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SYNTHESIS OF LYSOPHOSPHOGLYCERIDES

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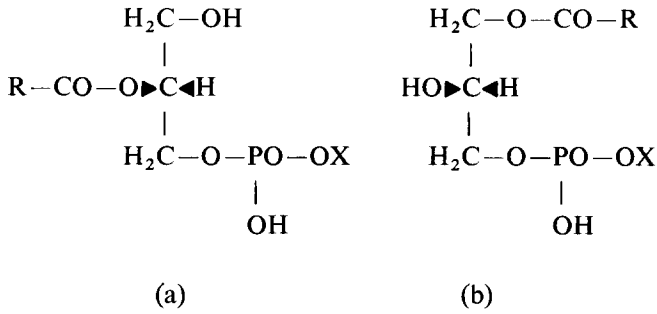
The chemical syntheses of 2-stearoyl-glycerol-1-phosphorylcholine, 2-[9,10–^{3}H]-stearoyl-glycerol-3-phosphorylcholine, (rac)-1- and 2-stearoyl-glycerol-3-phosphorylcholine, (rac)-1- and 2-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine and (rac)-1- and 2-stearoyl-glycerol-3-phosphate are described. These lysophosphoglycerides were prepared using (rac)-isomeric-0-benzyl-stearoyl-glycerol-3-(benzyl)phosphates as starting products. Hydrogenolysis of this latter compounds yielded the lysophosphatidic acids while reaction of the silver salts of the starting products with 2-bromo-ethyl N,N-dimethylamine picrate or 2-bromo-ethyl trimethylammonium picrate finally led to the isomeric N,N-dimethyl lysophosphatidyl ethanolamines and lysolecithins respectively. The (rac)-isomeric-0-benzyl-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamines could easily be converted with methyl iodide into the corresponding lecithins. Stereospecific degradation of (rac)-1-0-benzyl-2-stearoyl-glycerol-3-phosphorylcholine with phospholipase A (EC 3.1.1.4) gave 1-0-benzyl-glycerol-3-phosphorylcholine leaving 3-0-benzyl-2-stearoyl-glycerol-1-phosphorylcholine unhydrolysed. The latter product yielded upon hydrogenolysis 2-stearoyl-glycerol-1-phosphorylcholine. The first product viz. 1-0-benzyl-glycerol-3-phosphorylcholine was acylated with [9,10–^{3}H]-stearoyl chloride and after removal of the protecting benzyl group 2-[9,10–^{3}H]-stearoyl-lysolecithin was obtained.

Infrared spectra of the synthesized lysophosphoglycerides are reported. Some general properties of the lyso compounds e.g. their behaviour towards phospholipase A and their stability are discussed.

Several laboratories have established that monoacyl analogues of phosphoglycerides (lysophosphoglycerides) play an important part in phospholipid metabolism of a great variety of living cells (for reviews see ref. [1–3]). Although the enzymes catalysing the formation, the breakdown and the reacylation of lysophosphoglycerides have not been fractionated to a considerable extent it has become clear that both 1- and 2-acyl-glycerol-3-phosphate* derivatives (fig. 1a and b) are involved. Further work in this area may be facilitated by the use of substrates of well-defined structure, particularly the chemical synthesis of 2-acyl-glycerol-3-phosphate derivatives being desirable. As a continuation of our previous syntheses[4,5] of various isomeric lysophosphoglycerides we describe in the present paper the preparation of several 2-acyl-lysophosphoglycerides including a 2-acyl-lysolecithin containing a labeled fatty acid constituent.

* Notation according to H. Hirschmann, J. Biol. Chem. **235** (1960) 2762.

The pathways utilized in this work for the synthesis of 2-acyl derivatives are summarized in scheme 1. 1-0-benzyl-2-acyl-glycerol-3-(benzyl)phosphate (I) is an attractive starting material since it allows the preparation of analogues with different polar headgroups.



(a) (b)

Fig. 1. a = 2-acyl lysophosphoglyceride; b = 1-acyl lysophosphoglyceride; X = —H, —CH_2—CH_2—$N^+(CH_3)_3OH^-$, —CH_2—CH_2—$N(CH_3)_2$, —CH_2—CH_2—N^+H_3

Lysophosphatidic acid

As pointed out previously[4]) hydrogenolysis of I will furnish lysophosphatidic acid. This method is restricted to the preparation of compounds containing saturated fatty acid constituents. In the present work 2-stearoyl-glycerol-3-phosphate (II) was obtained in the racemic form. In principle, a compound having a stereochemical configuration identical to that of most natural phosphoglycerides can be obtained by using as a starting material I prepared from 1-0-benzylglycerol[6]). The method described above was also suitable for the preparation of 1-acyl-lysophosphatidic acid using as starting material racemic 1-acyl-2-0-benzyl-glycerol-3-(benzyl)phosphate (see experimental part). Very recently Stoffel and Wolff[7]) described a more elaborate synthesis of 1-acyl-lyso-phosphatidic acid containing a [^{3}H]-labelled palmitoyl residue.

