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THE SEPARATION OF MYO-INOSITOL PHOSPHATES BY ION-PAIR CHROMATOGRAPHY[1].

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The separation of myo-inositol phosphates by ion pair, reverse-phase high performance liquid chromatography has been investigated. The retention of the inositol phosphates is dependent on both the polarity of the hetaeron utilized and on the pH of the solvent. A method is presented which permits the isocratic separation of multiple forms of inositol phosphates including isomers of myo-inositol trisphosphate. This method appears to be superior to the anion exchange based systems currently employed because of smaller retention volumes, the low ionic strength of the solvent employed, the absence of a requirement for reequilibration, and the ability to perform separations isocratically.

Significant interest has been generated in inositol phosphate chemistry by the observation that the phosphatidyl inositol pathway plays an important and ubiquitous role in cell signal transduction (1-3). This interest has intensified as investigators have identified novel isomers of inositol phosphates many of which may have unique biological roles and which appear to be metabolized via distinct pathways (4,5).

Currently, several methods exist for separating inositol phosphates including open bed anion exchange (6), gas-liquid chromatography (7), and high voltage electrophoresis (8). These techniques are limited by either their inability to detect multiply phosphorylated compounds or to resolve multiple isomers of inositol phosphates. High performance liquid chromatography utilizing strong anion exchange resins has been successfully employed for resolving multiple forms of inositol phosphates (4,9). However,

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this approach is limited by several disadvantages including long run times, long reequilibration periods, complex gradients utilizing buffers of high ionic strength, and short column life.

Ion-pair chromatography has been successfully employed in the separation of both ionized and non-ionized compounds in reverse phase systems. Although widely exploited in the separation of both pharmaceuticals (10) and nucleic acid derivatives (11), this strategy has yet to be utilized in the separation of inositol phosphates. The application of ion pair chromatography to the study of inositol phosphate metabolism resulted in the development of the following method.

METHODS

Materials Myo-[2-^{3}H]inositol (19 Ci/mmole), myo-[2-^{3}H]inositol 1-phosphate (1.0 Ci/mmole), myo-[2-^{3}H]inositol 1,4-bisphosphate (1.0 Ci/mmole), and myo-[2-^{3}H]inositol 1,4,5-trisphosphate (1.0 Ci/mmole) were obtained from Amersham (Arlington Heights, Il). Myo-[2-^{3}H]inositol 1,3,4,5-tetrakisphosphate (5.0 Ci/mmole) was purchased from New England Nuclear (Boston, Massachusetts). Ion pairing agents including tetrabutyl ammonium phosphate, tetramethyl ammonium chloride, tetraethyl ammonium bromide, tetrapropyl ammonium bromide, and tetrapentyl ammonium chloride were obtained from Aldrich (Milwaukee, WI).

Chromatographic analysis Inositol phosphates were separated on a Waters high pressure liquid chromatography system consisting of a 501 pump, U6K injector, and a 481 Lambda-Max UV spectrophotometer (to monitor adenine nucleotides). A Waters uBondapak C_{18} column (0.5x30 cm) with a C_{18} precolumn insert was employed. Unless otherwise indicated, separations were performed utilizing an isocratic elution of [^{3}H]standards or cellular extracts with a solvent composed of acetonitrile:water, 19:81 (v/v), KH_2PO_4 0.04 M, and tetrabutyl ammonium phosphate 0.05 M, pH 3.5 adjusted with phosphoric acid. The flow rate was 0.75 ml/min and fraction size varied between 0.375 and 0.75 ml as indicated in the RESULTS. The elution of ATP was monitored at 260 nm. Eluted fractions were collected and mixed with 5 ml of aqueous counting scintillant (Amersham). The radioactivity was determined by liquid scintillation counting.

