphosphoryldichloride in the presence of 4.3 g (42 mmoles) of triethylamine in chloroform at 0°C. After the addition the mixture was kept at room temperature for 20 hr. The hydrolysis of the 2-hexadecanoylthio-1-ethyl-phosphorylmonochloride-ethyl-bromo-ester into 2-hexadecanoylthio-1-ethyl-phosphorylethyl-bromo-ester and the introduction of the trimethylamine group was carried out as described by Eibl *et al.* (*19*) except that during the introduction of the

trimethylamine group the temperature never exceeded 25°C. After evaporation of the solvent, the residue was taken up in chloroform–methanol–water (5 : 4 : 1, v/v/v) and percolated over a mixed Amberlite ion-exchange column (IRA-45 and IRC-50). The final purification was done on a silicic acid column, yielding 1.1 g of product IV (overall yield, 33%). On the silicic acid column the product behaved like lysolecithin, and the purified product gave a single phosphorus-positive spot upon silica gel thin-layer chromatography in chloroform–methanol–water (60 : 40 : 10, v/v/v) with an R_f-value identical to lysolecithin. On a weight basis the phosphorus analysis revealed a purity of 98%. The compound contained no free thiol groups, whereas after acid hydrolysis as described under Materials and Methods only 70% of the expected amount of thiol groups were recovered, presumably due to some oxidation of thiols. Enzymatic hydrolysis in the presence of an excess of phospholipase A_2 or lysophospholipase II resulted in the formation of 90–95% of the theoretical amount of thiols. A single element analysis gave the following data: *Anal.* Calcd. for $C_{23}H_{48}NO_5PS$ (MW, 482): C, 57.3; H, 10.1; N, 2.9; P, 6.4; S, 6.6. Found: C, 56.0; H, 10.3; N, 2.8; P, 6.4; S, 6.1.

B. 3-Hexadecanoylthio-1-propyl-phosphorylcholine (VIII)

This compound was synthesized because of its closer structural relationship to 1-acyl lysolecithin in comparison to compound IV. It has previously been shown (*21*) for rat liver lysophospholipase that the lack of a free hydroxyl group at carbon-2 of 1-acyl lysolecithin reduced the hydrolysis rate by only 10%. On the other hand the lack of this hydroxyl group facilitates for obvious reasons the synthetic processes. The reaction sequence for the synthesis of this thioester analog of lysolecithin is outlined in Scheme 2. Due to the fact that the starting material for this synthesis,



SCHEME 2. Route of synthesis of 3-hexadecanoylthio-1-propyl-phosphorylcholine. R stands for $-(CH_2)_{14}-CH_3$. V, 3-mercapto-1-propanol; VIa, 1-hexadecanoyloxy-3-propanethiol; VIb, 3-hexadecanoylthio-1-propanol; VII, 3-hexadecanoylthio-1-hexadecanoyloxy-propane; VIII, 3-hexadecanoylthio-1-propyl-phosphorylcholine (in text, thiodeoxylysolecithin).

3-mercapto-1-propanol, was not commercially available, it was synthesized from 3-chloro-1-propanol and thiourea according to the method of Clinton *et al.* (*22*). A colorless liquid was obtained by vacuum distillation, bp 39–40°C (0.7 mm); lit. bp 75–80°C (7 mm) (Ref. (*22*)). Since this material represented the starting product for our synthesis outlined in Scheme 2, it was carefully analyzed. An index of refraction n_D^{20} of 1.495 was found (lit. n_D^{22}: 1.492, Ref. (*23*)) as compared to an n_D^{20} of 1.446 for 3-chloro-1-propanol. The thiol group content of the synthesized 3-mercapto-1-propanol as determined with DTNB amounted to 102% of the theoretical value.