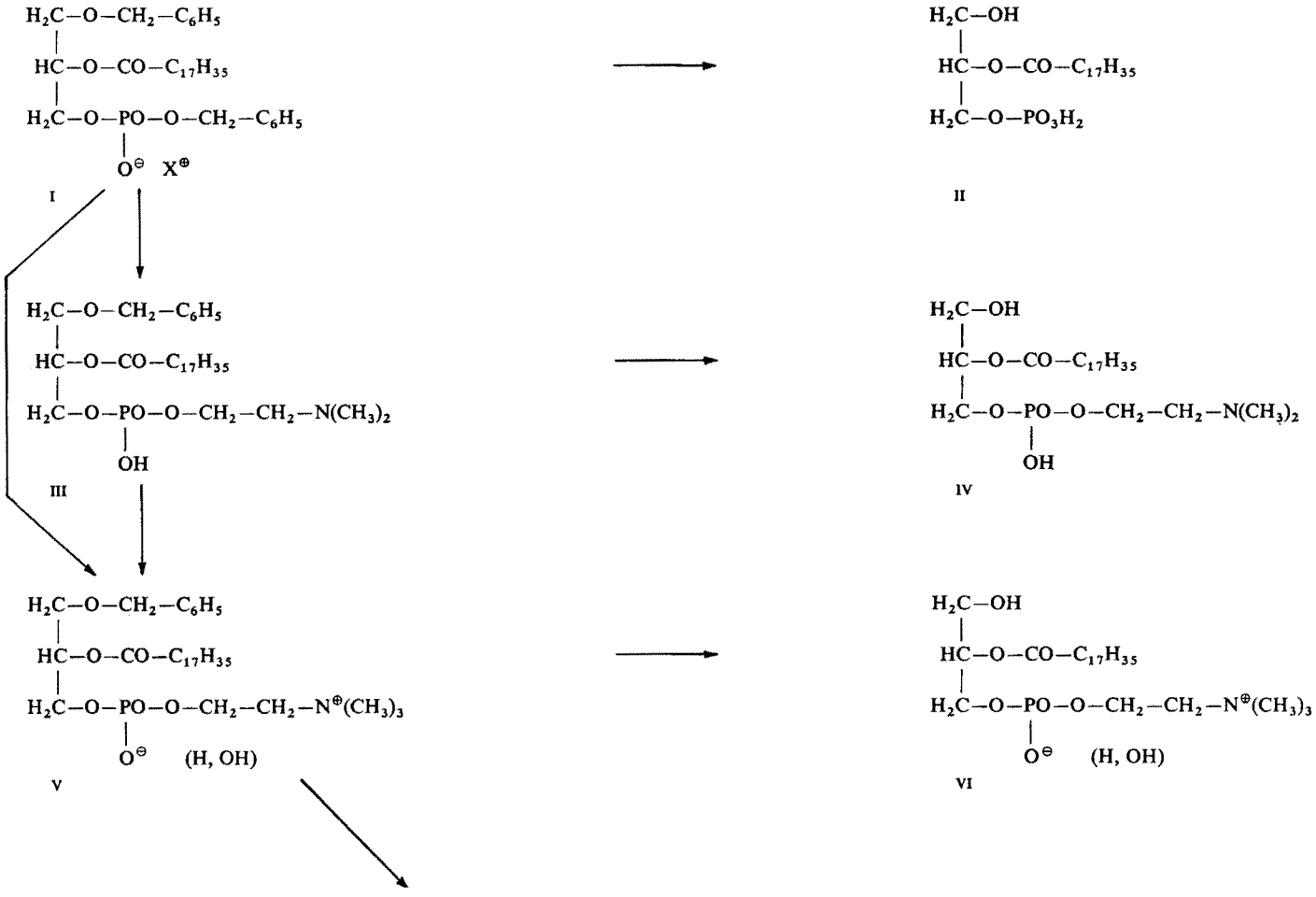
Lysophosphatidyl (N,N-dimethyl)ethanolamine

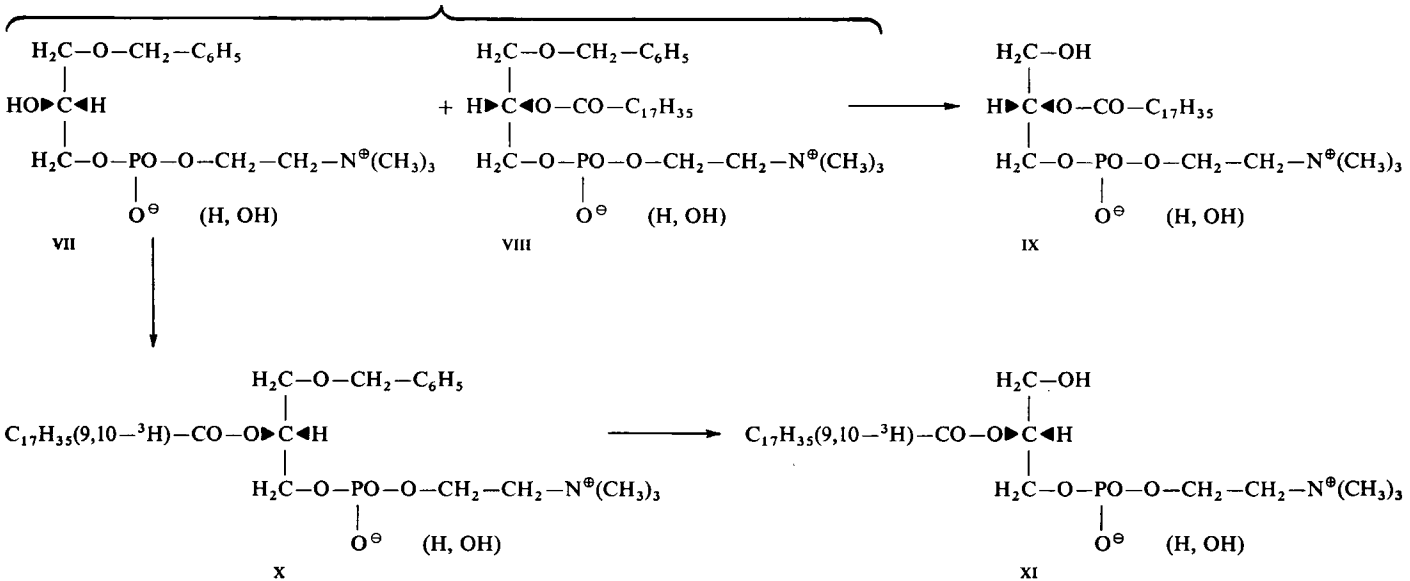
For the preparation of racemic 2-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (IV) the first attempts were directed to a reaction between the silver salt of I and 2-chloro-ethyl N,N-dimethylamine[8]). Although a small amount of the desired reaction product could be obtained an autodebenzylation due to the presence of the free dimethylamino group occurred, which gave rise to the formation of the N,N-dimethyl-N-benzyl derivative. For that reason the picrate of 2-bromo-ethyl N,N-dimethylamine[9]) was allowed to react with the silver salt of I. After debenzylation of the reaction product with lithium bromide we obtained 1-0-benzyl-2-stearoyl-

glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (III) in a good yield. This product was readily transformed by hydrogenolysis into racemic 2-stearoyl-3-phosphoryl-(N,N-dimethyl)-ethanolamine (IV). By a similar sequence of reactions starting from racemic 1-stearoyl-2-0-benzyl-glycerol-3-(benzyl)phosphate the isomer having the stearoyl residue esterified to the primary hydroxyl group was obtained (see experimental part).