Myo-inositol 1,3,4,-trisphosphate preparation Inositol 1,3,4-trisphosphate was prepared with erythrocyte 5 phosphomonoesterase following the protocol of Downes, et al. (12) In brief, permeable erythrocyte ghosts were prepared by the centrifugation of 30 ml of human blood at 2500 x g for 5 min. The plasma and leukocytes were removed by aspiration. Thereafter the cells were lysed by exposure to 2.5 mM Hepes/1 mM EGTA followed by washing x 4 with 10 vol of 1.5 mM Hepes, 0.154 M NaCl, pH 7.2. [^{3}H]Inositol 1,3,4-trisphosphate was generated by exposure of 20 nCi of myo-[2-^{3}H]inositol 1,3,4,5 tetrakiphosphate to 0.5 mg of erythrocyte protein in 0.5 ml of 30 mM hepes, 2 mM $MgCl_2$, pH 7.0 at 37°C for 15 min. The reaction was stopped by the addition of 10% trichloroacetic acid. Precipitated proteins were separated by centrifugation in an Eppendorf 5415 centrifuge, the supernatant removed and the acid extracted with 4 volumes x 4 of water-saturated diethyl ether. Samples were desalted by elution over a Dowex 50 H^+ column. Myo-inositol 1,4,5- and 1,3,4-trisphosphates were identified by periodate oxidation, borohydride reduction, and dephosphorylation of the radiolabeled peaks to produce respectively D-iditiol and L-altritol. These products were subjected to separation by descending paper chromatography on Whatman no. 1 paper and compared to authentic standards (13, 14). Myo-inositol tris- and tetrakiphosphate as well as putative myo-inositol bisphosphates were identified by migration on high voltage electrophoresis. The migration of these products was compared to that of a phytic acid hydrolysate. The electrophoretic separation utilized sodium oxalate, oxalic acid 0.06 M and followed the protocol of Seiffert and Agranoff (8).

Metabolism of myo-[2-^{3}H]inositol 1,4,5-trisphosphate The metabolism of radiolabeled inositol 1,4,5-trisphosphate was evaluated in rabbit renal papillary collecting tubule cells to discern the utility of the chromatographic method. Freshly isolated distal tubule cells were obtained from New Zealand white rabbits by collagenase digestion and hypotonic lysis as previously described. (15) The cells were permeabilized with saponin (75 ug/ml) in a cytosolic medium following the protocol of Tennes, et al. (16) Following permeabilization, aliquots of tubular cells (0.5 mg cell protein equivalent) were pelleted at 500 x g and resuspended in a buffer consisting of (in mM) NaCl 20, KCl 100, $MgSO_4$ 5.0, $Na\ H_2PO_4$ 0.96, $NaHCO_3$ 25, ATP 1.5, phosphocreatine 5, and creatine kinase (5 units/ml), pH 7.2, 37°C. At the initiation of incubation, 5 nCi of myo-[2-^{3}H] inositol 1,4,5-trisphosphate was added to 500 ul of cell suspension. At 15, 120, and 300 sec 500 ul of 10% trichloroacetic acid was added. The protein was precipitated and samples desalted as above.

RESULTS

The retentions of myo-inositol, myo-inositol 1-monophosphate, myo-inositol 1,4-bisphosphate, ATP, and myo-inositol 1,4,5-trisphosphate were evaluated by utilizing five distinct quarternary amines as counterions. Figure 1 displays the relationship between

