Separation of Glycerylphosphoryl Inositol and Related Compounds on Ion-Exchange Columns

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No adequate procedures are at present available for a complete separation of the various types of glycerophosphatides. An accurate determination of some of the compounds present in such a crude phospholipid mixture can be achieved, however, by means of a mild alkaline hydrolysis, followed by two-dimensional paper chromatography of the resulting phosphoric acid diesters (Dawson, 1954). This method has been used in various studies of the turnover-rate of phosphate in different glycerophosphatides (e.g. Dawson, 1955; Hokin & Hokin, 1956; Ansell & Dohmen, 1956). The inositol-containing phosphoric acid esters obtained by Dawson's method were never identified. Furthermore, the constant detection of glycerophosphate highly labelled with ^{32}P , thought to arise from phosphatidic acid, is difficult to reconcile with the findings of some workers (Dawson, 1957; Marinetti, 1956; Marinetti & Stotz, 1956), who were unable to detect phosphatidic acid in animal tissues.

The present work extends Dawson's method by the use of ion-exchange resins instead of paper chromatography for the separation of the diesters. In this way it has been possible to study the conditions of hydrolysis more carefully and to investigate the structure of certain products of phospholipid hydrolysis, particularly that from monophosphoinositide, which does not run well on paper chromatograms.

A short note of this work has already appeared (Hawthorne & Hübscher, 1958).

MATERIALS AND METHODS

Preparation of phospholipids. Phosphatidylserine was prepared from ox-brain kephalin by the method of Folch (1942). The combined fractions III and IV were used without dialysing them. Lecithin was prepared by passage of crude brain or egg lecithin in 95% ethanol through an alumina column, according to Hanahan, Turner & Jayko (1951). Liver monophosphoinositide was prepared from a sample of pig-liver lipid kindly supplied by Viobin Laboratories, Monticello, Ill., U.S.A. The crude lipid was suspended in chloroform (250 g. in 1 l.), and filtered. Lipid was precipitated from the filtrate with 3 vol. of absolute ethanol. The precipitate was redissolved in 500 ml. of chloroform and precipitated with 1.3 l. of acetone. This step was repeated and the precipitate then taken up in

500 ml. of chloroform saturated with water. The solution was passed through a cellulose-powder column (Whatman Standard Grade; size 2 cm. $\times$ 25 cm.) to remove water-soluble impurities and the lipid was precipitated by the addition of 2 l. of absolute ethanol to the eluate. Two final precipitations were made from 300 ml. of chloroform with 470 ml. of absolute ethanol. Such a preparation usually contained monophosphoinositide and phosphatidylserine in equal amounts, and is referred to as crude liver inositide. In some cases an alternative way of washing out water-soluble impurities was used. This was based on the work of Folch, Lees & Sloane-Stanley (1954). The lipid was dissolved in 400 ml. of chloroform-methanol (2:1, v/v) and shaken with 80 ml. of 0.01 M-CaCl₂ solution. The water layer was separated off, methanol was added to the lower layer to make up the volume to 400 ml. and the extraction was repeated. Preparations of monophosphoinositide of a higher degree of purity were obtained by further fractionation on silicic acid columns (Hanahan, Dittmer & Warashina, 1957).

Other phosphoric acid esters. β -Glycerophosphoric acid was a commercial preparation. Inositol monophosphate was prepared from phytic acid by the method of McCormick & Carter (1952) and purified on a resin column as previously described (Hübscher & Hawthorne, 1957).

Ion-exchange resins. In the early work Nalcite SAR (-80 + 200 mesh) was used. This is a monofunctional strong-base polystyrene anion exchanger, with about 10% cross-linking (Dow Chemical Co., U.S.A.). Since Dowex 2 is an equivalent resin which is more readily available, the behaviour of this resin (8% cross-linked, -100 + 200 mesh) was also studied. Dowex 1 (2% cross-linked, -100 + 200 mesh) was used with most of the compounds investigated. This resin has the slight disadvantage of swelling as the salt concentration of the eluent is reduced. The columns were usually repacked therefore, after washing with 3 M-ammonium formate. The anion-exchange resins were used in the formate form and the columns were 1 cm. in diameter and 15 cm. high.

For the removal of cations from various solutions, Zeo-Karb 225 (-16 + 50 mesh) was used. It was converted into the hydrogen form before use by washing with 2N-HCl and then water until the eluate was neutral. All distilled water used in this work was freed from ions by passage down a column of Bio-Deminrolit (The Permutit Co. Ltd.).

