REFERENCES

- Abrams, R. & Bentley, M. (1955). *J. Amer. chem. Soc.* **77**, 4179.
- Abrams, R. & Bentley, M. (1959). *Arch. Biochem. Biophys.* **79**, 91.
- Atkinson, M. R., Burton, R. M. & Morton, R. K. (1961). *Biochem. J.* **78**, 813.
- Atkinson, M. R., Jackson, J. F., Morton, R. K. & Murray, A. W. (1962). *Nature, Lond.*, **196**, 35.
- Beaven, G. H., Holiday, E. R. & Johnson, E. A. (1955). In *The Nucleic Acids*, vol. 1, p. 493. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
- Dixon, M. (1953). *Biochem. J.* **54**, 457.
- Doerr, I. L., Wempen, I., Clarke, D. A. & Fox, J. J. (1961). *J. org. Chem.* **26**, 3401.
- Fox, J. J., Wempen, I., Hampton, A. & Doerr, I. L. (1958). *J. Amer. chem. Soc.* **80**, 1669.
- Gehring, L. B. & Magasanik, B. (1955). *J. Amer. chem. Soc.* **77**, 4685.
- Hampton, A. (1962). *J. biol. Chem.* **237**, 529.
- Hampton, A. & Maguire, M. H. (1961). *J. Amer. chem. Soc.* **83**, 150.
- Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
- Kaplan, N. O. (1955). In *Methods in Enzymology*, vol. 3, p. 873. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Klouven, H. M. (1962). *J. Chromat.* **7**, 216.
- Lagerkvist, U. (1955). *Acta chem. scand.* **9**, 1028.
- Lagerkvist, U. (1958). *J. biol. Chem.* **233**, 138.
- Lowry, O. H., Bessey, O. A. & Crawford, E. J. (1949). *J. biol. Chem.* **180**, 399.
- McFall, E. & Magasanik, B. (1960). *J. biol. Chem.* **235**, 2103.
- Magasanik, B., Moyed, H. S. & Gehring, L. B. (1957). *J. biol. Chem.* **226**, 339.
- Montgomery, J. A. & Thomas, H. J. (1961). *J. org. Chem.* **26**, 1926.
- Nossal, P. M. (1953). *Aust. J. exp. Biol. med. Sci.* **31**, 583.
- Pabst Laboratories (1961a). *Research Biochemicals Specifications, Circular OR-17*. Milwaukee, U.S.A.
- Pabst Laboratories (1961b). *Ultraviolet Absorption Spectra of Pyridine Nucleotide Coenzymes and Coenzyme Analogs, Circular OR-18*. Milwaukee, U.S.A.
- Paterson, A. R. P. (1959). *Canad. J. Biochem. Physiol.* **37**, 1011.
- Paterson, A. R. P. (1960). *Canad. J. Biochem. Physiol.* **38**, 1117.
- Paterson, A. R. P. (1962). *Canad. J. Biochem. Physiol.* **40**, 195.
- Paterson, A. R. P. & Hori, A. (1962). *Canad. J. Biochem. Physiol.* **40**, 181.
- Salser, J. S., Hutchison, D. J. & Balis, M. E. (1960). *J. biol. Chem.* **235**, 429.
- Schmitz, H., Hart, W. & Ried, H. (1955). *Z. Krebsforsch.* **60**, 301.
- Spector, W. S. (1956). *Handbook of Biological Data*, p. 217. Philadelphia and London: W. B. Saunders Co.
- Turner, J. F. & King, J. E. (1961). *Biochem. J.* **79**, 147.
- Warburg, O. & Christian, W. (1942). *Biochem. Z.* **310**, 384.
- Wilkinson, G. N. (1961). *Biochem. J.* **80**, 324.

Biochem. J. (1963) **89**, 172

The Isolation of *myo*Inositol Pentaphosphates from Hydrolysates of Phytic Acid

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The mixture of inositol phosphoesters produced by the partial dephosphorylation of phytic acid by the enzyme 'phytase' was first investigated by Posternak & Posternak (1929), and later by Courtois (1951), by fractional-precipitation techniques. Subsequently Desjobert & Petek (1956) showed that *myo*inositol polyphosphate intermediates from the phytase reaction, as isolated by the methods of Posternak & Posternak (1929), gave discrete phosphate zones on paper chromatograms. They claimed to be able to separate the hydrolysate into seven zones, corresponding to hexa-, penta-, tetra-, tri-, di- and mono-esters, together with orthophosphate. However, their claim to be able to separate pentaphosphate from hexaphosphate was later

questioned by Preece, Grav & Wadham (1960), who were unable to achieve such a separation on paper chromatograms. The latter workers stated that it was uncertain whether a pentaphosphate had ever been isolated.

