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SYNTHESIS AND ENZYMIC HYDROLYSIS OF AN *O*-ALANYL ESTER OF PHOSPHATIDYL GLYCEROL

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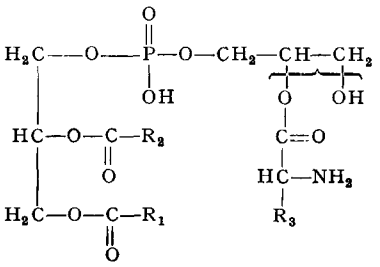
SUMMARY

A racemic *O*-alanyl ester of phosphatidyl glycerol, containing one saturated and one unsaturated fatty acid, was synthesized by a reaction between silver benzyl-(γ-oleoyl-β-palmitoyl)-DL-α-glycerol phosphate and DL-α-iodo-β-*tert.*-butyl-γ-(*N-tert.*-butoxycarbonyl)-DL-alanyl glycerol. The synthetic substance was hydrolysed by phospholipase A (EC 3.1.1.4), C (EC 3.1.4.3) and D. (EC 3.1.4.4). The results of the enzymic degradation and some other properties of this compound have been compared with those of amino acid derivatives of phosphatidyl glycerol from bacteria.

INTRODUCTION

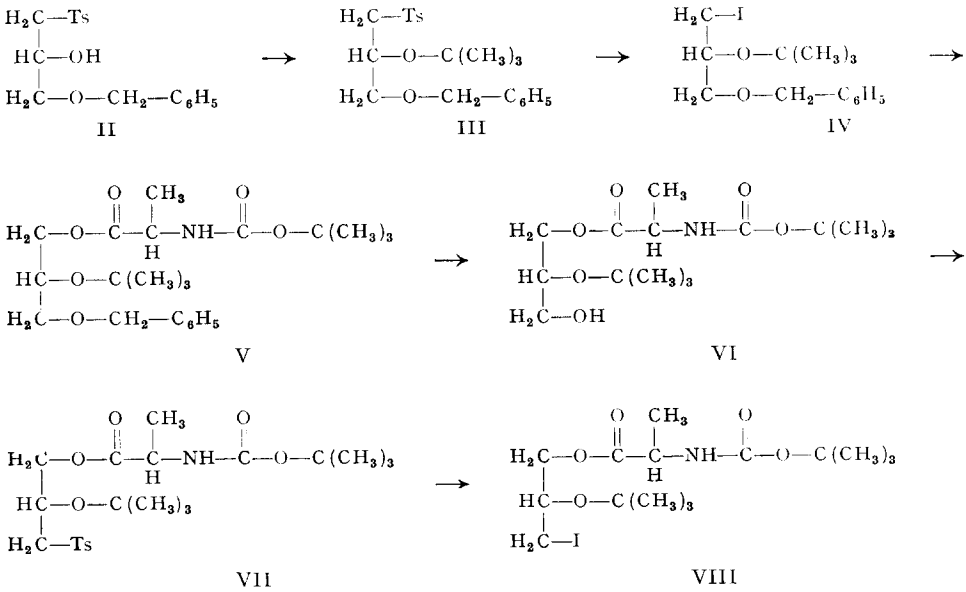
Recent studies on the phospholipids of bacteria have shown the existence of amino acid derivatives of phosphatidyl glycerol. MACFARLANE[1] found in *Clostridium welchii* a complex mixture of phospholipids of this type which could be separated into several fractions, one containing the alanyl ester of phosphatidyl glycerol as a major component. HOUTSMULLER AND VAN DEENEN[2,3] identified a compound in *Bacillus cereus* as an *O*-ornithine ester of phosphatidyl glycerol. Recently these authors[4] isolated in a pure state a lysine ester of phosphatidyl glycerol from *Staphylococcus aureus*, after inducing an accumulation of this phospholipid by means of the pH of the medium. The work in both laboratories indicated these phospholipids to have structure I, leaving so far unsettled the position of the amino acid-ester linkage.



Further investigation on the structure and properties of these compounds may be greatly facilitated by having at one's disposal synthetic substances of this phospholipid type. As a first approach in this direction an alanine ester of phosphatidyl glycerol was synthesized carrying the amino acid at a given position. Appropriate modifications may allow the preparation of the various possible enantiomers and structural analogues as well.