Removal of borate and formate from fractions containing organic phosphates. Since high concentrations of these ions interfere with the paper-chromatographic separation of the free inositol, glycerol and serine produced by acid hydrolysis of the phosphates (Hübscher & Hawthorne, 1957), they were removed. The pooled fractions were diluted with an equal volume of 0.005 M-sodium tetraborate and applied to a column of anion-exchange resin similar to the one from

which they were eluted. Under these conditions the phosphoric acid esters were retained on the column, but most of the borate passed through. The remaining borate was removed with 150 ml. of 0.01 M-formic acid and the phosphates were then eluted with the lowest concentration of formate which would easily remove them. This was usually 0.05 M-ammonium formate for the diesters. Five 50 ml. fractions were collected. Those containing phosphate were pooled and applied to a column of Zeo-Karb 225 (2 cm. $\times$ 30 cm.) to remove NH_4^+ ions. The column was washed with water until neutral. The total eluate was then concentrated *in vacuo* (at less than 20°) to about 50 ml. The remaining liquid was removed by freeze-drying.

Hydrolysis of phospholipids. The method is essentially that of Dawson (1954). An amount of phospholipid containing about 12 mg. of phosphorus was dissolved in 8 ml. of chloroform; 63 ml. of methanol, 9 ml. of water and 20 ml. of methanolic N-NaOH were added and the mixture was immediately incubated at 37° with occasional shaking. After 30 min. (or shorter times, according to the experiment) the hydrolysate was cooled in ice and diluted with 160 ml. of water. It was stirred with Zeo-Karb 226 (H^+ form) until neutral and the resin was removed by filtering through a funnel plugged with a little cotton wool. The resin was washed with two 50 ml. portions of water and the combined filtrate and washings were extracted with 250 ml. portions of carbon tetrachloride, ether and light petroleum (40–60°) successively. After the first or second extraction it was often necessary to centrifuge for a short time to clear the emulsion formed. A final extraction with 1 vol. of isobutanol and separation of the aqueous phase yielded a yellowish solution. This was filtered and made up to 500 ml. with water and sufficient sodium tetraborate solution to give a final borate concentration of 5 mM. Total phosphate was determined on this solution, which was then loaded directly on to a resin column. Inorganic phosphate was never detected.

Determination of glycerol. The chromatropic acid method was used, as described by Burton (1957), with omission of the chromatographic separation. Control experiments showed that inositol did not interfere and that HCl hydrolysates could be used directly, without removal of the acid. Glycerol standards were included with each determination.

Determination of nitrogen. The micro-Kjeldahl method was used, digestion being with a selenium-copper sulphate catalyst (K_2SO_4 , 2 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 4 g.; Se, 1.5 g.), and about 50 mg. of catalyst mixture with 2 ml. of conc. H_2SO_4 being used.

Other methods. Hydrolysis of phosphates, chromatography of serine, ethanolamine, choline and inositol, and determination of phosphate, borate and inositol have been described in a previous paper (Hübscher & Hawthorne, 1957).

RESULTS

Hydrolysis product of phosphatidyl choline

A sample of highly purified brain lecithin containing 12 mg. of P was hydrolysed as described, for 15 min., during which time 71% of the phospholipid P became water-soluble. When this solution was applied to a Nalcite SAR column in 5 mM-sodium borate, 86% of the total phosphate came straight through and the remaining phosphate was

eluted with the first 30 ml. of a solution containing 5 mM-sodium borate and 0.01 M-ammonium formate. Similar results were obtained with a Dowex-1 resin column. It can be concluded that glycerylphosphoryl choline is not adsorbed on anion-exchange resins, even in the presence of borate ions.

Separation of the hydrolysis products of crude monophosphoinositide

As it was hoped that the net charge of glyceryl-phosphoryl serine (GPS) might be sufficiently different from that of the hydrolysis product of monophosphoinositide for separation on a resin column, the formic acid-ammonium formate system was first used for elution. A 500 mg. portion of partially purified liver inositide containing roughly equal proportions of phosphatidyl serine and monophosphoinositide was hydrolysed for 15 min. and the water-soluble phosphoric acid esters were applied to a Nalcite SAR column. After washing with water the column was eluted with formic acid-ammonium formate solutions of increasing concentration (see Fig. 1). The main peak, eluted with 0.01 M-formic acid–0.01 M-ammonium formate, contained 86% of the phosphate applied to the column. An analysis of the peak showed that it contained, in addition to glycerol and phosphoric acid, both serine and inositol. With the second solvent (0.01 M-formic acid–0.03 M-sodium formate) monoesters of phosphoric acid (mainly glycerophosphate) were eluted. The third solvent (0.1 M-formic acid–0.5 M-sodium formate) would have eluted any diphosphates, but the small amount of phosphate ester obtained was not identified. Rechromatography of the main peak, either lower concentrations of formic acid and ammonium formate or ammonium formate at alkaline pH