The application of ion-exchange chromatography to the separation of inositol phosphates was first attempted by Smith & Clark (1952). Stepwise elution with hydrochloric acid of increasing concentration separated the hydrolysate into a number of components whose phosphorus:inositol ratios were determined. The identity of nine separated compounds, including two pentaphosphates, was tentatively established, but no attempt was made to isolate specimens of any of them for further

study. More recently this method has been applied by Tomlinson & Ballou (1962) who, by gradient elution with lithium chloride solution from a Dowex 1 (Cl^- form) column, separated di-, tri- and tetra-phosphate intermediates, but were unable to resolve the pentaphosphate and the residual phytic acid.

As a result of an examination of commercial 'phytin' preparations (Cosgrove, 1962, 1963), by gradient elution with hydrochloric acid from Dowex 1 (Cl^- form), certain components were obtained that were believed to be myoinositol pentaphosphates. This supposition has now been confirmed.

EXPERIMENTAL

Fractionation by ion-exchange chromatography. This has already been described (Cosgrove, 1963). For the examination of small quantities of material, containing 1–2 mg. of organic phosphate, the following method was used.

The solution, reduced in volume to about 5 ml., was applied to a column (0.2 cm. diam.) filled with Dowex AG 1 (X 8; Cl^- form; 200–400 mesh) to a height of 10 cm. The upper section of the column, above the surface of the resin, was of 1.4 cm. diameter to facilitate the addition of samples for examination. A gradient elution with hydrochloric acid was then carried out with an apparatus of the type described by Parr (1954), the mixer (2.9 cm. diam.) containing 95 ml. of water and the reservoir (2.36 cm. diam.) 60 ml. of 1.5N-hydrochloric acid. The approximate shape of the resulting elution gradient is indicated in Fig. 3. Fractions (2.1 ml.) were collected, at the rate of about 4 ml./hr., and analysed for total phosphorus.

For chromatography with a gradient elution with sodium chloride solution the apparatus described above was used. In place of 1.5N-hydrochloric acid 60 ml. of 1.0M-sodium chloride solution was used.

Barium phytate. Commercial 'calcium inositol hexaphosphate' (Ciba Ltd.) (0.3 g.) was suspended in water (50 ml.) and treated with an excess of Dowex AG 50W (H^+ form). After filtration the solution was made slightly alkaline with ammonia, evaporated *in vacuo* to about 10 ml. and resolved by ion-exchange chromatography on Dowex AG 1 (X 8; Cl^- form) resin.

The appropriate component, corresponding to I/A, (Fig. 1), was isolated as the barium salt (Cosgrove, 1963).

Hydrolysis of phytic acid at pH 4.0. Barium phytate (0.3 g.) was converted into a solution of the free acid by treatment with Dowex AG 50W (H^+ form). The solution was adjusted to pH 4.0 by the addition of 0.5N-sodium hydroxide solution and heated in a sealed tube at 110° for 100 min. The tube was cooled and opened, and the contents were adjusted to pH 7.0 and fractionated by ion-exchange chromatography.

Enzymic hydrolysis of phytic acid. Barium phytate (1.2 g.) was converted into a solution of the free acid which was then made up to 100 ml., and adjusted to pH 5.1 by the addition of 0.5N-sodium hydroxide solution. After the addition of wheat bran (1 g.) and toluene (1 ml.) the solution was incubated at 35° for 16 hr. The bran was filtered off and the solution treated with excess of Dowex AG 50W (H^+ form). After being filtered again the solution was made

alkaline with ammonia and concentrated to 20 ml. by evaporation *in vacuo*. Portions of 5 ml. were then fractionated by ion-exchange chromatography.

Analysis for phosphorus in chromatographic fractions. The method used, based on that of Smith & Clark (1952), has been described by Cosgrove (1963). In the small-scale separation each fraction was collected, evaporated, heated with perchloric acid and analysed in the same Pyrex test tube (6 in. $\times$ $\frac{5}{8}$ in.) throughout.