The infrared spectrum of the liquid indicated that the aliphatic C-Cl absorption bands of 3-chloro-1-propanol between 660 and 760 cm^{-1} had disappeared. Instead, a weak absorption band in the region of 2550 cm^{-1}, characteristic for the SH-group in 3-mercapto-1-propanol, had appeared. The proton nmr spectrum of the liquid showed a broad peak at δ 4.65, a triplet at δ 3.68 ($J = 6$ Hz), a triplet at δ 2.65 ($J = 6$ Hz) and a quintet at δ 1.83 ($J = 6$ Hz), with the areas of the last three peaks in a ratio of 2 : 2 : 3. When the compound was dissolved in D_2O, the OH-peak at δ 4.65 disappeared and the ratio of the areas of the other three peaks became 2 : 2 : 2. This allowed the conclusion that the SH-peak was hidden beneath the quintet at δ 1.83. After addition of some trifluoroacetic acid to the compound, a triplet at δ 1.47 ($J = 6$ Hz) due to SH-groups appeared, and the triplet at δ 2.65 became a quartet because of the extra splitting effect of the SH in an acidic medium. All these data indicate that pure 3-mercapto-1-propanol was obtained.

Procedure for synthesis of 3-hexadecanoylthio-1-propyl-phosphorylcholine (*VIII*). The synthesis of this compound according to the reactions in Scheme 2 was carried out under conditions identical to those described above for compound IV. After the acylation and crystallization step, the precipitate still contained some diacylated 3-mercapto-1-propanol. Since no free SH-groups could be detected in this fraction, it was concluded that the monoacyl product consisted entirely of compound VIb; and this preparation was used as such for the introduction of the phosphorylcholine moiety by the procedures described above for the synthesis of compound IV. After purification by silica column chromatography the final product appeared nearly pure on thin-layer chromatography in chloroform–methanol–water (60 : 40 : 10, v/v/v), giving a single molybdate-positive spot with an R_f-value identical to a 1-acyl lysolecithin reference. The phosphorus determination revealed a purity of 85% on a weight basis, and acid hydrolysis liberated 85–90% of the expected thiol groups on a weight basis. A single element analysis after correction for the 85% recovery found in the phosphorus analysis gave the following data: *Anal.* Calcd. for $C_{24}H_{50}NO_5PS$ (MW, 496): C, 58.1; H, 10.2; N, 2.8; P, 6.3; S, 6.5. Found: C, 57.5; H, 10.8; N, 2.7; P, 6.3; S, 6.5.

SYNTHESIS OF TRIBUTYRYL ESTER OF 3-MERCAPTO-1,2-PROPANEDIOL

To investigate the susceptibility of acylthioester bonds to hydrolysis by pancreatic lipase, a tributyrine analog containing an acylthioester bond was synthesized by acylation of 3-mercapto-1,2-propanediol with butanoylchloride. Butanoylchloride (300 mmoles) in dry ether was added to 50 mmoles of 3-mercapto-1,2-propanediol and 400 mmoles of pyridine in dry ether at 0°C. The mixture was kept 4 hr at room tem-

perature under stirring, after which the reaction was stopped by addition of water. The products were extracted several times with chloroform. The chloroform layer was evaporated and the residue was kept overnight in a desiccator over potassium hydroxide under high vacuum to remove traces of water and butanoic acid. The triacyl compound was purified by silicic acid column chromatography with *n*-hexane. The final product (yield, 20%) gave a single spot upon thin-layer chromatography with petroleum ether–ether–formic acid (60 : 40 : 1.5, v/v/v) as the developing solvent mixture and exhibited the same R_f-value as commercial tributyrine. Acid hydrolysis revealed the presence of 95–100% of the expected amount of thiol groups for 3-butyrylthio-1,2-dibutyryloxypropane.

ENZYMATIC HYDROLYSIS OF THIOESTER SUBSTRATES

The applicability of acylthioester analogs of substrates in measuring the activity of some lipolytic enzymes is demonstrated in the Figs. 2 and 3. Figure 2 is a photograph of a recorder tracing obtained from the spectrophotometer when phospholipase A from pig pancreas was incubated in a cuvette with thioglycol lecithin (IV). In agreement