Lysolecithin

For the preparation of monoacyl analogues of phosphatidyl choline two different methods have been applied. One procedure involved a reaction of racemic 1-0-benzyl-2-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (III) with methyl iodide in the presence of cyclohexylamine. This reaction proceeds under mild conditions so as to give a nearly complete conversion into the trimethyl derivative (V). After removal of the protecting benzyl group racemic 2-stearoyl-glycerol-3-phosphorylcholine (VI) was obtained. Similarly the 1-acyl isomer can be prepared in racemic form (see experimental part). This method may be suitable for the preparation of labelled lysolecithins through the use of [^{14}C]-methyl iodide. A second and more direct route for the synthesis of lysolecithins represents a reaction between the silver salt of I and 2-bromo-ethyl trimethylammonium picrate[10]). The condensation product was subjected to anionic debenzylation with lithium bromide and after removal of ions racemic 1-0-benzyl-2-stearoyl-glycerol-3-phosphorylcholine (V) was obtained in a fair yield. Hydrogenolysis gave racemic 2-stearoyl-glycerol-3-phosphorylcholine (VI). However, in addition racemic 1-0-benzyl-2-stearoyl-glycerol-3-phosphorylcholine (V) offered the possibility to prepare 2-acyl-lysolecithins of defined stereochemical configurations (IX and XI). To that end use was made of the stereospecific action of phospholipase A (EC 3.1.1.4) from *Crotalus adamanteus*[11]). This enzyme hydrolyses the fatty acid ester linkage at C_2 from phosphoglycerides derived from glycerol-3-phosphate but does not act on derivatives of glycerol-1-phosphate. Enzymatic hydrolysis of V gave a rapid decrease of acyl ester bonds[12]) to 50% of the original value and even after considerable continuation of the incubation time no further hydrolysis could be observed (fig. 2). This result is in agreement with the observations of de Haas and van Deenen[5]) who demonstrated that racemic 1-0-benzyl-2-acyl-glycerol-3-phosphorylcholine is converted by phospholipase A into 1-0-benzyl-glycerol-3-phosphorylcholine (VII) leaving 3-0-benzyl-2-stearoyl-glycerol-1-phosphorylcholine (VIII) unhydrolysed. The latter compound (VIII) yielded upon hydrogenolysis 2-stearoyl-glycerol-1-phosphorylcholine (IX). Further evidence concerning the structure and configuration of this lysolecithin will be given below. The other product of the enzymatic hydrolysis *viz.* 1-0-benzyl-


H2C–O–CH2–C6H5
HC–O–CO–C17H35
H2C–O–PO–O–CH2–C6H5
O⊖ X⊕
I
H2C–OH
HC–O–CO–C17H35
H2C–O–PO3H2
II
H2C–O–CH2–C6H5
HC–O–CO–C17H35
H2C–O–PO–O–CH2–CH2–N(CH3)2
OH
III
H2C–OH
HC–O–CO–C17H35
H2C–O–PO–O–CH2–CH2–N(CH3)2
OH
IV
H2C–O–CH2–C6H5
HC–O–CO–C17H35
H2C–O–PO–O–CH2–CH2–N⊕(CH3)3
O⊖ (H, OH)
V
H2C–OH
HC–O–CO–C17H35
H2C–O–PO–O–CH2–CH2–N⊕(CH3)3
O⊖ (H, OH)
VI



Scheme 1. Synthesis of (rac)-2-stearoyl-glycerol-3-phosphate (II), (rac)-2-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (IV), (rac)-2-stearoyl-glycerol-3-phosphorylcholine (VI), 2-stearoyl-glycerol-1-phosphorylcholine (IX) and 2-[9,10—^{3}H]-stearoyl-glycerol-3-phosphoryl-choline (XI)

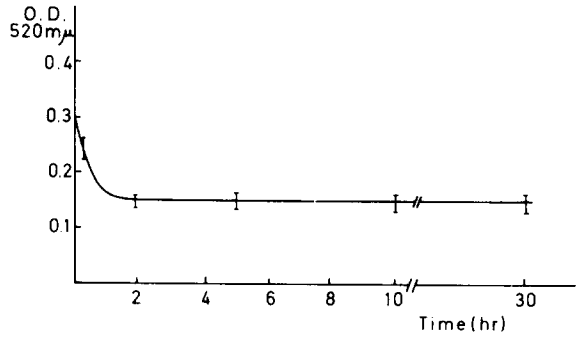
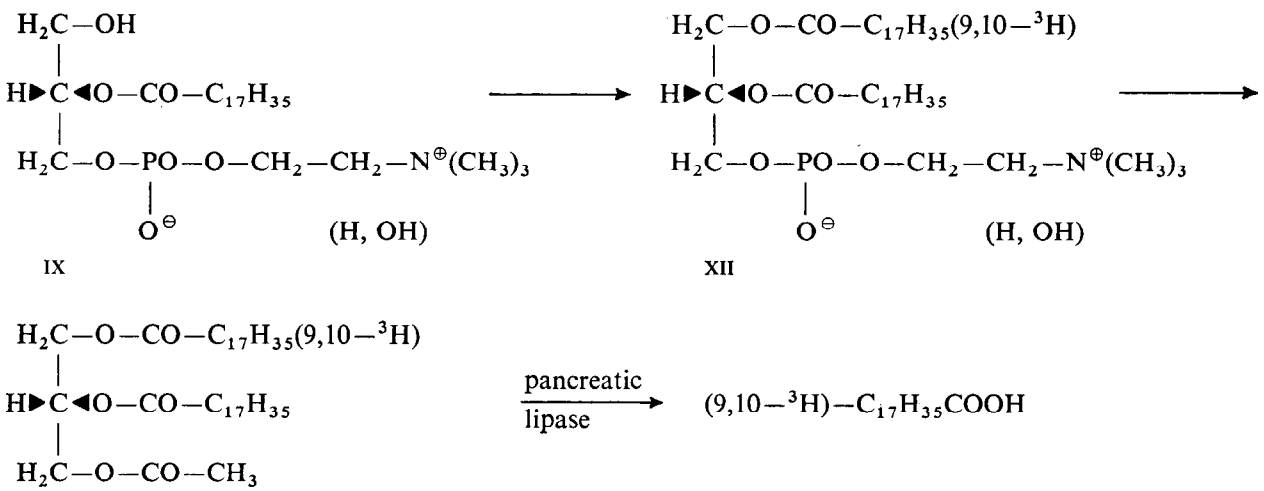


Fig. 2. Decrease of acyl ester bond content during the degradation of (rac)-1-0-benzyl-2-stearoyl-glycerol-3-phosphorylcholine (V) with phospholipase A measured by the hydroxamate method[12]). Abscissa: time (hr). Ordinate: optical density at 520 mμ.

glycerol-3-phosphorylcholine (VII) can be converted *via* acylation and removal of the protecting benzyl group into a 2-acyl-lysolecithin (XI) having the stereochemical configuration normally found in natural phosphoglycerides. This compound VII appears to be an attractive intermediate for the preparation of a labelled lysolecithin in as much as the procedure is limited to two conversions which proceed with yields of 40 and 100% respectively. In this way 2-[9,10-^{3}H]-stearoyl-glycerol-3-phosphorylcholine (XI) was obtained.

