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Enzymatic Fluorometric Assay for *myo*-Inositol Trisphosphate[1]

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The determination of *myo*-inositol trisphosphate by an enzymatic fluorometric assay is presented. The method involves the acid extraction of water-soluble inositol polyphosphates followed by separation by anion-exchange chromatography. Samples are subsequently neutralized by passage over a Dowex Cl^- resin and elution with lithium chloride. Samples are then desalted with ethanol. Following dephosphorylation with alkaline phosphatase, free *myo*-inositol is measured enzymatically via the NAD-dependent oxidation to *scyllo*-inosose with *myo*-inositol dehydrogenase. The efficiency of recovery, assay specificity, and an application to the measurement of inositol polyphosphates in hormone-stimulated tissue are discussed.

KEY WORDS: inositol polyphosphate; fluorometry; enzymatic assay.

The hydrolysis of phosphatidylinositol 4,5-bisphosphate with the formation of free inositol polyphosphates is now regarded as an important step in signal transduction with calcium-mobilizing effectors (1–3). Specifically, inositol 1,4,5-trisphosphate has been demonstrated to mobilize calcium from intracellular stores (4–6).

The study of effector-induced phosphatidylinositol metabolism, however, has been largely limited to radiotracer studies. In these reports the elaboration of inositol polyphosphates is determined by changes in the activity of [^{3}H]inositol-containing metabolites (often in the presence of lithium chloride to inhibit inositol 1-phosphatase activity). Alternatively, changes in inositol phosphates are inferentially determined by changes in the activity of ^{32}P-containing phospholipids (7) or by comparison to other phosphate-containing metabolites (8). The former technique is limited to those tissues which are able to incorporate [^{3}H]inositol; such studies usually assume that all [^{3}H]inositol-containing metabolites are at equal specific activity. This assumption has not been tested. The latter technique does not provide specific data on the individual inositol phosphates formed.

A practical method for determining inositol polyphosphate mass would therefore be useful. MacGregor and Matschinsky (9) have developed a sensitive enzymatic assay for the determination of *myo*-inositol mass. This method has been modified and utilized for the determination of inositol trisphosphate mass.

METHODS

Principles of the assay. The quantitative determination of inositol trisphosphate utilizes a multistep assay. It is based upon the

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separation of IP_3[3] by anion-exchange chromatography, dephosphorylation, and fluorometric enzymatic determination of the free inositol. Specifically, this involves eight distinct steps:

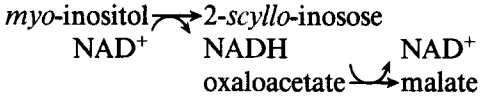
(1) Biological samples are extracted with trichloroacetic acid and neutralized by extraction of the trichloroacetic acid with diethyl ether.

(2) IP_1, IP_2, and IP_3 are separated by anion-exchange chromatography on a Dowex formate resin and a stepwise elution with an ammonium formate and formic acid gradient.

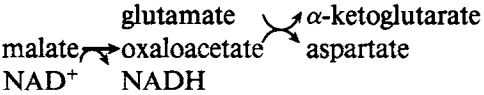
(3) To remove the ammonium formate and formic acid, the separated IP_3 is added to a second anion-exchange resin and eluted with 1.5 M lithium chloride. The sample is dried and most of the lithium chloride is removed with anhydrous ethanol. The lithium–IP_3 salt is insoluble in ethanol.

(4) IP_3 is dephosphorylated with intestinal alkaline phosphatase forming free *myo*-inositol.

(5) Free *myo*-inositol is converted to 2-*scyllo*-inosose with the *myo*-inositol dehydrogenase. The reaction is pulled to completion with malate dehydrogenase:

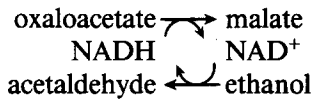


(6) The NADH and oxaloacetate are destroyed and the malate is converted back to oxaloacetate with malate dehydrogenase, yielding a stoichiometric amount of NADH. This reaction is pulled to completion with glutamate and aspartate transaminase:



[3] Abbreviations used: IP_1, *myo*-inositol monophosphate; IP_2, *myo*-inositol bisphosphate; IP_3, *myo*-inositol trisphosphate.

