

in certain [¹⁴C]amino acids has been investigated, together with the effect of insulin thereon. No important differences between the behaviour of the cut and intact preparations was observed.

2. With alanine, α -aminoisobutyric acid, glycine, leucine, lysine, ornithine and phenylalanine the ratio of concentration of radioactivity in tissue water to that in the medium exceeded unity. With glutamic acid and aspartic acid the ratio was below unity, probably because of the rapid metabolism of these amino acids in isolated rat diaphragm.

3. The addition of insulin to the medium significantly raised the ratio for glycine and α -aminoisobutyric acid, but not for the other amino acids under study. The effect of insulin was similar whether or not glucose was added to the medium. It was abolished by anoxia and by cooling the system to 12°.

4. Under anoxic conditions, both in the presence or absence of insulin, the ratio was significantly raised by the addition of glucose to the medium. A similar effect of glucose was not seen under any other conditions studied.

5. No substantial evidence has been obtained to suggest that insulin promotes the incorporation of amino acids into protein in isolated diaphragm by enhancing the rate of entry of amino acids into the cytoplasm of the cells from the medium.

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Phosphoinositides

1. CONFIGURATION OF THE INOSITOL PHOSPHATE IN LIVER PHOSPHATIDYLINOSITOL

By J. N. HAWTHORNE

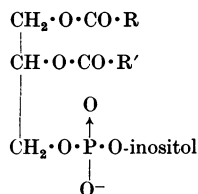
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Work from several different Laboratories suggests that the inositol-containing lipid which occurs in liver and heart has structure (I) (Faure & Morelec-Coulon, 1954; Hawthorne, 1955a; McKibbin, 1956; Hanahan & Olley, 1958). The α -

glycerophosphate structure, which might be expected from comparison with phosphatidylcholine, has not yet been rigidly proved. There is also doubt about the point of attachment of the phosphoric acid to the myoinositol ring, which is the

subject of this paper. The name 'monophosphoinositide' has been widely used for this compound. As the structure is now known to be analogous to that of phosphatidylcholine, phosphatidylinositol seems a more suitable name.



(I) Phosphatidylinositol (R and R' are long-chain alkyl residues)

The studies of Brown and his colleagues (e.g. Brown, Hall & Higson, 1958) and the unambiguous synthesis of glycerol 1-(inositol 2-phosphate) (Ellis & Hawthorne, 1959; Davies & Malkin, 1959), enable deductions to be made about the configuration of the inositol phosphate in phosphatidylinositol. These depend on a study of the proportions of the inositol and glycerol phosphates obtained by alkaline hydrolysis of the lipid. The diester from the lipid is referred to as glycerylphosphorylinositol. A preliminary account of some of this work has already appeared (Hawthorne & Hübscher, 1959*b*).

MATERIALS AND METHODS

Analytical methods. These have been described by Hawthorne & Hübscher (1959*a*).

Resin columns. Nalcite SAR (200–400 mesh) proved to be the most suitable resin for the separation of inositol and glycerol phosphates. This was obtained from the National Aluminate Corp., Chicago, 38, and not from the Dow Chemical Company as previously stated. Dowex 2 ($\times 8$, 200–400 mesh) is a similar anion-exchanger but was not quite so effective in the separation. This may have been partly due to the higher proportion of fine particles in the Nalcite SAR.

Preparation of phosphatidylinositol. This was obtained from ox liver. A crude ethanol-insoluble phospholipid fraction was first prepared (cf. Hawthorne, 1955*a*). The liver, obtained a few hours after the death of the animal, was minced and then homogenized for 1 min. in a high-speed mixer with chloroform-methanol (2:1, v/v). The volume was then made to 3 l./kg. of fresh liver with the same solvent. After 1 hr. the mixture was filtered at the pump and the residue re-extracted with a further 3 l./kg. of the chloroform-methanol. The upper layers of aqueous methanol were rejected. The combined extracts were then concentrated in a Cyclone evaporator (Quickfit and Quartz Ltd., Stone, Staffs.) at 30° until a viscous oil began to separate (approx. 0.1 vol.). Phospholipids were precipitated by the addition of 5 vol. of acetone to the concentrated extract. The mixture was then stored overnight at 4°. The original precipitate and any further lipid which came down on keeping were dissolved in 500 ml. of chloroform/kg. of fresh liver (subsequent volumes all refer to

1 kg. of fresh liver). The solution was dried over anhydrous Na_2SO_4 , filtered and phospholipid was reprecipitated with 3 vol. of acetone.