Although in a previous paper[5]) evidence was presented with respect to the validity of the conversion of V into the stereochemical isomers IX and XI it was considered essential to confirm and extend these investigations. In order to proof the stereochemical purity and position of the fatty acid ester linkage of both compounds and their intermediates use was made of phospholipase A degradation. This enzyme was found to give after a 1.5 hr incubation period a complete hydrolysis of X, all of the radioactivity being recovered in the fatty acid fraction.

In accordance with previous conclusions about the substrate specificity of phospholipase A, 2-[9,10-^{3}H]-stearoyl-glycerol-3-phosphorylcholine (XI) was found to be susceptible to the action of this enzyme although its hydrolysis proceeds much slower when compared with substrates containing two apolar residues. Using appropriate quantities of enzyme and a sufficiently long incubation period this lysolecithin was degraded for over 90% into [9,10-^{3}H]-stearic acid and glycerol-3-phosphorylcholine. That no 100% hydrolysis was obtained can probably be attributed to the fact that some migration of the fatty acyl moiety occurred during this incubation. In contrast, however, under similar conditions no fatty acid was released neither from the enantiomeric lyso-lecithin (IX) nor from its precursor (VIII). These



Scheme 2. Sequence of reactions applied for a structural proof of 2-acyl-glycerol-1-phosphorylcholine (IX).

observations support the conclusion that the lysolecithin XI is indeed a 2-acyl derivative of glycerol-3-phosphate. The structure of IX was further proven by subjecting this compound to an acylation with [9,10-^{3}H]-stearoyl chloride (scheme 2). The reaction product 3-[9,10-^{3}H]-stearoyl-2-stearoyl-glycerol-1-phosphorylcholine (XII) was found to have an optical rotation, $[\alpha]_D^{20} -5.9°$, being in fair agreement with values reported for lecithins derived of glycerol-1-phosphate[13–15]). This compound (XII) was dephosphorylated using a mixture of acetic acid and acetic anhydride[16]) so as to give 3-[9,10-^{3}H]-stearoyl-2-stearoyl-1-acetyl glycerol (XIII). This product was now treated with pancreatic lipase (EC 3.1.1.3) which enzyme is known to act preferentially at the fatty acid ester linkages with the primary hydroxyl groups of glycerol[17]). About 90% of the radioactivity was recovered in the liberated fatty acid fraction. Taking into account the various manipulations involved, this deviation from the theoretical value of the labelled stearic acid to be released (100%) hardly argues against the conclusion that IX is identical to 2-stearoyl-glycerol-1-phosphorylcholine. With respect to the structure of other lysophosphoglycerides described in this paper it can be recorded that racemic 2-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (IV) could be degraded for about 50% by the action of snake venom phospholipase A, this result being in good agreement with the known stereochemical specificity of this enzyme[11]). The corresponding structural isomer having the fatty acid residue esterified to the primary hydroxyl group appeared to resist completely the action of phospholipase A. No conclusions can be made with respect to the position of the fatty acid residues in the lysophosphatidic acids synthesized, since we have not been able to observe a degradation of these compounds by the preparation of snake venom phospholipase A used in this investigation.

Fig. 3 gives the I.R. spectra of the 2-acyl lyso derivatives of phosphatidic acid, phosphatidyl-(N,N)-dimethyl)ethanolamine and lecithin respectively. The spectra of the corresponding 1-acyl lyso-analogues were very similar and did not allow any distinction between 1- and 2-acyl lyso-derivatives with the same polar headgroup.

Furthermore some caution has to be taken again with respect to the handling of lysophosphoglycerides. Upon storage of 2-acyl lysolecithin (XI) in the solid state for 30 days at room temperature a considerable migration (30–40%) into the 1-acyl isomer was apparent by virtue of a reduced release of fatty acid by phospholipase A. Solutions of 2-acyl lysolecithin in chloroform–methanol revealed in this period a migration up to 25%. Chromatography on silicic acid even when carried out at 0 °C was found to give migration of at least 15–20%.

Finally, it has to be recalled that the synthetic procedures applied in the

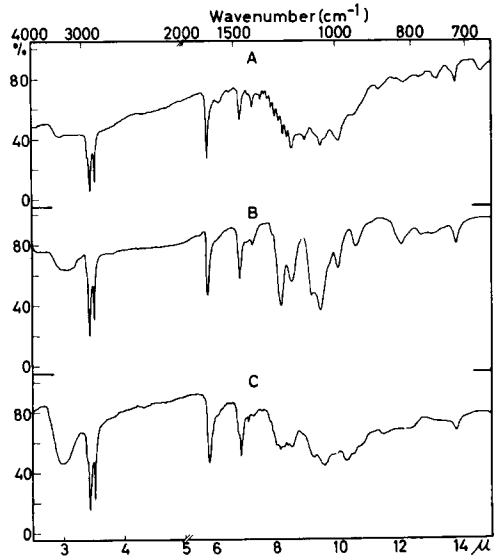


Fig. 3. I.R. spectra of some 2-acyl lysophosphoglycerides. A = (rac)-2-stearoyl-glycerol-3-phosphate (II); B = (rac)-2-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (IV); C = 2-[9,10—^{3}H]-stearoyl-glycerol-3-phosphorylcholine (XI). The samples were spread as thin films on a KBr disk. Ordinate: % transmission. Abscissa: wavelength.

present investigation are limited to the synthesis of saturated lysophosphoglycerides. The preparation of unsaturated 1-acyl-glycerol-3-phosphate derivatives, however, can be accomplished by the degradation of synthetic diacyl analogues with phospholipase A. This approach has been used routinely with good results in this laboratory also for 1-acyl lysolecithins containing polyunsaturated fatty acids. In principle the enzymatic approach may also be suitable for the preparation of 2-acyl lysolecithins since recent observations in several laboratories have led to the recognition of lipolytic enzymes acting on the 1-position of phosphoglycerides[18–22]).

EXPERIMENTAL PART

Materials and methods

Stearic acid (purity > 99.5%) was purchased from Fluka A.G. and converted into the acid chloride with oxalylchloride. Silver dibenzyl phosphate was prepared from dibenzyl phosphoric acid[23]) as described by Sheehan and Frank[24]). [9,10-^{3}H]-stearic acid was obtained from the Radiochemical Centre, Amersham, Great Britain. 2-Bromo-ethyl trimethylammonium picrate was prepared as described by Kabashima[10]). 2-Bromo-ethyl N,N-

dimethylamine picrate was prepared by a reaction of 2-bromo-ethyl N,N-dimethylamine hydrobromide[9]) with picric acid; m.p. 143.5–145.5 °C.

