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The Chemical Nature of the Products Obtained by the Action of Cabbage-Leaf Phospholipase D on Lysolecithin: the Structure of Lysolecithin

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1. Lysolecithin, prepared by the action of snake-venom phospholipase A on ovolecithin, when incubated with Savoy-cabbage phospholipase D, in the presence of Ca^{2+} ions, gave two degradation products (designated *A* and *B*) in the form of their calcium salts. 2. These calcium salts were separated quantitatively by solvent fractionation and converted into the corresponding sodium salts. 3. Substance *B* proved to be a lysophosphatidic acid of conventional structure (1-monoacyl-L-3-glycerophosphoric acid). When the phosphate group was removed by means of prostatic acid phosphomonoesterase, a 1-monoglyceride was formed quantitatively. Alkaline hydrolysis gave the theoretical yield of L-3-glycerophosphate. 4. Substance *A*, on the other hand, had all the properties expected for a cyclic phosphate of a 1-monoglyceride. It was unaffected by phosphomonoesterase. On alkaline hydrolysis, the acyl group was removed and ring opening of the presumed cyclic phosphate group gave an approximately equimolar mixture of 2- and L-3-glycerophosphates. 5. The structures of substances *A* and *B* confirm lysolecithin as 1-monoacyl-L-3-glycerylphosphorylcholine.

Early attempts to elucidate the structure of lysolecithin by Hanahan (1954) and by Long & Penny (1954) (see also Davidson, Long & Penny, 1955) involved oxidation by permanganate or chromic acid and appeared to demonstrate the presence of a carboxyl group in the reaction product. This result strongly suggested that lysolecithin possessed a primary alcoholic group and should be designated as a 2-monoacyl-L-3-glycerylphosphorylcholine. This conclusion was supported by Gray (1958). However, Marinetti, Erbland & Stotz (1958), also using an oxidation procedure, claimed that lysolecithin could exist in both the 1- and 2-monoacyl forms. Tattrie (1959) removed phosphorylcholine from lecithin by the action of phospholipase C and incubated the resulting 1,2-diglyceride with pancreatic lipase, which was assumed to act specifically on the 1-acyl group. He reported that the fatty acid liberated under these conditions was different from that released by the action of snake-venom phospholipase A on the original lecithin. This observation indicated that phospholipase A removed the acyl group from the 2-position of the lecithin and that the resulting lysolecithin should therefore be regarded as a 1'-monoacyl-L-3-glycerylphosphorylcholine. Hanahan, Brockenhoff & Barron (1960) confirmed this result and simultaneously De Haas, Mulder & van Deenen (1960), employing synthetic lecithins of known positional acyl structure, also supported Tattrie's (1959) hypothesis. [For a more complete review of this subject, see Marinetti (1962).]

In the present work, the problem has been examined in a different way. By the successive actions of phospholipase D and phosphomonoesterase on lysolecithin, it was planned to split off first the choline component and then the phosphate group. The final product would be either a 1- or 2-monoglyceride, and the structure of this material would establish the position of the acyl group in lysolecithin. This object has been achieved, with the result that the Tattrie (1959) structure has been confirmed. However, it has also been found that the action of phospholipase D on lysolecithin is more complex than might have been expected, in that a presumed cyclic phosphate compound is formed in addition to the predicted lysophosphatidic acid of conventional structure. The position of the acyl group in this cyclic compound has been established, and this also confirms the Tattrie (1959) configuration.

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Some of these results have already been reported briefly (Long, Odavić & Sargent, 1963).

EXPERIMENTAL

Materials

Lysolecithin. This was prepared from ovolecithin as previously described (Long & Penny, 1957; Long, Odavić & Sargent, 1967).

Solvents. Methanol was refluxed with KOH pellets and granulated Zn for 1hr.; it was then distilled through a fractionating column. Chloroform was washed by shaking several times with water; immediately after distillation, it was stabilized by addition of 2% (v/v) of methanol.

Buffers. Acetate buffers (Walpole) were prepared by mixing 0·2M-solutions of sodium acetate and acetic acid in appropriate proportions. Final pH values were determined by means of a glass electrode and pH meter. M-Citrate buffer, pH5·8, was prepared by mixing appropriate volumes of M-citric acid and M-trisodium citrate. In the enzymic (dehydrogenase) assay of L-3-glycerophosphate, hydrazine was used both as trapping reagent for dihydroxyacetone phosphate and as an effective alkaline buffer. It was prepared by adjusting a solution of hydrazine sulphate to pH9·2 with NaOH, and diluting to a final hydrazine concentration 1M.