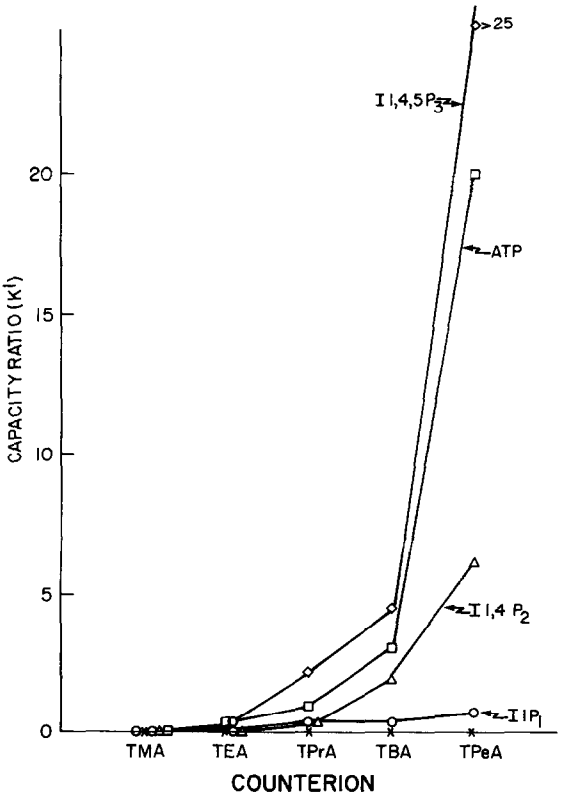

Figure 1. The observed capacity ratio of ATP and various tritiated inositol phosphates as a function of the carbon chain length of the hydrophobic moiety of 5 counterions. TMA, tetramethyl ammonium chloride; TEA, tetraethyl ammonium bromide; TPrA, tetrapropyl ammonium bromide; TBA, tetrabutyl ammonium phosphate; and TPeA, tetrapentyl ammonium chloride. Counterions were employed at a concentration of 0.05 M. I1-P, myo-inositol 1-monophosphate; I1,4-P_2, myo-inositol 1,4-bisphosphate and I1,4,5-P_3, myo-inositol 1,4,5-trisphosphate. X - denotes myo-inositol.

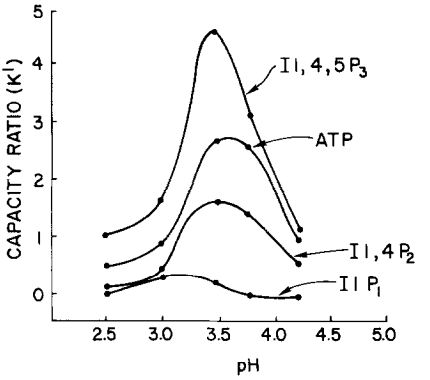

Figure 2. The observed capacity ratio of ATP and various tritiated inositol phosphates as a function of solvent pH. Tetrabutyl ammonium phosphate was employed throughout and the pH of the solvent adjusted with phosphoric acid. Abbreviations are as indicated in figure 1.

the retention of ATP and the tritium-labelled inositol phosphates as a function of the carbon chain length of the hydrophobic moiety of the counterion. The observed capacity ratio, defined as the difference of the retention volume and the void volume divided by the void volume, was directly correlated both to the hydrophobicity of the hetaeron and to the degree of phosphorylation of the sugar. Solvents consisted of acetonitrile 19%, water 81%, KH_2PO_4 0.04M, and counterion 0.05M, pH 3.35. Only counterions containing alkyl groups of 3 carbons or greater demonstrated any effect on the retention of those inositol phosphates studied. The uncharged myo-inositol always appeared in the void fractions and was not retained regardless of the polarity of the solvent composition utilized.

Figure 2 demonstrates the association between the pH of the solvent and the retention of ATP and of the tritiated inositol phosphates. During these experiments tetrabutyl ammonium phosphate was employed as the hetaeron and the pH was adjusted with phosphoric acid. The ionic strength was held constant by adjusting the concentration of KH_2PO_4. Under these conditions the maximum retention was observed at pH 3.5 with a narrow pH optimum. Because small changes in the pH of the solvent had dramatic effects on the retention of these standards, the pH of the solvent was routinely determined and adjusted just prior to use.

A sample chromatogram of myo-inositol 1 monophosphate, myo-inositol 1,4-bisphosphate, myo-inositol 1,4,5-trisphosphate, and myo-inositol 1,3,4,5