Found C 31.6 H 3.7 N 14.4

Calcd. for $C_{10}H_{13}BrN_4O_7$ (M = 381.15) C 31.51 H 3.44 N 14.70

Snake venom-phospholipase A (*Crotalus adamanteus*) was purchased from the Reptile Institute, Silver Springs, Florida. Silicic acid was a product from Mallinckrodt. The fraction between 60–140 mesh was used for column chromatography after activation overnight at 110 °C. The purity of all compounds was checked by thin-layer chromatography on micro-slides coated with silicic acid (Kieselgel G, Merck). After developing in the appropriate solvent system the spots were visualized by spraying 30% sulphuric acid followed by charring. Compounds containing phosphorus were detected by spraying with the ninhydrine reagent followed by the reagent of Hanes and Isherwood[25]). Melting points were determined on a Kofler hot plate and are uncorrected. Optical rotations were measured in a Lichtelektrisches Präzisions polarimeter 0.005°, Carl Zeiss. Radioactivity was measured with a liquid scintillation counter (Packard Tri-Carb). Micro-analyses were carried out in the Analytical Department of the Laboratory of Organic Chemistry, State University of Groningen (Groningen, The Netherlands) and in the Laboratory of Analytical Chemistry, University of Utrecht (Utrecht, The Netherlands). Infrared spectra were recorded in the Laboratory of Analytical Chemistry, University of Utrecht (Utrecht, The Netherlands) with a Beckmann I.R. Spectrophotometer I.R. 8.

(Rac)-1-0-benzyl-2-stearoyl-glycerol-3-(dibenzyl)phosphate

This product was prepared as described previously[4]).

Barium salt of (rac)-1-0-benzyl-2-stearoyl-glycerol-3-(benzyl)phosphate (I, $X^+ = \frac{1}{2}Ba^{++}$)

16.45 g of the phosphotriester and 7.0 g of dry barium iodide were refluxed in 100 ml of absolute acetone for 4.5 hr. After cooling, the precipitate was centrifuged and dissolved in chloroform. The chloroform solution was washed with water and evaporated *in vacuo*. The residue was crystallized from a chloroform-acetone mixture, yielding 11.52 g of I ($X^+ = \frac{1}{2}Ba^{++}$) (72%). This crude barium salt was directly converted into the free acid (I, $X^+ = H^+$).

(Rac)-1-0-benzyl-2-stearoyl-glycerol-3-(benzyl)phosphate (I, $X^+ = H^+$)

3.1 g of the crude barium salt (I) dissolved in chloroform was extracted twice with an ice-cold 0.5 N sulphuric acid solution and washed with water until neutral. The chloroform solution was evaporated to dryness *in vacuo*.

The last traces of barium sulphate were removed by centrifugation of an ether-tetrahydrofuran solution (1:1, v/v). After evaporation of the solvents *in vacuo* the residue was crystallized from ether at −20 °C. We obtained 2.5 g (90%) of the free acid I ($X^+ = H^+$) as colourless crystals, with a m.p. 51–52 °C. Thin-layer chromatography showed only one spot (chloroform–methanol–ammonia 70:20:1.5, v/v/v).

Found	C 68.1	H 9.0	P 4.8
Calcd. for $C_{35}H_{55}O_7P$ (M=618.77)	C 67.93	H 8.96	P 5.01

(Rac)-2-stearoyl-glycerol-3-phosphate (II)

0.15 g of the foregoing compound dissolved in 30 ml of dry ethyl acetate were shaken in the presence of a palladium catalyst in a hydrogen atmosphere under slightly more than atmospheric pressure. After about 2 hr the catalyst was filtered off, the solvent removed *in vacuo* and the residue crystallized from a mixture of chloroform, methanol and ether at −12 °C. A colourless crystalline precipitate amounting to 0.1 g (93%) was obtained (m.p. 74.5–75.5 °C). Thin-layer chromatography on silicic acid containing oxalic acid revealed only one spot (chloroform-methanol-hydrochloric acid 87:13:0.5 v/v/v). The I.R. spectrum of II is given in fig. 3.

Found	C 58.3	H 9.8	P 6.8
Calcd. for $C_{21}H_{43}O_7P$ (M=438.53)	C 57.51	H 9.88	P 7.06

(Rac)-1-stearoyl-2-0-benzyl-glycerol iodohydrin

This product was prepared as described previously[4]).

(Rac)-1-stearoyl-2-0-benzyl-glycerol-3-(dibenzyl)phosphate

A magnetically stirred suspension of 8.0 g of (rac)-1-stearoyl-2-0-benzyl-glycerol iodohydrin and 5.86 g of silver dibenzyl phosphate in 140 ml of absolute toluene was refluxed in the dark for 5½ hr. After cooling to room-temperature the precipitated silver iodide was centrifuged and washed twice with toluene. The combined toluene solutions were washed with a 5% potassium bicarbonate solution and twice with water. Drying of the organic phase over sodium sulphate and evaporating *in vacuo* gave 9.4 g of a colourless oil (94%). Thin-layer chromatography in the Marinetti system[26]) showed only one spot.

Barium salt of (rac)-1-stearoyl-2-0-benzyl-glycerol-3-(benzyl)phosphate

This compound was prepared as described for the isomer (I, $X^+ = \frac{1}{2}Ba^{++}$). 13.13 g of the above triester furnished 6.8 g of the barium salt (53%). Thin-layer chromatography in chloroform-methanol-ammonia (70:20:1.5, v/v/v) showed that no impurities were present.

Found	C 61.2	H 7.8	P 4.2
Calcd. for $C_{35}H_{54}Ba_{\frac{1}{2}}O_7P$ (M=686.43)	C 61.24	H 7.93	P 4.51

Sodium salt of (rac)-1-stearoyl-2-0-benzyl-glycerol-3-(benzyl)phosphate

9.4 g of (rac)-1-stearoyl-2-0-benzyl-glycerol-3-(dibenzyl)phosphate and 2.98 g of anhydrous sodium iodide were refluxed for 2 hr in 60 ml of absolute acetone. After cooling to −15 °C the precipitate was centrifuged and recrystallized from chloroform-acetone at −15 °C. 7.45 g (87%) of the sodium salt were isolated as a colourless solid with m.p. 88.5–89.9 °C. Thin-layer chromatography in chloroform-methanol-ammonia (70:20:1.5, v/v/v) revealed a pure compound to be present.

Found	C 65.5	H 8.4	P 4.7
Calcd. for $C_{35}H_{54}NaO_7P$ (M=640.75)	C 65.60	H 8.49	P 4.83

(Rac)-1-stearoyl-2-0-benzyl-glycerol-3-(benzyl)phosphate

4.13 g of the foregoing barium salt were converted into the free acid as described for the isomer (I, $X^+ = H^+$). We obtained 3.48 g of the acid (93%) as colourless crystals with m.p. 53–55 °C. No impurities could be detected on thin-layer chromatography (chloroform-methanol-ammonia, 70:20:1.5 v/v/v).

