ISOLATION AND PURIFICATION OF INOSITOL POLYPHOSPHATES

Thesis for the Degree of M. S.

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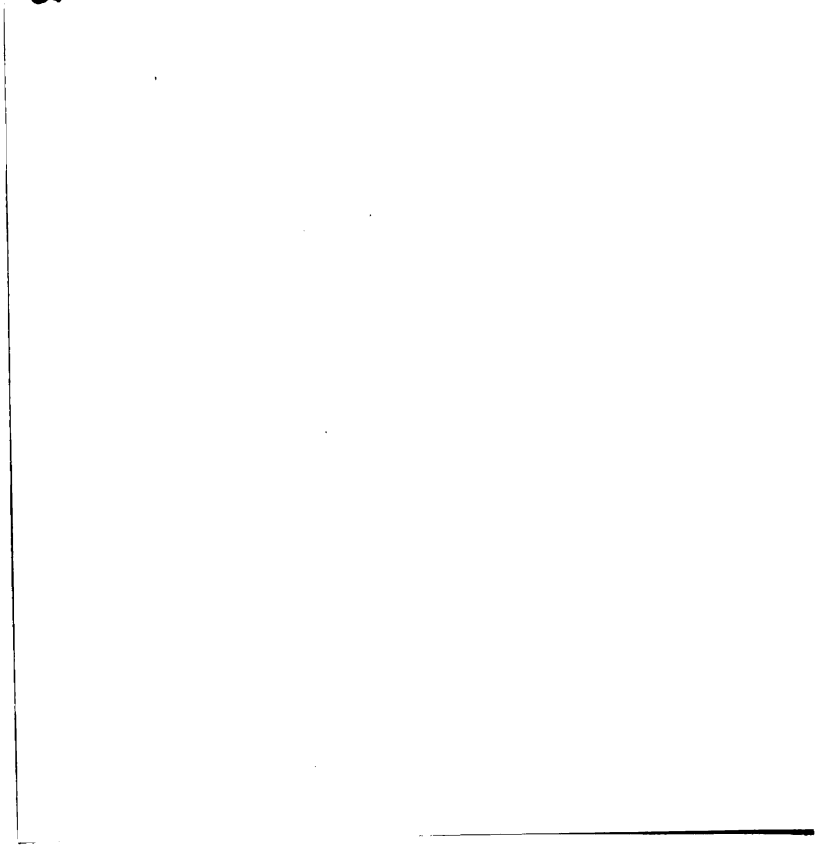
Mary Emma Barboni

1960



ISOLATION AND PURIFICATION OF INOSITOL POLYPHOSPHATES

BY

MARY EMMA BARBONI

A THESIS

Submitted to the College of Science and Arts of Michigan State University in partial fulfillment of the requirements for the degree of

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To Hugh

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INTRODUCTION

The purpose of this work has been to find suitable methods which could be used for the separation and isolation of pure phytic acid from various plant and animal tissues. This work was undertaken as part of a study of the biosynthesis of phytic acid and related compounds.

In order that preliminary studies of incorporation of C-14 labelled myo-inositol into phytic acid in maturing pea pods might be carried out, it was first necessary that methods be available for isolation of the phytic acid from the pods in a highly purified form. For the purposes of these experiments, it is much more important that the phytic acid be pure than that it should be recovered in good yield. It is also preferable that other closely related compounds, such as the other inositol polyphosphates, sugar phosphates, etc., which may be precursors of phytic acid, should be capable of separation and identification by the same methods. Another point which had to be borne in mind was the plan eventually to attempt to show biosynthesis of phytic acid in cell-free homogenates. In this case, one or more sources of phosphate groups (eg. ATP) must be added to the mixture and so both the added compound and its products must later be separated from the phosphorylated inositol products.

These requirements raise a number of problems which have not been faced by previous investigators. In all cases, they worked either with large quantities of tissues, or with large quantities of semi-purified compounds, or with synthetic materials. In the present case, it was necessary to develop methods which would permit working with very small amounts of tissue or homogenate, with extremely complex mixtures, and which would at the same time permit isolation of closely related compounds as well as phytic acid itself.

HISTORICAL

Phytic acid is one of the naturally occurring derivatives of myo-inositol which is characterized by having all of the hydroxyl groups esterified with phosphoric acid, as shown in Figure I. As such, it serves as a phosphorus reserve in

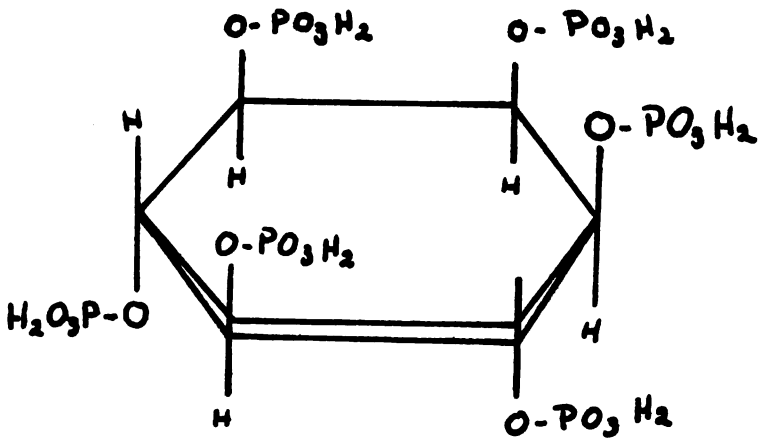


Figure I. Phytic acid.

normal plant tissues, and also as a resevoir for metallic elements such as calcium, magnesium, zinc, manganese, and possibly others. During the germination of various seeds, phytic acid is hydrolyzed, releasing both phosphorus and the metallic elements which are indispensible to the growth of the plant (1). Phytic acid is normally isolated as the calcium magnesium mixed salt, which is called "phytin".