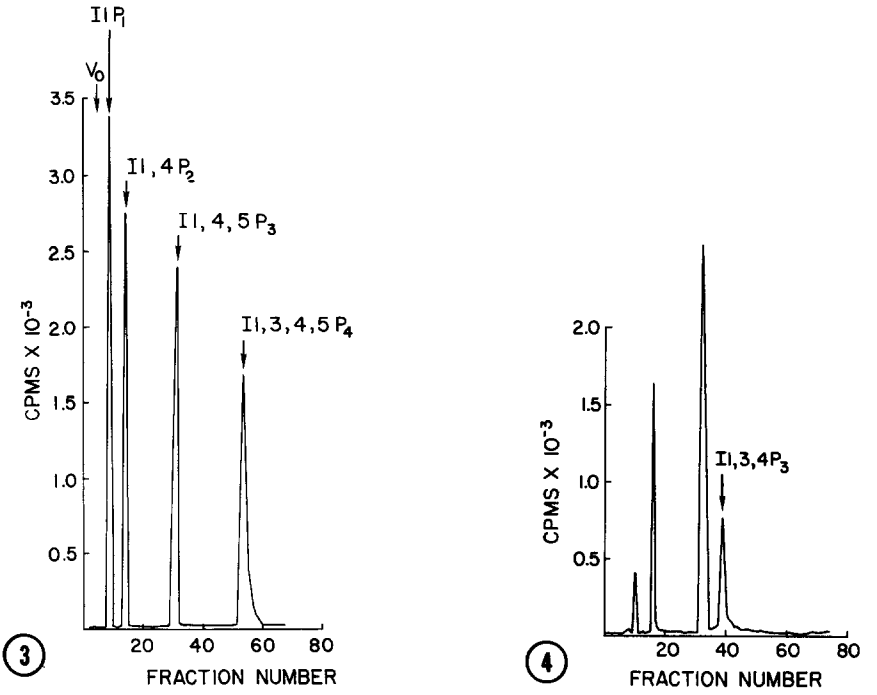

Figure 3. Representative chromatogram of 4 radiolabeled inositol phosphates including myo-inositol 1,3,4,5-tetrakisphosphate (I1,3,4,5-P_4). All other abbreviations are as indicated in figure 1. Fraction volume equaled 0.375 ml.

Figure 4. Chromatographic profile of myo [2-^{3}H]inositol 1,3,4,5-tetrakisphosphate and its reaction product following incubation with crude erythrocyte 5-phosphomonoesterase. The arrow indicates the migration of myo-inositol 1,3,4-trisphosphate. The product co-migrated with myo-inositol trisphosphate by anion exchange chromatography, but co-migrates with L-altritol following periodate oxidation, borohydride reduction and dephosphorylation. It is therefore tentatively identified as myo-inositol 1,3,4-trisphosphate. Fraction volume equals 0.375 ml.

tetrakisphosphate is shown in figure 3. Baseline resolution of all standards was apparent with complete elution of myo-inositol tetrakisphosphate by 30 minutes under the conditions employed (flow rate 0.75 ml/min, ambient temperature). Smaller retention volumes with equally good resolution are potentially possible with higher flow rates and elevated column temperatures but would require further evaluation. Of note is the observation that each standard was greater than 98% pure by these chromatographic criteria. However, myo-inositol 1,4,5-trisphosphate degraded at a rate of approximately 5% per month when stored at -20 C to a product which comigrated with inositol bisphosphate on open bed anion exchange chromatography but did not comigrate with myo-inositol 1,4-bisphophate by ion pair chromatography.

The chromatographic profile of the product of incubation of myo-inositol 1,3,4,5-tetrakisphosphate and crude erythrocyte 5 phosphomonoesterase is displayed in figure 4.

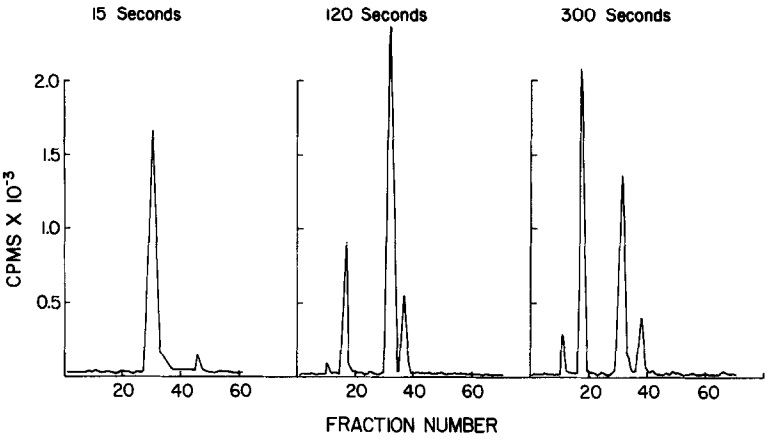

Figure 5. The time dependent metabolism of myo [2-^{3}H] inositol 1,4,5 trisphosphate in permeabilized rabbit papillary collecting tubule cells. At 15 seconds the major product co-chromatographs with authentic myo-inositol 1,4,5-trisphosphate. At 120 seconds another product with the identical retention volume as myo-inositol 1,3,4-trisphosphate generated from erythrocyte membranes is present. By 300 seconds, myo-inositol bisphosphate is the major product.