Determination of phosphorus: inositol ratios. The free phosphate esters, derived from the respective barium salts (50 mg.), were hydrolysed in 6N-hydrochloric acid in sealed tubes at 110° for 40 hr. The tubes were cooled and opened, and the contents were evaporated *in vacuo* almost to dryness at 40°. Removal of the last traces of hydrochloric acid was accomplished in a vacuum desiccator over sodium hydroxide pellets. Each sample was made up to 100 ml. in water and phosphorus determined on a suitable sample by the method of Truog & Meyer (1929).

myoInositol was determined by a modification of the periodic acid-oxidation method used by Böhm & Richarz (1954). The sample was mixed with 20 ml. of approx. 0.01M-periodic acid solution in a 250 ml. iodine flask, and sufficient water was added to bring the volume of liquid to 100 ml. The mixture was incubated at 50° for 2½ hr. and then cooled. The periodic acid remaining unused was determined by the method of Müller & Wegelin (1913).

The reliability of the method was tested by using standard mixtures of myoInositol and sodium phosphate, and ratios found were within 4% of the calculated values. For authentic samples of phytic acid the ratios of phosphorus to inositol tended to be low by up to 6%. However, ratios determined by microbiological methods are likely to be up to 10% in error (Grado & Ballou, 1961). For this reason the periodate method is preferable provided that sufficient material is available and no interfering substances are present.

Acid-catalysed migration of phosphate groups. Conditions under which the pentaphosphate components could be interconverted by the acid-catalysed migration of phosphate groups were studied in the following way.

The barium salt of the material (10 mg.) was converted into a solution of the free ester which was made up to 25 ml. Samples (2.5 ml.) were heated with 2N-hydrochloric acid (2.5 ml.) in sealed tubes at 100° for periods ranging from 5 to 60 min. (Pizer & Ballou, 1959; Tomlinson & Ballou, 1961). The tubes were cooled and opened, and the contents were evaporated *in vacuo* just to dryness at 35°. The residues were taken up in 5 ml. of water and examined by ion-exchange chromatography (small-scale separation).

RESULTS

The results obtained by partial hydrolysis at pH 4.0 of phytic acid from barium phytate (84 mg.), followed by fractionation, are illustrated in Fig. 1. Components I/A (i.e. residual phytic acid), I/B and I/C were isolated as barium salts from this type of fractionation.

A sample of the solution prepared by enzymic dephosphorylation of phytic acid was fractionated in the same way, giving the results shown in Fig. 2.

Material corresponding to component I/B is almost absent in this case. Components II/C and II/D were isolated as barium salts.

The phosphorus : inositol ratios were determined for components I/A (5.8), I/B (4.9), I/C (4.9), II/C

Fig. 1. Elution pattern from a Dowex 1 (Cl^- form) column of phytic acid, after partial hydrolysis at pH 4.0. Experimental details are given in the text. The broken line indicates the HCl gradient.

Fig. 2. Elution pattern from a Dowex 1 (Cl^- form) column of phytic acid, after partial hydrolysis with wheat phytase. Experimental details are given in the text.

Fig. 3. Elution pattern from a Dowex 1 (Cl^- form) column of component I/B (from 1.6 mg. of the barium salt), after partial hydrolysis at pH 4.0. Experimental details are given in the text. The broken line indicates the HCl gradient.

(4.9) and II/D (3.9). Although the results are somewhat less than the theoretical values there is little doubt that component II/D is a tetraphosphate and that components I/B, I/C and II/C are pentaphosphates. Cosgrove (1963) has already referred to the isolation of small quantities of material corresponding to components I/B and I/C. On the basis of their R_f values on paper and electrophoretic mobilities on paper, which in both cases were indistinguishable from phytic acid, these hydrolysis products were tentatively identified as pentaphosphates. The results of the phosphorus : inositol ratio determinations have thus confirmed this supposition.

Component II/C has now been examined by paper chromatography (Desjobert & Petek, 1956) and by paper electrophoresis (Arnold, 1956), and found to be indistinguishable from components I/B and I/C by these means.

The behaviour of components I/B, I/C and phytic acid on an ion-exchange column (small-scale separation) was investigated by using gradient elution with sodium chloride solution. Mixtures of any two, or all three together, were eluted as a single component by this system.

Heating in *N*-hydrochloric acid for 1 hr. transformed 17% of component I/B into material chromatographically indistinguishable from component I/C. Similarly component II/C was transformed into component I/B to the extent of 22%.