Fig. 1. Behaviour of crude liver inositide hydrolysate on a column of Nalcite SAR (–80 + 200 mesh, formate form), size 1 cm. $\times$ 20 cm. No borate was used in the elution. The large peak contained both GPI and GPS. Hydrolysis time, 15 min.

being used for elution, did not bring about a separation of the diesters contained in the main peak.

A separation of these diesters could be achieved by using the sodium tetraborate-ammonium formate system for elution (Khym & Cohn, 1954). In preliminary experiments it was found that the optimum concentration of borate for the separation of the various hydrolysis products was 5 mM. Such a separation is shown in Fig. 2. Peaks *B* and *C* were shown to be due to glycerylphosphoryl inositol (GPI) and GPS respectively. The isolation and analysis of these compounds are described below. Peak *D* contains glycerophosphate and there is some indication that peak *A* may be due to glycerylphosphoryl ethanolamine. In some experiments Nalcite SAR resin was used for the separation of the various phosphoric acid esters. Though essentially the same picture is obtained, slightly altered concentrations of ammonium formate were found necessary for elution. Thus GPI and GPS were eluted in the presence of borate with 0.06 M-ammonium formate and 0.08 M-ammonium formate respectively. Fig. 3 shows the isolation of GPI from purified monophosphoinositide and Fig. 4 the preparation of GPS from brain kephalin.

A time study of the hydrolysis was undertaken with the intention of explaining the presence of glycerophosphate (GP) in hydrolysates of tissue phospholipids. In Table 1 the relative amounts of the hydrolysis products are given. Three portions of partially purified liver inositide, each containing 10 mg. of phosphorus, were hydrolysed for periods

of 15, 30 and 45 min. respectively. The amount of phosphate which became water-soluble during these periods was determined and the hydrolysis products were then separated on a Dowex-1 column. As can be seen from Table 1, the percentage of phosphate becoming water-soluble increased with time. The relative amounts of GP decreased slightly with time. This was due to the fact that the absolute amounts of GP isolated (2.44, 2.42 and 2.57 % of the original phospholipid P at 15, 30 and 45 min. respectively) remained constant throughout the hydrolysis. Further breakdown of GPI and GPS to GP is thus not likely to occur under the experimental conditions used. Consequently, the GP observed in the 15 min. hydrolysate cannot have been derived from these diesters.

Identification and characterization of glycerylphosphoryl inositol and glycerylphosphoryl serine

The fractions containing either of the diesters were pooled and treated as described above for the removal of borate and formate. The dry material was taken up in about 1 ml. of water. GPS was precipitated from this as a white solid after addition of 3 vol. of acetone. GPI was first converted into the barium salt by addition of freshly prepared aqueous barium hydroxide to neutrality and then precipitated by the addition of 10 vol. of acetone.

An analysis of GPI isolated from liver inositide on Nalcite SAR resin gave molar ratios for inositol:phosphoric acid:glycerol of 1.1:1:1. A sample of GPI isolated as the barium salt after

Fig. 2. Crude liver inositide hydrolysate fractionated on a column of Dowex 1 $\times$ 2 (100–200 mesh, formate form), size 1 cm. $\times$ 20 cm. Eluted with the sodium borate-ammonium formate system. Peak *A* probably represents glycerylphosphoryl ethanolamine, peak *B* represents GPI, peak *C* GPS and peak *D* glycerophosphate. Hydrolysis time, 30 min.

separation on a Dowex-1 column gave the following analysis after drying to constant weight at 50° *in vacuo* over P₂O₅ (Found: P, 8.1; glycerol, 29.1%; molar ratio P:glycerol, 1:1.19. The anhydrous barium salt of GPI requires P, 7.7; glycerol, 22.9, molar ratio 1:1).

Paper chromatography with the solvents described below showed about 12% of glycerophosphate to be present as an impurity. This probably arose from partial hydrolysis of GPI during isolation. Use of much milder isolation conditions (after borate removal, concentration *in vacuo*

Fig. 3. Purified liver inositide hydrolysate fractionated on Nalcite SAR. Column details are as described in Fig. 1. The main peak is GPI. Hydrolysis time, 25 min.