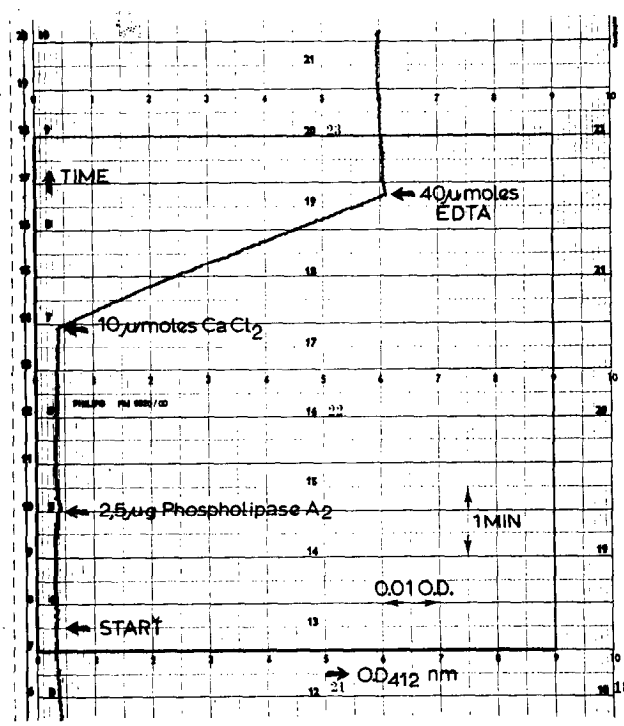


FIG. 2. Spectrophotometric assay of phospholipase A_2. Change in optical density with time of an incubation mixture containing 100 nmoles of 2-hexadecanoylthio-1-ethyl-phosphorylcholine, 1 µmole of DTNB and 0.6 µmole of EDTA in 1.0 ml of 200 m*M* Tris/maleic acid buffer (pH 7.5). Additions as indicated. The reference cuvette contained no substrate. The constant optical density before the addition of the enzyme indicates the high stability of the substrate.

with the requirement of this enzyme for Ca^{2+} ions (*24*), an increase in optical density as a consequence of thiol formation is observed only after addition of this cation. Enzymatic activity was blocked completely by addition of an excess of EDTA. This experiment clearly indicates that phospholipase A_2 can hydrolyse acylthioester bonds in its substrates. Thiodeoxylysolecithin (VIII) was not hydrolysed by phospholipase A_2, in agreement with the fact that this compound does not fulfill the minimal substrate requirements for phospholipase A_2 as deduced by de Haas *et al.* (*24*). Figure 3

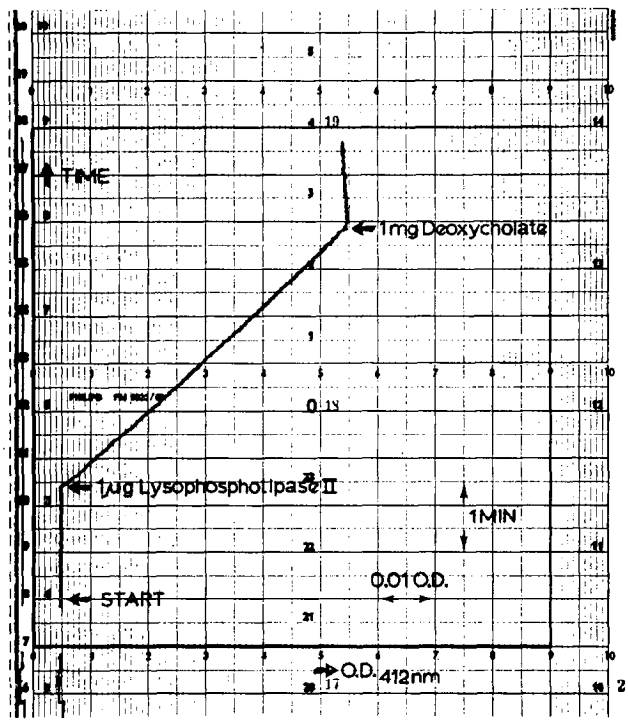


FIG. 3. Spectrophotometric assay of lysophospholipase. Change in optical density with time of an incubation mixture containing 200 nmoles of 3-hexadecanoylthio-1-propyl-phosphorylcholine in 1.0 ml of 1 m*M* DTNB in 20 m*M* potassium phosphate buffer (pH 7.5). Additions as indicated. The reference cuvette contained no substrate.

