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SYNTHETIC LECITHINS CONTAINING ONE SHORT-CHAIN FATTY ACID AND THEIR BREAKDOWN BY PHOSPHOLIPASE A

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SUMMARY

The synthesis is described of two structurally isomeric L- α -lecithins containing as fatty acid residues butyric acid and oleic acid. Phospholipase A (EC 3.1.1.4) from *Crotalus adamanteus* hydrolysed the β -fatty acid linkage irrespective of the nature of the fatty acid constituent, though (γ -oleoyl- β -butyryl)-L- α -lecithin was found to be much more readily attacked than (γ -butyryl- β -oleoyl)-L- α -lecithin.

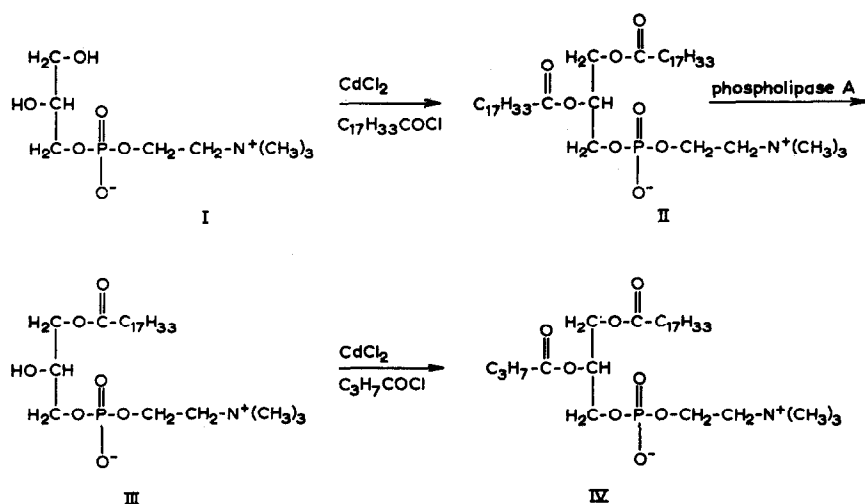
INTRODUCTION

Previous studies¹ have shown that water-soluble lecithins containing two short-chain fatty acid constituents are less susceptible towards the action of phospholipase A (EC 3.1.1.4) from *Crotalus adamanteus* than substrates containing long-chain residues. The results indicated that the micellar state and the nature of the fatty acid constituents are of importance for the interaction between the enzyme and substrate. Thus it became of interest to investigate the enzymic hydrolysis of phosphoglycerides containing one short-chain and one long-chain fatty acid. The present paper deals with the syntheses of (γ -oleoyl- β -butyryl)-L- α -lecithin and (γ -butyryl- β -oleoyl)-L- α -lecithin and a comparison has been made of their rate of hydrolysis with L- α -lecithins containing two molecules of oleic acid and butyric acid respectively.

EXPERIMENTAL

Synthetic substrates

(γ -oleoyl- β -butyryl)-L- α -Lecithin. The synthesis of the isomer carrying the short-chain fatty acid in β -position was carried out according to the principle applied by DE HAAS AND VAN DEENEN² and HANAHAN AND BROCKERHOFF³ for the synthesis of mixed-acid lecithins (Scheme 1). L- α -Glycerolphosphorylcholine (I) was prepared from egg lecithin by the very convenient method of HANAHAN⁴. This compound was converted into its CdCl₂ adduct ($[\alpha]_D^{20} = -1.15^\circ$ in water, *c* 10) and acylated with oleoyl chloride (from pure oleic acid donated by Unilever Research Laboratory,

