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Phosphoinositides

2. THE INOSITOL 1-PHOSPHATE STRUCTURE IN LIVER PHOSPHATIDYLINOSITOL

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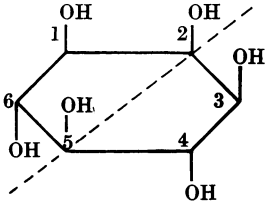
The hydrolysis studies reported in the preceding paper (Hawthorne, 1960) suggest that in liver phosphatidylinositol the phosphate group is linked to one of the *cis*-hydroxyl groups of the inositol, i.e. to positions 1(3)- or 2-. Until recently, the inositol monophosphate isolated from phosphoinositides was believed to be optically inactive (Iselin, 1949; Hawthorne, 1955) and so liver phosphatidylinositol was thought to have the 2-structure (Hawthorne & Hübscher, 1959*a*). It can be seen from the structure (I) that only the 2- or 5-phosphate has



(I)

a plane of symmetry. Pizer & Ballou (1959) however, have shown the inositol monophosphate from soya-bean phosphoinositide to be optically active. The same conclusion has been reached by Hanahan & Brockerhoff (1959) for liver phosphatidylinositol. The subject has therefore been reinvestigated with optical rotatory dispersion to study the optical activity of carefully purified inositol monophosphate from groundnut phosphatides, and an enzymic hydrolysis of liver phosphatidylinositol which should release inositol monophosphate without phosphate migration.

MATERIALS AND METHODS

Phospholipids. Liver phosphatidylinositol was prepared as described by Hawthorne (1960). Crude groundnut phosphoinositide, which is similar to the soya-bean product, was also made by the method of Hawthorne (1955).

Enzyme preparation. The phospholipase which hydrolyses phosphatidylinositol was prepared from rat liver as described by Kemp, Hübscher & Hawthorne (1959).

Experiments with Acetobacter. Attempts to oxidize various inositol phosphates were made with a strain of *Acetobacter suboxydans* (A.T.C.C. 621) in the Warburg apparatus (Magasanik & Chargaff, 1948). The most vigorous oxidation of inositol standards was obtained with bacteria grown for 3 or 4 days (not longer) on the sorbitol–yeast extract medium. For the oxidations, acetate buffer of pH 5·6 was used. Inositol phosphates in the form of *cyclo*hexylamine salts were first treated with Zeo-Karb 225 and the free acid so produced was neutralized with NaOH.

Other analytical methods and ion-exchange resins. These have been described by Hawthorne & Hübscher (1959*b*).

RESULTS

Preparation of inositol monophosphate from groundnut phosphoinositide. The material was hydrolysed with KOH according to Pizer & Ballou (1959). The *cyclo*hexylamine salt finally obtained was not recrystallized but applied directly to a column of De-Acidite FF (The Permutit Co. Ltd., London). The salt had 4·95 % of P and $[\alpha]_D^{20} + 26 \pm 2°$ in water (*c*, 2). It was Molisch-positive. A portion (1·15 g.) was applied to a column of resin (200–400 mesh; size 3 cm. × 30 cm.). Elution with 0·01 M-formic acid gave three peaks. The first two were due to sugar-containing compounds, but the third compound had only a trace of sugar. Rotations of this last compound, calculated as the *cyclo*hexylamine salt, were $[\alpha]_D^{20} - 2·7 \pm 0·2°$ (pH 1) and $[\alpha]_D^{20} + 5·6 \pm 0·2°$ after addition of *cyclo*hexylamine to pH 9 (*c*, 1·53).

For further purification, the fractions corresponding to the third peak were combined and sodium borate solution was added to give a final concentration of 5 mM. The combined fractions, containing 11 mg. of P, were then applied to a Dowex 2 (× 10) column (formate form, 100–200 mesh; 1·5 cm. × 35 cm.). All the phosphate was retained by the column and none was eluted with 500 ml. of 0·08 M-ammonium formate–5 mM-sodium borate, nor with 1 l. of 0·12 M-ammonium formate–5 mM-sodium borate. Organic P (8·5 mg.) was eluted as one peak with 0·15 M-ammonium formate–5 mM-sodium borate. This solvent removes inositol monophosphate. The central fractions of the peak were pooled, diluted with an equal volume of 5 mM-borate and applied to a similar Dowex 2 column. One main peak was then eluted with 0·05 M-formic acid–0·05 M-ammonium formate. Central fractions were pooled and cations removed with Zeo-Karb 225. The solution thus obtained was concentrated to 50 ml. *in vacuo* at 30° and then