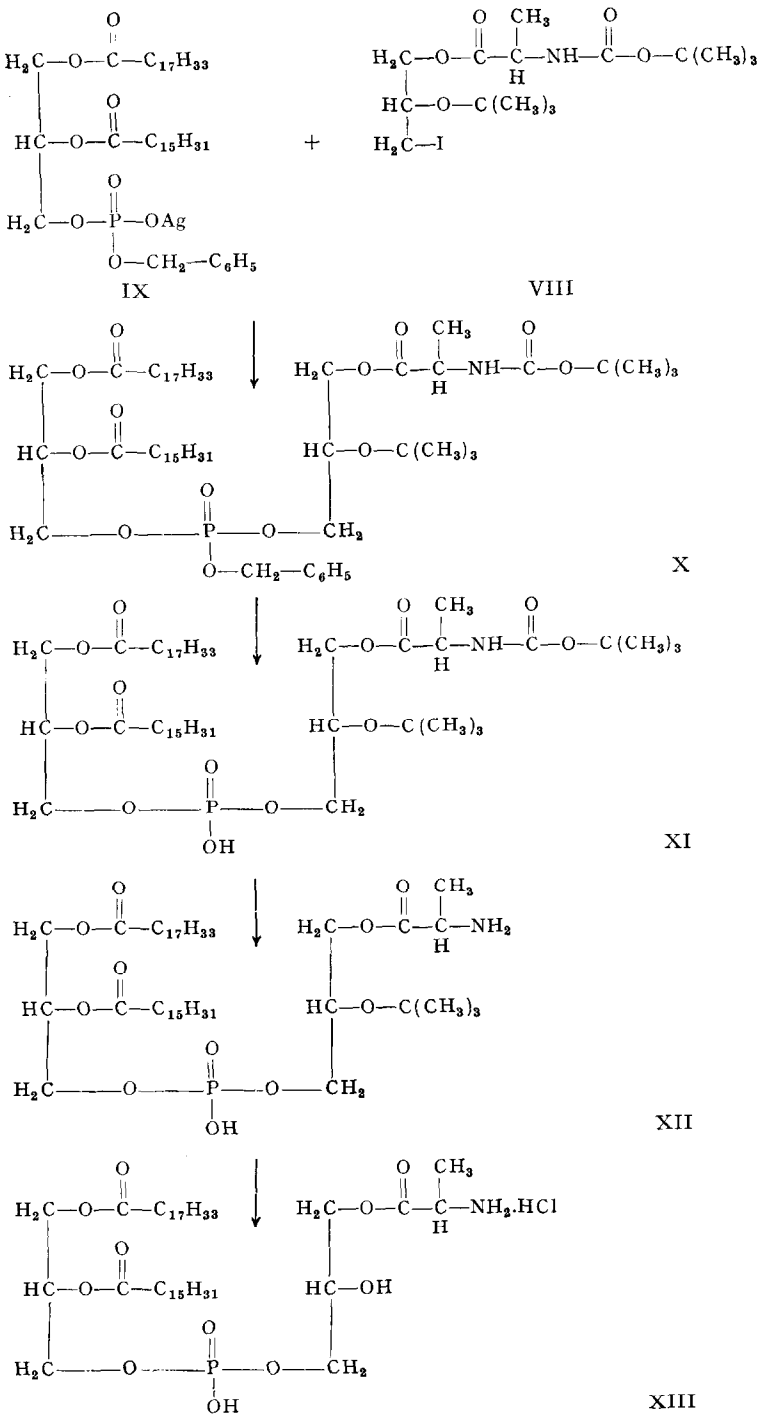
Synthetic methods

In view of the favourable results obtained in this laboratory in the synthesis of several classes of phosphoglycerides by a condensation between a silver salt and an iodo derivative, this principle has been applied in the present work. Envisaging the several modifications[5,6] of this method, preference was given to a reaction between a silver salt of monobenzyl phosphatidic acid and an amino acid ester of iodoglycerol containing appropriate protecting groups, allowing us to obtain an unsaturated phospholipid. For that purpose α-iodo-β-*tert.*-butyl-γ-(*N*-*tert.*-butoxy carbonyl)-alanyl glycerol (VIII) was synthesized (Scheme 1). Starting from α-benzyl-γ-*p*-toluene sulphonylglycerol[7] (II), the *tert.*-butyl ether group was introduced[8], and the reaction product III was converted by treatment with sodium iodide in acetone into α-benzyl-β-*tert.*-butyl-glycerol-γ-iodohydrine (IV). Using the principle of the acylation method described by MILLS *et al.*[9] V was prepared in a good yield by condensing IV with (*N*-*tert.*-butoxycarbonyl)-alanine. Removal of the benzyl group from V was effected by hydrogenolysis in ethanol with palladium as a catalyst, to give VI in a practically quantitative yield. After conversion of VI into VII with *p*-toluene sulphochloride, substitution of the *p*-toluene sulphonyl group with sodium iodide in acetone



Scheme 1

Synthesis of α-iodo-β-*tert.*-butyl-γ-(N-*tert.*-butoxycarbonyl)-alanyl glycerol.



Scheme 2

Synthesis of racemic γ-alanyl-α-phosphatidyl glycerol.

furnished α-iodo-β-*tert.*-butyl-γ-(*N*-*tert.*-butoxycarbonyl)-alanylglycerol (VIII) in an overall yield of 40%.

The sequence of reactions leading to γ-alanyl-α-phosphatidyl glycerol (XIII) Scheme 2 involves a silver iodide interchange reaction between the iodo compound (VIII) and silver benzyl-γ-oleoyl-β-palmitoyl-DL-α-glycerolphosphate (IX). The latter compound was prepared from γ-oleoyl-β-palmitoyl-DL-α-glycerol iodohydrine as described for other homologues [10, 11]. The coupling between VIII and IX proceeded less readily than in the synthesis of lecithins and cephalins, this being probably due to the presence of the bulky *tert.*-butyl ether group at the adjacent carbon atom. Prolongation of the reaction time, or raising the temperature, led to the formation of considerable quantities of by-products. Since debenzylation of the triester phosphate X with barium or sodium iodide did not result in a crystalline salt, use was made of *N*-methylmorpholine [12].

The chromatographic purification of XI offered some difficulties. Fractionation of the reaction mixture on silica columns with pure anhydrous chloroform as eluant caused removal of the *N*-*tert.*-butoxycarbonyl group, thereby giving a fair amount of XII. Both substances XI and XII could be freed from their protecting groups by means of anhydrous hydrochloric acid [13], giving after crystallization γ-alanyl-α-phosphatidyl glycerol as hydrochloric acid salt (XIII) in an analytically and chromatographically pure form, in an overall yield of 35%.