Chemicals. A sample of pure barium L-3-glycerophosphate was given by Professor E. Baer, University of Toronto, Canada; it was converted into the sodium salt as described by Long & Maguire (1953). Nicotinamide–adenine dinucleotide was a Sigma preparation. All other chemicals were the best available commercially.

Phospholipase D (phosphatidylcholine phosphatidohydrolase; EC 3.1.4.4). This enzyme was obtained from Savoy-cabbage leaves by the procedure of Davidson & Long (1958), and purified up to stage 3 (acetone-precipitation step; see Long *et al.* 1967).

Acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.2). Human prostate gland (42g.), frozen immediately after surgical removal, was cut into small pieces and homogenized with 200ml. of water. A few drops of toluene were added to the homogenate, which was stored overnight at 2°. After centrifuging, the supernatant was dialysed against water at 2°. The pH was adjusted to 5·6 with N-acetic acid, and after again centrifuging the clear supernatant was used as the source of enzyme. This is essentially the procedure of Schmidt (1955).

Muscle L-3-*glycerophosphate dehydrogenase* (L-*glycerol*-3-*phosphate–NAD oxidoreductase;* EC 1.1.1.8). This was purchased from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, West Germany.

Methods

Enzymic degradation of lysolecithin by phospholipase D. Lysolecithin (196μmoles) was dissolved in 4·5ml. of water. Acetate buffer, pH5·8 (0·2M; 12·5ml.) and M-$CaCl_2$ (3ml.) were added, followed by 5ml. of the phospholipase D preparation. The reaction mixture was incubated at 38° for 6hr., during which time a bulky white precipitate formed. This contained the calcium salts of the reaction products (substances *A* and *B*). Their separation by solvent fractionation and conversion into the sodium salts is given in the Results section.

Action of prostatic acid phosphomonoesterase. The substrate (sodium salts of substances *A* and *B*; about 0·1μmole), dissolved in 0·05ml. of water, was incubated with the enzyme (0·01ml.) and 0·2M-acetate buffer, pH5·8 (0·24ml.) for 60min. at 38°. Perchloric acid (72%, w/w; 0·125ml.) was then added, together with water to total volume 1ml. and the inorganic orthophosphate was determined. Control experiments with substrate alone or enzyme alone were made simultaneously. As a standard substrate, sodium DL-3-glycerophosphate (0·1μmole) was used. The amount of protein present in the enzyme preparation was so small that it was found unnecessary to remove it before determining inorganic phosphate.

Alkaline hydrolysis of substances A *and* B. Aqueous solutions of the sodium salts of substances *A* and *B* (about 5μmoles) were evaporated to dryness and hydrolysed by heating with 0·25ml. of 0·5N-NaOH on a boiling-water bath for 30min. After cooling, the hydrolysates were neutralized with N-HCl and diluted to 0·5ml. Fatty acids were removed by one extraction with 2ml. of $CHCl_3$–methanol (2:1, v/v), followed by two extractions with 1ml. of $CHCl_3$–methanol (17:3, v/v). The final aqueous methanolic phases were evaporated to dryness and dissolved in water; total vol., 2ml.

Action of L-3-*glycerophosphate dehydrogenase on alkaline-hydrolysis products of substances* A *and* B. The reaction mixture contained the substrate (0·1–0·3μmole), together with 1ml. of M-hydrazine buffer (pH9·2), NAD (1mg.), M-$MgCl_2$ (0·01ml.) and L-3-glycerophosphate dehydrogenase (0·05ml., containing about 50μg.); total vol., 3·5ml. After incubation at 38° for 60min., extinctions were read at 340mμ in 1cm. cells in a Unicam SP.500 spectrophotometer. A control without substrate and a series of standards containing different amounts of sodium L-3-glycerophosphate were done simultaneously. This is essentially the procedure of Bublitz & Kennedy (1954).

Determination of partition coefficients of the sodium salts of substances A *and* B *between aqueous methanol and chloroform–methanol at different pH values.* Aqueous solutions of the sodium salts (about 0·5μmole) were evaporated to dryness in a series of 5ml. centrifuge tubes with ground-glass stoppers. Water and 0·2M-acetate buffers (0·25ml.) of pH value between 5·7 and 7·9 or sodium acetate or $NaHCO_3$ or acetic acid were added together with sufficient 0·5M-NaCl to give final concn. 0·1M-Na^+ in a total volume 0·5ml. To each tube was then added 2ml. of $CHCl_3$–methanol (2:1, v/v). The contents were mixed thoroughly and centrifuged. Measured volumes of upper and lower phases were taken for determination of total phosphorus. Final pH values of the upper phases were determined with a glass electrode and pH meter on larger samples, in the absence of the sodium salts of substances *A* and *B*, after shaking with $CHCl_3$–methanol.