The first real investigations of phytic acid and its derivatives began with the independent works of R. Anderson and S. Postornak. In 1912 Anderson, using commercial samples of calcium phytate, prepared and described various salts of this compound (2). In a second paper he described his

unsuccessful attempts to synthesize phytic acid by the action of phosphoric acid on inositol. These attempts lead only to what was described as a tetraphosphoric acid derivative of inositol (3). Leaving this approach, he attempted a biological preparation from wheat bran (4) from which he obtained a compound $C_{20}H_{55}O_{49}P_9$, the structure of which was never clearly defined. In 1914 Anderson reported the isolation of inositol monophosphate from wheat bran (5) and in 1920, the crystalline barium salt of phytic acid from that source and from cotton-seed meal (6).

Posternak began work on phytic acid in 1903 (7), at which time he prepared and described several salts of phytic acid and attempted a chemical synthesis. In 1921, using phosphorus pentoxide as the dehydrating agent for the esterification of inositol with orthophosphoric acid, he obtained the pure crystalline calcium magnesium salt of phytic acid (8).

As interest in the connection of phytic acid to plant and animal nutrition grew, a number of investigators concerned themselves with the quantitative estimation of this compound in various foodstuffs. In 1926 Averill and King published their work (9) on the estimation of phytin from fifty-seven samples of various wheats, grains and nuts. The procedure this group followed for the determination of phytic acid required approximately eight grams of ground substance, which was extracted with 2.0% hydrochloric acid. A 0.3% solution of ammonium thiocyanate was used as an indicator and the phytic

acid titrated with a standard solution of ferric chloride. Again, in 1935, McCance and Widdowson (10) in a work involving sixty-four foodstuffs, found that phytin was a characteristic constituent of grains, whole cereals, nuts, legumes, vegetables, etc., and in some instances accounted for 40-50% of the total phosphorus present. Five to ten grams of the dried ground material was required for each determination. The phytic acid was extracted with 2.0 N HCl, filtered, and the filtrate heated with a known amount of ferric chloride which produced a precipitate of ferric phytate. Upon removal of the precipitate, excess iron was determined colorimetrically as the thiocynate.

As more became known about phytic acid, and methods for detecting its presence were developed, work in this area branched out considerably. Still the problem of overcoming the difficulty of separating phytic acid from the normal plant and animal tissue consitituents remained incompletely solved. With the necessity of working with micro quantities of phytic acid and derivatives, and with growing concern for the purity of samples, investigators turned their attention to paper chromatography and paper electrophoresis for the purification of the organic phosphorus compounds. A number of ion exchange resins and eluting procedures have also been used by different groups for separation of phosphate esters of inositol with varying degrees of success.

In a comparative study of the hydrolysis products of

phytic acid under acidic and alkaline conditions, Desjobert and Petak (11) found it necessary to do extensive work with solvent systems for paper chromatography. Out of the eleven systems reported, this group chose the isopropanol/ ammonium hydroxide/ water (5:4:1) system to be superior to the others. Using the descending technique for twenty-four hours and the ethanolic ferric chloride and salicylsulfonic acid spray, they were able to demonstrate the separation of all six esters, mono- through hexa-phosphates, from an acid hydrolyzate of phytic acid.

G. Anderson (12) reported particular success with the system composed of methanol/ 0.5 N ammonium hydroxide (70:30), if the paper had been previously washed with dilute hydrochloric acid, water, ammonium versenate and water, in that order.

In 1949 Hanes and Isherwood developed the acidic molybdate spray for the detection of phosphoric esters of organic compounds (13). Bandurski and Axelrod (14) later simplified the procedure for development by substituting ultraviolet radiation in the place of the hydrogen sulfide treatment used by Hanes and Isherwood.

Wade and Morgan (15) developed a phosphate spray which requires more time for development, but which may be more sensitive. This spray involves the use of ethanolic ferric chloride followed by ethanolic salicylsulfonic acid. The resulting chromatograms are stable indefinitely, and improve in clarity with age.

In 1956 Arnold (16) reported that he could separate the mono-, di-, tri-, and tetraphosphoric acid esters of inositol by using paper ionophoresis. The penta- and hexaphosphoinositols migrated at similar rates and could not be separated from each other.

While investigating the orthophosphate and phytin changes in maturing pea seeds, Fowler (17) found several solvent systems for paper chromatography which when used together produce an effective separation of several of the organic phosphorus compounds present in seeds. He obtained his pea extract by boiling 100 grams of fresh peas with 95% ethanol for several minutes. The peas were then filtered hot and allowed to dry twelve hours at 80° C., after which they were ground and treated either of two ways. The first consisted of treating the ground peas with 10% acetic acid for three hours, filtering, and vacuum distilling the filtrate to a small volume. The second procedure for extraction requires the use of 10% TCA on the ground peas for three hours, followed by filtration. The addition of 0.2 N barium hydroxide to a pH of 8.2 causes the formation of a precipitate, barium phytate. The precipitate was then dissolved in either 10% acetic acid or 2.0 % hydrochloric acid.