freeze-dried. The residue was taken up in 2 ml. of water and neutralized with saturated barium hydroxide solution. The barium salt of inositol monophosphate was precipitated with excess of acetone. The white solid was centrifuged and re-precipitated from water with acetone. To its solution in a little water the calculated amount of *cyclo*hexylamine sulphate solution was then added. Barium sulphate was spun down and the clear supernatant shaken with excess of butan-1-ol. Addition of 1 vol. of diethyl ether gave a semi-crystalline precipitate of *cyclo*hexylamine inositol monophosphate. This was recrystallized in the same way to give small crystals of the salt.

Inositol monophosphate from phytic acid. For comparison, the crystalline *cyclo*hexylamine salt of inositol monophosphate prepared enzymically from phytic acid was made (McCormick & Carter, 1952). This is believed to be the 2-phosphate (Hawthorne, 1955; Brown & Hall, 1959).

Rotation measurements. For the groundnut inositol monophosphate as the *cyclo*hexylamine salt, the value $[\alpha]_D^{20} + 3{\cdot}1 \pm 0{\cdot}3°$ was obtained. The phytic acid compound showed no optical activity. Samples of each salt were sent to Dr W. Klyne for rotational-dispersion measurements. The groundnut compound gave these molecular rotations (wavelengths are given in parentheses): +12° (600 mμ); +18° (500 mμ); −32° (400 mμ); −54° (350 mμ); −89° (300 mμ). At each of these wavelengths the phytic acid compound gave values of zero within the error of the instrument. In all the above-mentioned measurements water was used as solvent and calculations were based on the di*cyclo*hexylamine salts.

Studies with Acetobacter suboxydans. Attempts were made to oxidize synthetic (−)-inositol 3-phosphate (Kilgour & Ballou, 1958), *myo*inositol 2-phosphate (from phytic acid) and the groundnut inositol phosphate. In each experiment inositol standards were oxidized to make sure that the bacteria were active. No oxidation was observed with any of the inositol phosphates.

Enzymic hydrolysis of phosphatidylinositol. A sample of phosphatidylinositol equivalent to about 40 mg. of P was emulsified in water and dialysed to remove non-lipid phosphates. To this was added the enzyme extracted from five rat livers as described previously (Kemp *et al.* 1959); the precipitate obtained at 40% saturation of $(NH_4)_2SO_4$ was dissolved in 100 ml. of 0·01 M-2-amino-2-hydroxymethylpropane-1:3-diol–hydrochloric acid buffer, pH 7·3. 0·4 M-Sodium acetate buffer, pH 5·4 (20 ml.), and 20 ml. of 0·08 M-$CaCl_2$ were also added. The final volume was 220 ml. The mixture was incubated for 75 min. at 37° and then 25 ml. of 50% (w/v) trichloroacetic acid solution and some crushed ice were added. After 10 min. at 4°, 50 ml. of an aqueous 12·5% (w/v) suspension of Norit charcoal was added and the mixture shaken for 10 min. It was then centrifuged at 2000 rev./min. The precipitate was washed in the centrifuge with four 50 ml. portions of water. These were then added to the original supernatant.

The supernatant and washings were extracted four times with 100 ml. of diethyl ether to remove trichloroacetic acid. Excess of ether was removed by a stream of air, and cations were removed by shaking with Zeo-Karb 226 (H^+ form). Sodium borate solution and deionized water were then added to give a borate concentration of 5 mM in a final volume of 2 l.

Isolation and paper chromatography of inositol monophosphate. The above-mentioned solution was loaded on to a Nalcite SAR column which was then washed with 500 ml. of 5 mM-sodium borate. No phosphate was eluted with this or with 500 ml. of 0·06 M-ammonium formate–5 mM-sodium borate. Of the phosphate applied 90% was eluted as one peak as soon as the solvent was changed to 0·15 M-ammonium formate–5 mM-sodium borate. This peak corresponded in position with inositol monophosphate. The peak fractions were combined and re-applied to the same type of column for borate and formate removal (Hawthorne & Hübscher, 1959*b*). During elution from this second column a small peak corresponding to about one-tenth of the total P was seen in front of the main peak. It was rejected.