Found	C 67.9	H 8.9	P 5.6
Calcd. for $C_{35}H_{55}O_7P$ (M=618.77)	C 67.93	H 8.96	P 5.01

(Rac)-1-stearoyl-glycerol-3-phosphate

0.39 g of the free acid dissolved in absolute ethanol were converted into (rac)-1-stearoyl-glycerol-3-phosphate as described for the isomeric lysophosphatidyc acid II. We obtained 0.2 g (72%) of chromatographically pure (rac)-1-stearoyl-glycerol-3-phosphate with a m.p. 85–87 °C.

Found	C 57.5	H 9.9	P 7.2
Calcd. for $C_{21}H_{43}O_7P$ (M=438.53)	C 57.51	H 9.88	P 7.06

Silver salt of (rac)-1-0-benzyl-2-stearoyl-glycerol-3-(benzyl)phosphate (I, $X^+ = Ag^+$)

This compound was prepared as described previously[4]).

(Rac)-1-0-benzyl-2-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (III)

6.4 g of the silver salt (I, $X^+ = Ag^+$) and 6.65 g of 2-bromo-ethyl N,N-dimethylamine picrate dissolved in 475 ml of dry toluene were refluxed in the dark for 5 hr with magnetic stirring. The reaction mixture after cooling to room temperature, was centrifuged and the precipitate washed twice with toluene. Subsequently the combined toluene solutions were evaporated *in*

vacuo and after drying, the residue was dissolved in 250 ml of absolute acetone. 2.3 g of anhydrous lithium bromide were added and the mixture heated at 60 °C for 2 hr. The acetone solution was diluted to 1 l with a mixture of methanol and water (9:1, v/v). Removal of inorganic material was carried out by passing this solution over an ion-exchange column containing equal amounts of Amberlite IRC-50(H^+) and IR-45(OH^-). After washing the column the eluate was evaporated to dryness *in vacuo*. The residue was chromatographed on a silicic acid column with chloroform-methanol mixtures containing up to 30% of methanol. Crystallization from chloroform-acetone finally yielded 2.56 g of III (50%) as a colourless solid material with m.p. 116–118 °C. Thin-layer chromatography in chloroform-methanol-water (65:25:4, v/v/v) revealed only one spot.

Found	C 62.3	H 10.2	N 2.3	P 4.5
Calcd. for $C_{32}H_{58}NO_7P \cdot H_2O$ (M = 617.79)				
	C 62.21	H 9.79	N 2.27	P 5.01

(Rac)-2-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (IV)

0.2 g of III dissolved in absolute ethanol were hydrogenolysed in the presence of a palladium catalyst for 5 hr. The catalyst was removed by filtration and the filtrate evaporated *in vacuo*. Crystallization of the residue from chloroform-acetone at −12 °C gave 0.15 g of IV (86%) as a colourless solid melting at 208–210 °C. Thin-layer chromatographic control in chloroform-methanol-water (65:25:4, v/v/v) revealed only one spot. The I.R. spectrum of this lyso-compound is given in Fig. 3.

Found	C 58.2	H 10.1	N 3.0	P 5.7
Calcd. for $C_{25}H_{52}NO_7P$ (M = 509.65)				
	C 58.91	H 10.28	N 2.75	P 6.08

Silver salt of (rac)-1-stearoyl-2-0-benzyl-glycerol-3-(benzyl) phosphate

To a magnetically stirred solution of 7.26 g of the sodium salt of (rac)-1-stearoyl-2-0-benzyl-glycerol-3-(benzyl) phosphate in 125 ml of tetrahydrofuran and 50 ml of 15% acetone in distilled water was slowly added in the dark a solution of 1.93 g of silver nitrate in 30 ml of distilled water. After addition of more acetone the solution was cooled to 0 °C and the colourless precipitate (4.75 g, m.p. 75–77.5 °C) was centrifuged. Addition of more cold acetone to the filtrate yielded a second crop of silver salt (2.15 g, m.p. 72.5–74.5 °C). The total yield amounted to 84%.

(Rac)-1-stearoyl-2-0-benzyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine

5.15 g of the foregoing silver salt and 5.41 g of 2-bromo-ethyl N,N-di-

methylamine picrate dissolved in 350 ml of absolute toluene were refluxed in the dark for 2 hr with magnetic stirring. The reaction mixture was worked up as described for the isomer III. Debenzylation was carried out as described for III. After purification we obtained 2.64 g (62%) of (rac)-1-stearoyl-2-0-benzyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine (m.p. 163–169 °C). Thin-layer chromatography in chloroform-methanol-water (65:25:4, v/v/v) showed that a pure compound was present

Found C 61.1 H 9.7 N 2.2 P 5.0

Calcd. for $C_{32}H_{58}NO_7P \cdot H_2O$ (M = 617.79)

C 62.21 H 9.79 N 2.27 P 5.01

(Rac)-1-stearoyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine

0.4 g of the foregoing compound was hydrogenolysed as described for the isomer IV. We obtained 0.28 g of the lyso-compound as a colourless solid (82%) with m.p. 206–209 °C. Thin-layer chromatography in chloroform-methanol-water (65:25:4, v/v/v) showed no contaminant to be present.

Found C 54.7 H 9.9 N 2.7 P 6.7

Calcd. for $C_{25}H_{52}NO_7P$ (M = 509.65)

C 58.91 H 10.28 N 2.75 P 6.08

(Rac)-1-0-benzyl-2-stearoyl-glycerol-3-phosphorylcholine (V)

(a) To a solution of 1.5 g of III in 21 ml of methanol and 12 ml of tetrahydrofuran were added 0.71 g of methyl iodide and 0.47 g of cyclohexylamine in 6 ml of methanol. The reaction mixture was left at room temperature in the dark. Thin-layer chromatographic control (chloroform–methanol–water 65:25:4, v/v/v) showed that the reaction was complete in 4 hr. 80 ml of ice-cold 0.5 N sulphuric acid were added and the mixture was extracted twice with 90 ml of chloroform. The combined chloroform layers were extracted twice with 50% methanol and the resulting chloroform phase was evaporated *in vacuo*. The residue, dissolved in a mixture of 30 ml of chloroform, 80 ml of methanol and 10 ml of water, was treated with silver oxide (prepared from 2 g of silver nitrate and 0.5 g of sodium hydroxide) for 15 min. After centrifugation the clear supernatant was evaporated *in vacuo* and the residue chromatographed on a silicic acid column with chloroform-methanol mixtures containing up to 60% of methanol. Crystallization from chloroform-acetone yielded 1.3 g (82%) of a colourless solid, melting at 225–228 °C. Thin-layer chromatography in chloroform–methanol–water (65:25:4, v/v/v) gave one spot.

