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STRUCTURAL IDENTIFICATION OF ISOMERIC LYSOLECITHINS

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SUMMARY

1. Methods were developed for the preparation of 2-acyl-glycero-3-phosphoryl choline, 2-acyl-glycero-1-phosphoryl choline, 1-acyl-glycero-2-phosphoryl choline and 3-acyl-glycero-2-phosphoryl choline.

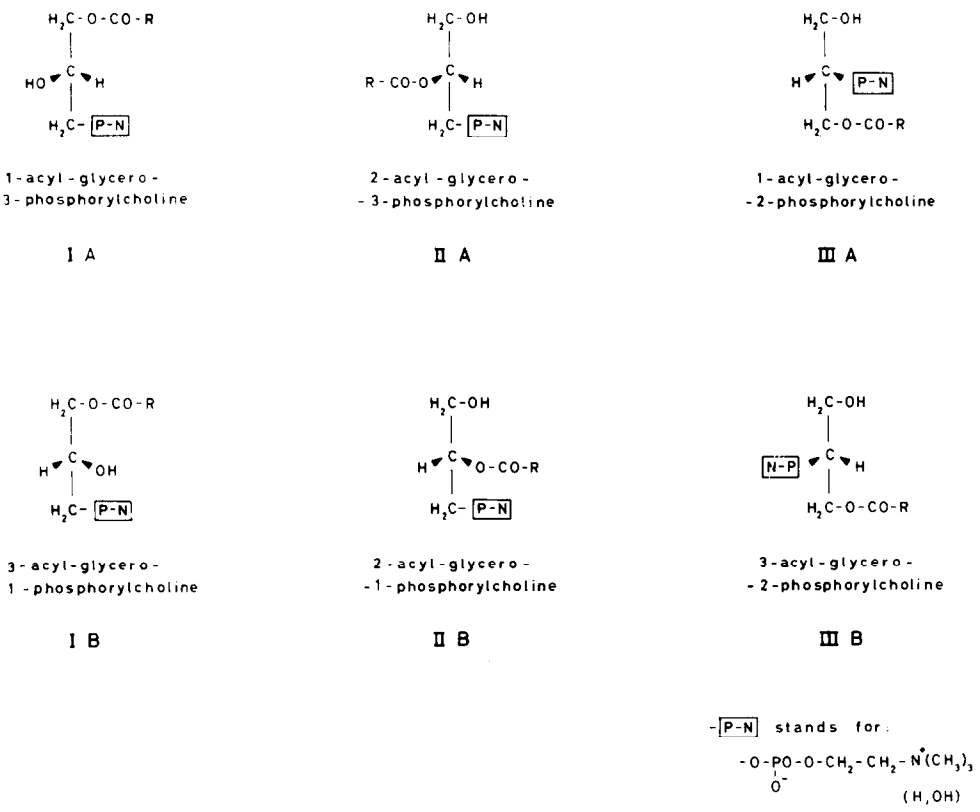
2. The chemical structure of different types of synthetic lysolecithins was confirmed by acylation and subsequent enzymic characterization of the lecithins formed. The latter method enables the recognition of phospholipids derived from glycero-1-phosphate, glycero-2-phosphate and glycero-3-phosphate, and is suitable for structural investigations on natural phosphoglycerides.

3. The four lysolecithins mentioned and 1-acyl-glycero-3-phosphoryl choline could be distinguished by virtue of their susceptibilities to phospholipase A (EC 3.1.1.4) and C (EC 3.1.4.3), combined with characterization of the enzymic hydrolysis products. This procedure was found to be suitable for the micro-analysis of mixtures of enantiomeric lysolecithins.

4. Chromatography on silica columns appeared to cause a migration of the acyl residue of 2-acyl-glycero-3-phosphoryl choline.

