Complete Characterization of the Myo-inositol Polyphosphates
from Beef Brain Phosphoinositide*

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The identification of a myo-inositol-containing phospholipid isolated from beef brain (1) as a triphosphoinositide has been reported in earlier publications from this laboratory (2) and in a note by Dittmer and Dawson (3). It has been established (2) by periodate oxidation that the myo-inositol moiety in the intact triphosphoinositide is substituted on the 1, 4, and 5 or 6 hydroxyl groups. When the phospholipid was hydrolyzed in base, several polyphosphate esters of myo-inositol were obtained (2). The mixture contained two triphosphates, two diphosphates, and one monophosphate. The major triphosphate ester was established as being L-myo-inositol 1,4,5 (or 6)-triphosphate, whereas it was shown that one of the diphosphates was either L-myo-inositol 4,5- or 1,6-diphosphate. The monophosphate was identical in properties, with the exception of the infrared spectrum, with synthetic n-myo-inositol 4-phosphate. Since it was optically active, the conclusion was that it must be one of the pure optical isomers of myo-inositol 4-phosphate.

We have also pointed out the interesting fact that the absolute configuration of the L-myo-inositol 1,4,5(6)-triphosphate is the same as that found for soybean monophosphoinositide (4, 5) and more recently for a monophosphoinositide from horse liver (6).

This paper presents further studies on the structures of the myo-inositol phosphate components from the brain phosphoinositide, and describes their complete characterization. The partially characterized di- and triphosphate esters obtained by base hydrolysis of the brain phosphoinositide now have been shown to be L-myo-inositol 4,5-diphosphate and L-myo-inositol 1,4,5-triphosphate. The two components whose structures were not elaborated in the earlier reports are shown to be myo-inositol 1,4-diphosphate and myo-inositol 2,4,5-triphosphate. The close structural relationships of all of these compounds suggest an interrelated biochemical role.

EXPERIMENTAL PROCEDURE

Analytical Methods—The method of Fiske and SubbaRow (7) was used for the determination of phosphorus. Myo-inositol assays were done microbiologically by the method of Atkins, Williams, Shults and Frey (5). Klebsiella brevis was used as the test organism rather than Saccharomyces carlsbergensis. Measurements of optical rotations were made on the cyclohexylamine salts in alkaline aqueous solution, with a Rudolph photoelectric polarimeter. Infrared spectra were determined in potassium bromide pellets with a Baird-Atomic infrared model 4-55 spectrophotometer.

Chromatography—Chromatographic solvents and spray developers were varied according to the compounds under examination. Myo-inositol mono- and polyphosphates were chromatographed on Whatman No. 1 filter paper in the solvent of Markham and Smith (9), 70 ml of isopropanol, 10 ml of NH₂OH, 20 ml of water, the descending technique at 30°. The monophosphate esters, which were well separated in 4 to 6 days, were located by the AgNO₃-NaOH dip of Anet and Reynolds (10), whereas the polyphosphate esters, which required 6 to 8 days development for good resolution, were detected by the molybdate spray of Axelrod and Bandurski (11) chromatography of the sugar polyols, which resulted from the periodate oxidation sequence, was carried out as described by Grado and Ballou (2).

Electrophoresis—This technique was used as a supplement to the chromatographic system (3) for identifying polyols. An aqueous solution of the unknown was subjected to paper ionophoresis in a water-cooled apparatus (12). It was run on Whatman No. 3MM paper along with authentic standard polyols in saturated aqueous boric acid adjusted to pH 6.1 with sodium hydroxide solution. The ionophoretogram was developed for 1½ to 2 hours at 2000 volts and a current of 15 ma. The polyols were detected with the periodate-benzidine spray (13).

Preparative Isolation of Myo-inositol Phosphate Isomers—This was accomplished by combining ion exchange and paper chromatography according to Grado and Ballou (2).

Characterization of Myo-inositol Polyphosphate Estersex—Characterization was achieved by the combined application of four general techniques: (a) periodate oxidation of the compound followed by suitable reactions to obtain a distinctive polyol; (b) partial dephosphorylation of the esters by alkaline hydrolysis to obtain a mixture of their component myo-inositol monophosphates; (c) comparison of the susceptibility of the isomers to phosphate migration under acid conditions; and (d) selective degradation of the triphosphate isomer to diphosphate compounds through the action of an alkaline phosphomonoesterase.