The sticky, yellowish precipitate was then dissolved in 300 ml. of chloroform and shaken well with 2 vol. of absolute ethanol. The resulting 'cephalin' precipitate was dissolved in 500 ml. of ether and stored at 4° overnight. The insoluble material was centrifuged off and the supernatant mixed with 3 vol. of absolute ethanol. The waxy precipitate so obtained was dissolved in 300 ml. of chloroform-methanol (1:1, v/v) and shaken well with 60 ml. of 1 mM- CaCl_2 to remove water-soluble impurities (Folch, Lees & Sloane-Stanley, 1957). The lower layer was then separated and mixed with 600 ml. of absolute ethanol. The precipitate was redissolved in 300 ml. of chloroform and crude phosphatidylinositol precipitated by the addition of 520 ml. of absolute ethanol. The lipid was dissolved in chloroform-methanol (4:1, v/v) for loading on to the column described below.

Silicic acid chromatography. The method of Hanahan, Dittmer & Warashina (1957) was followed, Mallinckrodt silicic acid (100 mesh, 'suitable for chromatography') was used mixed intimately with half its weight of Hyflo Supercel (Johns-Manville, U.S.A.). The lipids eluted with chloroform-methanol (4:1, v/v) were rejected. The fractions containing the first peak eluted by chloroform-methanol (3:2, v/v) were concentrated *in vacuo* to give a product with N:P molecular ratio approximately 0.2:1.0. Most of the pigmented material was removed in the early fractions obtained with chloroform-ethanol (4:1, v/v), so that the final product was almost white.

Hydrolysis of phosphatidylinositol and preparation of glycerylphosphorylinositol. The product from the silicic acid column was hydrolysed for 30 min. at 37° in methanolic 0.5N-NaOH and the diester prepared by chromatography on Dowex 1 as previously described (Hawthorne & Hübscher, 1959*a*). Only the central fractions of the peak corresponding to glycerylphosphorylinositol were worked up so as to avoid any contamination with diesters, such as glycerylphosphorylserine, which could give rise to glycerophosphate on hydrolysis. As will be seen below, in the final distillation and freeze-drying to remove formic acid, care was necessary to avoid partial decomposition of glycerylphosphorylinositol. It was essential to concentrate *in vacuo* at a bath temperature of 50° or less. When the volume had been reduced to 100 ml. the remaining water was removed by freeze-drying in a simple bench apparatus, consisting of a round-bottom 500 ml. flask attached by a ground joint to a trap cooled in solid CO_2 and acetone. This in turn was attached to a soda-lime trap, to absorb traces of formic acid, and finally to a high-vacuum pump.

The syrup obtained after freeze-drying was stored overnight in a vacuum desiccator over solid KOH to remove the last traces of formic acid and then dissolved in about 2 ml. of water/50 mg. of syrup. The solution was neutralized (pH 7.5) with saturated aqueous barium hydroxide and the barium salt precipitated by the addition of 10 vol. of acetone. After storage overnight at 4°, the white precipitate was centrifuged off and taken up in 2 ml. of water. Any insoluble material was spun down and rejected. The clear solution was again treated with acetone and stored overnight at 4°. The pure barium salt of glycerylphosphorylinositol was finally centrifuged down, washed with acetone and ether and stored in a desiccator.

RESULTS

Hydrolysis of different samples of glycerylphosphorylinositol

Since phosphate migration should not take place during the production of glycerylphosphorylinositol from phosphatidylinositol, the proportions of inositol monophosphate and glycerophosphate formed by hydrolysis of the diester can be used in deducing the structure of the original lipid. Hydrolysis with aqueous 0.5N-NaOH at 100° for 40 min. was chosen, because these conditions gave complete breakdown of glycerylphosphorylinositol to monoesters without further decomposition and release of inorganic phosphate.