EXPERIMENTAL PART

Materials and general methods

Palmitic acid was purchased from Fluka and had a purity of 99.6%. Oleic acid was purified as described previously [13]. Gas-liquid chromatographic analysis revealed a purity of at least 99.5%. *p*-Toluene sulphochloride and *N*-methylmorpholine were purchased from Fluka and purified by established procedures. *Tert.*-butoxycarbonylazide was prepared according to CARPINO *et al.* [14]. Liquid isobutene was obtained as described by KISTIAKOWSKY *et al.* [15]. Micro-analyses were carried out in the Analytical Department of the Laboratory of Organic Chemistry, University of Groningen. Melting points were determined on a Leitz Mikroscopheiztisch 350, and are uncorrected. Gas–liquid chromatographic analyses of the methyl esters of fatty acids were carried out as described previously [16]. The purity of intermediates and end-product was checked by thin-layer chromatography on microscope slides coated with silica according to PEIFER [17]. Methods of detection have been recorded previously [10, 13].

α-Iodo-β-tert.-butyl-γ-(N-tert.-butoxycarbonyl)-alanylglycerol (VIII). The synthesis of α-*p*-toluene sulphonyl-γ-benzylglycerol (II) has been described in detail [7]. *N*-*tert.*-butoxycarbonyl-DL-alanine was prepared according to the method outlined by WEYGAND AND HUNGER [18] from DL-alanine with *tert.*-butoxycarbonylazide in a yield of 50%. The compound had m.p. 111°. (Found: C, 50.6; H, 8.0; N, 7.2. $C_8H_{15}NO_4$ requires C, 50.78; H, 7.99; N, 7.40%).

α-Benzyl-β-tert.-butyl-γ-p-toluene sulphonylglycerol (III). To a solution of 8.6 g of α-benzyl-γ-*p*-toluene sulphonylglycerol (II) in 50 ml of anhydrous methylene chloride was added 100 ml of liquid isobutene and a solution of 0.5 ml of concentrated sulfuric acid in 25 ml of dry methylene chloride. After standing for 3 days at room temperature, the solution was cooled in an icebath, neutralized with a solution of

saturated potassium carbonate, and concentrated *in vacuo* to remove all organic solvents. The residual suspension was extracted with pentane. The pentane layer was washed twice with water and once with methanol–water (1:3, v/v), in order to remove a small amount of starting material. After drying over anhydrous sodium sulfate, the solvent was evaporated *in vacuo*. It was not possible to crystallize the residual colourless syrup (yield 95%), which was found to be pure on thin-layer chromatograms. (Found: C, 64.0; H, 7.2; S, 8.1. $C_{21}H_{28}O_5S$ requires C, 64.25; H, 7.19; S, 8.17%).

α-Benzyl-β-tert.-butyl-glycerol-γ-iodohydrine (IV). A solution of 9.5 g of III and 10.45 g of dry sodium iodide in 100 ml of anhydrous acetone was refluxed in the dark for 24 h. After removal of the precipitated sodium-*p*-toluene sulphonate (94%) the solution was concentrated *in vacuo*. The residue, dissolved in ether, was washed with a 5% sodium thiosulphate solution, then with water, and dried over anhydrous sodium sulphate. After evaporating the solvent *in vacuo*, the residue was subjected to chromatography in the dark on a silica column with benzene–ether mixtures. The chromatographically pure iodo compound IV was obtained as a nearly colourless oil in a 70% yield. (Found: C, 48.6; H, 6.1. $C_{14}H_{21}IO_3$ requires C, 48.29; H, 6.08%).

α-Benzyl-β-tert.-butyl-γ-(N-tert.-butoxycarbonyl)-alanylglycerol (V). A mixture of 5.75 g of IV, 6.42 g of *N-tert.*-butoxycarbonyl alanine (100% excess) and 3.42 g anhydrous triethylamine was heated for 4 h at 110° with occasional shaking. The cooled mixture was taken up in ether and washed twice with a sodium bicarbonate solution and water respectively. After drying over anhydrous sodium sulphate, the ether was evaporated *in vacuo*. The residue was purified by chromatography on a silica column with mixtures of benzene–ether as eluants. The desired compound V was obtained as a colourless syrup in a yield of 76%. (Found: C, 64,4; H, 8.7; N, 3.4. $C_{22}H_{35}NO_6$ requires C, 64.52; H, 8.61; N, 3.42%).

β-Tert.-butyl-γ-(N-tert.-butoxycarbonyl)-alanylglycerol (VI). Catalytic hydrogenolysis of V in absolute ethanol, using palladium as a catalyst, removed the benzyl group only, and, after removal of the catalyst and solvent, furnished a colourless oil, which showed only one spot on thin-layer chromatograms. A quantitative yield of VI was obtained. (Found: C, 56.1; H, 9.1. $C_{15}H_{29}NO_6$ requires C, 56.40; H, 9.15%).

α-p-Toluene sulphonyl-β-tert.-butyl-γ-(N-tert.-butoxycarbonyl)-alanylglycerol (VII). Introduction of a tosyl group in VI was effected by treatment of 2.8 g of VI with 2 g of *p*-toluene sulphonylchloride (20% excess) in anhydrous pyridine. The mixture was stirred for 48 h at room temperature, and then poured into ice-cold 0.5 N sulfuric acid and extracted with ether. The ether layer was washed twice with 0.5 N sulfuric acid, then with 5% sodium bicarbonate solution and water. From the ethereal solution chromatographically pure VII was obtained in a 90% yield as a colourless syrup, which could not be crystallized. (Found: C, 55.9; H, 7.6. $C_{22}H_{35}NO_8S$ requires C, 55.79; H, 7.45%).