Analytical methods. Acyl ester was determined by the hydroxamate method of Shapiro (1953), with pure ethyl palmitate as standard. Inorganic orthophosphate was determined by the method of Berenblum & Chain (1938), as modified by Long (1943), with $NH_4H_2PO_4$ as standard. Total P was measured as orthophosphate after wet-ashing with perchloric acid (Long & Staples, 1961). Total glycerophosphate was estimated as orthophosphate by the hot acid–periodate method of Burmaster (1946). Long-chain

aldehyde was measured by the procedure of Sloane-Stanley & Bowler (1962). Periodate oxidation of monoglyceride was done by the method of O'Dea & Gibbons (1953). Thin-layer chromatography was as described by Long *et al.* (1967). Sodium was determined by flame photometry.

RESULTS

Separation of substances A *and* B, *produced by the action of phospholipase D on lysolecithin*

Sodium salt of substance A. The enzymic incubation of lysolecithin (196 μmoles) with phospholipase D was carried out under the conditions described in the Experimental section. The reaction mixture was then treated with 100ml. of chloroform–methanol (2:1, v/v). After shaking vigorously and centrifuging, the lower chloroform-rich phase was removed quantitatively and set aside. The upper aqueous methanolic phase plus interfacial precipitate was extracted seven times by shaking with 70ml. portions of chloroform–methanol (17:3, v/v). Each of the eight lower phases was diluted to 75ml. with methanol and measured volumes were taken for determination of total phosphorus. The result is shown in Fig. 1. A total of 75·8 μg.atoms of phosphorus was recovered, corresponding to 38·5% of the original lysolecithin. The first three lower phases, which virtually contained the whole of the extracted phosphorus, were combined and evaporated to dryness. The residue, containing 74·4 μg.-atoms of phosphorus, was extracted four times with 5ml. portions of the lower phase, prepared by shaking together chloroform–methanol–water (8:4:3, by vol.). An insignificant amount of residue remained after the final centrifugation, and contained only 0·11 μg.atom of phosphorus.

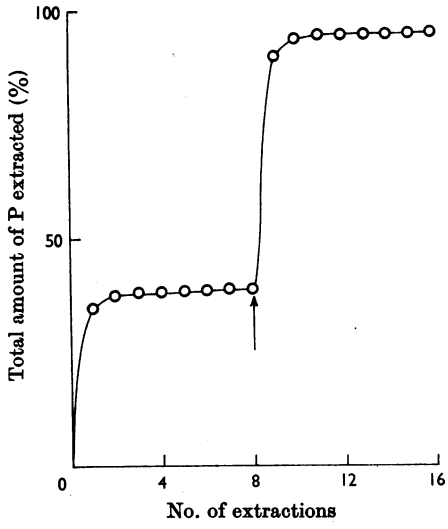


Fig. 1. Separation of products of hydrolysis of lysolecithin by phospholipase D. The enzymic reaction mixture contained lysolecithin (196 μmoles), phospholipase D (5ml.), M-$CaCl_2$ (3ml.) and 0·2M-acetate buffer, pH 5·8 (12·5ml.); total vol., 25ml. Incubation was for 6hr. at 38°. The first extraction (calcium salt of substance *A*) was made with 100ml. of $CHCl_3$–methanol (2:1, v/v); extractions 2–8, each with 70ml. of $CHCl_3$–methanol (17:3, v/v). At the point indicated by the arrow, M-sodium citrate buffer, pH 5·8 (10ml.) and methanol (10ml.) were added. Extractions 9–16 (sodium salt of substance *B*) were made with 70ml. portions of $CHCl_3$–methanol (17:3, v/v).

The calcium salt present in the combined extracts was converted into the sodium salt in the following way. The extract was treated with 6·5ml. of methanol, 5·5ml. of water and 1ml. of M-citrate buffer, pH 5·8, containing sodium as the cation. The system again became biphasic and after shaking and centrifuging, the lower phase was removed. The upper phase was washed five times with 19ml. portions of chloroform–methanol (17:3, v/v). Each of the six lower phases was diluted with methanol to 20ml., and measured volumes were taken for determination of total phosphorus. In all, 73·8 μg.-atoms of phosphorus were recovered (99% of original), mostly in the first three lower phases. These were combined and evaporated to dryness. The product is designated as the sodium salt of substance *A*.

Sodium salt of substance B. The upper aqueous methanolic phase plus interfacial precipitate, containing that part of the product of enzymic incubation which was not extractable by means of chloroform–methanol (see last section), was treated with 10ml. of methanol and 10ml. of M-citrate buffer (sodium form), pH 5·8, to convert the calcium salt into the sodium salt. The mixture was extracted eight times with 70ml. portions of chloroform–methanol (17:3, v/v). Each lower phase was diluted to 75ml. with methanol and measured volumes were taken for determination of total phosphorus. The results obtained are also shown in Fig. 1. A total of 110 μg.atoms of phosphorus was recovered, mostly in the first three extracts, corresponding to 56·3% of the original lysolecithin. These were combined and evaporated to dryness. This material is designated as the sodium salt of substance *B*.