Runeckles and Krotkov (18) presented a new technique for separating complex mixtures of phosphorus-containing compounds by using two-dimensional ionophoresis and

chromatography. This group experimented with thirty-five phosphorylated compounds, plus several organic acids, amino acids and sugars. Their procedure permits the separation of these compounds on a single sheet of paper.

Several investigators have reported successful separation of the inositol polyphosphates utilizing various ion exchange resins. Smith and Clark (19) seem to have had particular success using the weak base anion exchange resin De-Acidite (60-80 mesh) as absorbent and increasing acid strength solutions of hydrochloric acid as eluent.

EXPERIMENTAL

SPRAY TECHNIQUES

Two sprays were used for detection of phosphate-containing compounds on the chromatograms:

A. The acid molybdate procedure developed by Hanes and Isherwood (13) as modified by Bandurski and Axelrod (14). The dry papers are sprayed with the acid molybadate solution (5 ml. 60% perchloric acid, 25 ml. 4% ammonium molybdate, 10 ml. 1.0 N HCl, 60 ml. water) and dried at 90°C. to cause the hydrolysis of the esters. The liberated orthophosphoric acid reacts with the molybdate to form a phospho-molybdate complex. The Bandurski Axelrod modification requires at this point that the papers be exposed to ultraviolet radiation which reduces the complex to form a deeply blue colored compound. The original procedure of Hanes and Isherwood called for the treatment of the sprayed papers with hydrogen sulfide gas to cause the same reaction.

It has been found useful to re-hydrate the papers by steam treatment before exposure to ultraviolet light. This greatly speeds up the development of blue color and reduces background coloration.

B. The second procedure (15) consists of spraying the papers with 0.1% ferric chloride in 80% ethanol, allowing the papers to air dry and repeating the spraying with a 1.0%

salicylsulfonic acid solution in 80% ethyl alcohol. This technique depends first upon the fixation of the ferric ions by the phosphate esters, followed by reaction of the free uncomplexed ferric ions with salicylsulfonic acid. Phosphate-containing compounds appear as white spots on a pink to purple background. This technique permits recovery of the esters by elution from the papers if performed the same day as the spraying. It should be noted that although good development may be attained within four to twenty-four hours after spraying, the definition of the areas improves with time. The residual moisture in the paper must be within pH 1.5 to 2.5 for best results. Below a pH of 1.5 no color develops, while above a pH of 2.5 poor distinctions are observed.

Since the reactions involved require a free acidic group on the phosphate portion of the molecule, triesters of phosphoric acid are not detected.

C. The nucleotides such as ATP and AMP were also detected on the dry chromatogram or electrophoresis strip by examination under short-wavelength ultraviolet radiation. The nucleotides "quench" the natural fluorescence of the paper and appear as dark spots on the white fluorescent background.

PAPER CHROMATOGRAPHY

Various solvent systems were tested for both ascending and descending paper chromatography, in order to obtain a relatively fast and uncomplicated means of determining the major components of pea extracts, to effectively separate these components, and in order to purify the phytic acid if found in the mixture. It was also of importance to find a system which would separate all the phosphate esters of inositol, a necessity if pathway studies are to be continued.

A. Ascending Chromatography.

Whatman # 1 filter paper was used in all cases unless otherwise stated. In several instances Whatman # 41 H (acid-washed, hardened) paper was tried, but this did not significantly change the resolution of the compounds found in the mixture.

The standard compounds ATP, ADP, sodium dihydrogen phosphate, myo-inositol-2-phosphate, and sodium phytate, were spotted one and one half inches from the ends of the paper, spaced one inch apart. The papers were bent into the shape of cylinders, and stapled closed. The solvent front was allowed to travel approximately fourteen inches on papers seventeen inches long. Pyrex cylinders, 6 by 18 inches with ground tops were used, and were sealed with glass plates. One hundred milliliters of solvent was placed in the bottoms of the tanks and allowed to equilibrate.

The eight solvent systems tested were of the following compositions:

Isobutyric acid/ 0.5 N ammonium hydroxide (10:6)

Isopropanol/ ammonium hydroxide/ water (6:3:1)

n-Propanol/ ammonium hydroxide/ water (5:4:1)

Methanol/ formic acid/ water (80:15:5)

Methanol/ ammonium hydroxide/ water (7:3)

n-Butanol/ 30% acetic acid (1:1)

t-Butanol/ formic acid/ water (6:2:3)