INTRODUCTION

Lysolecithins and analogues are known to play an important part in the intermediary metabolism of phosphoglycerides. From a structural point of view six lysolecithins have to be considered (Scheme 1), which compounds differ with respect to the positions of the phosphoryl-choline moiety, the fatty acid ester linkage and the stereochemical configuration of the glycerol entity. Lysolecithins of structure I A are well known to be produced from naturally occurring lecithins by the action of phospholipase A (EC 3.1.1.4) from snake venom and pancreas. Recent studies of LLOVERAS *et al.*[1] and VAN DEN BOSCH AND VAN DEENEN[2] indicated the occurrence of phospholipases capable of catalyzing also the hydrolysis of the fatty acid ester linkage at the 1-position of natural phosphoglycerides so as to form lysolecithins of structure II A. Indirect evidence based on the fatty acid composition of isolated lysolecithins[3] appears to be in agreement with this view. Moreover this type of lyso compound will

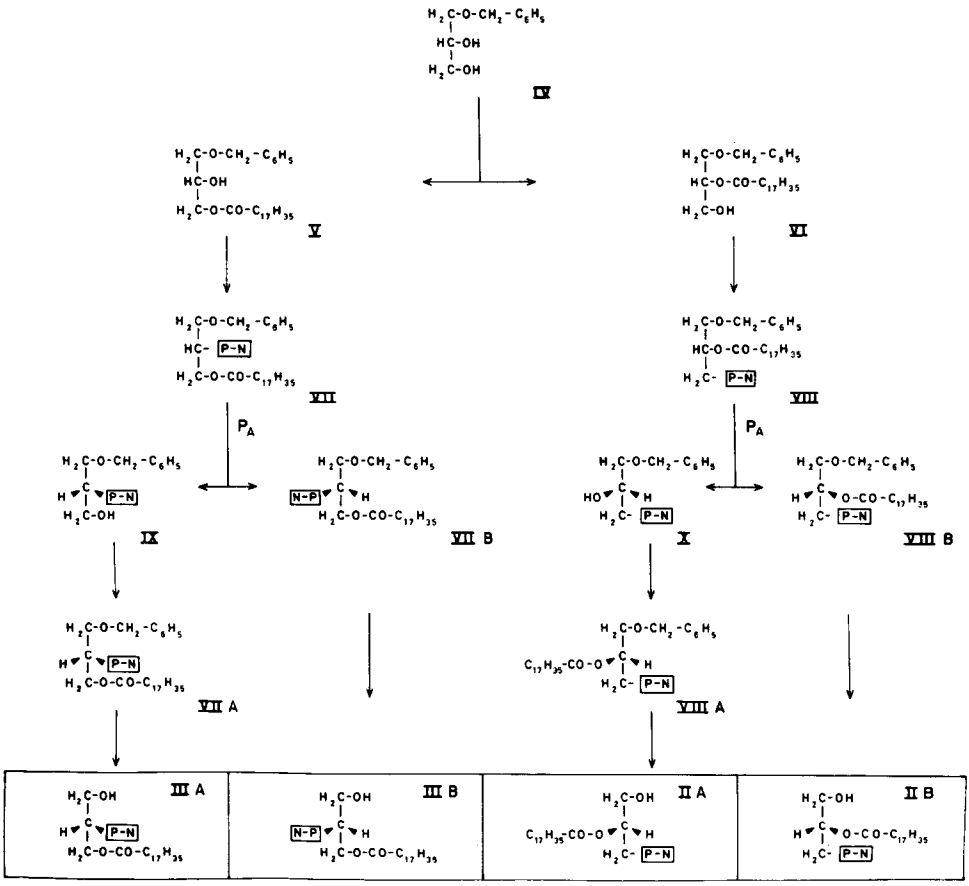


Scheme 1. Structural formulae of various types of enantiomeric lysolecithins. Nomenclature according to HIRSCHMANN[21].

be formed by the enzymic and chemical cleavage of the vinyl ether linkage of natural plasmalogens.

Although it is generally agreed that phosphoglycerides from higher animals and plants are derived from glycero-3-phosphate, recent studies of KATES *et al.*[4] on *Halobacterium cutirubrum* have shown that lipid derivatives of glycero-1-phosphate may also occur in nature. Lysolecithins having this configuration are represented by structures IB and IIB. Phosphoglycerides containing a glycero-2-phosphoryl moiety (*e.g.* so-termed *β*-lecithins) have been obtained by chemical synthesis only, but their possible occurrence in living material still appears to be under consideration[5]. The lysolecithins concerned are given by formulae IIIA and IIIB, one of which (IIIB) has been found in this laboratory to be formed after the hydrolysis of synthetic *β*-lecithins by snake-venom phospholipase A (ref. 6).