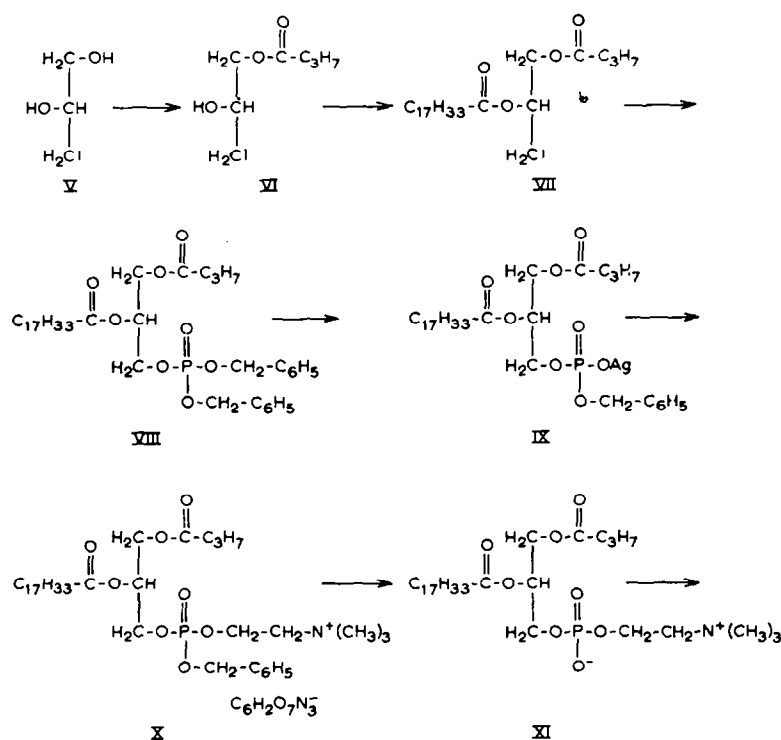


Scheme 1.

Vlaardingen), according to the procedure of BAER AND BUCHNEA⁵. After removal of the CdCl_2 , it was demonstrated by means of paper and thin-layer chromatography that there is also a considerable amount of lysolecithin formed as byproduct. After chromatography on silicic acid both the desired 1- α -(dioleoyl)-lecithin (II; $[\alpha]_D^{20} = +6.1^\circ$ in chloroform, *c* 10) and the mono-acyl analog were isolated in a pure form. As regards the chemical structure of the latter by-product, this product was subjected as CdCl_2 adduct to an acylation with palmitic acid and the oleoyl-palmitoyl- α -lecithin formed was almost completely degraded by snake venom. The fatty acids and lysolecithin formed were isolated by chromatography and gas-liquid chromatographic analysis of the fatty acid composition of each of these showed that the obtained lysolecithin must have been about 75% γ - and 25% β -oleoyl-1- α -lysolecithin. A similar method recently was reported by LETTERS AND SNELL⁶. The results were qualitatively confirmed by a breakdown of the lysolecithin preparation with phospholipase C (EC 3.1.4.3) and a separation of the 1- and 2-monoglycerides by thin-layer chromatography. Furthermore treatment with phospholipase A furnished glycerylphosphorylcholine in a 25% yield.

The dioleoyl lecithin (II) was hydrolysed enzymically by snake venom according to the procedure of HANAHAN⁷, giving the lysolecithin (III) in high yield. This product was chromatographically pure by thin-layer chromatography without recourse to column chromatography and gave $[\alpha]_D^{20} = -3.0^\circ$ in chloroform (*c* 10) and $[\alpha]_D^{20} = -1.17^\circ$ in chloroform-methanol (1:1, v/v).

0.6 g of the CdCl_2 complex of III was dissolved in 10 ml of dry chloroform at 0° and to this chilled solution 0.27 g of butyryl chloride in 1.8 ml anhydrous pyridine and 5 ml chloroform were slowly added. The mixture was stirred for 4 h at 25° when the reaction was stopped by chilling and adding a little water. The reaction mixture was then de-ionized by passing through a column containing a mixture of Amberlites IR-45 (OH⁻) and IRC-50 (H⁺) in chloroform-methanol-water (5:4:1, v/v). After silicic acid chromatography the desired lecithin (IV) was obtained in 63% yield



Scheme 2.