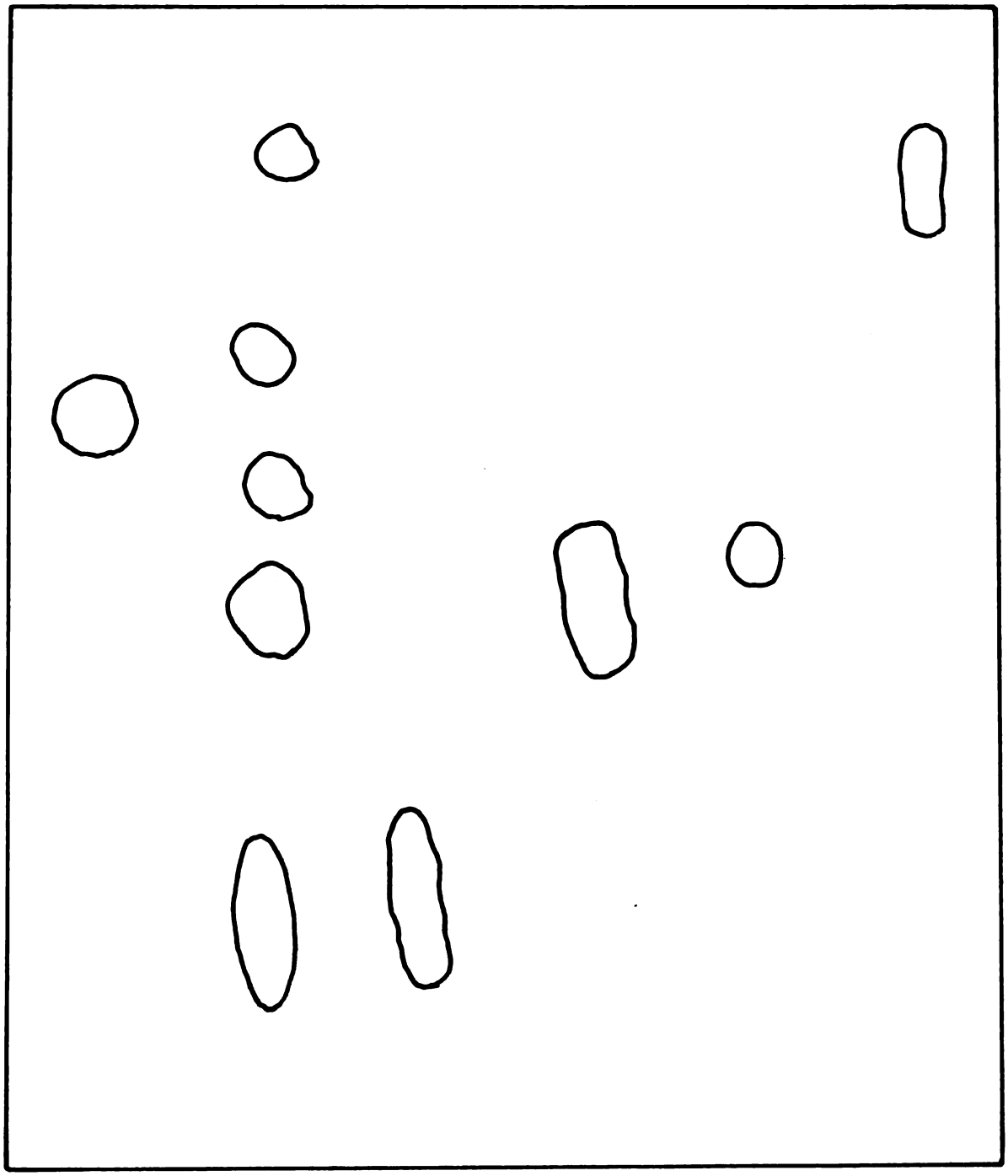
t-Butanol/ TCA/ water (80: 4g.:20)

Of these, five were found satisfactory for separations involving the standard compounds listed above. These were used as single solutions and together to form a standard mixture. The Rf values for these compounds in the five solvents are shown in Table I. Typical separations are shown in Figures 2-6.

Table I. Rf values of the standard compounds, sodium dihydrogenphosphate, ATP, AMP, myo-inositol-2-phosphate, and sodium phytate.

Solvent Systems:
1. t-Butanol/ formic acid/ water (6:2:3)
2. Isobutyric acid/ 0.5 N ammonium hydroxide (10:6)
3. t-Butanol/ TCA / water (80:4g.:20)
4. n-Propanol/ ammonium hydroxide/ water (5:4:1)
5. Methanol/ formic acid/ water (80:15:5)

	-Solvents-				
	1.	2.	3.	4.	5.
NaH_2PO_4	0.59	0.31	0.50	0.30	0.87
AMP	0.38	0.57	0.24	0.40	0.49
ATP	0.24	0.31	0.04	0.35	0.37
Na. Phytate	0.31	0.07	0.05	0.10	0.60
Myo-Inos.-2-phosphate	0.37	0.18	0.06	0.40	0.51



Na Phytate (purified) | Mixture | ATP | AMP | Inos-2-phosphate | NaH_2PO_4

Figure II. A typical chromatographic separation of the five standard compounds and a mixture of the five, in the solvent system Methanol, formic acid, water (80:15:5)

