SYNTHESIS OF THE HEXAPHOSPHATES OF MYO-, SCYLLO-, NEO- AND D-INOSITOL

By D. J. COSGROVE

Myoinositol hexaphosphate has been prepared by Posternak's method and purified from lower phosphate esters by ion-exchange chromatography. The product behaved differently from the natural hexaphosphate when used as a substrate for a plant phytase preparation. By modifying Posternak's original method it has been found possible to prepare a synthetic hexaphosphate which is hydrolysed at the same rate as its natural counterpart. The hexaphosphates of scyllo-, neo- and D-inositol have been prepared by the modified method and have been compared as substrates in phytase hydrolysis experiments.

Introduction

Plant phytin, a calcium–magnesium salt of myoinositol hexaphosphate, differs from similar material extractable from soil organic matter. Phytate from the latter source is a mixture of polyphosphates, mainly hexaphosphates, of DL-, scyllo-, neo- and myoinositol. Pure myoinositol hexaphosphate can be obtained easily from plant sources and freed from inorganic phosphate and lower esters by ion-exchange chromatography.[1] Soil organic matter is the only known source of the other inositol hexaphosphates; for this reason the preparation of pure synthetic materials is desirable.

Posternak[2] claimed to have prepared synthetic myoinositol hexaphosphate by phosphorylation of inositol with a mixture of phosphoric acid and phosphorus pentoxide. He separated the hexaphosphate from the resulting mixture of phosphate esters by fractional precipitation of the barium salts. Anderson[3] could not repeat this work and concluded that no ester higher than a tetraphosphate was formed by this reaction. This result confirmed his previous work.[4] Posternak[5] later defended his own method and appeared convinced that his product, obtained as a crystalline calcium–sodium salt, was identical with natural myoinositol hexaphosphate.

Fowler[6] has recently repeated Posternak's work but he was unable to prepare any crystalline salts of the hexaphosphate. He examined his product by paper chromatography and showed that it was probably a mixture of tetra-, penta- and hexaphosphate.

This paper describes a modification of Posternak's original method for the phosphorylation of myoinositol. The product after removal of lower phosphate esters by ion-exchange chromatography, was found to be identical with natural myoinositol hexaphosphate in its behaviour as a substrate for a wheat-bran phytase preparation. The hexaphosphates of D-, neo- and scylloinositol have been synthesised in the same way.

Experimental

Inositols

D-Inositol was obtained by demethylation of (+)-pinitol, neoinositol by the method of Angyal & Matheson[7]. Scylloinositol was prepared by Weissbach's[8] modification of Reymond's[9] method. Maximum yield of the diborate complex of scylloinositol was obtained by setting aside the inosose reduction-products for 60 h, not 24–30 h as recommended by Weissbach.

Phosphorylation of myoinositol

Method A

This was based on Posternak's[2] method. Orthophosphoric acid (90%; 3·5 g) was weighed into a small glass-stoppered flask and warmed on a steam bath. Phosphoric oxide (3·5 g) was added in portions, the flask being warmed and slowly rotated between each addition. When the contents of the flask were homogeneous, it was heated at 120° for 2 h. (The equivalent amount of commercial polyphosphoric acid may be used instead of the above.) The flask was cooled to room temperature and finely-powdered inositol (0·4 g) was added in portions and stirred into the polyphosphoric acid. The mixture was heated at 120° for 3 h, cooled and the resulting gum transferred in portions into 42 ml of 5N-sodium hydroxide solution. The mixture was heated on a steam bath in a porcelain basin until the weight of the solution was reduced to 42 g. The solution was then kept in the cold (5°) for 16 h and the crystals of sodium pyrophosphate formed were filtered off on a Buchner funnel, under vacuum, and washed with 5 ml of icewater. The filtrate was further evaporated until reduced to 15 g and left to crystallise as before. The crystals were again filtered off and washed with 3 ml of ice-water. The filtrate, about 10 ml, was transferred to a centrifuge tube and an equal volume of ethanol (95%) added. The mixture was shaken vigorously and kept in the cold as before for 3 h. The resulting gummy solid was recovered by centrifuging, washed with ethanol (50%; 20 ml) and dissolved in 6N-hydrochloric acid (15 ml). The solution was heated on a boiling water-bath for 1 h, cooled, adjusted to 0·5N in hydrochloric acid by the addition of an appropriate volume of 2N-sodium hydroxide solution, and ferric chloride solution (60%; w/v) added until no further precipitation took place. The precipitate was separated by centrifuging, washed with 0·5N-hydrochloric acid and water, and converted to the sodium salt by action of excess sodium hydroxide solution. By addition of barium acetate solution the barium derivative was precipitated. This was washed with water, ethanol and finally ether. It was then dried *in vacuo*. The product was phosphorylated myoinositol's barium salt (1·1 g).