The final aqueous methanolic phase plus interfacial material were found to contain 26·3 μg.atoms of phosphorus, of which the contribution of the enzyme preparation might be expected to amount to 17·9 μg.atoms, in the form of inorganic orthophosphate (Long *et al.* 1967). The overall recovery of lysolecithin phosphorus as the sodium salts of substances *A* and *B*, after allowing for samples taken for analysis, was 94·8%.

General properties of the sodium salts of substances A *and* B

Analytical. The sodium salts of substances *A* and *B* had ester:phosphorus ratios 1·02 and 0·96 respectively. In both cases the sodium:phosphorus ratios were close to unity. When examined by thin-layer chromatography with the solvent chloroform–methanol–water (65:25:4, by vol.), substance *A* had R_F about 0·5, whereas substance *B* did not move from the origin. In neither case was any trace of lysolecithin (R_F 0·10) found, and choline could not be detected in either product.

Substance *A* was inactive when tested as a substrate for prostatic acid phosphomonoesterase, whereas substance *B* was readily hydrolysed and formed 97% of the theoretical amount of inorganic phosphate. However, under the conditions given by Burmaster (1946), hot acid–periodate treatment of both substances *A* and *B* liberated all the phosphorus in the form of inorganic orthophosphate.

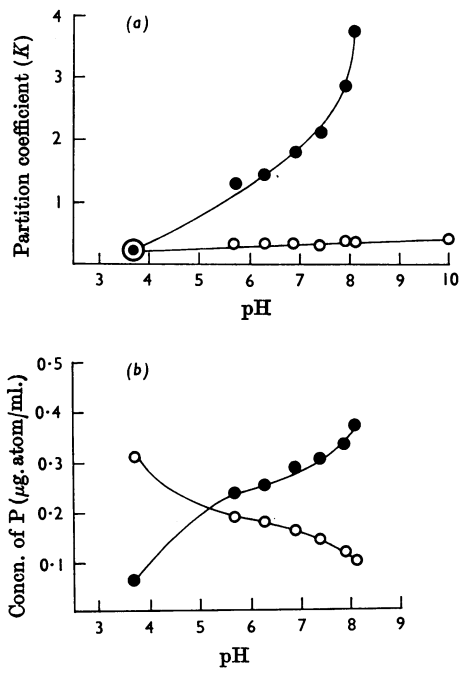


Fig. 2. (*a*) Partition coefficients (*K*), at different pH values, of the sodium salts of substances *A* (○) and *B* (●) between the immiscible phases obtained by shaking 1 vol. of aqueous solution with 4 vol. of $CHCl_3$–methanol (2:1, v/v). *K* is defined as concn. in upper phase/concn. in lower phase. In each case, the same quantity of sodium salt (approx. 0·5 μmole) was dissolved in a total aqueous volume 0·5 ml. and 2 ml. of $CHCl_3$–methanol (2:1, v/v) was added. (*b*) Absolute concentrations (μg.atom of total P/ml.) of substance *B* in: ●, upper phase; ○, lower phase.

Partition coefficients. The partition coefficients of the sodium salts of substances *A* and *B* were determined at different pH values under conditions described in the Experimental section. The two phases employed were those which separate spontaneously when 4 vol. of chloroform–methanol (2:1, v/v) are equilibrated with 1 vol. of aqueous solution. The results are shown in Fig. 2(*a*). From this it will be seen that the partition coefficient (*K* = concn. in upper phase/concn. in lower phase) for the sodium salt of substance *A* is low (about 0·3) and substantially constant at different pH values. By contrast, *K* for the sodium salt of substance *B* is appreciably higher and markedly pH-dependent. At pH 10·1 (not shown in Fig. 2) it had a value 13·1. This suggests that substance *B* can exist in two anionic forms at different pH values. When the actual concentrations of substance *B* in the upper and lower phases were plotted against pH (Fig. 2*b*), inflexions occurred in the region pH 6·0–6·5. Such a value suggests the secondary dissociation of a phosphate group.

Under comparable conditions, *K* for lysolecithin was 0·124 and was independent of pH.