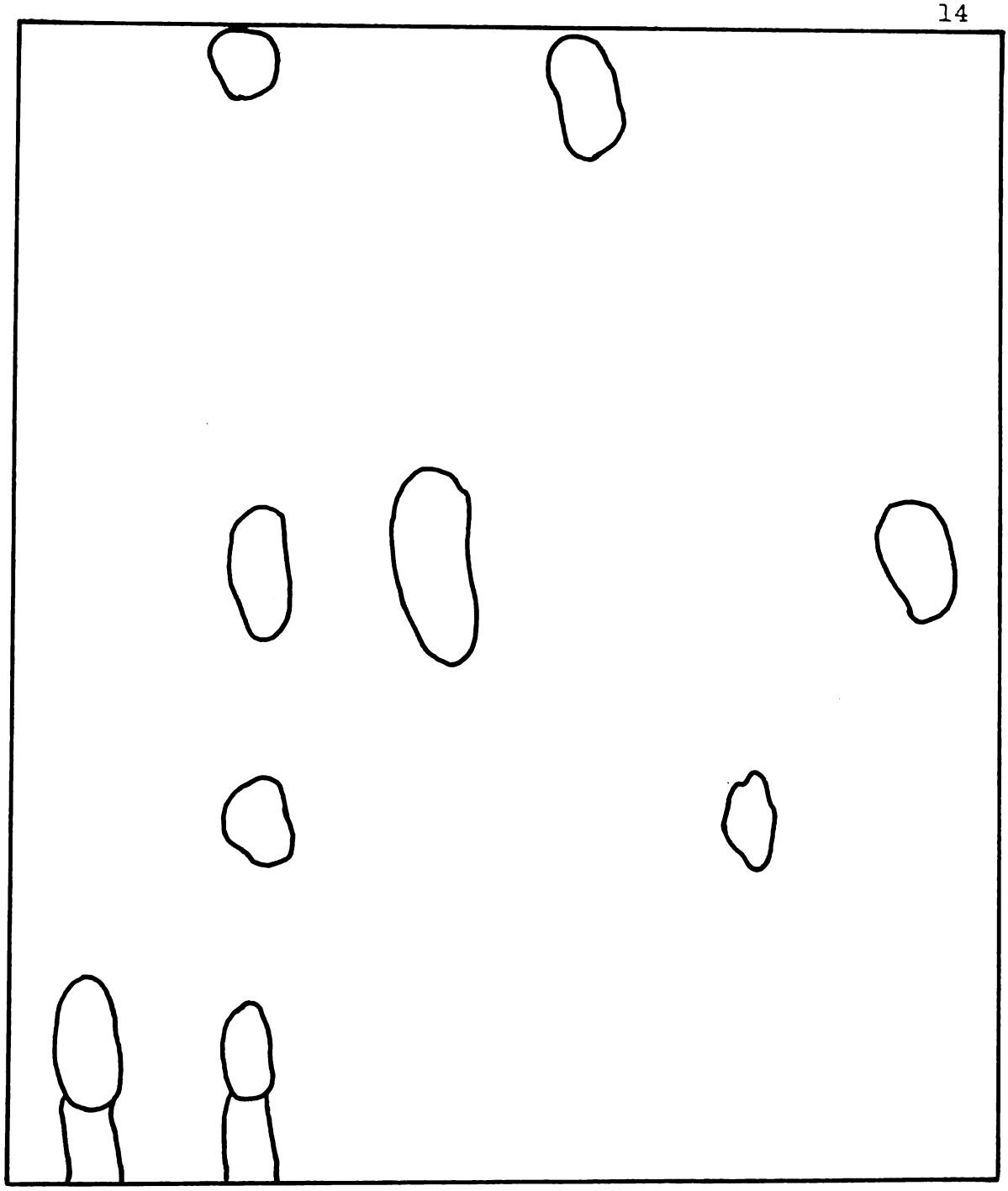


Figure III. A representative chromatogram of the five standard compounds and a mixture of the five, in the solvent system Isobutyric acid/ 0.5 N ammonium hydroxide (10:6)