shows the changes observed in optical density with time when lysophospholipase II from beef liver was incubated with thiodeoxylysolecithin (VIII). This enzyme has no requirement for bivalent cations (*18*) and thiol group release is observed without addition of Ca^{2+}. In confirmation of previous observations using 1-palmitoyl lysolecithin as substrate (*18*) the activity of this enzyme is completely inhibited by sodium deoxycholate at a concentration of 1.0 mg/ml. Similar results were obtained with thioglycol lecithin as substrate for the lysophospholipase II. The initial rates of hydrolysis as measured spectrophotometrically by the release of thiol groups from the respective substrates were linear functions of the amounts of enzymes (Fig. 4). To compare the rates of hydrolysis found for these thioester substrates with those for the corresponding oxyester substrates, the latter were synthesized in radioactive form

using [1-^{14}C]palmitoylchloride. In general, the thioester compounds appeared to be hydrolysed two- to threefold faster than the corresponding oxyester compounds (Aarsman and van den Bosch, unpublished observations). An exact comparison of the kinetic parameters for the hydrolysis of the thioester and oxyester compounds by these enzymes as well as by other purified lysophospholipases is currently under investigation.

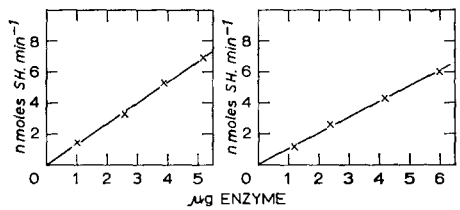


FIG. 4. Initial velocities as a function of enzyme concentration. Left: Hydrolysis of thioglycol lecithin as a function of phospholipase A_2 concentration. Conditions as described in the legend of Fig. 2, except that 10 m*M* Ca^{2+} and the indicated amounts of phospholipase A_2 were used. Right: Hydrolysis of thiodeoxylysolecithin as a function of lysophospholipase II concentration. Conditions as described in the legend of Fig. 3, except that 250 nmoles of substrate and varying amounts of enzyme were used.

The successful application of acylthioester hydrolysis by phospholipase A_2 and lysophospholipase in a spectrophotometric assay for these enzymes has led us to investigate whether pancreatic lipase is also able to split thioester bonds in triglycerides. Two reports in the literature claim that lipase is unable to hydrolyse acylthioester bonds in glyceride analogs. First, Brockman *et al.* (*25*) determined the rate constants for the enzymatic hydrolysis of monolayers of diglyceride analogs by pancreatic lipase and found that thioester analogs were not hydrolysed at measurable rates. Because of this observation the opinion prevailed that lipase is unable to hydrolyse thioester bonds (*26*). Second, Bovier-Lapierre (*27*) also did not succeed in detecting any activity of pancreatic lipase with 1,2-dihexanoyl dithioethane. On the other hand, Barber and Lands (*28*) used preparations of pancreatic lipase to hydrolyse the thioester bonds in various acyl-CoA derivatives in a quantitative assay to determine acyl-CoA concentrations. Although these authors could not exclude the possibility that other hydrolytic enzymes in the lipase preparation used were responsible for the observed hydrolysis of acyl-CoA esters, it appeared more likely that the splitting of the thioester bond was due to the action of the lipase itself. Recently, Jansen and Hülsmann (*29*) have shown that the long chain acyl-CoA hydrolase activity in postheparin serum is identical to clearing factor lipase and different from the carboxylesterase hydrolysing *p*-nitrophenylacetate. If lipases can hydrolyse acylthioester bonds in substrates like acyl-CoA it seemed unexpected that thioester bonds in the natural substrates for lipases, i.e., triglycerides, would be resistant. In view of the cited findings we have reinvestigated the subject of acylthioester hydrolysis by pancreatic lipases. The highly purified lipase preparation used in these studies was found to be not inhibited during a 10-min preincubation at room temperature in 1 m*M* diisopropylfluorophosphate, in agreement with the findings of Maylié *et al.* (*30*). The high hydrolysis rate of thiotributyrine by

this preparation can therefore not be due to possible traces of a contaminating esterase, as the latter is known to be inhibited very effectively by diisopropylfluorophosphate (*31*).