α-Iodo-β-tert.-butyl-γ-(N-tert.-butoxycarbonyl)-alanylglycerol (VIII). Substitution of the tosyl group in VII by iodine was effected in the same manner as described for IV. On thin-layer chromatograms some starting material appeared to be present. This was removed by chromatography in the dark on a silica column with benzene–ether (97:3, v/v) as eluant. The desired compound VIII (a slightly yellow syrup) could not be obtained in a crystalline form, yield 86%. (Found: C, 42.2; H, 6.8; N, 3.5. $C_{15}H_{28}INO_5$ requires C, 41.97; H, 6.57; N, 3.26%).

γ-Alanyl-α-phosphatidylglycerol (XIII). The synthesis of silver benzyl γ-oleoyl-

β-palmitoyl-α-glycerolphosphate (IX) was carried out by methods described in detail for other homologues[10,11].

α-[(γ-Oleoyl-β-palmitoyl)benzyl-α-glycerylphosphoryl]-β-tert.-butyl-γ-(N-tert.butoxycarbonyl)-alanylglycerol.(X). The coupling of 2.17 g of the iodo-compound VIII with 4.35 g of the silver salt IX was carried out in boiling benzene with stirring and under absolute anhydrous conditions. After 4 h reaction time the precipitated silver iodide was removed by centrifugation, the supernatant washed with a 5% sodium bicarbonate solution, a sodium thiosulphate solution and water. The benzene layer was dried over anhydrous sodium sulphate and evaporated *in vacuo*. Most of the unreacted silver salt IX was removed as sodium salt by crystallization of the reaction mixture from chloroform–acetone at −15°. The residue was chromatographed on a silica column with dry chloroform as eluant. This resulted in an almost colourless syrup in a yield of 45%. (Found: C, 65.6; H, 9.7; P, 2.8. $C_{59}H_{104}NO_{13}P$ requires C, 66.45; H, 9.83; P, 2.90%).

α-[(γ-Oleoyl-β-palmitoyl)-α-glycerylphosphoryl]-β-tert.-butyl-γ-(N-tert.-butoxycarbonyl)-alanylglycerol(XI). Debenzylation of the foregoing triphosphate X (1.04 g) was effected in *N*-methylmorpholine at 110° for 3 h, but the reaction did not go to completion. This was shown by thin-layer chromatograms, made after evaporating the excess of *N*-methylmorpholine and extracting the ethereal solution of the residue with ice-cold 0.5 N sulphuric acid and water. Yield 0.93 g (98%) of the crude phosphodiester XI. Purification of this product on a silica column failed. Some triester X could be removed with anhydrous chloroform as eluant. After increasing the methanol concentration of the eluant, only a small quantity of XI was recovered; in addition a substantial amount of XII, as well as a small amount of the end-product (VIII) were recovered. Apparently the condition applied caused cleavage of the protecting group.

α-[(γ-Oleoyl-β-palmitoyl)-α-glycerylphosphoryl]-γ-alanylglycerol (XIII). Both substances XI and XII could be freed from the protecting groups by bubbling a rapid stream of anhydrous hydrochloric acid through a solution in dry chloroform at icebath temperature. Thin-layer chromatograms made during the course of this reaction demonstrated, that, after 10 min, the *tert.*-butoxycarbonyl group was cleaved completely; the *tert.*-butyl group on the other hand required a reaction time of at least 2 h before its removal was complete. Most of the hydrochloric acid was removed by bubbling dry nitrogen through the solution for 30 min, after which the solution was evaporated *in vacuo*, and dried over caustic potash at 0.001 mm for 24 h. The resulting product, XIII (as a hydrochloric acid salt), was freed from a small amount of XII by crystallization from chloroform–acetone, thus giving a colourless hygroscopic powder, in a yield of 85% (calculated on XI and XII). Only a trace of phosphatidylglycerol could be detected on paper chromatograms, developed with the solvent system of Marinetti, namely di-isobutylketone–acetic acid–water (40:25:5, v/v). Determination of the fatty acid constituents revealed a ratio of palmitic to oleic acid of 1.00. The compound had m.p. 181–183°. (Found: C, 59.8; H, 10.0; P, 3.5. $C_{43}H_{83}ClNO_{11}P$ requires C, 60.29; H, 9.77; P, 3.62%).

RESULTS AND DISCUSSIONS

Some properties of the first synthetic member of the series of *O*-amino acid esters of phosphatidyl glycerol have been compared with the natural compounds

isolated from bacteria and with chemically related phospholipids, namely synthetic phosphatidyl ethanolamine[11], phosphatidyl glycerol isolated from bacteria[19] and from spinach leaves[20] and a synthetic preparation.

Chromatograms of these compounds developed on thin-layer plates and silica impregnated paper (Fig. 1) gave about the same results. The alanyl ester of phos-

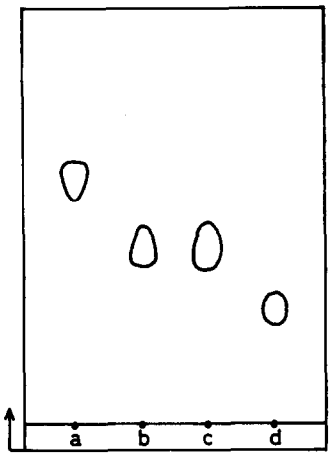


Fig. 1. Paper chromatogram demonstrating: a, synthetic phosphatidylethanolamine; b, synthetic phosphatidyl glycerol; c, synthetic alanyl ester of phosphatidyl glycerol; d, lysine ester of phosphatidyl glycerol, isolated from *S. aureus*. Paper chromatograms on silica impregnated paper were developed according to MARINETTI *et al.*[21]. The spots were made visible by spraying with the ninhydrin reagent (a, c and d), the reagent of HANES AND ISHERWOOD[22] for the phosphorus-containing components (a–d) the periodate–Schiff reagent[23] for detecting vicinal OH-groups (b), and the tricomplex-staining method according to BUNGENBERG DE JONG[24] (red spots for a, c and d, and a green one for b).