Hydrolyses of several different preparations of glycerylphosphorylinositol will be described since they illustrate the difficulty of arriving at the true ratio of monoesters liberated.

Hydrolysis of barium salt prepared by Hawthorne & Hübscher (1959 a). This sample contained 7.2% of P and had a molar ratio inositol:P:glycerol of 1.0:1:0.82. The barium salt (30 mg.) was hydrolysed with 6 ml. of 0.5N-NaOH for 40 min. at 100°. After cooling and diluting to 30 ml. with water, the hydrolysate was passed through a column of Zeo-Karb 225 (H⁺ form, -16+50 mesh; column size 2 cm. x 15 cm.) to remove cations. The column was washed with 200 ml. of water. The combined eluate and washings were made to 250 ml. with sodium tetraborate solution, so that the final

borate concentration was 5 mM, and loaded on to a Nalcite SAR column (formate form, 200-400 mesh; 1 cm. x 14 cm.). Elution with 250 ml. of 5 mM-sodium tetraborate-0.10M-ammonium formate removed no phosphate from the column. Fractions (25 ml.) were collected. Two compounds were eluted with 5 mM-sodium borate-0.15M-ammonium formate, in positions corresponding to inositol monophosphate and glycerophosphate (Fig. 1). Of the P eluted 39% appeared as inositol phosphate. No inorganic P was detected in either peak. The second (glycerophosphate) peak took up 0.39 mole of periodate/g. atom of P, which suggested that it contained 61% of β -glycerophosphate. The α - and β -glycerophosphate do not separate on the column used. Since only one inositol-containing peak was observed it seems that the isomers of inositol phosphate also run together.

Hydrolysis of the barium salt of glycerylphosphorylinositol and paper chromatography. In these estimations 0.3 ml. of a solution of the barium salt, containing about 250 μ g. of P/ml., was heated at 100° for 40 min. with 0.3 ml. of N-NaOH. After cooling, cations were removed with Zeo-Karb 225 and the hydrolysate was concentrated to a small volume for paper chromatography. An amount containing 30-40 μ g. of P was applied to each spot on a Whatman no. 54 paper. Markers of inositol 2-phosphate (from phytic acid) and β -glycerophosphate were also applied. Two papers were used for each hydrolysate, one chromatographic solvent being propan-1-ol-aq. NH₃ soln. (sp.gr. 0.88)-water (5:4:1, by vol.) and the other propan-2-ol-acetic acid-water (3:1:1, by vol.) After spraying by the method of Wade & Morgan (1953) the white phosphate-containing areas were cut out. The paper was shredded and washed with water until the pink background surrounding the spots had been completely removed. The extract was then boiled to a small volume and digested in the usual way for the estimation of P. Blanks on similar areas of the chromatogram where there were no spots revealed no P.

In the alkaline solvent both inositol phosphate and glycerophosphate from the hydrolysates ran as double spots. The phosphate determinations were done on the pair together in each case. The larger glycerophosphate spot moved faster and the larger inositol monophosphate spot moved more slowly. Comparison with synthetic α -glycerophosphate (kindly made by Mr B. Clark) showed that the slower glycerophosphate spot corresponded to the α -compound. From the results of Pizer & Ballou (1959) it seems probable that the faster inositol monophosphate spot is the 2-phosphate, the slower being the 1-phosphate. These double spots do not separate if the chromatograms are overloaded.

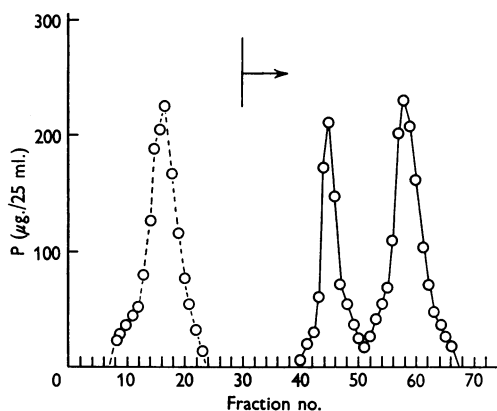


Fig. 1. Column behaviour of glycerylphosphorylinositol and its hydrolysis products. Nalcite SAR column, 25 ml. fractions. Before hydrolysis (broken lines): fractions 8-23, glycerylphosphorylinositol. Hydrolysis products: fractions 40-51, inositol monophosphate; fractions 51-66, glycerophosphate. Eluting mixtures: fractions 1-30, 5 mM-sodium tetraborate-0.06M-ammonium formate; fractions 31-70, 5 mM-sodium tetraborate-0.15M-ammonium formate.