(7) To obtain the necessary sensitivity, the NADH formed is amplified with the enzymatic cycling method of Kato *et al.* (10), which utilizes alcohol dehydrogenase and malate dehydrogenase:



(8) The malate formed is then determined, coincidentally, by the same reactions as in step 6.

A protocol suitable for measuring 1 to 10 pmol of inositol trisphosphate and its application to primary cultures of renal papillary-collecting tubule cells are given below. This protocol, however, is also applicable to other biologic samples.

Step 1: Renal cell culture, hormonal stimulation, and sample extraction. Primary cultures of rabbit papillary-collecting tubule cells were harvested and grown as previously described (11). Cell cultures (0.5 to 2.0×10^6 cells per dish) were rinsed three times with Krebs buffer and stimulated at 37°C with buffer alone or with bradykinin (10^{-7} M). Incubations were terminated by the rapid aspiration of buffer followed by the addition of 1 ml of ice-cold trichloroacetic acid (1 M). The precipitated protein and lipid were separated by centrifugation in an Eppendorf 5414 centrifuge. Protein was determined with the fluorescamine reagent (12). Aliquots (500 μl) of supernatant from the trichloroacetic acid-treated samples were placed in 13 × 100-mm test tubes and extacted with 2 ml of water-saturated diethyl ether. This extraction was repeated four times. The residual ether was evaporated by heating the samples to 65°C for 15 min. The remaining traces of trichloroacetic acid in the ether-extracted samples were neutralized with 1 M sodium bicarbonate (120 μl for a 500-μl sample).

Step 2: Chromatographic separation. The entire sample was applied to a 0.75 × 12-cm column containing Dowex AG 1- x8 formate

form, anion-exchange resin (bed volume 1 ml) (Bio-Rad, Richmond, CA). Inositol and the inositol phosphates were serially eluted with (a) water 4 ml 4×; (b) 0.025 M ammonium formate 4 ml 2×; (c) 0.2 M ammonium formate, 0.1 M formic acid 4 ml 2×; (d) 0.4 M ammonium formate, 0.1 M formic acid 4 ml 2×; and (e) 0.75 M ammonium formate, 0.1 M formic acid 4 ml 2×. These solvents eluted, respectively, inositol, glycerophosphatidyl inositol, IP_1, IP_2, and IP_3. The success of this separation procedure has been reported by previous investigators (13). Positional isomers of the inositol phosphates, however, were not separated. A reduced concentration of ammonium formate was utilized in (e) so as not to include inositol tetrakisphosphate in the IP_3 fraction (14).

Step 3: Desalting and neutralizing. Separated samples were desalted and neutralized by an adaptation of a recently reported protocol (15). Samples were diluted with 50 ml of water and added to a second anion-exchange column (Dowex AG 1- x8, 200–400 mesh, chloride form, column size 0.75 × 12 cm, bed volume 2 ml). The column was further washed with an additional 25 ml of water. The inositol phosphates were then eluted with 8 ml of 1.5 M LiCl. Samples were brought to dryness in a vacuum centrifuge (Speed Vac, Savant). Trapped water was removed by resuspension of the lithium salts in 4 ml of anhydrous ethanol and reevaporation. This was repeated three times. The dried pellet was resuspended in 8 ml of absolute ethanol. The remaining lithium chloride containing the insoluble lithium phosphate salts was pelleted by centrifugation at 4000*g* (Beckman J2-21 centrifuge). The supernatant was then removed. This procedure was repeated three times or until only a small pellet, barely visible, remained. Samples were then evaporated to dryness.

Step 4: Dephosphorylation of inositol polyphosphates. Samples were reconstituted in 50 μl of Tris buffer (50 mM, pH 9.0) containing 50 U/ml of bovine intestinal alkaline phosphatase and 5 mM $MgCl_2$ and were incubated for 2 h at 37°C. The alkaline phosphatase was reconstituted in 20 mM imidazole–HCl buffer (pH 7) containing 0.02% albumin before its addition to the Tris buffer. The alkaline phosphatase was then inactivated by heating the samples for 3 min in a boiling water bath.