The above quantities are the minimum that can be conveniently handled in this preparation.

Method B

This was based on the method used by Seegmiller & Horecker[10] for the phosphorylation of glucose and gluconic acid. Finely-powdered myoinositol (1·0 g) was mixed with water (1·2 ml) and polyphosphoric acid (80% P_2O_5; 17·5 g) added. The contents were mixed well and the glass-stoppered flask was left at room temperature for 60 h. The product was added to 5N-sodium hydroxide (105 ml) and worked up to a barium derivative as in method A (yield 3·1 g).

Method C

Polyphosphoric acid (80% P_2O_5; 17·5 g) was weighed into a glass-stoppered conical flask and heated to 150°. Finely-powdered myoinositol (1·0 g) was added and mixed well into the acid. The flask was returned to the oven and left at 150° for 6 h. The contents of the flask were cooled and then added to 5N-sodium hydroxide (105 ml) and worked up to the barium drivative as in Method A (yield 3·2 g). In a similar manner were prepared in the yield stated, (phosphorylated D-inositol barium salt (3·0 g); phosphorylated neoinositol barium salt (1·7 g); phosphorylated scylloinositol barium salt (2·4 g)).

Barium myoinositol hexaphosphate (natural)

Commercial calcium phytate (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 resolved by ion-exchange chromatography on Dowex AGI (X2; Cl form; 200–400 mesh) resin. The appropriate component (Fig. 2), was isolated as the barium salt[11] (yield 0·25 g).

Barium myoinositol hexaphosphate (synthetic, method A)

This was isolated from the phosphorylated inositol (0·3 g) by the method described in the previous paragraph (yield 0·12 g).

Barium inositol hexaphosphates (synthetic, Method C)

These were isolated from the phosphorylated inositols (0·3 g) by the method referred to in the previous paragraph. (Yields were: barium myoinositol hexaphosphate, 0·11 g,; barium D-inositol hexaphosphate 0·10 g; barium neoinositol hexaphosphate, 0·10 g; barium scylloinositol hexaphosphate, 0·11 g).

Wheat-bran phytase preparation

The method used was based on that described by Nagai & Funahashi,[12] the product being essentially similar to their Fraction II.

Wheat bran (200 g) was extracted with water (1000 ml) at 5° for 16 h and the mixture filtered through butter muslin. The filtrate was centrifuged (12,000 *g*) for 30 min. at 5° and the liquid (620 ml) retained. Ammonium sulphate (30 g/100 ml) was stirred in at room temperature and the resulting precipitate allowed to settle for 1 h. The precipitate (FI) was collected by centrifuging. The solution was saturated with ammonium sulphate, set aside for 1 h and the precipitate (FII) collected by centrifuging. FI and FII were redissolved in water (100 ml) and dialysed at 5° against several changes of distilled water; the residual solution from the ammonium sulphate fractionation was dialysed in the same way. The dialysed solutions were tested for phytase activity; most of the activity was found in FII. This fraction was centrifuged to remove insoluble matter and the solution was freeze-dried (2·0 g).

The phytase solution used in the hydrolysis experiments was made by mixing 0·1 g of the preparation with 40 ml of water. After storage for 16 h at 5° the solution was centrifuged and the clear supernatant made up to 50 ml.