phatidyl glycerol was found to have an R_F value close to that of phosphatidyl glycerol in the system used, a result also recorded by MACFARLANE[1] for the corresponding compound present in *Cl. welchii*. Both substances, however, can be readily distinguished by different staining properties *e.g.* by treatment with the ninhydrin and periodate–Schiff reagents. The R_F values of the synthetic alanine compound and the ornithine and lysine analogues isolated from bacteria are significantly different. Recently VORBECK AND MARINETTI[25] reported the occurrence of alanine, lysine and glycine esters of phosphatidyl glycerol in *Streptococcus faecalis*. In this laboratory a phospholipid fraction prepared from this bacterium was found by HOUTSMULLER* to give, after mild alkaline hydrolysis, phosphatidyl glycerol and amino acids, *e.g.* lysine and glycine. Chromatograms of the intact phospholipid on silica-impregnated paper, however, gave only one major spot, having a mobility corresponding to that of the lysine ester of phosphatidyl glycerol. Therefore, further studies on the structure of this substance (or substances) are of interest.

The infrared spectra of γ-analyl-α-phosphatidyl glycerol, of the lysine ester of phosphatidyl glycerol and of phosphatidyl ethanolamine were almost identical in the region 2–10 μ (Fig. 2 A, B and C). For the alanyl ester an additional weak absorption peak was found for the hydrochloric acid salt of the amino group at 4.86 μ. The

* Unpublished observations.

spectra do not give any information about the free hydroxyl groups because of the coincidence of these absorption bands with those of the phosphorus group. More differences are to be noted between the spectra mentioned above and the infrared spectrum of phosphatidyl glycerol (Fig. 2 D), especially the absence of the characteristic NH_3^+, NH_2 and NH vibrations.

The highly specific action of various phospholipases have been used in many studies on structural problems of phospholipids, and the natural amino acid esters of phosphatidyl glycerol have also been investigated by this method[2]. Further extension of our knowledge on the enzymic hydrolysis of this phospholid species, particularly when obtained on defined synthetic substances, may be of great help for the determination of the precise structures and configuration of natural specimens. The synthetic compound was demonstrated to be a good substrate for phospholipases A, C and D, and the different hydrolysis products have been characterized (compare Scheme 3).

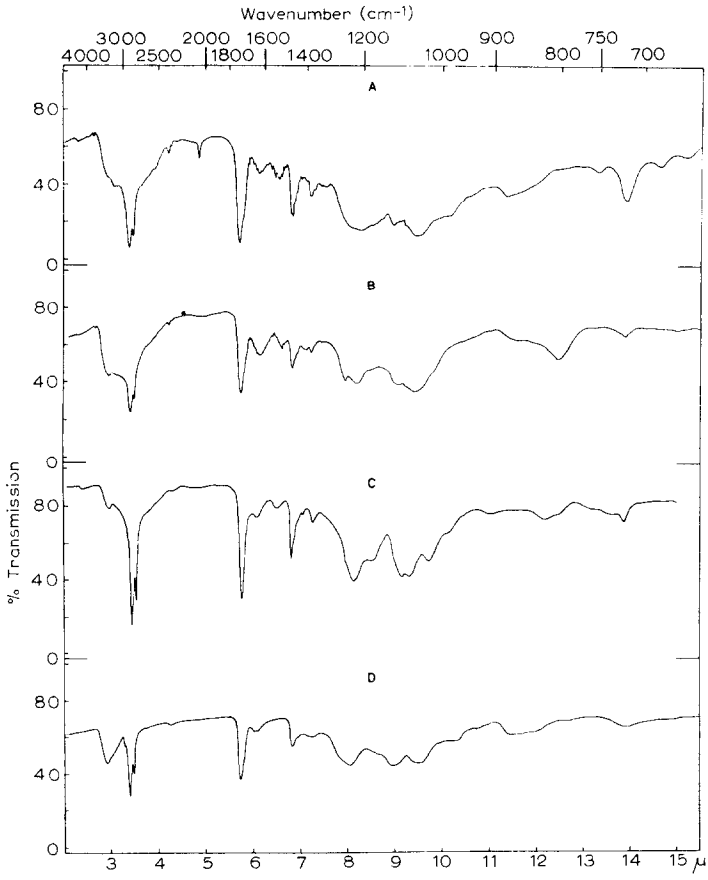
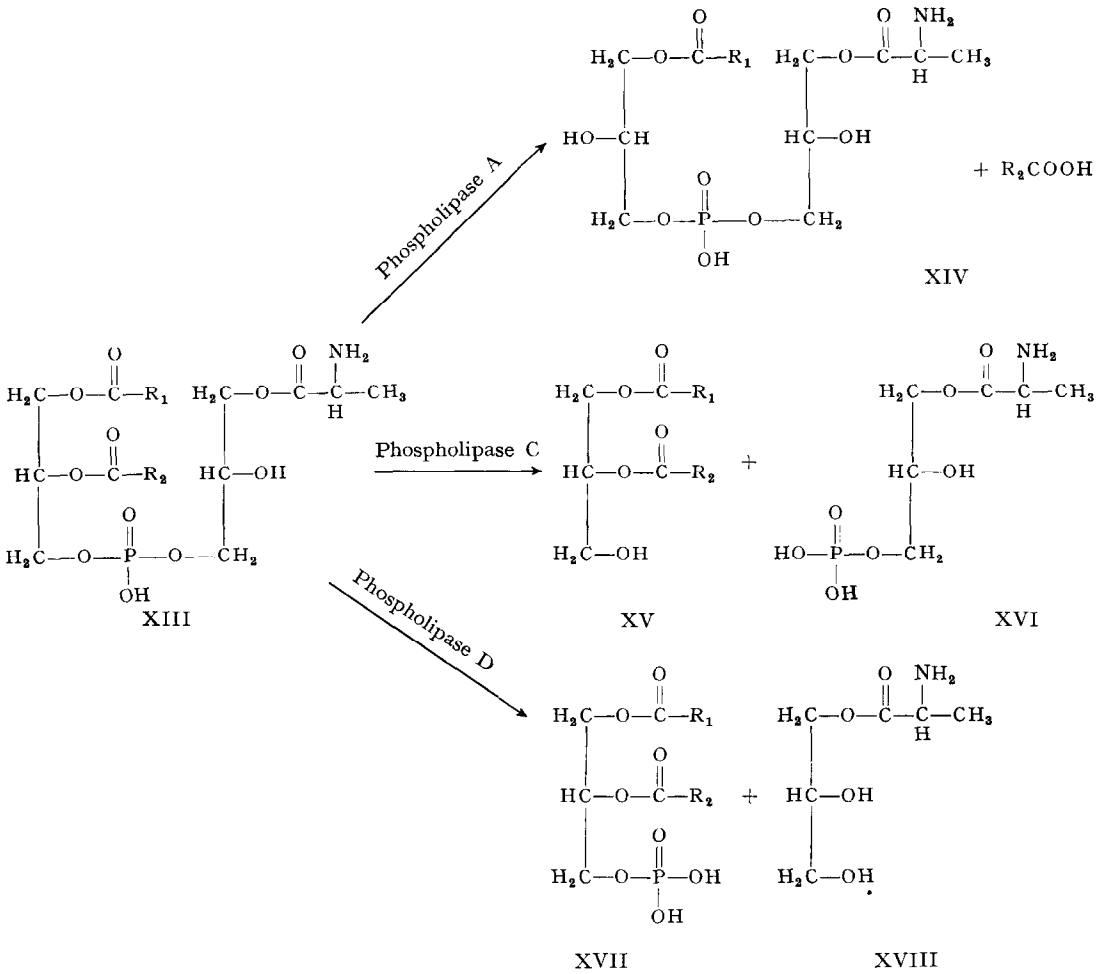