Found C 42.9 H 6.7 N 1.5 P 3.5

Calcd. for $(C_{33}H_{62}NO_8P)_2(CdCl_2)_3$ (M = 1813.60)

C 43.71 H 6.88 N 1.54 P 3.42

(b) A magnetically stirred solution of 13.4 g of the silver salt I ($X^+ = Ag^+$) and 14.9 g of 2-bromo-ethyl trimethylammonium picrate in 225 ml of absolute tetrahydrofuran was refluxed for 24 hr in the dark. The reaction mixture after cooling to room temperature, was centrifuged and the precipitate washed twice with tetrahydrofuran. Removal of the solvent *in vacuo* gave a residue which was dissolved in absolute acetone. Subsequently 4.7 g of anhydrous lithium bromide were added and the solution heated at 60 °C for 3 hr. The mixture was evaporated *in vacuo* and the residue dissolved in 2 l of a mixture of chloroform-methanol-water (15:30:20, v/v/v) was passed through a mixed ion-exchange column, containing equal amounts of Amberlite IRC-50(H^+) and IR-45(OH^-). After washing the column with another 2 l of the solvent mixture the combined eluates were evaporated *in vacuo*. Purification of the residue was carried out by chromatography on a silicic acid column with increasing percentages of methanol in chloroform up to 70%. Crystallization from chloroform-acetone yielded 5.5 g of V (47%) with m.p. 220–221 °C which was pure on thin-layer chromatography.

(Rac)-2-stearoyl-glycerol-3-phosphorylcholine (VI)

0.18 g of V dissolved in absolute ethanol were hydrogenolysed in the presence of a palladium catalyst at slightly more than atmospheric pressure for 2 hr. The catalyst was removed by filtration and the filtrate evaporated *in vacuo*. The residue was crystallized from chloroform-acetone at −15 °C. We obtained 0.13 g of chromatographically pure lysolecithin (VI) (85%) with m.p. 249–254 °C.

Found	C 58.3	H 10.3	N 2.6	P 5.6
Calcd. for $C_{26}H_{56}NO_8P$ (M = 541.68)				
	C 57.64	H 10.42	N 2.58	P 5.71

(Rac)-1-stearoyl-2-0-benzyl-glycerol-3-phosphorylcholine

2.05 g of (rac)-1-stearoyl-2-0-benzyl-glycerol-3-phosphoryl-(N,N-dimethyl)ethanolamine was treated with methyl iodide as described for the isomer V. 1.3 g of the wanted product were isolated as colourless crystals (60%) with m.p. 225–227 °C which on thin-layer chromatography in chloroform-methanol-water (65:25:4, v/v/v) behaved as a single component.

Found	C 62.2	H 9.8	N 2.3	P 5.1
Calcd. for $C_{33}H_{62}NO_8P$ (M = 631.81)				
	C 62.73	H 9.88	N 2.22	P 4.90

(Rac)-1-stearoyl-glycerol-3-phosphorylcholine

0.87 g of the foregoing product was hydrogenolysed as described for VI. After purification we obtained 0.7 g of (rac)-1-stearoyl-glycerol-3-phosphoryl-

choline (93%) with m.p. 246–252 °C which was chromatographically pure.

Found C 56.7 H 10.3 N 2.7 P 5.6

Calcd. for $C_{26}H_{56}NO_8P$ (M = 541.68)

C 57.64 H 10.42 N 2.58 P 5.71

1-0-benzyl-glycerol-3-phosphorylcholine (VII) and
3-0-benzyl-2-stearoyl-glycerol-1-phosphorylcholine (VIII)

To 1775 ml of a borate buffer (*p*H = 7, 0.2 M) containing 5×10^{-3} M calcium chloride and 177.5 ml of ether were added with continuous stirring 5.52 g of the (rac)-"benzyllecithin" (v) followed by 180 mg of phospholipase A. The mixture which was initially clear rapidly became turbid. The degradation was followed by measuring the decrease of fatty acid ester bond by the hydroxamate method[12]). At certain time intervals a sample of 0.5 ml of the reaction mixture was removed. To this sample were added 4 ml of a mixture of ethanol-ether (3:1, v/v), 0.5 ml 2 M hydroxylamine hydrochloride and 0.5 ml 3.5 N sodium hydroxide solution. The mixture was left at room temperature for 20 min. Subsequently 0.6 ml of 3.3.N hydrochloric acid was added followed by 0.5 ml of a 0.37 M ferric chloride solution in 0.1 N hydrochloric acid. The optical density was read at 520 mμ (Beckman Spektral Fotometer DU, Modell G 2400) 12 min after addition of the ferric chloride solution (Fig. 2). After 4 hr at room temperature the reaction mixture was extracted three times with 30% methanol in chloroform. The chloroform layer was extracted once with 10% methanol in water. Thin-layer chromatography showed the presence of free fatty acid and 3-0-benzyl-2-stearoyl-glycerol-1-phosphorylcholine (VIII) in the chloroform layer while the aqueous phase contained the 1-0-benzyl-glycerol-3-phosphorylcholine (VII) and the enzyme.

3-0-benzyl-2-stearoyl-glycerol-1-phosphorylcholine (VIII) was isolated by evaporation of the chloroform solution *in vacuo* and the residue dissolved in 70% ethanol in water. This solution was extracted three times with pentane to remove free fatty acid. The resulting ethanolic solution was evaporated *in vacuo* and the residue chromatographed on a silicic acid column. Crystallization from chloroform-acetone yielded 2.15 g (78%) of VIII (m.p. 217–220 °C) which was pure on TLC (chloroform-methanol-water, 65:25:4, v/v/v) and on silica impregnated paper in the Marinetti system[26]); $[\alpha]_D^{20} + 0.7°$ in chloroform (c 9.5).

Found C 61.7 H 9.9 N 2.6 P 4.9

Calcd. for $C_{33}H_{62}NO_8P$ (M = 631.81)

C 62.73 H 9.88 N 2.22 P 4.90

Purification of 1-0-benzyl-glycerol-3-phosphorylcholine (VII) was made by passing the aqueous solution through a mixed Amberlite column con-

taining equal amounts of IRC-50(H^+) and IR-45(OH^-). The eluate was evaporated *in vacuo*. Small amounts of the enzyme were removed by treatment with absolute ethanol, centrifuging and evaporation *in vacuo*. The finally obtained clear solution was evaporated *in vacuo* and the residue chromatographed on a silicic acid column with methanol in chloroform up to pure methanol. After crystallization from chloroform we obtained 1.16 g of chromatographically pure VII (73%) as colourless crystals (m.p. 164–166 °C). [Chromatography on Whatmann paper M-1 with isopropanol-ammonia-water (7:1:2, v/v/v) and on silica impregnated paper in the Marinetti system[26])]. $[\alpha]_D^{20} -11.2°$ in chloroform–methanol (9:1, v/v) (c 9).