(0.28 g) based on the CdCl_2 adduct of III; $[\alpha]_{\text{D}}^{20} = +4.9^\circ$ in chloroform (c 10). (Found: C, 41.03; H, 7.04; $(\text{C}_{30}\text{H}_{50}\text{O}_6\text{NP})_3(\text{CdCl}_2)_2$ requires C, 40.72; H, 6.83%). Other properties of the lecithin are discussed together with its structural isomer.

(\gamma-butyryl- β -oleoyl)-L- α -Lecithin. While L- α -(dibutyryl)-lecithin can be obtained starting from glycerylphosphorylcholine⁸, this short-chain lecithin cannot serve for the synthesis of the desired mixed-acid lecithin because of its limited susceptibility towards the phospholipase A from snake venom¹. For this reason use was made of the sequence of reactions (Scheme 2) applied previously for the synthesis *de novo* of lecithins⁹, which was, however, modified to give an appreciably higher yield (Scheme 2).

A solution of 2.13 g butyryl chloride (20 mmoles) in 20 ml dry chloroform was added over 30 min to a mixture of 4.04 g of L- α -iodoglycerol¹⁰ (V; 20 mmoles) and 2 ml of dry pyridine (24 mmoles) cooled to -10° . The mixture was allowed to stand in the dark for 3 days at 0° , then 4 h at 25° . The reaction mixture was diluted with ether, washed with ice-cold 0.1 N H_2SO_4 and then with water; all these operations being effected in the dark. After drying and evaporation of the solvents *in vacuo* the product was recrystallized three times from hexane at -70° to yield 4.0 g (73%) of (γ -butyryl)-L- α -iodoglycerol (VI), a colourless, but very light-sensitive, liquid. Elemental analysis was made difficult by the extreme light sensitivity of the compound.

By thin-layer chromatography the product gave one single spot (Fig. 1) and this together with the infrared spectrum obtained precludes the existence of more than a trace of the unwanted β -butyryl-iodoglycerol. The mother liquor from the recrystallisation contained a small amount of material, presumably the dibutyl-iodoglycerol (R_F 0.9 in the ether-hexane system).

For the conversion into the diacyl derivative a solution of 4.8 g of oleoyl chloride (10% excess) in 10 ml dry chloroform was slowly added to a mixture of 3.6 g of VI and 1.6 ml (65% excess) dry pyridine in 5 ml of chloroform at 0°. After standing for 3 days at room temperature in the dark the pyridine was removed by the acidic wash described for VI, leaving on removal of the solvents the product contaminated with oleic acid. This material was purified by silicic acid column chromatography,

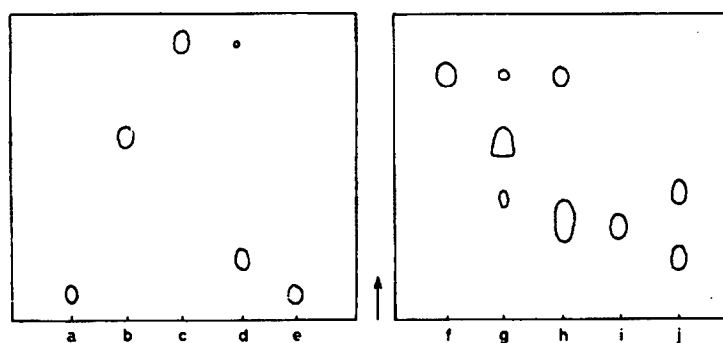


Fig. 1. Reproduction of thin-layer chromatograms of synthetic lecithins and intermediates. Left-hand plate developed in hexane-ether (1:1, v/v). (a) Glycerol- L - α -iodohydrin (V). (b) γ -Butyryl- L - α -iodoglycerol (VI). (c) γ -Butyryl- β -oleoyl- L - α -iodoglycerol (VII). (d) Dibenzyl phosphatidic acid (VIII) with trace of VII as contaminant. (e) Barium salt of monobenzyl phosphatidic acid. Right hand plate developed in chloroform-methanol-water (65:25:4, v/v). (f) Salts of monobenzyl phosphatidic acid (IX). (g) Benzyl lecithin picrate (X) with benzyl phosphatidic acid and bromocholine picrate. (h) Lecithin (XI) with benzyl phosphatidic acid as by-product. (i) Lecithin IV and XI. (j) Dioleoyl lecithin and lyso-(oleoyl)-lecithin reference compounds.