Fig. 2. Infrared spectra. A Perkin-Elmer (model 21) double beam spectrophotometer (sodium chloride prism) was used. Each sample was spread as a thin film on a KBr disc. Ordinate: transmission, %. Abscissa: wave length in microns.
A, γ-alanyl-α-phosphatidyl glycerol; B, lysine ester of phosphatidyl glycerol isolated from *S. aureus*; C, γ-oleoyl-β-stearoyl-L-α-phosphatidylethanolamine; D, phosphatidyl glycerol isolated from spinach leaves.



Scheme 3
Enzymic hydrolysis of γ-alanyl-α-phosphatidyl glycerol.

The hydrolysis with phospholipase A (EC 3.1.1.4) was carried out with *Crotalus adamanteus* venom in the 2,4,6-collidine–ether system (pH 6.5) introduced by MAGEE AND THOMPSON[26]. In view of the lability of the amino acid esters of glycol[27], nucleosides[28] and glucose [29], this slightly acidic buffer was preferred. According to expectation, the action of phospholipase A did not effect a complete hydrolysis of the DL-compound under discussion (Fig. 3). Previous studies on synthetic lecithins had already shown that D-α-derivatives resist the action of this enzyme[30]. Under the conditions applied only free fatty acid and the lyso-derivative of alanyl phosphatidyl glycerol were demonstrated to be formed. HOUTSMULLER AND VAN DEENEN[2] reported that in addition phosphatidyl glycerol and lysophosphatidyl glycerol were produced during phospholipase A hydrolysis of the *O*-ornithine ester of phosphatidyl glycerol from *B. cereus*. The conditions used at that time are likely to have caused a non-enzy-

mic cleavage of the amino acid-ester linkage. Afterwards it became evident that no free amino acid was formed when a slightly acidic buffered system was used for the phospholipase A hydrolysis of the natural compound*. In order to establish the site of attack of phospholipase A on this phospholipid type, the products remaining after the enzyme hydrolysis were separated on chromatoplates coated with silica. After

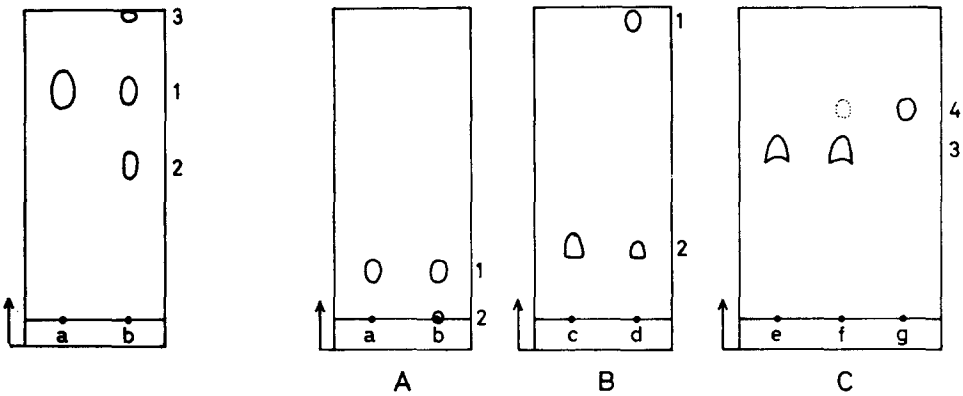


Fig. 3. Thin-layer chromatographic demonstration of the action of snake-venom phospholipase A. The chromatograms were developed in chloroform–methanol–water (65:35:4, v/v). Detection of the spots was accomplished by charring after spraying with a 30% solution of sulfuric acid.
a, γ-alanyl-α-phosphatidyl glycerol (XIII); b, XIII after incubation with phospholipase A for 2 h.
1. γ-alanyl-α-phosphatidyl glycerol (XIII); 2. lyso-compound of 1 (XIV); 3. liberated fatty acid.