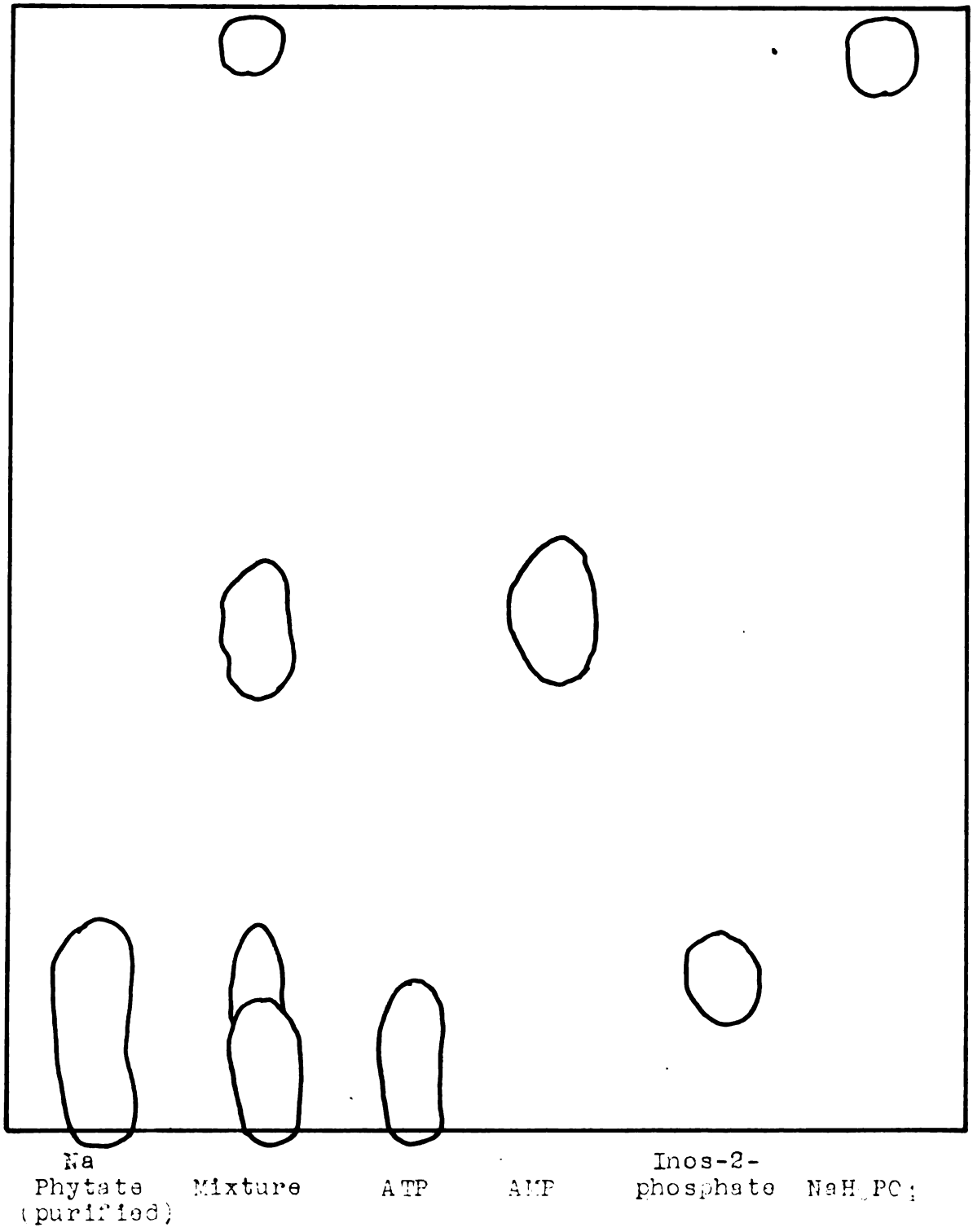


Figure IV. A paper chromatogram of the standard compounds and a mixture of the compounds, run in the solvent system t-Butanol, TCA, water (80:4 g: 20)