Here 5 nCi of [^{3}H] myo-inositol 1,3,4,5-tetrakisphosphate were incubated with 100 ul of enzyme preparation (20 ug membrane protein) for 20 min at 37 C in the presence of 20 mM LiCl to inhibit trace 1-phosphomonoesterase activity. The retention period of the major product of this reaction was clearly longer than of the inositol 1,4,5-trisphosphate standard. This product was collected, treated with sodium periodate, sodium borohydride and bovine intestinal alkaline phosphatase as described in the METHODS. The reaction product was found to co-chromatograph with L-altritol by descending paper chromatography. Of note was the existence of other products of this reaction including a product which co-chromatographed with authentic myo[2-^{3}H]inositol 1,4,5-trisphosphate. These results are indicative of the presence of other contaminating phosphatases in erythrocyte ghosts and are contrary to previous reports (12). Figure 5 displays the sequential chromotogram from permeabilized renal papillary collecting tubule cells incubated with myo-[2-^{3}H] inositol 1,4,5-trisphosphate. Within 2 minutes a large peak co-migrating with myo-inositol 1,3,4-trisphosphate is evident as well as myo-inositol mono- and bisphosphates. Of note is the ability to resolve to baseline both isomers of inositol trisphosphate under the conditions employed. Additionally, within 15 seconds, a smaller peak with a larger retention volume than both myo-inositol 1,4,5- and 1,3,4-trisphosphate is present. This product however elutes as an inositol trisphosphate

by both anion-exchange chromotography and high voltage electrophoresis. The structural analysis of this product is currently under investigation.

DISCUSSION

The present study investigates the use of ion-pair chromatography in the resolution of inositol polyphosphates. Although high performance liquid chromatography utilizing strong anion exchangers has now been widely reported in the separation of these products, such methodology has several limitations. These include short column life, complex gradients employing solvents of high ionic strength, poor baseline resolution of inositol phosphate isomers, long retention times and equally long reequilibration periods. These problems are not apparent with the current method.

The present study demonstrates the ability to perform ion-pair separations of inositol phosphates utilizing a reverse phase isocratic system. A single C_{18} column has been used for all of the studies to date. Superior resolution with significantly shorter retention volumes is apparent with this method. Baseline resolution of several inositol phosphate isomers is also observed (most importantly inositol 1,3,4 and 1,4,5-trisphosphates). Finally, there is effectively no reequilibration time required between runs.

Several variables affect the retention of inositol polyphosphates by ion-pair chromatography two of which have been evaluated in detail in the present paper. The hetaeron employed significantly alters the retention of the inositol polyphosphates. Of the quarternary amines tested, only tetrabutyl ammonium phosphate appears to have general utility in separating the inositides having the greatest range of phosphorylation. However, other potential ion-pair reagents exist including mono-, di- and trialkyl ammonium salts; N',N'-dimethyl protryptiline; and N-methyl imipramine. These counterions are worthy of further study. In addition, the zwitterion 11 aminoundecanoic acid has been employed in preliminary studies and is also capable of resolving inositol phosphates. (Data not presented.)

Solvent pH profoundly affects the retention of the inositol phosphates. With the current method the assiduous control of the solvent pH was found to be important in the maintainence of reproducible retention times. Other variables which potentially could also be exploited to improve the resolution of the inositol phosphates include the ionic

strength of the solvent, the concentration of the counterion, the carbon content of the column, the column temperature, and the polarity of the solvent. This last variable was less formally evaluated. It was observed that significant changes in the retention volumes of inositol mono- and bisphosphate were observed only when the acetonitrile concentration was less than 10% and that all inositol phosphates were indefinitely retained if the acetonitrile was completely absent.

A comprehensive model fully describing the mechanistic basis of ion-pairing of inositol phosphates is unavailable at present. The observations reported in the current study are consistent with the "dynamic exchange" model. (17) This model postulates that the reverse phase column is effectively transformed into an anion exchanger by the adsorption of the counterion with subsequent binding of the eluate. In support of this model is the observation that the retention of the inositol phosphates is directly correlated to the degree of phosphorylation that is the number of charged sites present. Also in support of the anion exchange model was the observation that the same order of retention was observed when the counterion was eliminated from the solvent and the standards run over a column previously exposed to the tetrabutyl ammonium phosphate. These are conditions under which ion pairs would not form in the solvent. Further work is clearly required to define more fully the mechanisms at work in the present system.

In summary, ion-pair chromatography has been successfully employed in the resolution of inositol phosphates by high performance liquid chromatography. Several technical advantages in the present method render this system superior to the traditional anion-exchange based chromatography currently employed.

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