Most of the structural and enzymic work to be carried out in the near future on lysolecithins will undoubtedly concern compounds IA and IIA. However, this work and an understanding of the specificity of the enzymes involved in the metabolism of lysoderivatives will be greatly promoted by studying all enantiomeric lysolecithins. As a continuation of our synthetic work on lysophosphatides[7] and their characterization by enzymic means[6,7], the stereoisomers of II and III have been



Scheme 2. Pathways for the preparation of enantiomeric acyl-glycero-2-phosphorylcholines (III A, III B) and 2-acyl-glycero-phosphorylcholines (II A, II B).

prepared. A comparison was made of various properties of the five enantiomeric lysolecithins now available.

SYNTHETIC METHODS

The sequence of reactions applied for the preparation of four enantiomeric lysolecithins is indicated in Scheme 2. Starting from racemic glycerol-1-benzylether (IV), monoacylation at low temperature with stearoyl chloride yielded a mixture of the isomeric "diglycerides" V and VI in a molar ratio of about 15:1. After separation of both positional isomers by silica-chromatography and crystallization, nuclear magnetic resonance analysis* showed the preparation of V to be completely free of VI, while the fraction VI appeared to be contaminated with only a negligible trace of V. Both V and VI were converted to the corresponding "lecithins" VII and VIII

* Infrared spectroscopy showed no difference between the two isomers.

according to the method of HIRT AND BERCHTOLD[9]. The isomeric racemic "benzyl-lecithins" VII and VIII were converted to their enantiomeric forms VIIA, VIIB and VIIIA, VIIIB respectively with the aid of phospholipase A from snake venom. This enzyme acts stereospecifically and attacks only one enantiomer of VII and VIII, yielding the optically active "benzyl-lysolecithins" IX and X, whereas the enantiomers VIIB and VIIIB are not hydrolysed by this enzyme. Reacylation of IX and X with stearoyl chloride then afforded the stereoisomers of VIIB and VIIIB, namely VIIA and VIIIA. Finally catalytic hydrogenolysis with Pd of VIIA, VIIB, VIIIA and VIIIB furnished the wanted lysolecithins IIIA, IIIB, IIA and IIB.

MATERIALS AND GENERAL METHODS

Crystalline racemic glycerol-1-benzyl ether was prepared according to the method of HOWE AND MALKIN[10]. The compound had m.p. 34–36° and upon periodate titration showed a value in accord with theory. The synthesis of 1- and 2-monostearine was carried out according to the methods described by VAN LOHUIZEN AND VERKADE[11]. The purity of both monoglycerides was checked by periodate oxidation. Thin-layer chromatography was carried out on microscope slides covered with silicic acid as described previously[12]. The separation between 1- and 2-monostearines was achieved by thin-layer chromatography on hydroxylapatite as reported by HOFMAN[13]. Optical rotations were measured in a Lichtelektrisches Präzisions polarimeter 0.005°, Carl Zeiss. Nuclear magnetic resonance spectra were measured with a 60 mc (Model A-60 Varian Associates, Calif.) spectrometer. Lysolecithins were dissolved at ^2H_2O in a concentration of 10–20%. The isomeric "benzyl-glycerides" were measured in carbon tetrachloride solution (20%) containing tetramethyl silane as an internal reference standard. Chemical shifts are expressed as ppm relative to tetramethyl silane taken as 0 ppm.

Infrared absorption spectra were measured with a Perkin-Elmer (Model 21) double beam spectrophotometer in a sample concentration of 3 mg of compound per 1.0 g of KBr. Degradation of the various substrates with phospholipase A from snake-venom was carried out under conditions described earlier[8]. Enzymic hydrolysis experiments with phospholipase C (EC 3.1.4.3) from *Bacillus cereus* and phospholipase D (EC 3.1.4.4) from Brussels sprouts were done as described by HAVERKATE AND VAN DEENEN[14].