Structure of the sodium salt of substance B

Action of prostatic acid phosphomonoesterase. Enzymic removal of the phosphate group gave rise to a monoglyceride, which was isolated as follows. The sodium salt (4·6 μmoles), dissolved in 0·5 ml. of 0·2 M-acetate buffer, pH 5·8, was incubated with 0·5 ml. of the enzyme preparation at 38° for 2 hr. The reaction mixture was then extracted three times with chloroform–methanol. The upper phase contained 4·3 μg.atoms of inorganic phosphate and was free from acyl ester. The lower phase contained 4·43 μmoles of acyl ester and was free from phosphorus, indicating that hydrolysis to inorganic phosphate and monoglyceride was complete. The chloroform-rich lower phase was evaporated to dryness.

Structure of the monoglyceride. Equal portions of the monoglyceride were emulsified in water and subjected to periodate oxidation at room temperature under the conditions of O'Dea & Gibbons (1953). The amount of monoglyceride taken was assessed by its acyl ester content. After stated intervals up to 120 hr., the extent of oxidation was measured by the production of formaldehyde. The results of this experiment are shown in Fig. 3. After 120 hr., the reaction rate had fallen markedly and 1·05 mol.prop. of formaldehyde had been produced. This is close to the theoretical value to be expected for the behaviour of a 1-monoglyceride.

The other product of periodate oxidation of a 1-monoglyceride was presumed to be the acyl ester of glycolaldehyde ($R{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CHO$). The

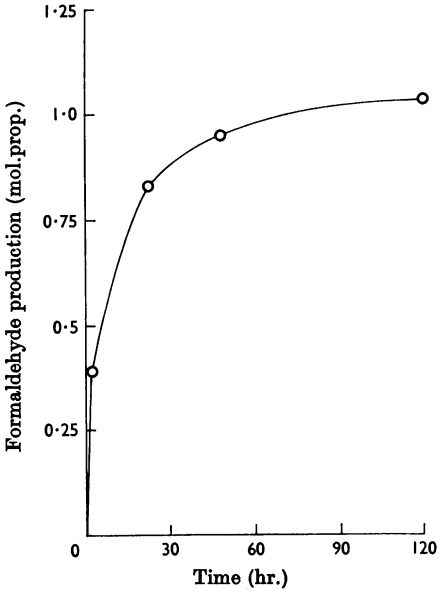


Fig. 3. Periodate oxidation of the monoglyceride formed by the action of acid phosphomonoesterase on the sodium salt of substance *B*. The experimental conditions were those of O'Dea & Gibbons (1953).

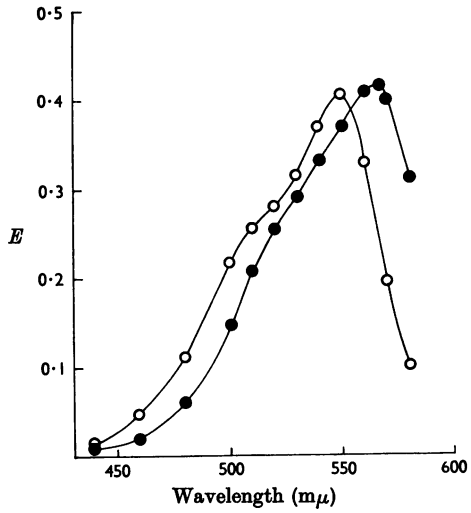


Fig. 4. Absorption spectra of chromogens produced by reaction of Schiff's reagent with myristaldehyde (0·081 μmole; ○) and periodate-oxidation product of 1-monoglyceride (0·089 μmole; ●), under the conditions of Sloane-Stanley & Bowler (1962); total vol., 4ml. Periodate oxidation of 1-monoglyceride was for 120hr. by the procedure of O'Dea & Gibbons (1953); the acyl glycolaldehyde produced was extracted with chloroform–methanol before reaction with Schiff's reagent.

aldehydic properties of this material were shown in the following way. A separate experiment was carried out for 120hr., and the reaction mixture was extracted three times with chloroform–methanol. The combined chloroform-rich phases were tested for long-chain aldehyde by the procedure of Sloane-Stanley & Bowler (1962). The extinction curve of the red product was examined in a Unicam SP.500 spectrophotometer and compared with that formed from an authentic sample of myristaldehyde (Fig. 4). For the latter, $\lambda_{max.}$ was at 550mμ; for the product of periodate oxidation of α-monoglyceride it was at 566mμ. This observation suggests that the fuchsine–SO_2–acylglycolaldehyde complex does not break down to free fuchsine ($\lambda_{max.}$ 550mμ) on standing, in contrast with the behaviour of the fuchsine–SO_2–long-chain fatty aldehyde complexes.