Periodate Oxidation, Reduction, and Dephosphorylation—Between 4 and 40 μmoles of myo-inositol phosphate was dissolved in 1 to 2 ml of 0.1 m sodium periodate. The reaction, which was left at room temperature, was followed by the change in absorbancy at 260 μ which the solution was diluted to 3.0 ml with water. When periodate consumption had ceased, 100 mg of sodium borohydride were added and the mixture was allowed to stand for 5 to 8 hours. Destruction of the excess borohydride was accomplished by reducing the pH...
to 2 with 1 N HCl. After hydrogen evolution had ceased, the pH was adjusted to 9.2 with 1 N NaOH. The total volume was increased to 15 ml with distilled water and 1 mg of MgCl₂ was added in addition to 5 mg of Armour's alkaline intestinal phosphomonoesterase. Both inorganic and total phosphate determinations were made on 0.2 ml aliquots at this stage. The solution was then incubated at 40° until all of the phosphate had been liberated from the polyol phosphate. The solution was concentrated to dryness under vacuum, and the residue was distilled several times with 1% methanolic HCl to remove the boric acid. The residue was again dissolved in water and deionized by a batchwise treatment with a mixed-bed ion exchange resin. The filtrate, after removal of the resin, was concentrated and the polyols present were identified by chromatography (2) and electrophoresis (12).

Partial Dephosphorylation of Polyporphosphate Esters by Alkali—The optimal conditions for partial dephosphorylation of polyphosphate esters by this technique were determined empirically. A 1 to 2 μmole quantity of the ammonium salt of myo-inositol di- or triphosphate gave satisfactory chromatograms on which the myo-inositol monophosphates could be detected with the AgNO₃-NaOH dip technique. An aqueous solution of the compound was placed in a small glass tube (0.5 X 12 cm) and the solution was concentrated to dryness at 90° in an oven. The tube was then cooled, and after 0.4 ml of 10 N NH₄OH had been added, it was sealed and heated at 110°. For diphosphate isomers, 18 hours at this temperature was satisfactory, whereas the triphosphates were heated for 24 hours. After the heating period, the tubes were cooled and opened, and the contents were chromatographed directly without further treatment. Half of the solution, 0.15 to 0.2 ml, was applied to each of two chromatograms. One chromatogram was developed for 3 days, whereas the other was allowed to run for 6 days to achieve maximal resolution of the monophosphate esters.

Acid-catalyzed Phosphate Migration—The conditions for these experiments were chosen on the basis of the results from migration studies with myo-inositol monophosphates. Migration of the 1- and 2-phosphate esters (across cis hydroxyl groups) was apparent after 5 to 10 minutes of heating at 100° in 1 N HCl; 20 minutes of heating caused migration across trans-oriented hydroxyls, i.e., onto position 4. Comparative lability among polyphosphate esters was studied in the following way. About 1 to 2 μmoles of each of the two isomers under examination was dissolved in 0.25 ml of water. After samples had been removed at this stage, the aqueous solutions were put in a water bath at 100° and an equal volume of hot 2 N HCl was added. Samples for chromatography were removed after 5, 10, and 20 minutes. These samples were all chromatographed in a comparative manner with the isopropanol-NH₄OH-water solvent system (9). A variation of the technique was used to distinguish between two triphosphate isomers, both of which underwent migration. From 3 to 5 μmoles of compound were heated for 10 minutes in 1 N HCl after which the solution was immediately neutralized and evaporated to dryness under vacuum. The acid-treated compound was oxidized with sodium periodate and the polyol products were compared to those obtained from the sample before acid treatment.

Partial Dephosphorylation of Triphosphate Isomers by Alkaline Phosphomonoesterase—The myo-inositol triphosphate sample was converted to its ammonium salt. The salt was dissolved in an appropriate amount of water to yield a concentration of 0.75 to 1.0 mm, and the pH of the solution was adjusted to 9 by the addition of concentrated NH₄OH. The only further addition was alkaline intestinal phosphomonoesterase (2 to 4 mg). No Mg²⁺ ion was added. The mixture was incubated at 40° and the liberation of phosphate was determined in 0.2-ml aliquots of the solution. When these tests indicated that one-third of the phosphate had been released, the enzyme was inactivated by boiling the incubation mixture and subsequently evaporating it to dryness under vacuum. The results were assessed by chromatography.

RESULTS AND DISCUSSION

Polyphosphate esters of myo-inositol can be characterized by their responses to a series of tests. The four tests which we have developed exploit some unique property of each ester. Their uses are discussed below.