Fig. 4. Demonstration of the degradation of γ-alanyl-α-phosphatidyl glycerol (XIII) with phospholipase C on thin-layer chromatograms with silica.
(A) The chromatogram was developed in ether–hexane (1:1, v/v).
a, α, β-Diglyceride; b, ether layer of the degradation.
(B) Development in chloroform–methanol–concentrated-NH_3 (70:20:1, v/v).
c, Alanyl ester of phosphatidyl glycerol; d, ether layer of the degradation.
(C) Developement in methanol–water (1:1, v/v).
e, γ-Alanyl-α-glycerophosphate; f, water layer of the degradation (see text); g, alanine.
1. α, β-Diglyceride; 2. alanyl ester of phosphatidyl glycerol; 3. γ-alanyl-α-glycerophosphate; 4. alanine.

detection of the spots with Rhodamine 6 G under ultraviolet irradiation, the zones were scraped off, and subjected to fatty acid analysis. The lyso-compound (Scheme 3 XIV) was found to contain 98.6% of oleic acid. In accordance with the results obtained on other mixed-acid phosphoglycerides only the β-attached fatty acid appeared to be released.

Phospholipase C (EC 3.1.4.3) is known to catalyse the hydrolysis of phosphoglycerides into a diglyceride and the water-soluble polar headgroup. A similar conversion (Scheme 3) was observed after incubating the alanyl ester of phosphatidyl glycerol with phospholipase C from *B. cereus* in a system containing equal volumes of 2.4.6.-collidine buffer (pH 6.5) and ether. After 1 h incubation the water layer was extracted three times with ether in order to remove the lipid components. Thin-layer chromatograms of this fraction (Fig. 4) showed the presence of an α,β-diglyceride (XV) together with the non-degraded phospholipid. After addition of three volumes of ethanol to the water layer and centrifugation, the clear supernatant was brought to

* Unpublished observations of HOUTSMULLER.

dryness *in vacuo* and subsequently dissolved in ethanol. Thin-layer chromatograms of this fraction, when stained with ninhydrin, showed the presence of two spots (Fig. 4), the minor one being identical with alanine. The major component revealed a positive reaction both with the ninhydrin and the molybdic acid reagent. In view of the established mode of action of phospholipase C this hydrolysis product was expected to be identical with γ-alanyl-α-glycerophosphate (XVI). Comparison of the chromatographic behaviour of the breakdown product with synthetic γ-alanyl-α-glycerophosphate confirmed this assumption (Fig. 4). The enzymic hydrolysis of the *O*-ornithine ester of phosphatidyl glycerol was also expected to furnish the corresponding ornithine analogue, but instead glycerophosphate was detected as a major water-soluble

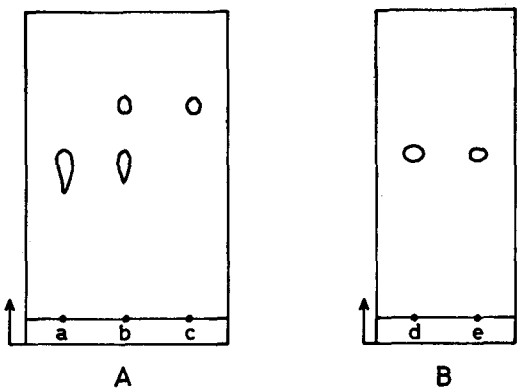


Fig. 5. Thin-layer chromatographic demonstration of the action of phospholipase D on γ-alanyl α-phosphatidyl glycerol (XIII).
(A) Chromatoplate, coated with silica, impregnated with 0.5 M oxalic acid, was developed in the MARINETTI system, namely di-isobutyl ketone–acetic acid–water (40:25:5, v/v). Detection was effected by charring after spraying with a 30% solution of sulfuric acid.
a, Alanyl ester of phosphatidyl glycerol (XIII); b, XIII after incubation with phospholipase D during 3 h (ether layer); c, phosphatidic acid.
(B) Chromatoplate, coated with silica, was developed in propanol–concentrated ammonia–water (7:1:2, v/v). Detection with the ninhydrin reagent.
d, α-Alanylglycerol; e, water layer of the degradation of XIII with phospholipase D.

phosphorus component[2]. Apparently, in those experiments the amino acid-ester linkage was cleaved completely, and it is worth noting that prolongation of the incubation period with the synthetic compound also increased the amount of free alanine accompanied by the formation of glycerophosphate. In this context it is of interest to record that after 1 h incubation about half the amount of the racemic phospholipid was converted by the enzyme, while after that period further degradation proceeded at a decreased rate. This observation is in accordance with the finding that phospholipase C acts less readily on D-α-phosphoglycerides than on L-α-isomers.