Position of the phosphate group and configuration of the glycerol portion of the molecule. To ascertain the complete structure of substance *B*, it was necessary to determine the location of the phosphate group and the stereochemistry of the glycerol moiety. Since the compound contains a monoesterified phosphate group, the acyl ester group may be removed by alkaline hydrolysis without the simultaneous isomerization which is known to occur with phosphodiesters of glycerol (Baer & Kates, 1948; Brown & Todd, 1952; Long & Maguire, 1953). The sodium salt (5μmoles) was deacylated by alkaline hydrolysis and the resulting glycerophosphate was isolated as already described.

This product was shown to be 3-glycerophosphate. When treated with periodate at room temperature under the conditions of O'Dea & Gibbons (1953), 1mol.prop. of formaldehyde was produced. The results are shown in Table 1, with authentic L-3-glycerophosphate and the alkaline hydrolysis product of the sodium salt of substance *A* for comparison; the latter observation will be referred to later.

Samples of the 3-glycerophosphate were incubated with L-3-glycerophosphate dehydrogenase and NAD under the conditions given in the Experimental section, and the increase in extinction at 340mμ was determined. Authentic L-3-glycerophosphate was used as a standard substrate. The results obtained are shown in Table 2. The behaviour of the sodium salt of substance *A* after alkaline hydrolysis is also included and will be referred to later. With L-3-glycerophosphate as standard and by using the molecular extinction $6{\cdot}22 \times 10^6$ (Horecker & Kornberg, 1948) for $NADH_2$, the value of *E* (340mμ)/μg.atom of phosphorus in total volume 3·5ml., with a 1cm. light-path, may

Table 1. *Periodate oxidation of* L-3-*glycerophosphate and of the alkaline hydrolysis products of substances* A *and* B

The experimental conditions of O'Dea & Gibbons (1953) were followed.

Substance	Amount taken (μg.atom of P)	Formaldehyde produced (μmole)	Formaldehyde production (μmole/μg.atom of P)
L-3-Glycerophosphate	0·214	0·200	0·94
Alkali-hydrolysed substance *A*	0·248	0·120	0·48
Alkali-hydrolysed substance *B*	0·230	0·231	1·00

Table 2. *Action of* L-3-*glycerophosphate dehydrogenase on* L-3-*glycerophosphate and on the alkaline hydrolysates of substances* A *and* B

The reaction mixtures had the composition given in the Experimental section. After incubation at 38° for 60 min., extinctions were read at 340 mμ against a control without substrate.

Substrate	Amount taken (μg.atom of P)	*E*	*E*/μg.atom of P		Ratio (L-3-glycerophosphate = 1)
L-3-Glycerophosphate	0·105	0·174	1·66	1·64	1
	0·210	0·339	1·61		
	0·314	0·515	1·64		
Alkali-hydrolysed substance *A*	0·117	0·087	0·74	0·75	0·46
	0·233	0·174	0·75		
Alkali-hydrolysed substance *B*	0·124	0·203	1·64	1·63	0·99
	0 248	0·402	1·62		

be calculated to be 1·78 for complete conversion into dihydroxyacetone phosphate,

L-3-glycerophosphate + NAD ⇌ dihydroxyacetone phosphate + $NADH_2$

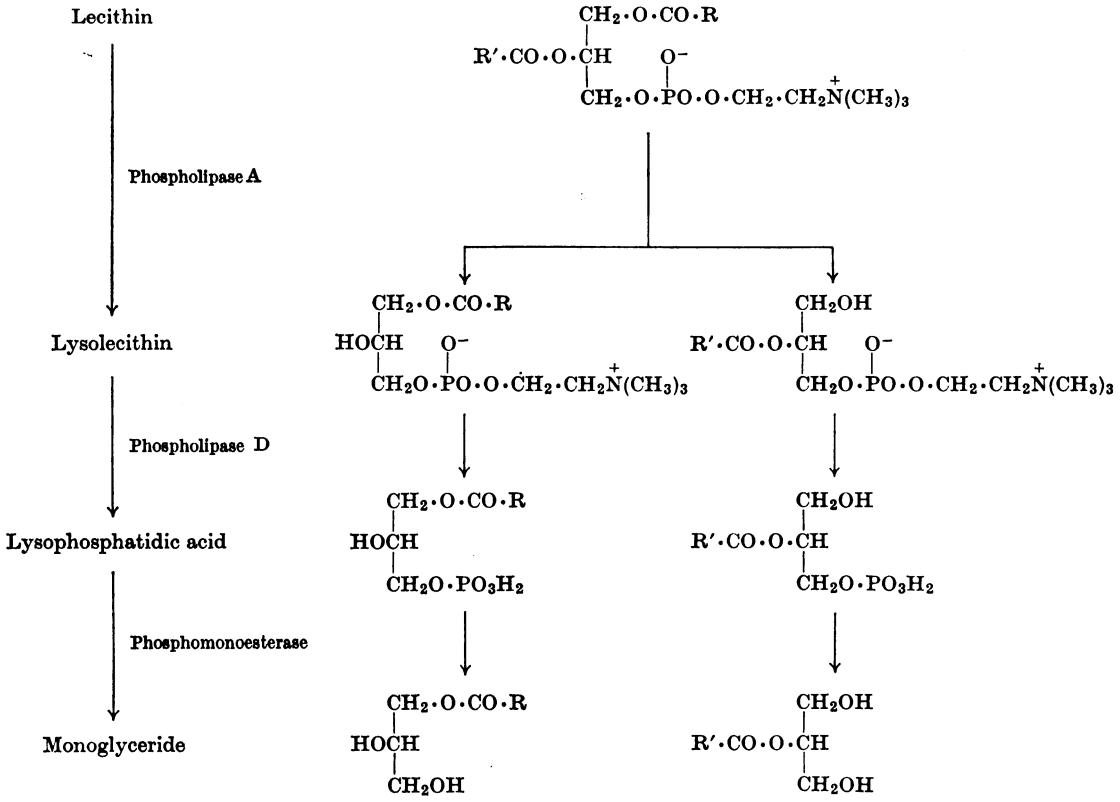
The observed mean value was found to be 1·64, suggesting that the above reaction was 92% complete from left to right. If the extent of dehydrogenation of authentic L-3-glycerophosphate be arbitrarily set at unity, then the behaviour of the alkaline hydrolysis product of substance *B* is identical with this value. Hence the alkaline hydrolysis of substance *B* gives rise exclusively to L-3-glycerophosphate, and the parent substance must be designated structurally as 1′-monoacyl-L-3-glycerophosphoric acid.