Ion-exchange chromatography, paper chromatography, analysis for phosphorus

The methods used have been described elsewhere.[1,11]

Enzymic hydrolysis of hexaphosphates

The barium salt of the hexaphosphate (0·074 g) was suspended in water (15 ml) and shaken for 4 h with excess Dowex AG-50W (H^+ form). The resin was removed by filtration and the solution adjusted to pH 7·0 by addition of 0·5 N-sodium hydroxide. The hexaphosphate solution was made up to 50 ml and total phosphorus determined on an aliquot. The progress of hydrolysis was studied at 40° using a mixture of 5 ml of the hexaphosphate solution, 1·0 ml of enzyme solution, 4 ml of 0·5 M-sodium acetate buffer solution (pH 5·2) and 10 ml of water.[12] At intervals of 15 min., 1·0-ml portions were withdrawn and immediately added to a mixture of 0·1 ml of perchloric acid (70%) and 4·0 ml of water. These solutions were analysed for free orthophosphate.[1]

Enzymic hydrolysis, larger scale

The comparison of a partial hydrolysate of natural myoinositol hexaphosphate with similar material prepared from a synthetic sample (Method A), was made in the following way. The barium salt of the hexaphosphate (0·037 g) was suspended in water (10 ml) and treated with excess Dowex AG-50W (H^+ form) resin as before. The phosphate solution (15 ml; pH 7·0) was mixed with enzyme solution (5 ml) and buffer solution (5 ml) and incubated at 40° for 30 min. in the case of the natural ester, and 45 min. in the case of the synthetic ester. After incubation, each was heated at 100° for 5 min., the mixture cooled, centrifuged (12,000 *g*), made alkaline (pH 9·0) with ammonia solution and evaporated to small volume. The products were examined by ion-exchange chromatography on a column (10 cm × 1·2 cm dia.) of Dowex AG-1 (X2; Cl^- form; 200–400 mesh) resin.[1,11]

Results

Synthetic barium phytate (Method A; 0·050 g) was converted to the free acid and fractionated, by gradient elution with hydrochloric acid, on a column (10 cm × 1·2 cm dia.) of Dowex AG-1 (X2; Cl^- form; 200–400 mesh) resin.[1,11] The results are shown in Fig. 1. Results obtained in a similar experiment with a commercial sample of barium phytate (0·050 g) are shown in Fig. 2.

Progress curves for the hydrolysis of natural and synthetic myoinositol hexaphosphate (Method A) are shown in Fig. 3 and curves for synthetic myo-, D-, neo- and scylloinositol hexaphosphate (Method C) are in Fig. 4.

An attempt to fractionate synthetic barium phytate (Method B) by ion-exchange chromatography was not successful. Phosphate was eluted from the column in an irregular manner over the whole period of the experiment, that is, up to an acid strength of about 1M. Synthetic barium phytate (Method C) was fractionated by ion-exchange chromatography and gave an elution pattern very similar to that

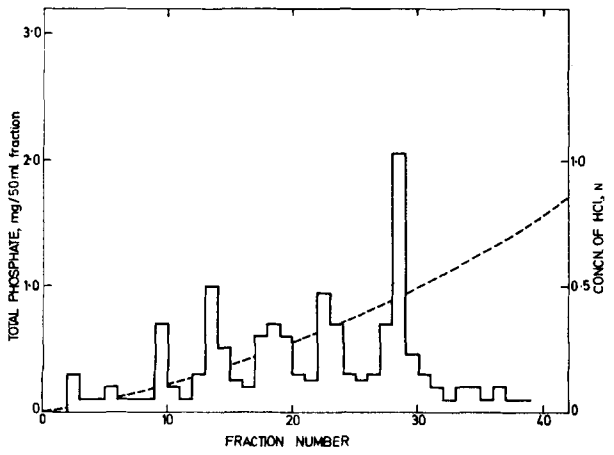

FIG. 1. *Elution pattern from a Dowex-1 (Cl^- form) column of phosphorylated myoinositol (Method A)*
(0·050 g of the barium salt used. The broken line indicates the HCl gradient)