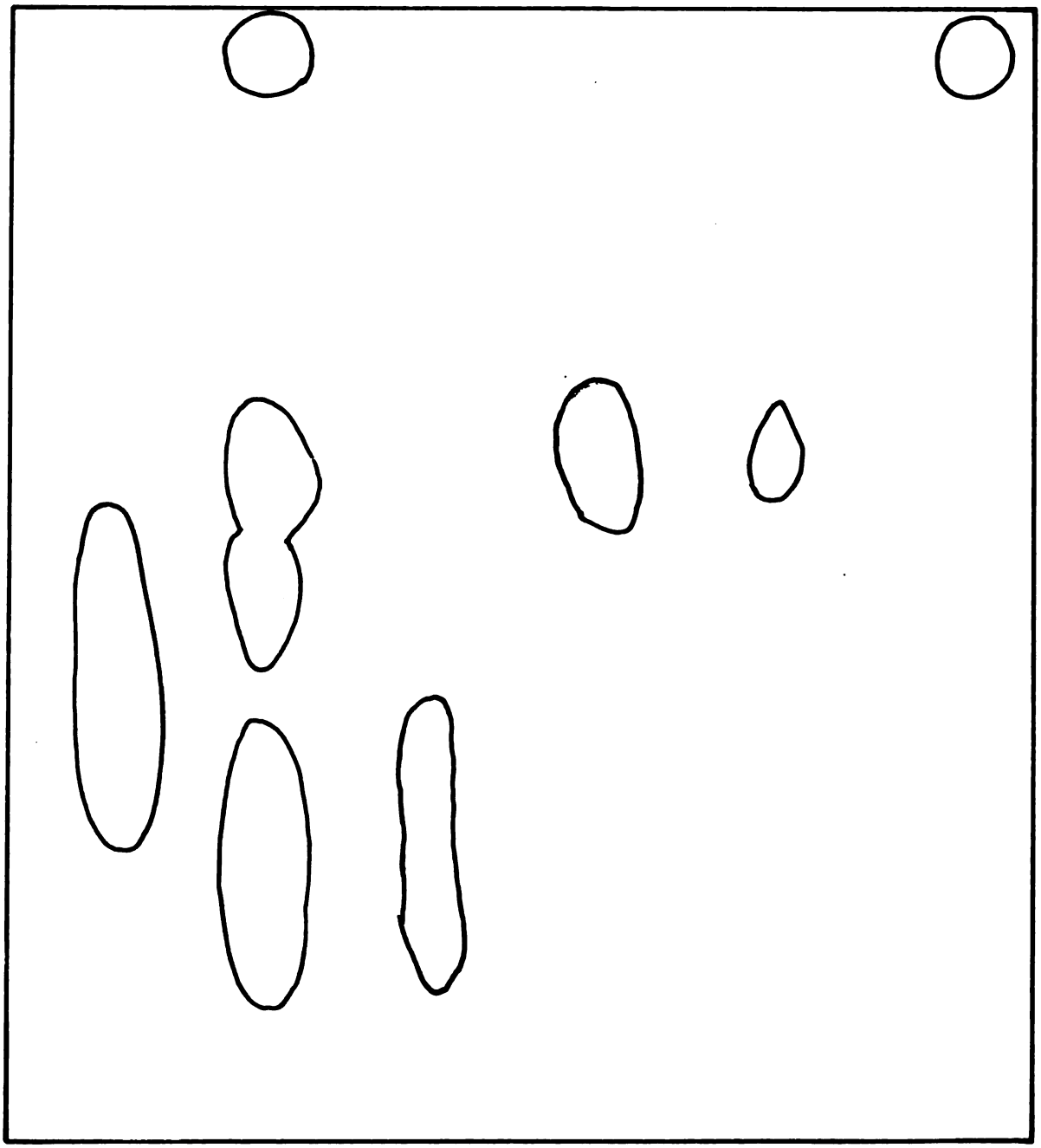


Figure V. A typical paper chromatogram of the five standard phosphorus-containing compounds and a mixture of the five.

Solvent system: t-Butanol, formic acid, water (6:2:3)

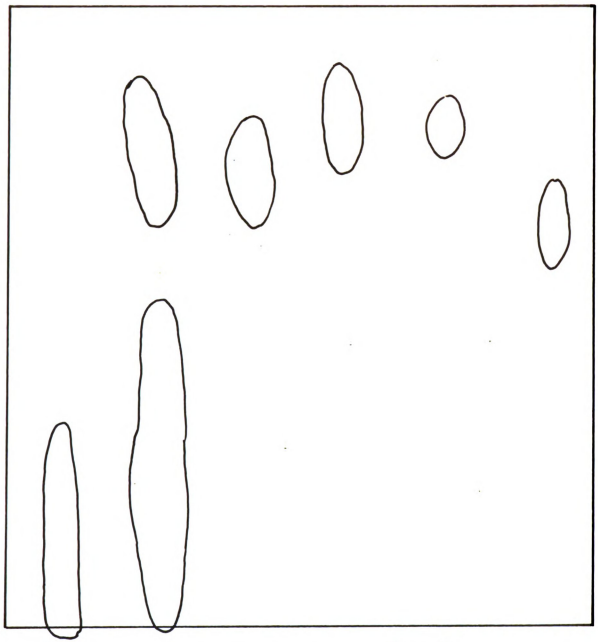


Figure VI. A representative paper chromatogram of the five standard compounds and a mixture of the five, run in the solvent system n-Propanol/ ammonium hydroxide/ water (6:4:1)

These solvent systems all require different times to run fourteen inches. Table II shows the approximate times for each of the five solvents.

Table II. Approximate time required for each of the five solvent systems used to travel fourteen inches under standard ascending conditions.

Note: These papers were run on Whatman # 1 paper, at 25'C. as described above.

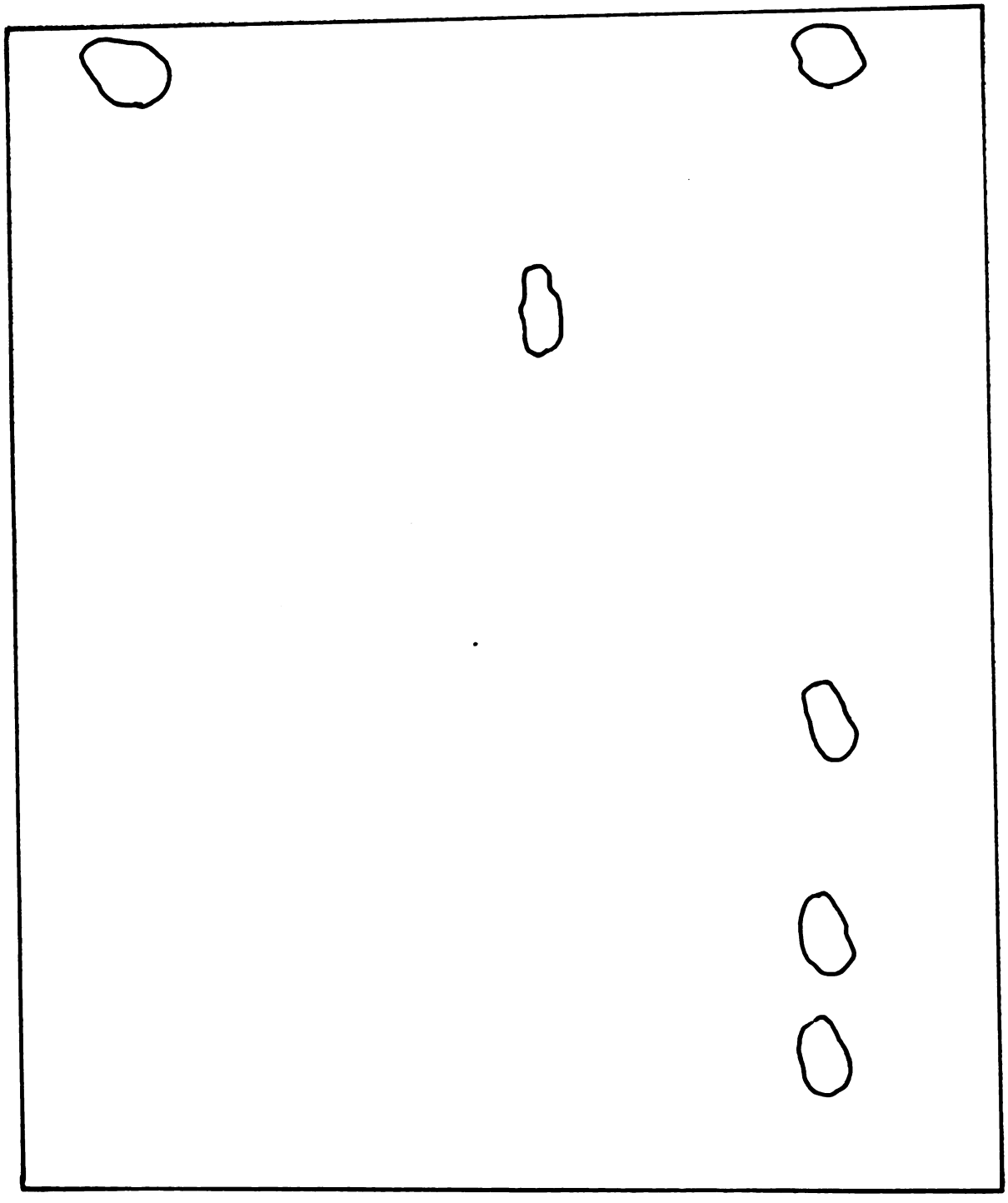
	1.	2.	3.	4.	5.
Time:	25 hr.	19 hr.	48hr.	17 hr.	7 hr.