the fatty acid being pre-eluted with 40% benzene in hexane while the desired compound was recovered with 60% benzene in hexane. A yield of 5.5 g (78% on VI) of γ -butyryl- β -oleoyl- L - α -iodoglycerol (VII) was obtained; this colourless liquid was somewhat light sensitive. Thin-layer chromatography revealed one single spot (Fig. 1); $[\alpha]_D^{20} = +3.1^\circ$ in chloroform (c 10).

5 g of VII were refluxed 3 h in the dark with 4.4 g of silver dibenzyl phosphate in dry benzene. The reaction mixture was filtered hot, the filtrate evaporated *in vacuo*, dissolved in pentane and cleared by centrifuging. After evaporation of the solvent, 6.3 g of *O,O*-dibenzyl phosphatidic acid (VIII) were obtained containing a trace of the starting material (Fig. 1). The compound was debenzylated by refluxing 3 h with 50% excess anhydrous BaI_2 in dry acetone. The barium salt was precipitated on cooling to 0° and was crystallized once more from acetone giving 4.7 g (76% on VII) of a white solid, m.p. 158–159°, which was chromatographically pure (Fig. 1). The compound was converted to the more soluble sodium salt by shaking a solution in chloroform with aq. Na_2SO_4 . The sodium salt was converted in the usual way* into

the silver salt of L-(γ -butyryl- β -oleoyl)(O-benzylphosphoryl)-glycerol (IX) of which 4.0 g were obtained.

An amount of 3 g of IX was refluxed 6 h with 3.4 g (100% excess) bromocholine picrate in 250 ml dry tetrahydrofuran in the dark. The reaction mixture was filtered hot to remove AgBr, and the filtrate evaporated *in vacuo*. The mixture was then extracted with dry benzene leaving a residue of unreacted picrate. The evaporated extract (3.9 g of yellow solid) was shown to be largely O-benzyl lecithin picrate (X) together with small amounts of bromocholine picrate and a benzyl phosphatidic acid derivative (Fig. 1).

By contrast to the previously described procedure this crude material was now directly debenzylated by refluxing 2 h in dry acetone with 1.1 g (200% excess) of anhydrous LiBr. After evaporation of the solvents *in vacuo* excess lithium salts were removed by centrifuging from an ethereal solution and the product was then completely de-ionized by passage through a mixed IR-45 and IRC-50 Amberlites column in 90% ethanol. The colourless product was shown by thin-layer chromatography to contain only benzyl bromide and a benzyl phosphatidic acid as well as the desired lecithin (XI). The mixture (3 g) was then purified by silicic acid chromatography to give as a main by-product 0.7 g of benzyl phosphatidic acid and 1.6 g (64% on IX) of pure (γ -butyryl- β -oleoyl)-L- α -lecithin (X), as a white solid, m.p. 208–210°; $[\alpha]_D^{20} = +5.9^\circ$ in chloroform (*c* 10). (Found: C, 40.68; H, 6.74; $(C_{80}H_{100}O_6NP)_2$ (CdCl₂)₂ requires C, 40.72; H, 6.83%.)

Both lecithins (IV and XI) were soluble in most organic solvents including 80% alcohol, but insoluble in acetone, and in hexane at 0°. However the lecithin XI gave a clear solution in a borate buffer (pH 7), whereas IV produced a turbid dispersion. By thin-layer chromatography in a chloroform–methanol–water system (Fig. 1) and paper chromatography on silica-impregnated paper both compounds display a similar behaviour having a R_F value between that of dioleoyl lecithin and lyso-oleoyl lecithin.