After removal of the last traces of formic acid in a vacuum desiccator over KOH, the inositol monophosphate was dissolved in a little water. Portions corresponding to 10 μg. of P were applied to Whatman no. 54 papers for descending chromatography in propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88)–water (6:3:1, by vol.). The solvent was allowed to run off the end of the papers for several hours. Markers of inositol 2-phosphate and the mixture of inositol 1- and 2-phosphate produced by acid-catalysed migration (Posternak, 1959) were applied. Only a trace of 2-phosphate was seen in the enzymically produced inositol monophosphate. The bulk of the compound ran to the inositol 1-phosphate position. The 2-compound may have been produced during the formic acid removal.

DISCUSSION

The results suggest that the inositol monophosphate from groundnut phosphoinositides is optically active. The values obtained ($[\alpha]_D + 3{\cdot}1°$ for the *cyclo*hexylamine salt) are similar to those of Pizer & Ballou (1959) for the soya-bean compound (+3·4°). In view of the other evidence supplied by these authors it seems likely that both these phosphoinositides have the inositol 1-phosphate structure.

Since a vigorous alkaline hydrolysis is used in the preparation of the monophosphates, some phosphate migration to the 2-position occurs. However, as Pizer & Ballou show, the inositol 2-phosphate can be removed by repeated crystallization because its *cyclo*hexylamine salt is more soluble than that of the 1-phosphate. Under acid conditions (Posternak, 1959), the 2-phosphate itself undergoes phosphate migration, giving a mixture of 1(3)- and 2-phosphate. This further migration would reduce the optical activity of the soya-bean and groundnut products by producing 1(3)-phosphate, but fortunately it does not occur readily under alkaline conditions.

Strongly dextrorotatory sugar phosphates are also present in hydrolysates of these lipids (Klenk & Sakai, 1939; Hawthorne & Chargaff, 1954). The presence of traces of such phosphates could easily mask the small rotation of inositol monophosphate, since the free acid is laevorotatory, and this is presumably why in earlier work (Iselin, 1949; Hawthorne, 1955) the inositol monophosphate was thought to be optically inactive.

Although phosphatidylinositol may occur in soya-bean phosphoinositides (Hörhammer, Wagner & Hölzl, 1958), more complex inositol-containing lipids are also present (Folch, 1952). Conclusions about the inositol monophosphate from groundnut or soya-bean phosphatides therefore cannot be applied directly to liver phosphatidylinositol.

Acetobacter suboxydans of the strain used in these experiments can oxidize (−)-inositol to a diketone, attacking the two axial hydroxyl groups. The bacteria are unable to oxidize (−)-inositol 3-phosphate, even though in this compound the axial hydroxyl groups remain free. This confirms the earlier view (Hawthorne, 1955) that the phosphate group prevents enzymic oxidation whether it is linked to the hydroxyl group that is usually oxidized or not. Since neither *myo*inositol 1- nor 2-phosphate will be attacked, the bacteria are of no use in structural studies.

Alkaline hydrolysis of phosphatidylinositol or glycerylphosphorylinositol gives a mixture of inositol monophosphate and glycerophosphate in proportions suggesting that the inositol of the original lipid is phosphorylated in the 1- or 2-position (Hawthorne, 1960). Paper chromatography of such hydrolysates shows the presence of inositol 1- and 2-phosphate. By analogy with the behaviour of glycerophosphate the most probable explanation is the intermediate formation of a cyclic inositol 1:2-phosphate during hydrolysis and then 'non-specific' ring opening. Thus it is impossible to decide on the original position of the phosphate group from the results of alkaline hydrolysis.

Similar conditions for cyclic ester formation are present during the action of ribonuclease on ribonucleic acid and a cyclic phosphate mechanism has been proposed for the enzymic hydrolysis. The final products, however, include only nucleoside 3-phosphates and not a mixture of the 2- and 3-compound. It was hoped that a similar specificity might be found in the action of the phospholipase attacking phosphatidylinositol, which is also a phosphodiesterase. This proved to be so. The isolation of inositol 1-phosphate from the enzymic digest therefore provides further evidence that liver phosphatidylinositol has the 1-structure. The work of Hanahan & Brockerhoff (1959), on periodate oxidation of glycerylphosphorylinositol from the same lipid, also leads to this conclusion.

SUMMARY

1. Carefully purified inositol monophosphate from an alkaline hydrolysate of groundnut inositides was optically active.

2. This inositol monophosphate, inositol 2-phosphate and (−)-inositol 3-phosphate were all resistant to oxidation by *Acetobacter suboxydans* (A.T.C.C. 621).