With the same sample of glycerylphosphoryl-inositol, five different chromatograms gave 41.1, 41.7, 37.2, 38.4 and 40.9 % of total P of hydrolysate present as inositol monophosphate.

Hydrolysis of glycerylphosphoryl-inositol without isolation as barium salt. Experiments on partly hydrolysed samples indicated that slight hydrolysis of glycerylphosphoryl-inositol during its isolation might lead to inaccurate figures for the composition of the final NaOH hydrolysate. Such samples contain inositol monophosphate and glycerophosphate. After acetone precipitation of the barium salts, the former is preferentially precipitated with the glycerylphosphoryl-inositol.

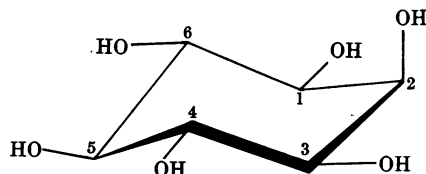
To avoid any decomposition, glycerylphosphoryl-inositol was separated from a phosphatidylinositol hydrolysate by chromatography on Dowex 1 as described under Materials and Methods. No attempt was made to isolate glycerylphosphoryl-inositol as a solid. Instead the central peak fractions were pooled, made 0.5N with respect to NaOH and boiled for 40 min. After cooling, cations were removed with Zeo-Karb 225. The resin was washed to avoid losses. The pH of the combined hydrolysate and washings was adjusted to 7 with sodium bicarbonate. Sodium borate solution was then added to give a final borate concentration of 5 mM and a volume of 500 ml. The solution, containing 6.25 mg. of P, was loaded on to the usual Nalcite SAR column. No phosphate was eluted with 0.06M-ammonium formate-5 mM-sodium borate (500 ml.). Two peaks, corresponding to inositol monophosphate and glycerophosphate, were eluted with 0.15M-ammonium formate-5 mM-sodium borate. Of the total P 34 % appeared as inositol monophosphate.

Hydrolysis of synthetic glycerylphosphoryl-inositol. A sample of the barium salt of glycerylphosphoryl-inositol was kindly supplied by Mr J. H. Davies and Dr T. Malkin (Davies & Malkin, 1959). It contained 7.3 % of P and the glycerol:P ratio was 1.03:1. Chromatography on Dowex 2 ($\times 10$, 200-400 mesh, formate form), by using 0.06M-ammonium formate-5 mM-sodium borate for elution, gave a sharp peak in the first few fractions. This tailed considerably, however, possibly because of hydrolysis on the column. Only the fractions corresponding to the peak were used in further studies. These fractions were combined and hydrolysed directly, as described above. The chromatographic separation was done on a Dowex 2 ($\times 10$) column. Of the total P 31 % appeared as inositol monophosphate. A second experiment gave 35 %.

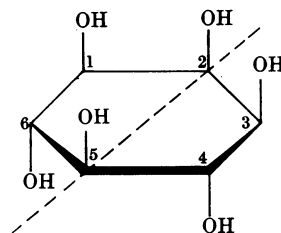
A synthetic sample made by a different method (Ellis & Hawthorne, 1959) was hydrolysed in the same way. It gave 31 % of total P as inositol monophosphate.

DISCUSSION

From the principles of conformational analysis (Klyne, 1954; Barton & Cookson, 1956), the preferred conformation (II) of *myoinositol* would be expected to be the chair form with five equatorial hydroxyl groups and one axial hydroxyl group (that on C-2). The common convention for inositol derivatives depicts the cyclohexane ring as a planar hexagon (e.g. III) with the substituents projecting above or below the plane of the ring.