Step 5: myo-Inositol oxidation. Initial steps were performed in 6 × 50-mm borosilicate, nitric acid-washed tubes. All enzymes suspended in ammonium sulfate were pelleted by centrifugation and resuspended in imidazole–HCl buffer (pH 7.0), containing 0.02% bovine serum albumin. An exception was *myo*-inositol dehydrogenase; this enzyme was reconstituted in 0.02 M potassium phosphate buffer, pH 7.0 (see Results and Discussion).

myo-Inositol dehydrogenase reagent (2 μl) was added to a 10-μl aliquot of sample from step 4. This reagent consisted of Tris-HCl buffer (350 mM, pH 9.0), oxaloacetate (7 mM), malate dehydrogenase (15 U/ml), NAD^+ (1.5 mM), and *myo*-inositol dehydrogenase (1.0 U/ml). After incubating at 25°C for 40 min, excess oxaloacetate was destroyed by adding 0.5 μl of 13 mM H_2O_2 and heating in a water bath at 75°C for 20 min.

Step 6: Malate oxidation. NADH was generated next by the oxidation of malate to oxaloacetate with 10 μl of malate dehydrogenase reagent. This consisted of 2-amino 2-methyl 1-propanol–HCl buffer (200 mM, pH 9.9) containing malate dehydrogenase (35 U/ml), glutamate (50 mM), glutamic oxaloacetic transaminase (5 U/ml), and catalase (2000 U/ml). To permit the amplification of the NADH formed by enzymatic cycling, the excess NAD^+ was destroyed by adding 5 μl of NaOH (0.35 N) and heating for 20 min at 75°C.

Step 7: Amplification. The cycling of NADH was performed with the method of Kato *et al.* (10). The enzyme concentrations were adjusted to cycle 1000 times. Cycling was performed on 5 μl of the 27.5-μl incuba-

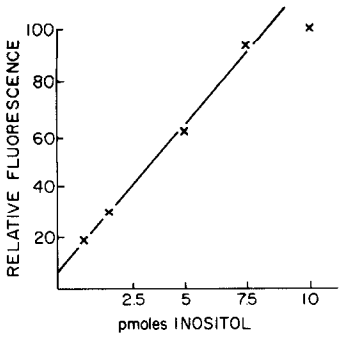


FIG. 1. Standard curve for *myo*-inositol determination. The points represent the means of triplicate samples.

tion mixture. Samples were cycled in 10 × 75-mm borosilicate tubes with 100 μl of cycling reagent. This reagent was added to samples while on ice and within a 5-min time span. The rack of samples was then incubated for 1 h in a 25°C water bath, and cycling was terminated by heating for 3 min in a boiling water bath.

The assay was completed by measuring the malate formed during cycling with a reagent consisting of 2-amino 2-methyl 1-propanol buffer (pH 9.9, 50 mM), malate dehydrogenase (5 U/ml), glutamate (10 mM), glutamic oxaloacetic transaminase (2 U/ml), and NAD (200 μM). One milliliter of this reagent was added to the cycled samples. Fluorescence was determined in a Farrand filter fluorometer following a 20-min incubation at 25°C.

Calculations were based on a standard curve for *myo*-inositol (1–10 pmol) introduced at step 5. This is depicted in Fig. 1. Recoveries of IP_3 were assessed either by parallel determinations of known quantities of authentic IP_3, assayed following exposure to trichloroacetic acid, or by spiking samples with [^{3}H]IP_3 at step 1. The observed recoveries for the overall determination of IP_3 and for the individual steps are discussed below.

Materials. Biochemicals, including enzymes, were purchased from Sigma Chemical Co. (St. Louis, MO) with the following exceptions. Inositol 1,4-bisphosphate was the generous gift of Dr. William Sherman (Washington University Medical School, St. Louis, MO). Malate dehydrogenase and catalase were obtained from Boehringer-Mannheim (Indianapolis, IN). *myo*-[2-^{3}H]Inositol 1,4,5-trisphosphate was purchased from Amersham (Niles, IL). Inositol 1,2-cyclic monophosphate was synthesized by the method of Pfizer and Ballou (16) as previously described (17).