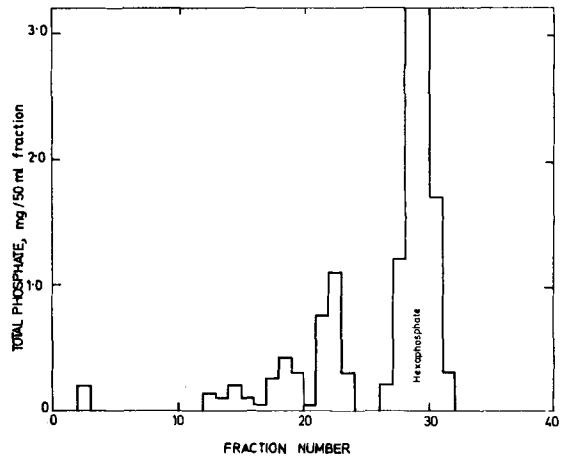

FIG. 2. *Elution pattern from a Dowex-1 (Cl^- form) column of natural phytic acid*
(0·050 g of the barium salt used)

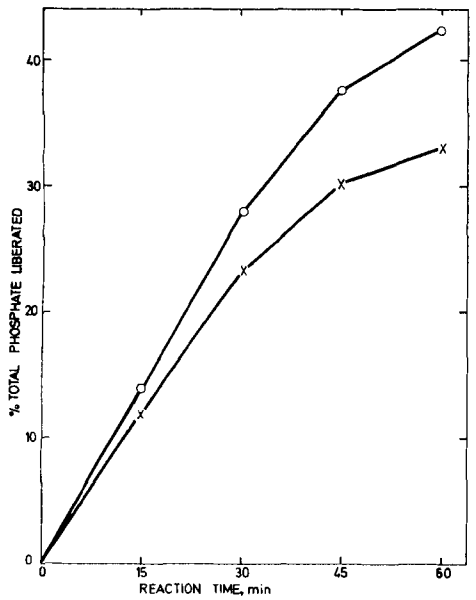

FIG. 3. *Progress curves for the dephosphorylation of natural,* (o) *and synthetic* (x) *myoinositol hexaphosphate (Method A)*

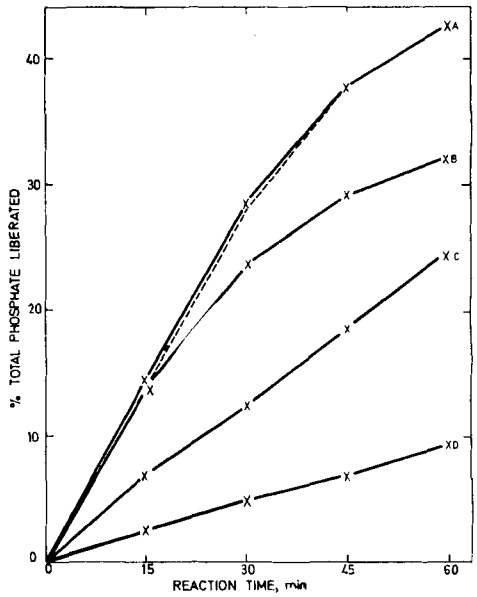

FIG. 4. *Progress curves for the dephosphorylation of synthetic, hexaphosphates (Method C)*
A. myoinositol B. neoinositol
C. D-inositol D. scylloinositol
(The broken line is the curve for natural myoinositol hexaphosphate, shown where it differs from Curve A)

shown by commercial (natural) barium phytate (Fig. 2). Products obtained by phosphorylation (Method C) of D-, neo- and scylloinositol were examined in the same way. Each product consisted of a mixture of phosphate esters that was clearly resolved by chromatographic fractionation. In each case the last component to be eluted from the column was the main constituent of the mixture.

Discussion

The results shown in Figs 1 and 2 are similar in the sense that each sample of barium phytate is made up of a number of phosphate-containing components. The last to be eluted from the column is, in each case, the main constituent. This is known to be the hexaphosphate in the case of natural barium phytate.[1] The synthetic product (Fig. 1) contains a high proportion of lower esters, whereas the natural material is mainly the hexaphosphate.