Structure of the sodium salt of substance A

Certain observations, already referred to, provided a clue to the structure of substance *A*. First, the inability of acid phosphomonoesterase to split this compound strongly suggested that the phosphate group was not attached to the glycerol portion of the molecule by a simple monophosphate ester linkage. Secondly, the evidence from thin-layer chromatography indicated that substance *A* was much less polar than was substance *B*. Finally, the absence of a pH effect on the partition coefficient was not consistent with the presence of a secondary phosphate dissociation. The likelihood that substance *A* was a monoacyl derivative of cyclic glycerophosphoric acid had therefore to be considered.

Location of the phosphate group. When the acyl group of substance *A* was removed by alkaline hydrolysis and the liberated fatty acid separated by extraction with chloroform–methanol, as already described for substance *B*, the water-soluble product was treated with periodate at room temperature under the conditions of O'Dea & Gibbons (1953) to determine the proportion of 3-glycerophosphate present. This result is given in Table 1, from which it is clear that rather less than 0·5 μmole of formaldehyde is produced per μg.atom of phosphorus. This observation is to be contrasted with the release of one mol.prop. of formaldehyde from the alkaline hydrolysis product of substance *B* under identical conditions. The yield of formaldehyde is consistent with a cyclic structure for substance *A*, since alkaline hydrolysis would remove the acyl group and simultaneously open the cyclic phosphate ring structure to give a mixture of 1- and 2-glycerophosphate; periodate would react only with the 1-isomer.

Configuration of the 3-*glycerophosphate.* The



Scheme 1.

3-glycerophosphate present in the alkaline hydrolysate of substance *A* was incubated with L-3-glycerophosphate dehydrogenase in the presence of NAD (Table 2). Of the total glycerophosphate, 46% can be accounted for as L-3-glycerophosphate. This value is very close to the 48% of total 3-glycerophosphate found by periodate oxidation (Table 1).

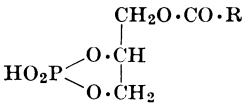
DISCUSSION

The original aim of the present investigation was to determine the structure of lysolecithin by an enzymic degradative procedure not hitherto attempted. Successive removal of the choline component by phospholipase D and of phosphate by phosphomonoesterase should give a monoglyceride, the structure of which could readily be ascertained. The two possible routes for monoglyceride formation are given in Scheme 1.

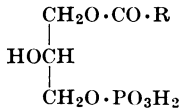
However, phospholipase D was found unexpectedly to degrade lysolecithin with the formation of two distinct and separable products, substances *A* and *B*. They differed from one another in the following respects: (1) the calcium salt of substance *A*, but not of *B*, could be extracted from an aqueous medium by chloroform–methanol (Fig. 1); (2) the sodium salt of substance *A* was more readily extractable into chloroform–methanol than was the sodium salt of substance *B*, as shown by the magnitude of the partition coefficients (Fig. 2); (3) on thin-layer chromatography, salts of substance *A* had R_F value about 0·5 in chloroform–methanol–water, whereas salts of substance *B* remained at the origin; (4) substance *B* was hydrolysed by acid phosphomonoesterase but substance *A* was not. Observations (2) and (3) demonstrated that substance *A* was much less polar than was substance *B*; in fact, by thin-layer chromatography it was considerably less polar than was lysolecithin (R_F 0·10).

