The Structure of the Naturally Occurring Phosphoglycerides

2. EVIDENCE DERIVED FROM A STUDY OF THE ACTION OF PHOSPHOLIPASE C

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(Received 10 December 1953)

Previous investigations on the structure of the naturally occurring phosphoglycerides were based mainly on the results of chemical hydrolysis and have been reviewed in the first paper of this series (Long & Maguire, 1953*a*). It was pointed out that alkalis first split off the fatty-acid radicals so as to allow the formation of unstable cyclic phosphate triesters from glycerylphosphorylcholine; these then lose choline, whereupon further splitting of the diesters leads to the formation of isomeric glycerophosphates. The net effect is a partial migration of the phosphate group. However, by comparing the proportions of β -, D - α - and L - α -glycerophosphates formed on alkaline hydrolysis of naturally occurring phosphoglycerides and of synthetic phosphoglycerides of known structure and optical configura-

tion of the latter, should yield glycerophosphate, the structure of which would directly correspond to that of the original phosphoglyceride.

Macfarlane & Knight (1941) have shown that culture filtrates of *Clostridium welchii* type A contain an enzyme which splits ovolécithin into a diglyceride and phosphorylcholine. In the present work we have isolated the diglyceride formed in this way and, after catalytic hydrogenation to saturate the fatty-acid radicals, have phosphorylated it to give the corresponding saturated phosphatidic acid. On alkaline hydrolysis the latter has yielded pure L - α -glycerophosphoric acid, thus confirming our previous conclusion that ovolécithin belongs to the L - α series. The sequence of reactions may be represented as follows:

tion, it was shown that the naturally occurring phosphoglycerides in all probability belong to the L - α series.

It has been established by Baer & Kates (1950*a*) that phosphatidic acids, i.e. phosphoglycerides from which the nitrogen-containing component has been removed, are hydrolysed by alkali without phosphate migration to yield the corresponding glycerophosphates. This may be explained by the fact that loss of the fatty-acid radicals leads directly to glycerophosphate which, once formed, cannot yield an intermediate cyclic phosphate diester. It is clear therefore that the conversion of a naturally occurring phosphoglyceride into the corresponding phosphatidic acid, followed by alkaline hydrolysis

In the course of this work we have also extended the substrate-specificity studies of Macfarlane (1948*a*), and have concluded that the presence of an unsaturated fatty-acid radical is necessary for enzyme action to proceed.

A preliminary report of this work has already been published (Long & Maguire, 1953*b*).

EXPERIMENTAL

Materials

Phosphoglycerides. Ovolécithin was prepared by the method of Pangborn (1951). The following samples of synthetic phosphoglycerides were also used: dimyristoyl L - α -lecithin (Baer & Kates, 1950*b*), dipalmitoyl DL - α -lecithin, dimyristoyl DL - α -kephalin and dimyristoyl β -kephalin (Malkin & Bevan, 1951).

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Phospholipase C. A dried preparation of *Cl. welchii* type A α -toxin (Wellcome Research Laboratories) was used directly as enzyme. (In accordance with the suggestion of Zeller (1951), we refer to this enzyme as a 'phospholipase' rather than as a 'lecithinase'.)

Analytical methods

Determinations of total N, total P and the isomeric glycerophosphates were carried out as previously described (Long & Maguire, 1953a). Iodine values were determined according to Norris & Buswell (1943) and 'acetyl' values by the procedure described by Mann & Saunders (1947).