Several observations indicate that lipase can hydrolyse acylthioester bonds in glyceride analogs. First of all, when 1 μmole of 2-hexadecanoylthio-1-ethanol (IIb) was incubated with 40 μg of lipase in 1.0 ml of 10 m*M* Tris/HCl buffer (pH 7.8) containing 10 m*M* $CaCl_2$ and 0.5 mg of sodium deoxycholate for 1 hr at 37°C, spectrophotometric thiol determinations on aliquots of the incubation medium revealed 60% hydrolysis. A control incubation without lipase contained no free thiol groups. The remainder of the incubation mixtures was extracted with chloroform, and the extracts were subjected to thin-layer chromatography. Figure 5 clearly shows the formation of fatty acid and

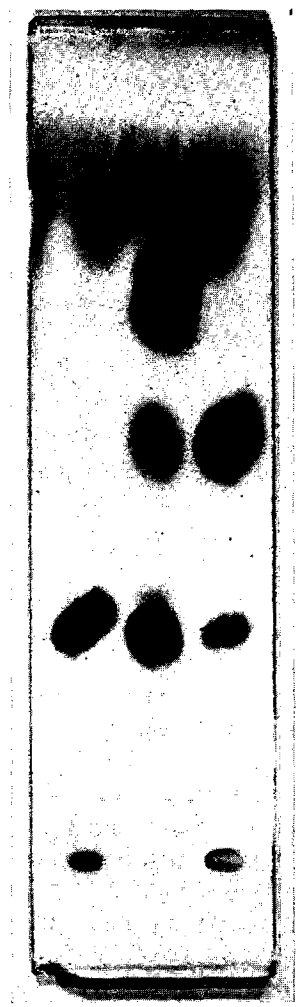


FIG. 5. Action of pancreatic lipase on 2-hexadecanoylthio-1-ethanol. Thin-layer chromatogram of chloroform extracts obtained from an incubation of 2-hexadecanoylthio-1-ethanol without (left lane) and with lipase (right lane). Developing solvent and reference compounds (middle lane) as in Fig. 1.

a decrease in the amount of 2-hexadecanoylthio-1-ethanol in the incubation mixture containing lipase, in agreement with the result of the thiol determination. The spots at the origin and at the solvent front were also found when a solution of only sodium deoxycholate was extracted with chloroform. When 2-hexadecanoylthio-1-ethanol was incubated with lipase in the presence of 1 m*M* DTNB a linear increase of the optical density at 412 nm with time was observed. On the other hand, such an increase in absorbance with 2-hexadecanoylthio-1-hexadecanoyloxy-ethane as substrate for the lipase in the presence of 10% (v/v) isopropyl alcohol was observed only when the temperature was raised to about 50°C. A similar phenomenon, suggesting that the substrate should be in a liquid state to permit hydrolysis, was observed by Luddy *et al.*

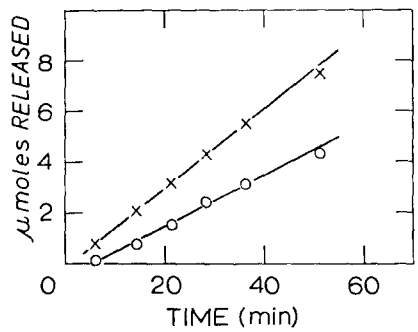


FIG. 6. Determination of released acyl and thiol groups during incubation of thiotributyrin with pancreatic lipase. The incubation mixture in the titration vessel at 37°C consisted of an emulsion of 16 μmoles of thiotributyrin in 4.0 ml of 0.3 m*M* Tris/HCl buffer (pH 8.0) containing 0.2 *M* sodium chloride, 1.6 mg of bovine serum albumin, and 1.6 mg of sodium deoxycholate. After addition of 0.05 μg of lipase the released acid was continuously titrated at a constant pH of 8.0 under nitrogen with 0.01 *M* sodium hydroxyde with a Radiometer pH-Stat TTT_2 equipment. At the indicated times aliquots were withdrawn for immediate spectrophotometric determination of released thiols. Corrections were made for the change in turbidity during the incubation period. (×—×), Release of acyl groups; (o—o), release of thiol groups.