3. The enzymic hydrolysis of liver phosphatidylinositol released inositol 1-phosphate.

4. It is concluded that liver phosphatidylinositol has the inositol 1-phosphate structure.

We wish to thank Professor A. C. Frazer for his continued interest. Dr W. Klyne, of the Postgraduate Medical School, London, kindly undertook the rotatory-dispersion studies. Mrs Janet Day made the measurements. We are indebted to Dr C. E. Ballou and Dr G. L. Kilgour, Biochemistry Department, University of California, for the generous sample of (−)-inositol 3-phosphate. Miss M. Bason provided valuable technical assistance and Mr M. G. Rumsby made the *Acetobacter* studies.

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Mitochondrial Preparations from the Leaves of Tobacco (*Nicotiana tabacum*)

2. OXIDATIVE PHOSPHORYLATION*

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A sucrose solution is suitable for the isolation of functional mitochondria from some animal tissues (Hogeboom, Schneider & Pallade, 1948), but not sufficient for use in the isolation of mitochondria from leaves. Smillie (1955), working with pea leaves, added ethylenediaminetetra-acetic acid; James & Das (1957), working with bean leaves, added phosphate and magnesium as well; Pierpoint (1959), working with tobacco leaves, increased the buffering power and added various substances, of which citrate was the most important. Even with these additions the tobacco mitochondria appeared to be somewhat functionally damaged and were heavily contaminated with chloroplast fragments.

Attempts are being made both to assess the damage done to these tobacco-leaf mitochondria and to purify them further. This paper provides information about their respiratory quotients and demonstrates that the oxidations catalysed by them are 'loosely' coupled to the phosphorylation of adenosine nucleotides.

EXPERIMENTAL

Mitochondrial preparations. Two modifications were made to the procedure already described (Pierpoint, 1959). The pH of the extraction medium (sucrose, 0·4M; 2-amino-2-hydroxymethylpropane-1:3-diol (tris), 0·2M; potassium phosphate, 0·01M; ethylenediaminetetra-acetic acid, 5 mM; sodium citrate, 0·02M) was decreased to 7·7, and the amount of medium used was increased so that 25–30 g. of leaves was disrupted in 100–130 ml.

Oxygen uptake. Oxygen uptake was measured in the conventional Warburg apparatus. Details of the procedure, including the incubation temperature (30°), were the same as those described by Pierpoint (1959).

In experiments in which only gas exchanges were being followed the samples of the mitochondrial preparations were added to manometer flasks containing: substrate acid, 40 μmoles; sucrose, 0·3 m-mole; phosphate buffer, pH 7·3, 0·1 m-mole; magnesium sulphate, 10 μmoles; adenosine triphosphate (ATP), 2 μmoles; cytochrome *c*, 0·018 μmole; diphosphopyridine dinucleotide (DPN), 0·2 μmole; thiamine pyrophosphate, 0·2 μmole; coenzyme A (CoA), 0·025 μmole. The final volume was 1·5 ml. and the pH was 7·2–7·3.

When both phosphorylation and oxygen uptake were being followed, the medium in the manometer flasks contained: substrate acid, 40 μmoles; sucrose, 0·4 m-mole; phosphate buffer, pH 7·3, 12 μmoles; magnesium sulphate, 10 μmoles; manganese sulphate, 0·1 μmole; sodium fluoride, 15 μmoles; ATP, 2 μmoles; cytochrome *c*, 0·018 μmole; DPN, 0·2 μmole; thiamine pyrophosphate, 0·2 μmole; glucose, 10 μmoles; hexokinase (Sigma Chemical Co., type 11), 1 mg. CoA (0·025 μmole) was also present in most experiments. The final volume varied a little, but was generally 2·0–2·1 ml.

Evolution of carbon dioxide. Evolution of the gas was measured manometrically by the 'direct method' (Umbreit, Burris & Stauffer, 1957).

Orthophosphate disappearance. Samples (1 ml.) were taken from the contents of manometer flasks and added to centrifuge tubes containing 1 ml. of cold trichloroacetic acid (5%, w/v). The tubes were cooled in ice–water for 10 min. and then centrifuged at 1500 *g* for the same length of time. The supernatant solution was decanted, diluted with four times its volume of sodium acetate (0·1M) and portions were taken for phosphate estimation.

Phosphate esterification occurred in the manometer flasks during the equilibration period, before it was possible to follow oxygen uptake. In measuring P/O ratios (μmoles of P esterified/μg.atom of oxygen uptake) it was necessary

* Part 1: Pierpoint (1959).