Fig. 4. Ox-brain phosphatidyl serine hydrolysate. Nalcite SAR column, as in Fig. 1. The main peak is GPS. Hydrolysis time 30 min.

Table 1. *Time study of phospholipid hydrolysis*

Samples of partially purified liver inositide containing roughly 10 mg. of phosphorus were hydrolysed as described under Methods. The figures represent percentages of the total water-soluble phosphates obtained after hydrolysis. 'GPE' has not been identified with certainty (see text).

Time of hydrolysis (min.)	Phospholipid hydrolysed (%)	'GPE' (%)	GPI (%)	GPS (%)	GP (%)	P not eluted (%)
15	42	8.5	45.7	24.2	5.8	15.8
30	57.5	6.1	49.0	26.3	4.2	14.4
45*	66	8.8	42.2	27.2	3.9	12.7

* An additional compound to those listed above was eluted with 5 mM-sodium borate and 0.09M-ammonium formate, representing 5.2% of the total water-soluble phosphates.

only to 200 ml., then freeze-drying to remove the remaining water and formic acid) yielded a barium salt which showed no glycerophosphate on chromatography. (Found: N, none detected; P, 7.2; molar ratio inositol:P:glycerol, 1.0:1.0:82. ($C_9H_{12}O_{11}P$)₂Ba.4H₂O requires P, 7.1 %).

Optical activities of two samples of GPI (free acid) were measured in aqueous solutions containing about 1 mg. of P/ml. and the specific rotations were calculated for the anhydrous compound.

$$[\alpha]_D^{20} = -16 \pm 2^\circ \text{ (sample containing GP)}$$

$$[\alpha]_D^{20} = -18 \pm 2^\circ \text{ (no GP detected)}$$

Hydrolysis of GPI (barium salt) with 0.5N-KOH at 100° for 40 min. completely decomposed the diester. Glycerophosphate and inositol monophosphate were produced and identified by chromatography in (a) propan-2-ol-acetic acid-water (3:1:1, by vol.) and (b) propan-1-ol-aq. NH₃ soln. (sp.gr. 0.88)-water (5:4:1, by vol.). R_f values relative to GP were as follows: solvent (a) IMP, 0.3; GPI, 0.3; GP, 1.0; solvent (b) IMP, 0.5; GPI, 1.0; GP, 1.0. A more detailed study of this hydrolysis, and its implications relating to the structure of monophosphoinositide, will be reported (cf. Brown, Hall & Higson, 1958).

GPS was isolated from hydrolysates of ox-brain phosphatidylserine after separation on a column of Dowex 1. Its glycerol content could not be determined by the chromotropic acid method used for GPI, because periodate oxidation of the serine also produced formaldehyde, which caused interference. However, hydrolysis with 3N-HCl at 100° for 90 min. gave only one phosphate, which was identified as glycerophosphate by chromatography in solvents (a) and (b) (Found: N, 5.2; P, 10.9; N:P molar ratio, 0.95:1.0. Anhydrous GPS requires N, 5.4; P, 11.96 %). Hydrolysis followed by chromatography revealed serine as the only ninhydrin-reactive spot. It was present in equimolar amounts to P. The specific rotation was so small that it could not be accurately measured on the dilute solutions available, but it seemed to be of the order of +1.9° for the free acid in water.

Paper chromatography of glycerylphosphoryl inositol and glycerylphosphoryl serine. For comparison with the work of Dawson (1954), chromatograms of the free acids were prepared in solvents (a) and (b) above, (c) phenol-aq. NH₃ soln. and (d) *tert.*-butanol-water (62:38, v/v) containing 10 % (w/v) of trichloroacetic acid. In solvent (a) R_f values relative to GP were: GPI, 0.3; GPS, 0.5; GP, 1.0, and in solvent (b) both diesters moved to the same position as GP. In phenol, GPI did not move and GPS had an R_f value less than 0.05. With solvent (d) R_f values were: GPI, 0.14; GPS, 0.32; GP, 0.61.

Separation of glycerophosphate, inositol monophosphate and inorganic phosphate

A separation of these three compounds on Nalcite SAR resin was reported previously (Hübscher & Hawthorne, 1957). All attempts failed to separate the compounds on Dowex-1 (×2) resin. Only a partial separation of them was obtained with Dowex-2 (×8) resin which is very similar to Nalcite SAR.