Hydrolysis by phospholipase C

The phosphoglyceride (1 mg.) was emulsified with 1 ml. of distilled water in a 15 ml. conical centrifuge tube. 0.2M Aminotris(hydroxymethyl)methane (2-amino-2-(hydroxymethyl)-1,3-propanediol) buffer (Gomori, 1946), pH 7.8 (0.4 ml.) and 0.2M-CaCl₂ (0.1 ml.) were added, followed by 0.5 mg. enzyme in 0.5 ml. water. The reaction mixture was incubated at 38° for 4 hr., after which 0.1 ml. of 72% HClO₄ was added. The precipitate was filtered off on a Whatman no. 5 paper. A 0.3 ml. sample of the filtrate was ashed with 0.25 ml. 72% HClO₄ for 2 hr. at 160° and the resulting inorganic orthophosphate estimated. From this value the amount of the original phosphoglyceride P converted into acid-soluble form was calculated.

Preparation of the diglyceride from ovoidlecithin

Ovoidlecithin (500 mg.), emulsified in 40 ml. of 0.15M aminotris(hydroxymethyl)methane buffer, pH 7.8, and made 0.01M with respect to CaCl₂, was treated with 50 mg. *Cl. welchii* α -toxin and incubated at 38°. After 12 hr. a further 25 mg. of toxin was added and the incubation continued for another 24 hr., to ensure complete hydrolysis. The diglyceride was isolated from the reaction mixture by extraction with two successive 25 ml. portions of diethyl ether. The combined ethereal extracts were concentrated by evaporation to 3 ml., and 30 ml. of acetone were added to precipitate any unchanged ovoidlecithin. After standing at 0° for 1 hr., the small flocculent precipitate which had formed was filtered off. The filtrate was dried *in vacuo* yielding 350 mg. of a yellow oil.

Catalytic hydrogenation

Platinum oxide (Adams & Shriner, 1923) was used as catalyst. Ovoidlecithin or diglyceride (200–400 mg.) was

dissolved in ethanol to give a final concentration of 1% (w/v). The solution was vigorously shaken with 10 mg. of catalyst in an atmosphere of hydrogen, uptake of gas being generally complete after 90 min. Any hydrogenated material which separated from solution was redissolved by addition of 20 ml. ethanol and warming. The hot solution was then filtered with suction to remove the platinum black, and the filtrate dried *in vacuo*.

Phosphorylation of the diglyceride

The method of Baer (1951), employing diphenylphosphoryl chloride as phosphorylating agent, was used. To 125 mg. of the dry hydrogenated diglyceride, dissolved in 1.25 ml. dry pyridine, was added diphenylphosphoryl chloride (60 mg.) dissolved in 0.3 ml. dry pyridine. The mixture, protected from moisture, was incubated at 38° for 24 hr. The reaction product was then precipitated by addition of crushed ice and ice water. After standing at 0° for 2 hr. it was filtered and the precipitate thoroughly washed with ice water. It was purified by dissolving in light petroleum, b.p. 40–60°, filtering the solution and evaporating to dryness *in vacuo*. The yield was 50–70%, based on the amount of diglyceride taken. Hydrogenolysis of the diphenyl ester in glacial acetic acid (3 ml.) using Adams's catalyst (20 mg.) was complete in 1 hr. The free phosphatidic acid, which separated from solution during the hydrogenolysis, was redissolved by addition of CHCl₃. The solution was then filtered to remove platinum black and the filtrate dried *in vacuo*. The overall yield of phosphatidic acid was usually about 50%, based on the diglyceride taken.

RESULTS

Substrate specificity of phospholipase C

In addition to its action on lecithin, phospholipase C also slowly splits phosphorylcholine from sphingomyelin; it has no action on glycerylphosphorylcholine, phosphatidylethanolamine, phosphatidylserine or lysolecithin (Zamecnik, Brewster & Lipmann, 1947; Macfarlane, 1948a). It was therefore to be expected that neither of the synthetic cephalins used in this work would be acted upon by phospholipase C, while the synthetic lecithins would be hydrolysed to an extent depending on whether or not the enzyme exhibits an absolute specificity for one particular isomer. The experimental results shown in Table 1, however, indicate that no enzymic

Table 1. Action of phospholipase C on phosphoglycerides

Phosphoglyceride (1 mg.) was incubated at 38° for 4 hr. in 0.04M aminotris(hydroxymethyl)methane buffer, pH 7.8, and 0.01M-CaCl₂ with 0.5 mg. *Cl. welchii* α -toxin; final vol., 2 ml. The reaction mixture was treated with 0.1 ml. 72% HClO₄ and 0.3 ml. of filtrate was taken for determination of acid-soluble P. The ovoidlecithin and hydrogenated ovoidlecithin had iodine values of 58 and 1.7, respectively.