Periodate Oxidation Sequence—Periodate oxidation of a myo-inositol polyphosphate ester, followed by reduction of the resulting dialdehyde and dephosphorylation of the polyol polyphosphate ester, will yield a polyol, if the starting compound possesses free vicinal hydroxyl groups. The polyol formed is a function of the number of phosphate groups and their positions on the myo-inositol ring. Myo-inositol monophosphates are completely oxidized by periodate with the formation of inorganic phosphate, formic acid, and probably carbon dioxide (14-16). We have found that a myo-inositol p-diphosphate reacts in a similar manner (Table I). α-Diphosphates yield a tetritol (2) and m-diphosphates yield a pentitol. Myo-inositol triphosphates will yield either a pentitol or hexitol by the reaction se-

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**TABLE I**

Comparison of periodate consumption and phosphate liberation between component IIb and myo-inositol 1,4-diphosphate

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sodium periodate consumed (μmol/mole of compound)</th>
<th>Phosphate liberated (μmol/mole of compound)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(±) Myo-inositol 1,4-diphosphate</td>
<td>3.7</td>
<td>8.0</td>
</tr>
<tr>
<td>Component IIb</td>
<td>4.2</td>
<td>8.1</td>
</tr>
</tbody>
</table>

* Kindly supplied by Dr. S. J. Angyal, University of New South Wales, Sydney, Australia.

**TABLE II**

Polysols from periodate oxidation, reduction, and dephosphorylation of myo-inositol polyphosphates

<table>
<thead>
<tr>
<th>Polysol</th>
<th>Component IIa</th>
<th>Component IIb</th>
<th>Component IIIa</th>
<th>Component IIIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Threitol*</td>
<td>Threitol</td>
<td>Sorbitol</td>
<td>Iditol</td>
<td>d-Iditol(2)</td>
</tr>
</tbody>
</table>

* Described previously (2) and confirmed herein.
† See Table I.

1 R. V. Tomlinson and C. E. Ballou, unpublished observations.
find that extrapolation to myo-inositol polyphosphates is valid due to the slowness of the initial attack compared to the rate of decomposition in acid and yield new isomers (Table III). By contrast, those isomers not having a phosphate group on either of these two positions show a greater relative stability under the same conditions, although migration can be made to occur even across vicinal trans groups. The products of periodate oxidation after acid migration under mild conditions can be used to distinguish between two triphosphate isomers, both of which have one of the phosphate groups on positions 1 or 2 but which differ in other ways. For example, structures A and B would give

\[
\begin{align*}
\text{A} & : \quad \text{Iditol, Sorbitol, Xylitol} \\
\text{B} & : \quad \text{Myo-inositol} 
\end{align*}
\]

\[\text{PO}_1 \text{PO}_2 \text{PO}_3\]

\[\text{PO}_1 \text{PO}_2 \text{PO}_3\]

...
The myo-inositol-containing hydrolysis products of the brain phosphoinositide used in this study showed the same ion exchange column elution pattern and chromatographic characteristics as those described in the earlier paper (2) (Fig. 1).

Component IIb from the mixture of products is known to be an asymmetrically substituted myo-inositol diphosphate, since its phosphorus to myo-inositol ratio was 2 and it had a specific rotation of +3.3° (2). When oxidized with periodate, it gave inorganic phosphate in 90% yield (Table I). This response is characteristic of monophosphate esters of myo-inositol, and in the case of a diphosphate it requires a para orientation of the phosphate groups. Only myo-inositol 1,4-diphosphate could possess both para orientation and optical activity. This structure for IIb was supported by the results of both alkaline hydrolysis and acid migration. Partial hydrolysis by alkali yielded only myo-inositol 1-phosphate and myo-inositol 4-phosphate (Table IV), whereas mild treatment with acid converted it in part to two new isomers (Fig. 2 and Table III). The ease with which this migration was accomplished is characteristic of a phosphate group situated on positions 1 or 2 of myo-inositol, but not of one on positions 4 or 5. The characterization was confirmed by a comparison of component IIb with authentic synthetic (±)-myo-inositol 1,4-diphosphate, with which it was identical both in chromatographic properties and in infrared spectrum. The compounds showed the following characteristic peaks expressed in cm⁻¹: synthetic 1,4-diphosphate, 955 (strong), 940 (shoulder), 860 (medium), 735 (medium), 685 (medium); component IIb, 967 (strong), 940 (shoulder), 855 (medium), 735 (medium), 680 (medium).

Component IIa also has a phosphorus to myo-inositol ratio of 2 and is optically active. The product of periodate oxidation, reduction, and dephosphorylation has been shown to be β-threitol (2). Only two myo-inositol diphosphates could give this polyol; namely, the 4,5- and the 1,6-diphosphate, or a mixture of the two. The possibility that IIa was a mixture of these two isomers was excluded when we were able to synthesize a second chromatographically distinct, threitol-yielding diphosphate ester of myo-inositol. When these two isomers were subjected to partial hydrolysis by alkali, component IIa yielded myo-inositol 4- and 5-monophosphate, whereas the synthetic compound gave myo-inositol 1-phosphate and myo-inositol 4-(or 6)-phosphate (Table IV). These results are consistent with their relative susceptibility to acid migration. Compound IIa was more resistant to acid migration than was the synthetic myo-inositol 1,6-diphosphate. By virtue of its chromatographic distinction from myo-inositol 1,6-diphosphate, its periodate oxidation product (β-threitol), its partial degradation products (myo-inositol 4-phosphate and myo-inositol 5-phosphate), and its relative
resistance to acid migration, component IIa must be L-myo-inositol 4,5-diphosphate.