The lack of agreement between the progress curves in Fig. 3 shows that the hexaphosphate fraction prepared from synthetic barium phytate (Method A) behaves differently from its natural counterpart when used as a substrate for a wheat-bran phytase preparation. When the products of a larger scale enzymic hydrolysis of each hexaphosphate were examined by ion-exchange chromatography, however, the elution patterns were found to be qualitatively similar (See ref.,[1] Fig. 2).

Figs 1 and 2 are not identical in all respects. The separation shown in Fig. 1 is less sharp, the components are not as well

separated from one another. Again, in Fig. 1 phosphorus is shown to be continually eluted from the column long after the hexaphosphate fraction has been collected. Fractions 34–40 from this separation were combined, evaporated to 5 ml and heated in a sealed tube of 110° for 40 h. Paper chromatography[11] of the hydrolysate showed the presence of myoinositol. The nature of the material in these later fractions was not investigated further. It is likely that similar material is eluted throughout the ion-exchange chromatographic separation; its presence in the hexaphosphate fraction from synthetic phytate (Method A) could account for the lack of agreement between the progress curves shown in Fig. 3.

The conditions used in Method B have evidently favoured side-reactions at the expense of the production of the expected range of inositol phosphate esters. The latter may be present in the products from Method B but it is impossible to resolve them by the ion-exchange methods used in this investigation.

The elution pattern obtained by ion-exchange chromatography of the product from Method C was very similar to that given by commercial (natural) barium phytate (Fig. 2) although the proportion of lower esters present was greater. The phosphate-containing components were clearly separated and no phosphate was eluted after fraction number 32 had been collected.

Progress curves for the enzymic hydrolysis of natural barium myoinositol hexaphosphate and its synthetic counterpart (Method C) are shown in Fig. 4. They are virtually identical. No attempt was made to compare natural with synthetic samples of the hexaphosphate by elementary analysis. Phosphate analyses on barium derivatives of the natural product were often made during the progress of this investigation and appreciable variations between samples were found. No crystalline derivatives of definitely-established composition are known—a crystalline sodium salt can be obtained with difficulty, but its composition and degree of hydration are uncertain. The results of phosphorus/inositol ratio determinations are not sufficiently precise[1,13] to be useful in deciding whether a sample of an inositol hexaphosphate is pure or not.

Until better criteria are available, it is reasonable to assess the identity and purity of a synthetic sample of barium myoinositol hexaphosphate by comparison with the natural counterpart of its behaviour on ion-exchange resin columns and as a substrate for phytase preparations.

The results of the ion-exchange chromatography of phosphorylation products (Method C) of D-, neo-, and scylloinositol can usefully be compared with the elution diagram of a soil phytate preparation.[11,14] In the case of D-inositol esters, the main constituent was found to be chromatographically similar to myoinositol hexaphosphate. This was an expected result as the main constituent of soil phytate is an inseparable mixture of DL- and myoinositol hexaphosphate.[11] The main constituent of the neoinositol phosphate mixture was eluted more easily, again in keeping with the observation that the highest naturally-occurring phosphate of neoinositol is inseparable from the myoinositol pentaphosphates present in soil phytate.[14] The most highly-phosphorylated product in the scylloinositol phosphate mixture was chromatographically identical with the natural hexaphosphate.[11]

Under the conditions chosen for the hydrolysis-rate measurements, the progress curves (Fig. 4) are of two types. In the case of the myoinositol ester the velocity of the reaction begins to fall after 30 min.; the neoinositol ester is hydrolysed as rapidly for 15 min. but the reaction velocity falls with time after this. D-Inositol hexaphosphate is hydrolysed more slowly, but the velocity remains almost constant over the period studied; scylloinositol has a similar progress curve but is even more resistant to hydrolysis.