Enzymic hydrolysis

The lecithins were dissolved or emulsified by sonic vibration to a concentration of 0.02 M in a borate buffer (pH 7.0) containing $2.5 \cdot 10^{-3}$ M calcium acetate, and 1 mg of *Crotalus adamanteus* was added. Use was made of a batch of snake venom having moderate phospholipase A activity. The enzymic reaction was stopped by the addition of ethanol–acetic acid (20:3, v/v). Aliquots were subjected to chromatography on silica-impregnated paper using diisobutyl ketone–acetic acid–water (40:25:5, v/v) as solvent system¹¹. In the case of (dibutyryl)-lecithin the chromatograms were made on Whatman paper No. 1 with butanol–ethanol–water (5:5:2, v/v). The spots of the lecithins and lysolecithins were eluted by treating three times for 30 min with methanol at 60°. After evaporation of the solvent, phosphorus analyses were carried out according to the procedure of BÖTTCHER *et al.*¹².

RESULTS AND DISCUSSION

Both isomeric lecithins were found to act as excellent substrates for phospholipase A. After complete enzymic hydrolysis of (γ -oleoyl- β -butyryl)-L- α -lecithin the silica-paper chromatograms revealed only one phosphorus-containing spot, the R_F

value of this lysolecithin being identical to that of γ -oleoyl-L- α -glycerylphosphorylcholine (Fig. 2). Similarly, the structural isomer after incubation with the snake venom furnished one breakdown product moving only a short distance on the silica paper. Chromatograms developed on non-impregnated paper showed that this hydrolysis product was, as expected, identical with butyryl-L- α -glycerylphosphorylcholine (Fig. 2). By virtue of the nature of the lyso derivatives formed it can be concluded that phospholipase A from *Crotalus adamanteus* hydrolyses the β -ester linkage, thus confirming and extending the view that this enzyme acts in a positionally specific way irrespective of the nature of the fatty acid constituents of the substrate. The

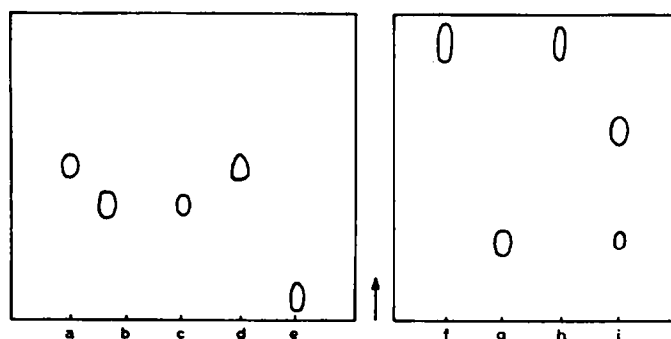


Fig. 2. Paper-chromatographic demonstration of the action of phospholipase A (*Crotalus adamanteus*) on synthetic lecithins containing one short-chain fatty acid. Left hand chromatogram developed on silica-impregnated paper. (a) (γ -oleoyl- β -butyryl)-L- α -Lecithin (IV) control. (b) After hydrolysis of IV with snake venom. (c) Oleoyl-lysolecithin reference compound. (d) (γ -butyryl- β -oleoyl)-L- α -Lecithin (XI) control. (e) After hydrolysis of XI with snake venom. Right hand chromatogram developed on Whatman paper No. 1 with butanol-ethanol-water. (f) control of XI. (g) After enzymic hydrolysis of XI. (h) Oleoyl-lysolecithin reference compound. (i) Uncomplete hydrolysis of (dibutyryl)-L- α -lecithin.

anomalies encountered during the hydrolysis by pancreatic lipase (EC 3.1.1.3) of triglycerides containing long-chain fatty acids and butyric acid^{13,15} appear not to apply to the phospholipase studied. Although both synthetic substrates are readily hydrolysed by phospholipase A a significant difference between their hydrolysis rates was to be noted (Fig. 3). The isomer carrying butyric acid in the β -position appeared to be the best substrate out of the series of synthetic and natural substances so far investigated in this laboratory¹. The results obtained confirm our previous observations that the susceptibility of different lecithin species towards the action of the snake-venom enzyme depends on the nature of the fatty acid constituents but in addition show that their position in the phosphoglyceride molecule is also important. Recent studies of MOORE AND WILLIAMS^{16,17} on the phospholipase A hydrolysis of egg lecithin, consisting of a heterogeneous population of lecithin species, also demonstrated such an effect.