Rac.-3-stearoyl-glycero-1-benzylether (V)
Rac.-2-stearoyl-glycero-1-benzylether (VI)

12.2 g of racemic glycero-1-benzylether (IV) and 3.17 g of dry pyridine were dissolved in 100 ml of dry chloroform, and the solution was cooled to —15°. To this solution was slowly added under stirring a chilled solution of 12.2 g stearol chloride in 100 ml of dry chloroform. After stirring for two h at —15° thin layer chromatography showed a heavy spot of V while only traces of IV,VI and the diacyl compound were present. Alter treatment of the chloroform solution with cold 0.5 N sulphuric acid and water, the solvents were evaporated and the residue was dissolved in hexane and subjected to chromatography on a silicic acid column using ether–hexane (20:80, v/v as eluent. After elution of a small amount of the diacyl compound and of free fatty acid the desiaed 1^63 isomer V was obtained in a yield af 65% based on glycero-

1-benzylether. This material was crystallized twice from hexane to remove a trace of VI yielding colourless crystals of V, m.p. 36–37°. Thin-layer chromatography as well as nuclear magnetic resonance analysis (5 ppm) showed this material to be free of the 1,2 isomer VI. (Found: C, 75.0; H, 10.7. Required: C, 74.95; H, 10.78.) After elution of the 1,3 isomer (V) and of a small fraction consisting of a mixture of V and VI, the pure 1,2 isomer was obtained from the column in a yield of 1.31 g, as a colourless waxy material; m.p. 32–33°. On thin-layer chromatograms no 1,3 isomer could be detected while nuclear magnetic resonance analysis (4.1 ppm) revealed only a trace of the isomer V.

Rac.-1-O-benzyl-3-stearoyl-glycero-2-phosphorylcholine (VII)
Rac.-1-O-benzyl-2-stearoyl-glycero-3-phosphorylcholine (VIII)

The phosphorylation of V and VI with β-bromoethyl-phosphoryldichloride and the subsequent reaction with trimethylamine was carried out as described by HIRT AND BERCHTOLD[9], yielding the isomeric racemic "benzyl-lecithins" VII and VIII. After chromatographic purification on silicic acid and crystallization from ether both lecithins were obtained as colourless crystals in yields of about 55%; m.p. (VII) 218–221°; m.p. (VIII) 218–220°. Both lecithins were analysed as cadmium chloride adducts (Found for VII: C, 43.7; H, 6.9; N, 1.9%. Found for VIII: C, 43.5; H, 7.1; N, 1.9%. Required: C, 43.70; H, 6.89; N, 1.55%.) Both "lecithins" appeared to be very soluble in methanol, ethanol and chloroform, less soluble in ether and insoluble in hexane and acetone.

3-O-Benzyl-1-stearoyl-glycero-2-phosphorylcholine (VIIA)
1-O-Benzyl-3-stearoyl-glycero-2-phosphorylcholine (VIIB)

4.3 g of the racemic "benzyl-lecithin" VII dispersed in a mixture of 400 ml of ether and 400 ml of calcium containing borate buffer (pH 7) was incubated at 20° with 230 mg of lyophilized *Crotalus adamanteus* venom. After 7-h and 12-h incubation an additional amount of 50 mg of venom was added, and after 24 h the reaction was stopped. Most of the organic solvent was removed *in vacuo* and the aqueous phase was extracted with 500 ml of chloroform containing 20% of methanol. The chloroform phase, after being washed with water, was evaporated *in vacuo* and the residue partitioned between ethanol (containing 30% of water) and pentane. Upon evaporation the pentane phase yielded pure stearic acid, m.p. 68.5–69.5° (yield 98% of theory). The unhydrolysed enantiomeric "benzyllecithin" VIIB was recovered from the aqueous ethanol phase. After crystallization from ether, VIIB was obtained in the form of colourless crystals in a yield of 90%, m.p. 217–219°, $[\alpha]_D^{20}$ +4.3° in chloroform (c, 9). (Found: P, 4.9%. Required: P, 4.92%.) Upon renewed incubation with phospholipase A no further degradation could be observed indicating the absence of the stereoisomer VIIA.

The aqueous methanol phase, which contains the enantiomeric "benzyl-lysolecithin" IX was freed from buffer and traces of enzyme by percolation through a mixed amberlite column (IRC −50 H^+; IR −45 OH^-). The eluate was evaporated *in vacuo*, and the residue crystallized from chloroform-acetone. 3-*O*-Benzyl-glycero-2-phosphoryl choline (IX) was obtained as colourless hygroscopic crystals in a yield of 95%, m.p. 167–168°, $[\alpha]_D^{20}$ −2.0° in chloroform–methanol, 9:1 v/v (c, 11). (Found: N, 2.3; P, 4.7%. Required: N, 2.19; P, 4.84%.) Upon thin-layer chromatography with

methanol–water (1:1, v/v) or propanol–ammonia–water (6:3:1, v/v) no contaminants appeared to be present.