(II)



(III)

Although these formulae clearly indicate the steric relationships of substituents and are convenient for the application of rules of symmetry, they do not reveal the true shape of the molecule, and care must be exercised in the interpretation of reactions on the basis of such formulae. In this discussion, however, the convention in which the ring is considered planar will be used and the hydroxyl groups described as being in *cis* or *trans* relationship; certain points then become clearer. Use of the non-planar convention does not affect the argument (e.g. Angyal, 1957).

Since the *myoinositol* molecule is symmetrical about the plane perpendicular to the broken line in (III), there are two possible optically inactive monophosphoric acids, the 2- and 5-compounds. The other monophosphoric acids will be pairs of stereoisomers, the 1(3)- and 4(6)-compounds. If it is assumed that the inositol monophosphate isolated from phosphoinositides is optically inactive (see below), it should be the 2- or 5-compound (Iselin, 1949; Hawthorne & Chargaff, 1954). The latter workers considered that cyclization and possible migration of the phosphate group was unlikely to occur in the hydrolysis of phosphoinositides. Recent evidence is that such cyclization does occur (Posternak, 1958; Pizer & Ballou, 1959).

Borate-complex formation with pyranose sugar derivatives which contain vicinal *cis*-hydroxyl groups flanked by a *cis*-methoxyl group is hindered (Foster, 1957). Certain neighbouring group reactions of some sugar acetates are hindered (Lemieux & Brice, 1956), since they involve intermediates structurally related to (IV). A detailed interpretation of these steric effects is given elsewhere (Foster, 1957; Lemieux & Brice, 1956).

Such a steric effect operating in the hydrolysis of glycerol 1-(inositol 2-phosphate) would oppose the formation of an inositol cyclic phosphate and hence divert the hydrolysis along the alternative pathway involving a glycerol cyclic phosphate. Significantly less inositol monophosphate would result from glycerol 1-(inositol 2-phosphate) than *cyclohexane cis*-1:2-diol phosphate from the *cyclohexane cis*-diol analogue.

The observed figures of 30–40% of inositol monophosphate are therefore quite probably in keeping with the *cis* structure for the inositol phosphate of phosphatidylinositol. Confirmation of this comes from the hydrolysis figures for synthetic glycerol 1-(inositol 2-phosphate.) These agree well with the figures from the natural glycerylphosphorylinositol. Hydrolysing phosphatidylinositol itself, which should give a similar ratio since the fatty acids are quickly removed by alkali, Hanahan & Olley (1958) found 35% of inositol monophosphate. Lower figures would be expected for a *trans* structure. In this case glycerylphosphorylscyllitol should provide an ideal model compound.

The chemical evidence suggests that the inositol phosphate of liver phosphatidylinositol has its phosphate group on one of the *cis*-hydroxyl groups. It must therefore be the 1-(D- or L-) or 2-phosphate. These structures would give similar hydrolysis results and so cannot be distinguished without further evidence. Until recently the inositol monophosphate isolated from lipids was thought to be optically inactive (Iselin, 1949; Hawthorne, 1955*b*). Pizer & Ballou (1959) have now isolated an inositol monophosphate with a small but definite rotation from soya-bean inositide. From their chemical studies and from this rotation they conclude that soya-bean inositide has the 1-structure. Since this vegetable lipid has strongly dextrorotatory sugar phosphates associated with it (Hawthorne, 1955*b*) it was thought advisable to obtain further evidence on the rotation (and if possible other independent evidence), before deciding between the 1- and 2-structures (see Hawthorne, Kemp & Ellis, 1960).

SUMMARY

1. Liver phosphatidylinositol has been hydrolysed to give glycerylphosphorylinositol, alkaline hydrolysis of which gives a mixture of inositol monophosphate (30–40%) and glycerophosphate.

2. Hydrolysis of synthetic glycerol 1-(inositol 2-phosphate) gives a similar mixture.

3. Steric effects in the hydrolysis are discussed and it is concluded that liver phosphatidylinositol has the inositol 1- or 2-phosphate structure.

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