From the evidence presented, it has been concluded that substance *B* is a lysophosphatidic acid of conventional structure, whereas substance *A* has all the physical and chemical properties to be expected for a cyclic phosphate compound. The

structures of the free acids are considered to be represented by



Substance *A*

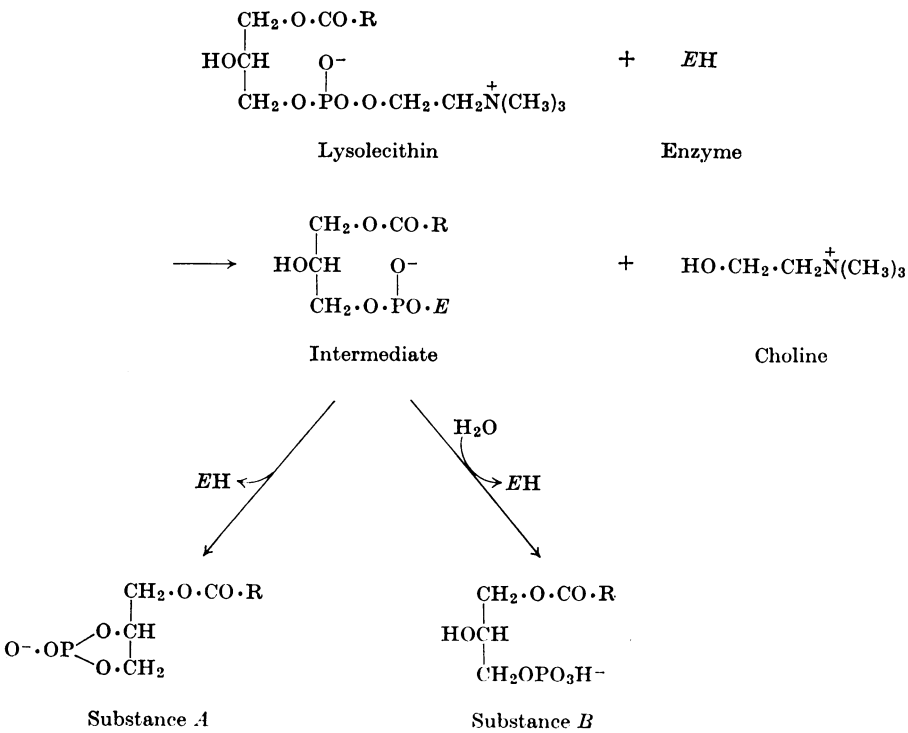


Substance *B*

In both cases the acyl group is attached to the 3-position of the glycerol moiety, thus confirming the structure for lysolecithin first proposed by Tattrie (1959).

Substance *A* is taken to be a monobasic acid containing one primary phosphate group, and the sodium salt was found to possess a sodium:phosphorus ratio of unity, as expected. Substance *B*, on the other hand, would be a dibasic acid containing both primary and secondary dissociable hydrogen atoms, and might be supposed to exist in the form of mono- and di-sodium salts. However, the sodium salt of substance *B*, when extracted from aqueous medium into chloroform–methanol at pH 5·8 (Fig. 1), also possessed a sodium:phosphorus ratio of unity, suggesting that only the monosodium salt was extractable, although at this pH an appreciable amount of the disodium salt might also be expected to be present in the aqueous phase. It seems probable that the strongly polar bivalent anion is not at all extractable into chloroform–methanol, since at higher pH values, where the proportion of univalent anion would be smaller, the partition coefficient in favour of the upper aqueous phase was correspondingly increased (Fig. 2).

With regard to the mechanism of action of phospholipase D on lysolecithin, the alternative pathways shown in Scheme 2 are proposed. It is suggested that the enzyme reacts with lysolecithin to release choline and form an enzyme–lysophosphatidic acid intermediate. The latter may then undergo an intramolecular ring closure of the phosphate group whereby the enzyme is regenerated and substance *A* is formed; alternatively, it may undergo hydrolysis to release the enzyme and form substance *B* (lysophosphatidic acid). A different mechanism in which substance *A* is postulated to be an obligatory intermediate which may undergo partial hydrolysis to give substance *B*, as has been established for pancreatic ribonuclease, is thought to be unlikely. Such a mechanism could not explain the production of phos-



Scheme 2.

phatidic acid from lecithin, since the presence of the 2-acyl group prevents the possibility of forming such an analogous cyclic intermediate.

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