The "benzyl-lysolecithin" IX in the form of its cadmium chloride addition compound was reacylated with stearoyl chloride (5 moles) and pyridine (5 moles) under conditions* described earlier.[15] After a reaction time of 7 h, the chloroform solution was extracted three times with 0.5 N sulphuric acid (containing 30% ethanol) and subsequently twice with 90% aqueous ethanol to remove pyridine, starting material (IX) and cadmium chloride. The chloroform solution, containing the wanted "benzyl-lecithin" VIIA and excess of stearic acid, was evaporated *in vacuo*, and the residue partitioned between 70% aqueous ethanol and pentane. From the aq. ethanol layer the wanted lecithin was recovered in a yield of 35%. After crystallization from ether, colourless crystals were obtained, m.p. 217–219°, $[\alpha]_D^{20} -3.9°$ in chloroform (c, 13). (Found: P, 4.9%. Required: P, 4.92%.) Upon hydrolysis with phospholipase A this enantiomeric "benzyl-lecithin" was converted to the extent of over 95% to the benzyl-lysolecithin (IX), indicating that only traces of the stereoisomer VIIB were present.

1-Stearoyl-glycero-2-phosphorylcholine (IIIA)
3-Stearoyl-glycero-2-phosphorylcholine (IIIB)

Catalytic hydrogenolysis of VIIA and VIIB carried out in absolute ethanol with palladium as catalyst afforded in high yield the lysolecithins IIIA and IIIB. After removal of the catalyst both lysolecithins were crystallized from chloroform–acetone. The compound IIIA had m.p. 248–250°; $[\alpha]_D^{20} -3.7$ in chloroform–methanol (9:1, v/v) (c, 11). The compound IIIB had m.p. 247–249°; $[\alpha]_D^{20} +4.2$ in chloroform–methanol (9:1, v/v) (c, 12). Analyses were performed on the cadmium chloride adducts (Found for IIIA: C, 38.2; H, 6.9; N, 1.9; P, 3.8. Found for IIIB: C, 38.4; H, 6.9; N, 1.7; P, 3.7. Required: C, 38.23; H, 6.91; N, 1.71; P, 3.79).

1-O-Benzyl-2-stearoyl-glycero-3-phosphorylcholine (VIIIA)
3-O-Benzyl-2-stearoyl-glycero-1-phosphorylcholine (VIIIB)

The degradation by phospholipase A of the racemic "benzyl-lecithin" VIII was carried out as described for the isomeric compound VII. After a similar work-up the unhydrolysed enantiomeric "benzyl-lecithin" VIIIB was obtained as a colourless waxy material in quantitative yield m.p. 223–225°. Upon renewed incubation with phospholipase A no formation of lysolecithin was observed, indicating the absence of the stereoisomer VIIIA. The optically active hydrolysis product of the enzymic treatment, the "benzyl-lysolecithin" X, was isolated in a similar manner to that described for the structural isomer IX. After crystallization from chloroform–acetone, 1-*O*-benzyl-glycero-3-phosphoryl choline was obtained in the form of colourless crystals in a yield of 90%, m.p. 154–156°. The compound was converted to its cadmium chloride adduct and reacylated with stearoyl chloride and pyridine so as to form the optically active "benzyl-lecithin" VIIIA. Isolation and purification were carried out as indicated for the structural isomer (VIIA). Yield 30%, m.p. 221–223°. Again a quantitative conversion of VIIIA to the "benzyl-lysolecithin" X was found upon incubation of VIIIA with phospholipase A.

* In contrast to the reacylation of normal lysolecithins which proceeds in high yield, the introduction of an acyl chain in these "benzyl-lysolecithins" is more difficult.