Phosphoglyceride	P content of phosphoglyceride taken (μ g.)	Acid-soluble P content of HClO ₄ filtrate (μ g.)	Hydrolysis (%)
Ovoidlecithin	39.6	37.2	94
Hydrogenated ovoidlecithin	38.1	0.76	2
Dimyristoyl L- α -lecithin	44.5	Nil	0
Dipalmitoyl DL- α -lecithin	41.2	Nil	0
Dimyristoyl DL- α -cephalin	46.0	Nil	0
Dimyristoyl β -cephalin	46.0	Nil	0

splitting of either of the synthetic lecithins occurred under conditions which caused the complete hydrolysis of ovolecithin. Although the latter reaction was always complete after 2 hr., in routine hydrolysis studies the time of incubation was never less than 4 hr.

Since our earlier work (Long & Maguire, 1953a) had provided convincing evidence that naturally occurring lecithins possess the L- α configuration, the inactivity of phospholipase C towards synthetic dimyristoyl L- α -lecithin was unexpected. The explanation of this result, however, is suggested by the observation, also shown in Table 1, that fully hydrogenated lecithin is not acted upon. Hence it seems likely that the enzyme requires an unsaturated fatty-acid radical in its substrate. It was therefore of interest to determine the effect of phospholipase C on partially hydrogenated samples of ovolecithin. These were prepared by interrupting the hydrogenation process at different times before saturation was complete. The iodine value and the extent of hydrolysis by phospholipase C were then determined for each sample. While the unsaturated natural lecithin was almost completely hydrolysed, the hydrogenated samples were only partially so, even after prolonged incubation. The results shown in Fig. 1 indicate that the extent of hydrolysis varies linearly with the iodine value. This supports the view that only lecithins containing unsaturated fatty-acid radicals can serve as substrates for phospholipase C.

Preparation and alkaline hydrolysis of the phosphatidic acid corresponding to ovolecithin

The yields of diglyceride obtained after incubation of ovolecithin with phospholipase C, as described in the Experimental section, were practically quantitative. For ease of handling, the oily product was catalytically hydrogenated to give a white waxy solid, the acetyl value of which was in accordance with the presence of one free hydroxyl group per molecule.

In several experiments using diphenylphosphoryl chloride as phosphorylating agent, followed by catalytic removal of phenyl groups, the final yield of purified phosphatidic acid amounted to about 50%. Analytical values for phosphorus were in the range 4.9–5.0% (calculated for dipalmitoyl phosphatidic acid, P = 4.8%).

Samples of phosphatidic acid, ovolecithin and hydrogenated ovolecithin were hydrolysed by heating with N-NaOH for 2 hr. at 110° (Baer & Kates, 1950a; Long & Maguire, 1953a). The glycerophosphate formed was analysed for total, α and L- α isomers as previously described (Long & Maguire, 1953a). The results shown in Table 2 demonstrate that on alkaline hydrolysis a typical phosphatidic acid sample yielded pure L- α -glycero-

Fig. 1. The extent of hydrolysis of partially saturated lecithins by phospholipase C, as a function of iodine value. The extent of hydrolysis is given by the percentage of the lecithin P rendered acid-soluble after prolonged treatment with *Cl. welchii* α -toxin.