Found C 50.6 H 7.8 N 3.9 P 9.0

Calcd. for $C_{15}H_{28}NO_7P$ (M = 365.36)

C 49.80 H 7.72 N 3.83 P 8.47

1-0-benzyl-2-[9,10−^{3}H]-stearoyl-glycerol-3-phosphorylcholine (X)

To 1.17 g of VII dissolved in absolute ethanol were added 0.96 g of cadmium chloride monohydrate dissolved in 96% of ethanol. The solution was evaporated *in vacuo* and the residue thoroughly dried. This cadmium chloride adduct was suspended in 20 ml of absolute chloroform. To this stirred suspension at 0 °C were added subsequently 9.65 g of [9,10−^{3}H]-stearoyl chloride (spec. act. 4.84×10^9 cpm/mMol) in 10 ml of dry chloroform and 2.52 g of dry pyridine in 10 ml of dry chloroform. After stirring 15 min at 0 °C the mixture was stirred for 2 hr at room temperature and then washed three times with icecold 0.5 N sulphuric acid, three times with 5% sodium bicarbonate solution and finally three times with water. The resulting chloroform layer was evaporated *in vacuo*, the residue dissolved in 70% ethanol and extracted with pentane. The pentane phase was extracted twice with 70% ethanol and the combined ethanol extracts were washed twice with pentane. The pentane solution, containing free fatty acid, was discarded, while the ethanolic solution was evaporated *in vacuo*. The residue was chromatographed on a silicic acid column with 0 to 40% of methanol in chloroform. The combined pure "benzyllecithin" fractions were recrystallized from chloroform-acetone. Yield 0.85 g (42%) of chromatographically pure 1-0-benzyl-2-[9,10−^{3}H]-stearoyl-glycerol-3-phosphorylcholine (X) with m.p. 215–216 °C (spec. act. 7.8×10^8 cpm/mMol). $[\alpha]_D^{20} -1.33°$ in chloroform (c 10.4)

Found C 60.8 H 10.0 N 1.9 P 4.6

Calcd. for $C_{33}H_{62}NO_8P$ (M = 631.81)

C 62.73 H 9.88 N 2.22 P 4.90

2-[9,10−^{3}H]-stearoyl-glycerol-3-phosphorylcholine (XI)

0.15 g of X dissolved in absolute ethanol were hydrogenolysed as described

for VI. After crystallization from chloroform-acetone 0.13 g of XI were obtained (99%), m.p. 250–254 °C. (spec. act. 7.4×10^8 cpm/mMol). $[\alpha]_D^{20} + 3.05°$ in chloroform-methanol (9:1 v/v) (c 8.7). The I.R. spectrum is given in Fig. 3.

2-stearoyl-glycerol-1-phosphorylcholine (IX)

0.49 g of VIII dissolved in absolute ethanol were hydrogenolysed in the presence of a palladium catalyst as described for VI. After crystallization from chloroform-acetone we obtained 0.35 g (84%) of lysolecithin IX, m.p. 246–249 °C. $[\alpha]_D^{20} - 3.89°$ in chloroform-methanol (9:1 v/v) (c 8.2).

3-[9,10—^{3}H]-stearoyl-2-stearoyl-glycerol-1-phosphorylcholine (XII)

A solution of 0.22 g of IX and 0.12 g of cadmium chloride monohydrate in 96% ethanol was evaporated *in vacuo* and the adduct thoroughly dried. To a suspension of this adduct in 5 ml of absolute chloroform were added with continuous stirring at 0 °C 1.21 g of [9,10—^{3}H]-stearoyl chloride in 5 ml of absolute chloroform followed by 0.32 g of dry pyridine in 5 ml of absolute chloroform. After stirring for 15 min at 0 °C the mixture was stirred for 2 hr at room temperature. At the end of the reaction more chloroform was added and the solution washed with ice-cold 30% ethanol being 0.5 N in sulphuric acid, followed by three washings with 30% ethanol. The chloroform solution was then evaporated *in vacuo* and the residue purified by chromatography on silicic acid with methanol-chloroform mixtures containing 0 up to 50% of methanol. After crystallization from hot chloroform-acetone we obtained 0.14 g (43%) of the distearoyllecithin (XII), m.p. 232–234 °C and $[\alpha]_D^{20} - 5.9°$ in chloroform (c 5.8).

Dephosphorylation of 3-[9,10—^{3}H]-stearoyl-2-stearoyl-glycerol-1-phosphorylcholine (XII) followed by degradation with pancreatic lipase

20 mg of 3-[9,10—^{3}H]-stearoyl-2-stearoyl-glycerol-1-phosphorylcholine (XII) were dephosphorylated as described by Bevan *et al.*[16]). We obtained after crystallization 10 mg of a product with m.p. 52.5–54 °C (cf. [16]) m.p. 55–56.5 °C for (rac)-1,2-distearoyl-3-acetylglycerol), which on thin-layer chromatography behaved as a pure compound (30% ether in hexane, v/v). Degradation of this compound (XIII) with pancreatic lipase following the conditions of Luddy *et al.*[27]) at 48 °C showed a complete conversion into free fatty acids and monoglyceride. The mixture was separated by thin-layer chromatography (petroleum ether–ether–formic acid 60:40:1.6, v/v/v) and the radioactivity estimated. It was found that 90% of the radioactivity was located in the free fatty acid fraction.

Enzymatic degradations with phospholipase A

5 mg of substrate were emulsified in 0.5 ml of a borate buffer (pH = 7, 0.2 M) and 0.1 ml of ether, which was 5.10^{-3} M in calcium chloride. After addition of 1–5 mg of phospholipase A the incubation mixture was vibrated at room temperature. The enzymatic degradation was followed by thin-layer chromatography in the appropriate solvent system. Substrates containing labelled fatty acid were applied to silicic acid plates (5 × 20 cm) when the conversion was optimal. The plates were developed first with chloroform-petroleum ether-acetic acid (65:33:2, v/v/v) and afterwards with chloroform-methanol-water (65:25:4, v/v/v). After locating the spots by scanning (Dünnschicht-Scanner LB 2721, Berthold) as well as by Rhodamine 6G staining, the spots were scraped off and the radioactive material eluted twice with 50 ml of 80% methanol in chloroform. The solvents were removed *in vacuo* and the radioactivity measured.

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