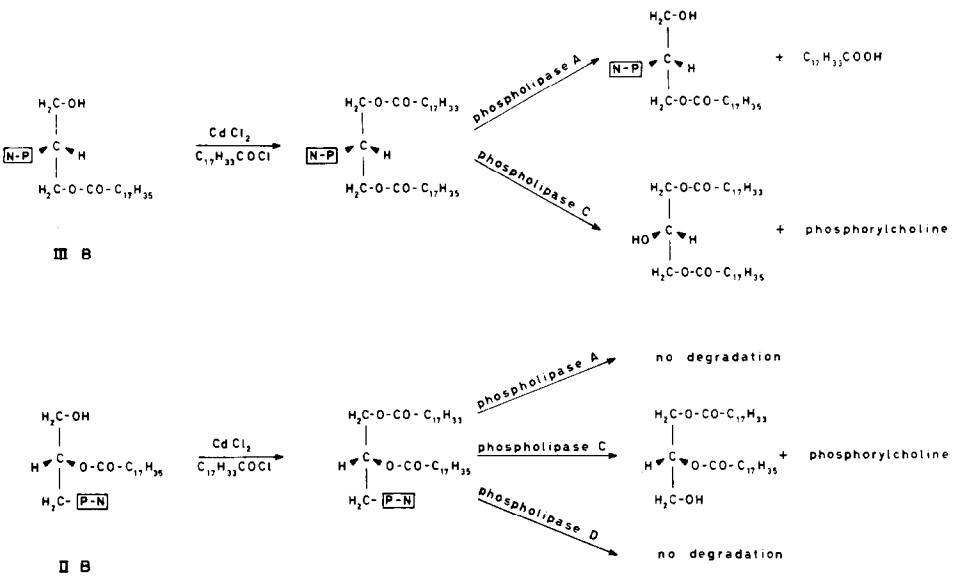
2-Stearoyl-glycero-3-phosphorylcholine(II A)
2-Stearoyl-glycero-1-phosphorylcholine(IIB)

Catalytic hydrogenolysis of VIII A and VIII B in absolute ethanol with palladium as catalyst yielded the wanted stereo isomeric lysolecithins II A and II B. Final purification of both lysolecithins was achieved by crystallization from chloroform–acetone; compound II A had m.p. 249–250°; compound II B, m.p. 247–249°; $[\alpha]_D^{20}$ — 3.4° in chloroform–methanol, (9:1, v/v), (c, 10).

RESULTS AND DISCUSSION

Structural proof

Before studying differences in (bio)chemical behaviour of lysolecithins of types I, II and III, it is desirable to give additional evidence that the newly prepared compounds indeed possess the structure that we initially set out to synthesize. For that purpose one lysolecithin of type III and one of type II were converted to the corresponding mixed-acid diacyllecithins the structure of which could be proved with the aid of phospholipases A, C and D (compare Scheme 3). As regards compound III B,



Scheme 3. Structural proof of the isomeric lysolecithins III B and II B.

acylation of its cadmium chloride adduct with oleoyl chloride afforded a mixed-acid lecithin containing equimolar amounts of stearic and oleic acid. The optical rotation $[\alpha]_D^{20} = 0.0°$ already indicated that the lecithin obtained indeed belongs to the 2- or β-series. In addition phospholipase A hydrolysis of this enantiomeric mixed-acid β-lecithin yielded a lysolecithin composed almost exclusively of stearic acid, thus indicating that oleic acid is attached to the glycero-C_1 and stearic acid to the glycero-C_3 position[6].

Hydrolysis of this mixed-acid β-lecithin with phospholipase C gave a quanti-

tative conversion to the corresponding 1,3-diglyceride. This diglyceride upon thin-layer chromatography appeared to be completely free of the isomeric 1,2-diglyceride thus again proving that the lecithin was not contaminated with derivatives of glycero-1-phosphate or glycero-3-phosphate. Experiments on known mixtures of enantiomeric lecithins demonstrated that this method enables about 5–8% of either 1- or 2-lecithins to be detected. Hence this technique may be useful for studying the nature of isolated phospholipids.