RESULTS AND DISCUSSION

Recovery. The separation of IP_1, IP_2, and IP_3 by anion-exchange chromatography was confirmed by the addition of authentic inositol phosphate standards to columns containing Dowex formate resin and by the determination of *myo*-inositol mass following desalting and dephosphorylation. *myo*-Inositol could be detected only in fractions eluting with 0.2 M ammonium formate, 0.1 M formic acid for IP_1; 0.4 M ammonium formate, 0.1 M formic acid for IP_2; and 0.75 M ammonium formate, 0.1 M formic acid for IP_3. The recovery of authentic IP_3 added to 1 M trichloroacetic acid and chemically measured by the eight-step assay varied between 75 and 95%. These measurements were performed on either 100 pmol or 1 nmol of IP_3 in duplicate during three separate determinations.

When the recovery of authentic standard was assessed at each step, it was determined that the greatest loss occurred during the removal of the LiCl (step 3). Whereas greater than 95% of [^{3}H]IP_3 added to the Dowex chloride column at step 2 could be recovered by LiCl elution, only 70 to 95% of the labeled standard remained in the pellet following ethanol extraction. Losses at this step were even more substantial if care was not taken to remove trapped water prior to LiCl removal. This problem could possibly be lessened by eluting IP_3 with LiCl at a lower concentration. However, the recovery of [^{3}H]IP_3 from the Dowex chloride column was sub-

stantially lower when 1.0 M LiCl or less was used. The recovery of 100 pmol of IP_3 was the smallest amount assessed. For assay of smaller amounts of IP_3, recovery would need to be determined.

The recovery of [^{3}H]IP_3 from the Dowex formate column (step 2) and following dephosphorylation (step 4) was greater than 98%. The recovery of authentic unlabeled inositol as determined by the enzymatic fluorometric assay (steps 5 through 8) was greater than 95% and was determined with samples as small as 1 pmol.

Dephosphorylation. The concentration dependence of dephosphorylation with alkaline phosphatase is shown in Fig. 2. Here, [^{3}H]IP_3 was subjected to alkaline phosphatase hydrolysis over a wide range of enzyme concentrations. Following a 2-h incubation, samples were separated by anion-exchange chromatography. The formation of IP_1, IP_2, and IP_3 was assessed by counting the samples eluting with each buffer. The results indicate that the rates of hydrolysis are very different for the successive compounds. The curves are consistent with removal of the first phosphate at a (first-order) rate of 50% per hour with about 0.5 U/ml of enzyme, removal of the second phosphate at a tenth this rate (50% per hour with about 5 U/ml), and removal of the last phosphate at a rate intermediate between the two. These data are probably indicative of the inefficiency of hydrolysis of vicinal phosphates by alkaline phosphatase. Similar observations have been reported previously for glycerophosphatidylinositides (15).

myo-Inositol determination. The fluorometric measurement of *myo*-inositol follows the protocol of MacGregor and Matschinsky (9) with some modifications. First, the initial oxidation of malate (step 6) is performed without the addition of NAD^+. Under the conditions employed for oxaloacetate destruction, sufficient NAD^+ remains from the inositol oxidation step (step 5) to allow for the complete oxidation of malate.

A second change included the use of a lower concentration of oxaloacetate during the *myo*-inositol oxidation step.

It was observed that if *myo*-inositol dehydrogenase was dissolved in Tris buffer (pH 8.1 or 9.0) or in 2-amino 2-methyl 1-propanol (pH 9.9), the oxidative activity was lost. If instead the enzyme was reconstituted

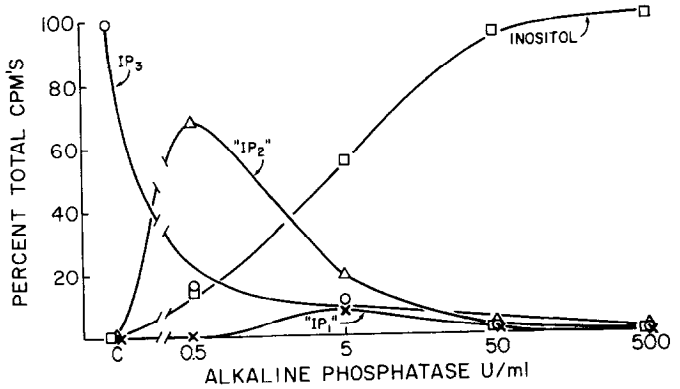


FIG. 2. The dephosphorylation of [^{3}H]IP_3 as a function of bovine intestinal alkaline phosphatase concentration. [^{3}H]IP_3 (5 μCi) was subjected to alkaline phosphatase digestion as described in the text. Samples were then separated by anion-exchange chromatography. The data are expressed as the percentage of total ions. Quotation marks denote the fact that the precise structures of these compounds are unknown. Each point represents the mean of three experiments.