Myoinositol hexaphosphate is hydrolysed most readily, scylloinositol hexaphosphate is most resistant, but the parent inositols differ in structure only at position C-2 (Fig. 5). The lack of an axial group in the scylloinositol ester evidently prevents the phytase enzyme from attacking this molecule by the preferred mode of operation, i.e., that used to deal with myoinositol hexaphosphate. It is of interest to recall that the 2-phosphate group in myoinositol hexaphosphate is the most resistant; the major penultimate product of enzymic hydrolysis is myoinositol 2-phosphate.[15]

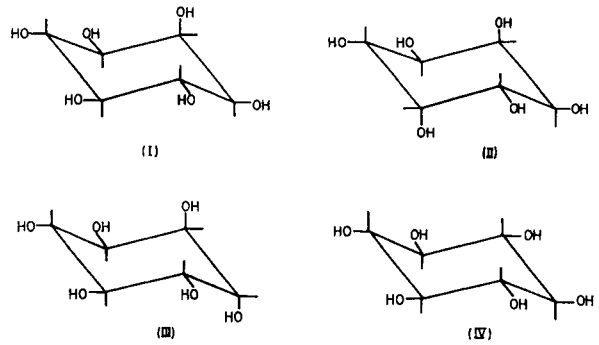

FIG. 5. *Conformation formulas for inositols*
I. myo II. neo III. D IV. scyllo

Neoinositol hexaphosphate is initially hydrolysed as rapidly as the myoinositol ester, but the velocity falls after about 15 min. (Fig. 4). This corresponds to a degree of dephosphorylation of about 15%, at which stage an appreciable proportion of the substrate will be pentaphosphate. The velocity differences in the later stages of hydrolysis may be due to differences between the ease of hydrolysis of myoinositol pentaphosphate and that of the corresponding neoinositol ester. There is evidence[15] that enzymic hydrolysis of myoinositol hexaphosphate is initially at position C-4—in both neoinositol and myoinositol this group is *trans* to the trio of *cis*-groups at C-1, C-2 and C-3 (Fig. 5).

The availability of synthetic counterparts of all the naturally-occurring inositol hexaphosphates makes possible studies such as their relative rates of hydrolysis by phytase preparations from soil organisms. Such investigations are at present being undertaken.

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SOLUBLE OXALATES, ASCORBIC AND OTHER CONSTITUENTS OF RHUBARB VARIETIES

By R. M. ALLISON*

(1) The petioles of ten commercial lines of rhubarb have been analysed for dry matter, ascorbic acid, titratable acidity, soluble and insoluble oxalate and soluble carbohydrate at three times during the 1958–59 harvest season.
(2) Strong varietal characteristics were noted for acidity, oxalates and soluble carbohydrates.
(3) Acidity and oxalate content were strongly influenced by the harvest date.
(4) Ascorbic acid content while influenced by variety did not behave consistently with respect to harvest date.
(5) The distribution of acid function with maturity and senescence is discussed and merit evaluations on the varieties attempted.

Introduction

The petiole of the cultivated rhubarb, *Rheum rhaponticum* L., is widely used as an acid fruit for cooked desserts, and as a filler in the jam-making industry. The sweetening of such preparations masks their acidity, which, when caused in part by oxalic acid may present a health hazard to growing children, nursing mothers, and others whose calcium balance may be precarious.[1] Lovelace *et al.*,[2] using rats, showed that addition of soluble oxalates to their diet produced a stoicheiometric immobilisation of calcium. However, rhubarb is well established in the human diet and may supply ascorbic acid at times when other natural sources are insufficient. Substantial oxalate and ascorbic acid differences have been established between varieties.[3,4] The amounts of these constituents present in locally established varieties should be known. The work reported here extends that of Blundstone & Dickinson[3] who did not report constituents other than organic acids and were mainly concerned with the corrosion of cans used in the food preservation industry.

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Materials and methods

Clones of ten named varieties (Table I) collected from commercial growers in New Zealand were grown at Lincoln in a randomised block layout with four replications. Plots consisting of four plants were established in 1956. Since the effect of light on the accumulation of acid in rhubarb is well established, each harvest was made in the morning on bright days, the first harvest being made as soon as marketable petioles could be obtained from all varieties in 1958. Harvest dates are shown in Table I.

Sampling method

Samples from the first harvest were obtained from each plant in each replication and each replication was analysed separately. Samples from later harvests were drawn from the bulked petioles (one from each plant) from all four replications. Each sample of petioles was deep-frozen in a polythene bag within half an hour of being pulled. For chemical analysis the central third was removed from each of the frozen petioles, and cut into 1-cm sections. The accumulated sections were mixed thoroughly and subsampled by quartering