(*32*). In another experiment to show acylthioester hydrolysis by lipase to the tributyryl ester of 3-mercapto-1,2-propanediol (thiotributyrine) was used. Total hydrolysis was monitored by continuous titration of fatty acids by the pH-Stat technique. Release of thiol groups was determined at intervals by using aliquots of the assay medium for reaction with DTNB. The results shown in Fig. 6 indicate that the ratio of total fatty acid to thiol release is roughly 2 : 1. No correction was made for alkali utilization due to neutralization of the slightly acidic thiol group. Since, after extraction of the incubation medium with chloroform, only the formation of dibutyrin could be detected on thin-layer chromatograms and since lipase is known to be specific for the primary 1,3-positions in triglycerides (*33*), we conclude from this experiment that the thioester bond is hydrolysed at about the same rate as the oxyester bond.

DISCUSSION

The results described in this paper clearly indicate the potentiality of using acylthioester analogs of substrates in convenient and sensitive assays of phospholipase A_2

from pig pancreas and lysophospholipase II from beef liver. This method may very well be applicable to other lipolytic enzymes as well. In this respect it is worthwhile to mention that homogeneous lysophospholipase I from beef liver (*18*) and lysophospholipase from beef pancreas (*34*) also were able to hydrolyse the acylthioester bond in thioglycol lecithin and thiodeoxylysolecithin (unpublished observations). The possibility of using the thioester analogs for continuous spectrophotometric determination of lipolytic activities has great advantages for investigators in those research and clinical laboratories where determination of lipolytic enzymes is a routine matter, especially since initial velocities can easily be obtained. Whether such a spectrophotometric assay can be developed depends, of course, on the sensitivity of the lipolytic enzyme under investigation to dithionitrobenzoic acid and on its pH optimum. Best results can be expected for enzymes with a pH optimum between pH 7 and 9, where the molar extinction coefficient of 5-thio-2-nitrobenzoate is highest and air oxidation of 5-thio-2-nitrobenzoate represents no serious problem. On the other hand a partial inhibition of the enzyme by the DTNB concentration used does not necessarily mean a decrease in sensitivity in comparison to methods using substrates with acyloxyesters. The hydrolysis of acylthioester bonds may be several times more rapid than that of the corresponding oxyester, as we have observed for phospholipase A_2 and beef liver lysophospholipase II. Some of these limitations due to the use of DTNB might be circumvented by use of pyridyl disulfides as described by Grassetti and Murray (*35*).

Obviously, the measurement of a specific lipolytic enzyme in crude systems by use of a thioester substrate analog can be disturbed by the possible presence of nonspecific thioesterases, just as methods employing oxyester substrates for the given lipolytic enzyme can be disturbed by the action of nonspecific oxyesterases. Of course, when studying a lipolytic enzyme in crude enzyme systems, it is absolutely necessary to establish that the hydrolysis of a thioester substrate is affected by environmental factors in a manner similar to the hydrolysis of the corresponding oxyester substrate. Likewise, during the initial purification of a given lipolytic enzyme, the results obtained by assaying with a thioester analog of the substrate should be compared with results obtained by using the oxyester substrate in order to be sure that the same enzyme is being purified. In this respect it is worth noting that a crude beef liver extract, which gave two well-separated peaks of lysophospholipase activity on DEAE-Sephadex chromatography when assayed with 1-[1-^{14}C]palmitoyl lysolecithin (Ref. (*18*)), yielded the same two peaks when assayed with a thioester analog of the substrate, i.e., thioglycol lecithin.

The acylthioester analogs of lysolecithin may be useful in several other aspects of research on lysophospholipases, and these methods can probably be extended to other lipolytic enzymes as well. Presumably, the question whether these enzymes act by *O*-alkyl or *O*-acyl cleavage can easily be answered by using the thioester analogs. In addition, the release of free thiol groups during hydrolysis may be applied in cytochemical localization of lysophospholipases by the methods developed by Higgins and Barrnett (*36*) for acyl-CoA-glycero-3-phosphate acyltransferases. Finally, lysophospholipases may be stained in polyacrylamide disc gels by incubation of the gel in the presence of thioester substrate and *p*-nitro blue tetrazolium chloride. This method of detection was very recently described by Farmer and Hageman (*37*), who used *N*-benzoyl-*L*-tyrosine thiobenzylester as a substrate for α-chymotrypsin and subtilisin.

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