TABLE 1

OXIDATION OF INOSITOL PHOSPHATES BY *myo*-INOSITOL DEHYDROGENASE

Inositol	100
Inositol 1,2-cyclic phosphate	0
Inositol 2-monophosphate	3
Inositol 1,4-bisphosphate	0
Inositol 1,4,5-trisphosphate	0

Note. Data are expressed as the percentage of expected malate production from the oxidation of inositol and inositol phosphates by *myo*-inositol dehydrogenase.

in 0.02 M potassium phosphate buffer (pH 7.0), then oxidative activity was preserved.

Previous reports have detailed the specificity of *myo*-inositol dehydrogenase for other sugars and sugar alcohols (9). Of the sugars tested, only glucose demonstrated significant reactivity. This is not problematic in the presently detailed method since glucose elutes with free *myo*-inositol in the chromatographic system employed. *myo*-Inositol dehydrogenase appears to be completely inactive toward the inositol phosphates (Table 1). The small amount of reactivity with IP_1 probably reflects contamination with *myo*-inositol.

Inositol trisphosphate measurements in renal cells. Preliminary determinations of IP_3 mass were made in a biological system where radiolabeled IP_3 has been demonstrated to increase in response to a polypeptide hormone. Specifically, when exposed to bradykinin, rabbit renal papillary-collecting tubule cells exhibit increased radiolabeling of IP_3 with parallel changes in intracellular calcium mobilization and prostaglandin E_2 formation (11,18).

Cells were incubated with or without bradykinin (10^{-7} M) for 10 s. Duplicate samples were measured in four separate experiments. Tissue blanks omitting *myo*-inositol dehydrogenase were 20 to 30% of the total observed fluorescence. Under these conditions, IP_3 rose from 0.16 ± 0.05 to 0.34 ± 0.08 pmol/μg protein ($p < 0.05$ by paired t analysis). When intracellular volume was measured from the distribution of ^3H_2O and [^{14}C]inulin, intracellular IP_3 was found to rise from 7.5 μM under basal conditions to 16 μM following bradykinin stimulation.

IP_3, a product of the phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate, is now regarded as a potentially important second messenger in the mobilization of intracellular calcium. However, few data presently exist to confirm whether endogenous levels of IP_3 are of sufficient magnitude to account for the calcium release observed with the addition of exogenous IP_3 either to permeabilized cells or to intact cells via micropipets. The data detailed above are of comparable magnitude to similar observations made in thrombin-stimulated platelets (19). Moreover, the observed concentration of IP_3 under kinin-stimulated conditions is close to the reported K_m for the 5-phosphatase which degrades IP_3 to IP_2 (20,21). However, both the basal and stimulated values of IP_3 in renal cells are in excess of those concentrations demonstrated as necessary for the mobilization of intracellular calcium in permeabilized cells (4). On the one hand, this difference in concentration may reflect the failure to resolve *myo*-inositol 1,4,5-trisphosphate from the other inositol trisphosphate isomers under the separation conditions employed in the present study. On the other hand, IP_3 concentrations may be regulated in intact cells (e.g., by compartmentalization) in ways not apparent in permeabilized cells.

In summary, a sensitive enzymatic fluorometric assay for the determination of inositol trisphosphate mass has been described. The application of this method should permit the chemical determination of *myo*-inositol trisphosphate levels in freshly isolated samples, tissues not amenable to radiolabeling, and small samples. Moreover, by simultaneously measuring radiolabeling and mass, it should be possible to determine the true specific activity of radiolabeled inositol phosphates. In

this manner, a more precise determination of the kinetics of inositol phosphate formation and degradation will be possible.

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