As is to be expected, both the synthetic myo-inositol 1,6-diphosphate and synthetic myo-inositol 1,4-diphosphate gave the same products on base hydrolysis (Table IV) and the same isomers as a result of acid migration (Fig. 2). However, they differ characteristically in their response to periodate oxidation (Tables I and II).

Component IIIb of the hydrolysis mixture was shown previously to have a phosphorus-myo-inositol ratio of 3 and a specific rotation of -27.4°, and to yield n-iditol on periodate oxidation. Thus, component IIIb yielded component IIa, which we have shown is myo-inositol 4,5-diphosphate. Therefore, component IIIb must be L-myo-inositol 1,4,5-triphosphate. This structural assignment was confirmed by the nature of the products of partial alkaline hydrolysis, myo-inositol 1-phosphate, myo-inositol 4-phosphate, and myo-inositol 5-phosphate (Table IV).

Component IIIa of the hydrolysis mixture had a phosphorus-myo-inositol ratio of 3 and a specific rotation of -15.3° (2). The periodate oxidation product of this compound was converted to sorbitol, a result compatible with only a 2,3,5- or a 2,4,5-triphosphate ester of myo-inositol. Partial dephosphorylation with a phosphomonoesterase yielded a diphosphate that was chromatographically identical with component IIa. This diphosphate from component IIIa gave threitol by the periodate oxidation sequence, and can only be myo-inositol 4,5-diphosphate. Therefore, component IIIa must be L-myo-inositol 2,4,5-triphosphate. The products of partial hydrolysis by alkali support this structural assignment. They are myo-inositol 2-phosphate, myo-inositol 4-phosphate, and myo-inositol 5-phosphate (Table IV). The presence of myo-inositol 2-phosphate and absence of the 1-phosphate in the products of partial alkaline hydrolysis alone makes it mandatory that IIIa be the 2,4,5-triphosphate, since component IIIa is optically active.

Acid migration studies also confirm this as the structure. Component IIIa underwent migration in hot acid to yield an isomer chromatographically identical with component IIIb. Iditol and xylitol, as well as the original sorbitol, were obtained by the periodate oxidation of the acid treated compound (Table III). This change would result from the following reaction sequence:

\[
\text{PO} \quad \text{PO} \quad \text{PO} \\
\text{O} \quad \text{O} \quad \text{O} \\
\text{IIIa} \\
\text{PO} \quad \text{PO} \quad \text{PO} \\
\text{O} \quad \text{O} \quad \text{O} \\
\text{IIIb} \\
\text{PO} \quad \text{PO} \quad \text{PO} \\
\text{O} \quad \text{O} \quad \text{O} \\
\text{IIa} \\
\text{PO} \quad \text{PO} \quad \text{PO} \\
\text{O} \quad \text{O} \quad \text{O} \\
\text{Iditol} \\
\]

From these studies, it is now possible to write the following structures for the myo-inositol phosphate esters obtained by base hydrolysis of beef brain polyphosphoinositide. The same numbering system is used as in previous reports (2) so that the structures can be easily related back to the components in those publications. Components IIa, IIIa, and IIIb all have in common the location of phosphate groups on positions 4 and 5. They differ in a way that suggests either migration or elimination of a phosphate group that occupied position 1 in the original lipid. Components Ib and IIb both have a phosphate group on position 4, whereas IIa has the additional phosphate on position 1. These relationships have important implications concerning the structure of the brain phosphoinositide(s), and further work underway in this laboratory will define in more detail the significance of these similarities.

1. Four methods are described which, when used in conjunction, can lead to the characterization of complex myo-inositol polyphosphate esters. These methods involve periodate oxidation, acid-catalyzed migration, and partial dephosphorylation by ammonia and by a phosophoesterase.

2. The four myo-inositol polyphosphate components obtained by alkaline hydrolysis of a beef brain phosphoinositide preparation have been characterized as L-myo-inositol 1,4,5-triphosphate, myo-inositol 2,4,5-triphosphate, L-myo-inositol 4,5-diphosphate, and myo-inositol 1,4-diphosphate.

REFERENCES

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