The structure of the isomeric lysolecithin II B was ascertained in the same way. Acylation of its cadmium chloride addition compound with oleoyl chloride furnished a mixed-acid lecithin composed of equimolar amounts of both fatty acid constituents. The optical rotation $[\alpha]_D^{20} = -5.9°$ in chloroform ($[\alpha]_D^{20}$ of 1-lecithins: $-6.1°$) proved that only traces of the stereoisomer II A were present. This mixed-acid lecithin, however, appeared to be hydrolysed neither by phospholipase A from snake venom nor by phospholipase D from Brussels sprouts, which again demonstrated that the compound was a derivative of glycero-1-phosphate. In order to obtain a rapid hydrolysis of the lecithin by phospholipase C (from *Bacillus cereus*), which enzyme is less stereospecific, a large amount of a highly active enzyme preparation had to be used. After an incubation period of 5 h at room temperature thin-layer chromatograms showed that the substrate was hydrolysed completely into the corresponding 1,2-diglyceride. No contamination with the isomeric 1,3 diglyceride could be detected, thus indicating the absence of lecithins derived from glycero-2-phosphate.

Physical characteristics

The infrared spectra (Fig. 1) did not enable any clear-cut distinction to be made between lysolecithins of types I, II and III. Nuclear magnetic resonance at 4 ppm allowed us to distinguish lysolecithins IIA and IIB containing a 2-acyl-glycero group from members of both other types. Unfortunately the amounts required appear at present seriously to limit the application of this method for the identification of natural lysoderivatives. A comparison of the values of the optical rotation of the enantiomeric lysolecithins (see experimental part) shows that even for pure compounds a distinction will be rather difficult. Furthermore the optical rotation of the various lysocompounds turned out to be highly dependent on the solvent system used.

A reliable method for the investigation of the structure of lysolecithins on a microscale became apparent through the use of phospholipases.

Enzymic hydrolysis

A characterization of isolated lysolecithins in principle may be carried out by the methods used in the present study to confirm the structure of the compounds synthesized. However, a conversion of the lysolecithins to the diacyl analogues followed by a characterization of the enzymic hydrolysis products obtained from these lecithins is rather cumbersome and requires substantial amounts of material. In view of the favourable results already obtained for the characterization of some enantiomeric lysoderivatives by a direct hydrolysis with phospholipase A and C[6,7], this approach was pursued. Phospholipase A from *Crotalus adamanteus* has been demonstrated by the present authors to hydrolyse a fatty acid ester linkage adjacent to the phosphoryl alcohol ester bond provided that in an asymmetric compound a given stereochemical configuration is present. The high degree of positional and stereo-

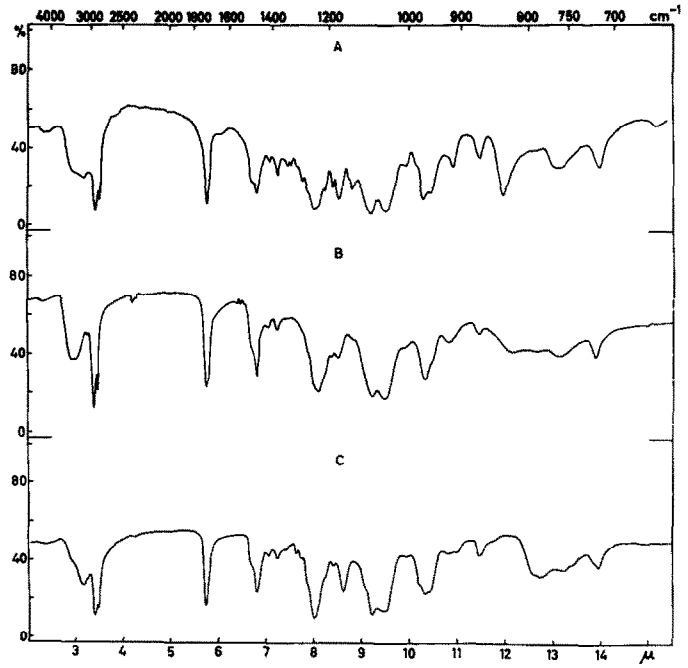


Fig. 1. Infrared spectra of isomeric lysolecithins: (A) 1-stearoyl-glycero-3-phosphorylcholine; (B) 2-stearoyl-glycero-1-phosphorylcholine; (C) 3-stearoyl-glycero-2-phosphorylcholine.