Table 2. Composition of the glycerophosphates formed on alkaline hydrolysis of phosphatidic acid, ovolecithin and hydrogenated ovolecithin

The material (40 mg.), dissolved in 0.5 ml. ethanol, was hydrolysed by heating with 1 ml. N-NaOH for 2 hr. at 110°. The glycerophosphates were isolated and analysed for total, α and L- α isomers as previously described (Long & Maguire, 1953a). A and B are duplicate samples of phosphatidic acid carried through the complete hydrolysis procedure.

	Analysis of glycerophosphates			
	β	α	L- α	D- α
Phosphatidic acid A	0	100	98	2
Phosphatidic acid B	0	100	96	4
Ovolecithin	53	47	41	6
Hydrogenated ovolecithin	55	45	43	2

phosphate, in contrast to the mixture of β , D- α and L- α isomers formed from the lecithin samples. Since no phosphate migration occurs on alkaline hydrolysis of a phosphatidic acid (Baer & Kates, 1950a), it follows that the latter has the L- α configuration. The same must also be true of the original ovolecithin, since the configuration of the β -carbon atom of the glycerol moiety remains unchanged during the sequence of reactions which have been described.

It should be pointed out that this conclusion assumes that no Walden inversion occurs at the β -carbon atom of the glycerol moiety during alkaline hydrolysis. This would scarcely be expected on general grounds, but in any case it is excluded by the work of Baer & Kates (1950a) who showed that when synthetic L- α -phosphatidic acid was hydrolysed by alkali, the product was pure L- α -glycerophosphate and contained no D isomer.

DISCUSSION

As a general method for the elucidation of phosphoglyceride structure, the procedure described in this paper is of limited application because of the high degree of specificity of the enzyme. Only lecithins containing unsaturated fatty-acid radicals appear to serve as substrates for the phospholipase C of *Cl. welchii*. Other bacterial enzymes which also split phosphorylcholine from phosphoglycerides have been described (Macfarlane, 1948b; Miles & Miles, 1950; Lewis & Macfarlane, 1953), but no detailed study has yet been made of their substrate specificities.

In view of the low overall yields of L- α -phosphatidic acid (about 50 %) which we have obtained, it is necessary to consider whether or not a selective phosphorylation of part of the diglyceride, not representative of the whole, could have taken place. This possibility seems to us to be unlikely for other workers (Malkin & Bevan, 1951; Baer, 1951) have observed no noticeable differences in the ease of phosphorylation of the isomeric diglycerides. Our low yields are almost certainly due to the small scale on which the work was carried out and the difficulty of working under completely anhydrous conditions. In view of this, it seems reasonable to conclude that this enzymic approach to the elucidation of phosphoglyceride structure has provided unequivocal evidence in support of the L- α configuration.

An alternative and more direct procedure for obtaining the phosphatidic acid from a phosphoglyceride is by the action of phospholipase D, found in cabbage leaves and carrots (Hanahan & Chaikoff, 1948), spinach (Kates, 1953) and in the latex of *Hevea brasiliensis* (Smith, 1954). This enzyme may prove to be less exacting in its substrate requirements and therefore of more general application. Work in this field is at present in progress.

SUMMARY

1. Alkaline hydrolysis of the phosphatidic acid, prepared from ovoidlecithin by the action of phospholipase C and phosphorylation of the resulting diglyceride, gave only L- α -glycerophosphate, thus confirming the L- α configuration of ovoidlecithin.

2. The presence of an unsaturated fatty-acid radical in the lecithin substrate seems to be essential for the activity of *Cl. welchii* phospholipase C.

The authors wish to thank Prof. W. O. Kermack, F.R.S., for his interest in this work, and to express their gratitude to Dr E. Baer, for a sample of synthetic dimyristoyl L- α -lecithin; Dr T. Malkin, for synthetic dimyristoyl DL- α -lecithin, dipalmitoyl DL- α -kephalin and dimyristoyl β -kephalin; and Prof. B. C. J. G. Knight and the Wellcome Research Laboratories for two samples of *Cl. welchii* α -toxin.

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