Phospholipase D (EC 3.1.4.1.) is well known to release the polar endgroup from phosphoglycerides so as to form phosphatidic acid. In analogy alanyl phosphatidyl glycerol (Scheme 3) was expected to give rise to phosphatidic acid (XVII) and alanyl glycerol (XVIII). The synthetic substance was incubated with an enzyme preparation from Brussels sprouts in an ether–water system (pH 5.5). By extraction with ether the enzymic hydrolysate was separated into lipid and water-soluble fractions, which were subjected to thin-layer chromatography (Fig. 5). The lipid frac-

tion was found to contain unreacted XIII and phosphatidic acid, while the water-soluble part contained a component which was identical in chromatographic behaviour with a synthetic sample of α-alanyl glycerol (XVIII). As regards the stereochemical specificity of the enzyme concerned, about the same properties were observed as mentioned above for phospholipase C. The relative stereospecificity was also demonstrated with enantiomeric lecithins. Whereas a complete breakdown of (γ-oleoyl-β-stearoyl)-L-α-lecithin through the action of phospholipase D was obtained after a 2-h incubation, under similar conditons a D-α-isomer revealed the first detectable amounts of phosphatidic acid only after a 6-h incubation period. The difference in breakdown of both enantiomers appeared to be more pronounced than suggested by the observations of DAVIDSON AND LONG[31] on the slower degradation of DL-α-lecithins in comparison to the L-α-analogues.

In summary the enzymic hydrolysis of amino acid derivatives of phosphatidyl by phospholipase C and particularly by phospholipase D allow the production of the water-soluble entities of these complex lipids, thereby facilitating the determination of the structure and configuration of these moieties in the naturally-occurring species.

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REFERENCES

1 M. G. MACFARLANE, *Nature*, 196 (1962) 136.
2 U. M. T. HOUTSMULLER AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 70 (1963) 211.
3 U. M. T. HOUTSMULLER AND L. L. M. VAN DEENEN, *Koninkl. Ned. Akad. Wetenschap, Proc. Ser. B*, 66 (1963) 236.
4 U. M. T. HOUTSMULLER AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 84 (1964) 96.
5 P. E. VERKADE, *Bull. Soc. Chim. France*, (1963) 1993.
6 L. L. M. VAN DEENEN AND G. H. DE HAAS, *Advan. Lipid Res.*, 2(1964) 168.
7 A. J. SLOTBOOM, G. H. DE HAAS AND L. L. M. VAN DEENEN, *Rec. Trav. Chim.*, 82 (1963) 469.
8 G. W. ANDERSON AND F. M. CALLAHAN, *J. Am. Chem. Soc.*, 82 (1960) 3359.
9 R. H. MILLS, M. W. FARRAR AND O. J. WEINKAUFF, *Chem. Ind. (London)*, (1962) 2144.
10 G. H. DE HAAS AND L. L. M. VAN DEENEN, *Rec. Trav. Chim.*, 82 (1963) 1163.
11 G. H. DE HAAS AND L. L. M. VAN DEENEN, *Rec. Trav. Chim.*, 80 (1961) 951.
12 J. BADDILEY, V. M. CLARK, J. J. MICHALSKI AND A. R. TODD, *J. Chem. Soc.*, (1949) 815.
13 G. H. DE HAAS, H. VAN ZUTPHEN, P. P. M. BONSEN AND L. L. M. VAN DEENEN, *Rec. Trav. Chim.*, 83 (1964) 99.
14 L. A. CARPINO, C. A. GIZA AND B. A. CARPINO, *J. Am. Chem. Soc.*, 81 (1959) 955.
15 G. B. KISTIAKOWSKY, J. R. RUHOFF, H. A. SMITH AND W. E. VAUGHAN, *J. Am. Chem. Soc.*, 57 (1935) 876.
16 G. H. DE HAAS, F. J. M. DAEMEN AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 65 (1962) 260.
17 J. J. PEIFER, *Microchim. Acta*, (1962) 529.
18 F. WEYGAND AND K. HUNGER, *Chem. Ber.*, 95 (1962) 1.
19 F. HAVERKATE, U. M. T. HOUTSMULLER AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 63 (1962) 547.
20 F. HAVERKATE AND L. L. M. VAN DEENEN, *Biochem. J.*, 88 (1963) 42P.
21 G. V. MARINETTI, J. ERBLAND AND J. KOCHEN, *Federation Proc.*, 16 (1957) 837.
22 C. S. HANES AND F. A. ISHERWOOD, *Nature*, 164 (1949) 1107.
23 J. BADDILEY, J. G. BUCHANAN, R. E. HANDSCHUMACHER AND J. F. PRESCOTT, *J. Chem. Soc.*, (1956) 2818.
24 H. G. BUNGENBERG DE JONG, *Koninkl. Ned. Akad. Wetenschap. Proc. Ser. B.*, 64 (1961) 467.
25 M. L. VORBECK AND G. V. MARINETTI, *Federation Proc.*, 23 (1964) 375.

26 W. L. MAGEE AND R. H. S. THOMPSON, *Biochem. J.*, 77 (1960) 526.
27 Z. A. SHABAROVA, N. A. HUGHES AND J. BADDILEY, *Biochem. J.*, 83 (1962) 216.
28 D. H. RAMMLER AND H. G. KHORANA, *J. Am. Chem. Soc.*, 85 (1963) 1997.
29 N. K. KOCHETKOV, V. A. DEREVITSKAYA AND L. M. LIKHOSHERSTOV, *Izv. Akad. Nauk. SSSR*, (1963) 688.
30 L. L. M. VAN DEENEN AND G. H. DE HAAS, *Biochim. Biophys. Acta*, 70 (1963) 538.
31 F. M. DAVIDSON AND C. LONG, *Biochem. J.*, 69 (1958) 458.