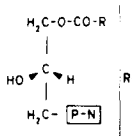
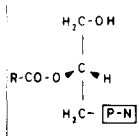
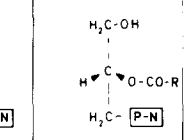
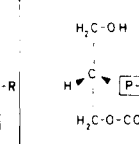
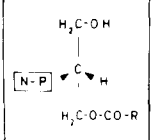
chemical specificity enabled a first distinction to be made between the five lysolecithins under investigation, but in addition a second tool was required. Although phospholipase C from *Bacillus cereus* does not act in a completely stereospecific way, conditions can be applied which allow a clear-cut distinction to be made between stereoisomeric lysolecithins. Furthermore determination of the position of the fatty acid constituent in the monoglyceride formed can supply additional information. This combination of methods facilitates in a simple way the recognition of five enantiomeric lysolecithins (Table I).

The lysolecithin IA which represents the hydrolysis product of phospholipase A action on diacyl-glycero-3-phosphorylcholine is not susceptible to this enzyme. Recently DOERY AND PEARSON [16] made the interesting observation that snake-venoms may contain, in addition to phospholipase A, phospholipase B or lysophospholipase (EC 3.1.1.5) activity. The venom of *C. adamanteus* under the conditions used did not give any breakdown of IA, but bee venom has already been reported to be less suitable for this purpose [17]. The compound under discussion was susceptible to the action of phospholipase C, being quantitatively converted to a monoglyceride. By virtue of the optical activity, the behaviour on thin-layer plates made of hydroxy-apatite, and periodate titration this hydrolysis product was shown to be identical with glycerol-1-stearate.

Lysolecithin IIA was almost completely converted during incubation with snake venom to glycero-3-phosphorylcholine, as identified by paper chromatography and the periodate–Schiff reaction. Treatment with phospholipase C gave a rapid and

TABLE I

HYDROLYSIS OF (STEREO)ISOMERIC LYSOLECITHINS BY PHOSPHOLIPASE A AND PHOSPHOLIPASE C

Type of lysolecithin	I A	II A	II B	III A	III B
Structure					
Degradation by phospholipase A	−	+	−	+	−
Degradation by phospholipase C	+	+	−	−	+

+, Indicates complete or nearly complete hydrolysis.
−, Indicates no degradation under conditions of complete hydrolysis of the stereoisomer. Sometimes, especially with the less stereospecific phospholipase C, a slow hydrolysis is observed in the presence of very high enzyme concentrations or after a prolonged incubation time.

quantitative breakdown of II A, and thin-layer chromatography on hydroxy-apatite showed that glycerol-2-stearate was the only glyceride formed.

Neither phospholipase A nor phospholipase C attacked the lysolecithin II B under conditions giving a complete breakdown of II A.

Hydrolysis of III A with snake venom produced a quantitative conversion to a water-soluble compound chromatographically identical with glycero-phosphoryl choline. This breakdown product failed to give any reaction with the periodate–Schiff reagent and apparently belongs to the 2- or β-series. The lysolecithin III A was not appreciably hydrolysed by phospholipase C under the conditions utilized. Lysolecithin III B exhibited a reversed behaviour *viz.* no degradation by phospholipase A but a complete breakdown by phospholipase C. The monoglyceride formed was isolated in quantitative yield and found to be identical with glycero-3-stearate (L-α-monostearine), m.p .75–76°; $[\alpha]_D^{20}$ −4.3° in pyridine. Thin-layer chromatography confirmed the absence of 2-monoglyceride.

This approach again established the identity of the various lysolecithins prepared, and provides a rapid procedure for analysing preparations consisting of enantiomeric lysolecithins. Model experiments on mixtures of I A and II A showed that it is possible to recognize the presence of about 10% of either isomer. It appeared possible to establish by this method the nature and proportions of the lyso derivatives formed during the synthesis of lecithins by means of an acylation of the cadmium chloride adduct of glycero-3-phosphoryl choline[18]. The procedure was utilized also to investigate migration of the fatty acid constituent of lysolecithin during chromatography on silica acid columns. The observations of SLOTBOOM *et al.*[7] that under these conditions lysophosphoglycerides of type II can be converted to a considerable extent into the isomer I were confirmed. Therefore structural investigations on lysolecithins[19]

purified by chromatography on silica columns appear not to warrant any final conclusion. The duration of the chromatographic procedure determines the extent of migration, and thin-layer chromatography may limit the extent of these unwanted reactions. Lysolecithin obtained from rat liver after a separation of the phospholipids on chromatoplates when studied with the aid of phospholipase A and C was found to consist of two isomers[20].

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