The results obtained by partial hydrolysis at pH 4.0 of component I/B are shown in Fig. 3.

DISCUSSION

It is now well established that hydrolysis of phytic acid either chemically or enzymically gives as the penultimate product *myo*inositol 2-phosphate, suggesting that the 2-phosphate group is more resistant to hydrolysis than the others.

Tomlinson & Ballou (1962) have shown that enzymic hydrolysis of phytic acid is initially at position 4. If this is so, then component II/C is *L*-*myo*inositol 1,2,3,5,6-pentaphosphate.

In the absence of evidence to the contrary it must be assumed that hydrolysis by heating at pH 4.0 is likely to be initiated with equal facility at all positions other than position 2. This would give rise to a mixture of *DL*-*myo*inositol 1,2,3,5,6-pentaphosphate, *DL*-*myo*inositol 1,2,4,5,6-pentaphosphate and *myo*inositol 1,2,3,4,6-pentaphosphate. The first of these is presumably present in component I/C but the disposition of the other two, if both are present, is uncertain.

As the acid-catalysed transformations of one component into the other do not readily occur, they are evidently due to migration of a phosphate group across *trans*-oriented vicinal hydroxyl groups (Pizer

& Ballou, 1959; Tomlinson & Ballou, 1961, 1962). Migration of the phosphate groups at either position 3 or position 5 to the vacant position 4 in *L-myo*-inositol 1,2,3,5,6-pentaphosphate (component II/C) would be a transformation of this type. Similar reasons can be deduced for the slow transformation of component I/B into component I/C.

Phosphomonoesters can be hydrolysed at pH 4.0 without appreciable phosphate migration unless there exists a favourable steric disposition of the phosphate and an adjacent hydroxyl group (Baddiley, Buchanan & Letters, 1958; Pizer & Ballou, 1959). Hydrolysis of either *DL-myo*inositol 1,2,4,5,6-pentaphosphate or *myo*inositol 1,2,3,4,6-pentaphosphate would be unlikely to involve simultaneous transformation into *DL-myo*inositol 1,2,3,5,6-pentaphosphate. This is in agreement with the fact that component I/C is absent from hydrolysates of component I/B (Fig. 3).

SUMMARY

1. *myo*Inositol pentaphosphate fractions have been isolated from hydrolysates of phytic acid by gradient-elution chromatography on an anion-exchange resin, Dowex AG 1 (X8). Partial dephosphorylation with wheat phytase liberated only one pentaphosphate component but acid hydrolysis gave rise to two.

2. The structures of these esters have not been established, but possibilities are discussed in the light of certain of their properties.

REFERENCES

- Arnold, P. W. (1956). *Biochim. biophys. Acta*, **19**, 552.
Baddiley, J., Buchanan, J. G. & Letters, R. (1958). *J. chem. Soc.* p. 1000.
Böhm, P. & Richarz, G. (1954). *Hoppe-Seyl. Z.* **298**, 110.
Cosgrove, D. J. (1962). *Nature, Lond.*, **194**, 1265.
Cosgrove, D. J. (1963). *Aust. J. Soil Res.* (in the Press).
Courtois, J. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1075.
Desjobert, A. & Petek, F. (1956). *Bull. Soc. Chim. biol., Paris*, **38**, 871.
Grado, C. & Ballou, C. E. (1961). *J. biol. Chem.* **236**, 54.
Müller, E. & Wegelin, G. (1913). *Z. anal. Chem.* **52**, 755.
Parr, C. W. (1954). *Biochem. J.* **56**, xxvii.
Pizer, F. L. & Ballou, C. E. (1959). *J. Amer. chem. Soc.* **81**, 915.
Posternak, S. & Posternak, T. (1929). *Helv. chim. acta*, **12**, 1165.
Preece, I. A., Grav, H. J. & Wadham, A. T. (1960). *J. Inst. Brew.* **66**, 487.
Smith, D. H. & Clark, F. E. (1952). *Proc. Soil Sci. Soc. Amer.* **16**, 170.
Tomlinson, R. V. & Ballou, C. E. (1961). *J. biol. Chem.* **236**, 1902.
Tomlinson, R. V. & Ballou, C. E. (1962). *Biochemistry*, **1**, 166.
Truog, E. & Meyer, A. H. (1929). *Industr. Engng Chem. (Anal.)* **1**, 136.