Separation of hydrolysis products obtained from freshly prepared liver phosphatides

The livers of three rats were removed as soon as possible after death and homogenized in 5 % trichloroacetic acid. After centrifuging, the washed precipitate was extracted with chloroform-methanol (1:1, v/v). The extracted lipids were subjected to the usual alkaline hydrolysis and the phosphate esters produced were applied in 5 mm-sodium borate to a Dowex-1 column. Much of the phosphate passed right through the column, as would be expected since glycerylphosphoryl choline is a major product of hydrolysis. Elution with the formate-borate solutions described above gave several peaks, two of which corresponded in position with glycerylphosphoryl inositol and glycerylphosphoryl serine. A further peak is thought to be glycerylphosphoryl ethanolamine, since hydrolysis and paper chromatography showed ethanolamine in about equimolar amounts to P, and a fourth remains unidentified. As with brain and pancreas (Hokin & Hokin, 1956) small amounts of glycerophosphate were observed. More detailed studies on liver and other tissues are in progress.

DISCUSSION

The present method can be applied to crude phospholipids freshly prepared from tissues. It should thus be possible to determine the relative amounts of the various glycerophosphatides in animal tissues more accurately than with the other methods so far available. The resin method is also more suitable than paper chromatography for the study of minor unidentified constituents of phospholipid hydrolysates. Little is known yet, for instance, about the hydrolysis product of liver polyglycerophosphatide (McKibbin & Taylor, 1952). Study of ³²P uptake into this and other phosphatides should be facilitated by the use of resin columns.

Several workers (e.g. Dawson, 1954; Hokin & Hokin, 1956) have shown that in certain tissues the ³²P turnover of inositol phosphatides is much higher than that of the other phospholipids. However, the hydrolysis products on which these results depended were not well characterized. The

present work establishes the structure of the inositol-containing ester from liver phosphatides as glycerylphosphoryl inositol. Previous results suggested that this grouping was present in monophosphoinositide (Faure & Morelec-Coulon, 1954; Hawthorne & Hawthorne, 1955; McKibbin, 1956), but the evidence was not conclusive. It now seems clear that this lipid, which is probably present in most tissues other than nervous tissue, has a structure analogous to that of lecithin, choline being replaced by myoinositol.

It is of some interest that inositol monophosphate, glycerophosphate and inorganic phosphate behaved differently on the three resins used, in spite of the fact that each resin has the same ionic groupings (quaternary ammonium). This suggests that adsorption is determined partly by the structure of the resin matrix, resins with a higher degree of cross-linking giving better separations.

Further hydrolysis of glycerylphosphoryl inositol releases both glycerophosphate and inositol monophosphate. In view of the possibility of 'cyclization' (cf. Brown *et al.* 1958) with the adjacent ring hydroxyls of the inositol, as well as with the β -hydroxyl group of the glycerol, it was at first thought that this diester would be unstable. Its breakdown under the mild alkaline conditions of Dawson's method might then have explained the presence of small amounts of glycerophosphate in hydrolysates of liver and pancreas phospholipids which include monophosphoinositide. The results, however, show that glycerophosphoryl inositol is stable under these conditions and that it differs little in stability from glycerylphosphoryl serine, which possesses only one hydroxyl group vicinal to the phosphate group. The observed glycerophosphate thus cannot arise from monophosphoinositide. This is confirmed by the effects of longer hydrolysis times (30 and 45 min. as compared with Dawson's 15 min.). No further release of glycerophosphate was observed after 15 min. In addition, both Dawson (1954) and Hokin & Hokin (1956) found much higher specific activities for the glycerophosphate than for the inositol ester. This would also exclude a common origin.

The glycerophosphate must therefore arise from phosphatidic acid, as Dawson suggested, or from some phosphatide much less stable than the known glycerophosphatides. The present work does not directly exclude polyglycerophosphatide, but its structure suggests that it would not hydrolyse rapidly and completely to glycerophosphate in 15 min., but rather that a more complex ester would be first formed. Though brain tissue contains diphosphoinositide, not monophosphoinositide, the structures are sufficiently similar for this compound also to be excluded as a source of glycerophosphate.

SUMMARY

1. The mild alkaline hydrolysis of certain phospholipids has been studied.
2. Glycerylphosphoryl inositol and glycerylphosphoryl serine have been separated from one another and from other phosphates, on anion-exchange columns.
3. The two diesters have been isolated by this method and analysed.
4. Glycerylphosphoryl choline is not retained by the columns used.
5. It is suggested that the glycerophosphate observed in hydrolysates of tissue phospholipids cannot arise from any known lipid except phosphatidic acid.

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