Comparison of the behaviour of both isomeric lecithins with that of L- α -(dibutyryl)-lecithin shows that the presence of one long-chain fatty acid significantly improves the substrate properties. This result fits in well with the previous conclusions of VAN DEENEN AND DE HAAS¹ that a certain molecular orientation of the phospholipid molecules forms a requisite for their enzymic hydrolysis. On the other

hand both mixed-acid lecithins under the given experimental conditions were hydrolysed also faster than L- α -(dioleoyl)-lecithin (Fig. 3). It has to be noticed, however, that a complete hydrolysis of the latter compound is attainable in a 3-h period when emulsions are prepared in the presence of ether. Probably the nature of the substrate micelles is one of the factors determining the accessibility of the lecithins towards the enzyme. Such an effect may be involved also in bringing about the distinction in the velocity of breakdown of both isomeric lecithins. In this respect it is of interest to recall that (γ -oleoyl- β -butyryl)-L- α -lecithin, the "best-substrate", furnished turbid emulsions, whereas under the conditions used a clear solution of (γ -butyryl-

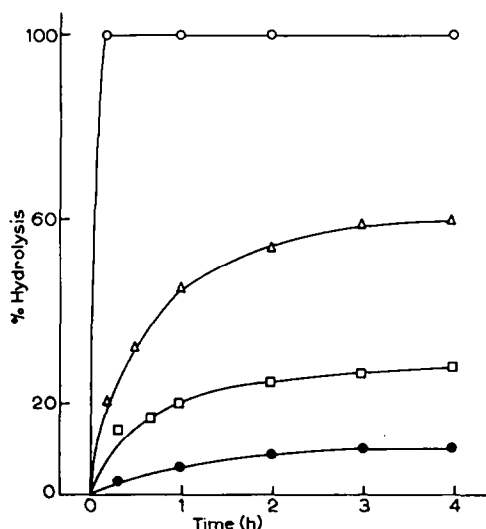


Fig. 3. Hydrolysis of synthetic lecithins by phospholipase A (*Crotalus adamanteus*): \circ — \circ (γ -oleoyl- β -butyryl)-L- α -lecithin (IV); Δ — Δ , (γ -butyryl- β -oleoyl)-L- α -lecithin (XI); \square — \square (dioleoyl)-L- α -lecithin; \bullet — \bullet , (dibutyryl)-L- α -lecithin.

β -oleoyl)-L- α -lecithin was obtained. Studies on the micellar properties of both lecithins may be of help for a further understanding for some of the differences noted. Also the interfacial behaviour of both lecithins appeared to be significantly different as demonstrated by current investigations of Mr. R. A. DEMEL and Dr. B. A. PETHICA (Unilever Research Laboratory, Port Sunlight) on monomolecular layers of these substrates at the oil-water interface. The distinction in molecular orientation does raise the question whether some quantitative differences may exist in the ability between both substrates to interact with the enzyme. In order to account for the steric and positional specific course of phospholipase A catalysed reactions by a three-points interaction the possible involvement of both the fatty acid ester bond at the β -position and the oxygenalkyl function at the γ -position have been discussed¹. With a view to these speculations it can be argued on one side that the more rapidly hydrolysed isomer having a short-chain fatty acid at the β -position may more easily render this ester linkage to the enzyme while on the other hand the presence of the long-chain function at the γ -position may improve the substrate-enzyme interaction. The facts available, so far, do not permit any precise evaluation of the relative importance

of both elements forwarded in this dualistic consideration. At the present stage the various observations appear to justify the conclusion only that a reversed position of two different fatty acids in the lecithin molecules can affect their interfacial properties and may bring about a difference in the rate of breakdown by phospholipase A. These results may have certain implications with respect to the significance of the non-random distribution of fatty acids among both ester positions of natural phosphoglycerides in connection to their interaction with proteins present in biological structures.

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