B. Descending Chromatography.

Whatman # 1 paper and Whatman # 41 H paper was used, but as in the ascending technique, the 41 H paper did not improve the separation of the standard compounds. Two tanks were used: one, 12 by 24 inches, which contained two glass troughs, supported on steel rods capable of holding four, seven by fourteen inch papers. Using this tank, it was possible to run similar papers 24, 48, 72 and 96 hours under identical conditions. In all cases, the solvents was allowed to flow off the ends of the papers, which had been serrated to permit even flow. The second tank, 6 by 18 inches, contained one five inch glass trough supported on glass rods which was capable of containing one four inch paper. This tank was used for runs in which the temperature was held at 37'C.

Using the isobutyric acid/ 0.5 N ammonium hydroxide

system for descending chromatography, it was possible to separate some of the myo-inositol polyphosphates. This required from three to four days, allowing the solvent to flow continuously off the end of the paper. The time was shortened by placing the chromatography tank in an incubator held at 37'C. Using this procedure, four separate phosphate-containing areas in addition to the inorganic phosphate spot were found from a crude sodium phytate sample. Very little difference was noted using washed (0.01 % EDTA) Whatman # 1 paper. A typical separation is shown in Figure VII.



NaH_2PO_4 Myo-Inos.-2-Phosp. Na Phytate

Figure VII. Descending chromatogram of a crude preparation of sodium phytate, and the standards myo-inositol-2-phosphate and sodium dihydrogen phosphate, after running three days in the solvent system Isobutyric/ 0.5 N ammonium hydroxide (10:6).

PAPER IONOPHORESIS

The procedure of Arnold (16) for paper ionophoresis was followed with a few minor changes, in an attempt to separate the five compounds, ATP, ADP, myo-inositol-2-phosphate, sodium dihydrogen phosphate, and sodium phytate. The buffer used, was made by adding ten parts of a 0.2 N acetic acid solution to one part of a 0.2 N sodium acetate solution, giving a buffer of pH 3.6. The Model R Spinco electrophoresis unit was used at 230 volts for three hours at room temperature. Longer times at this voltage caused some of the compounds to migrate off the paper strips.

Each of the five standard compounds, inositol, and a mixture of the five compounds, were run on separate strips. Overloading the paper strips had to be avoided in order to obtain satisfactory resolution of the mixture. By using two separate strips for the mixture, and spraying each with a different reagent, it was possible to get a good picture of the compounds present in the mixture.

At 230 volts and three hours running time, at room temperature, the compounds were located at the following distances from the origin:

Sodium phytate	18.4 cm.
Sodium dihydrogen phosphate	16.2 cm.
Myo-inositol-2-phosphate	12.1 cm.
Adenosinetriphosphate	11.0 cm.
Adenosinemonophosphate	5.0 cm.
Myo-inositol	4.2 cm.

ADP and ATP were detected on the dry strips by examination under ultraviolet light, where they appear as dark blue

areas or bands on a light background. One strip containing the mixture was then sprayed with 0.1% ferric chloride, allowed to dry and sprayed with 1.0% ethanolic salicylsulfonic acid and allowed to darked for approximately four to twenty-four hours. The phosphorylated compounds appeared as white bands on a pink background. The second strip containing the mixture was sprayed with the acid molybdate reagent of Hanes and Isherwood (13), and then heated at 90°C. until dry. Inorganic phosphate appeared as a bright yellow band immediately after spraying. By exposing the strip to ultraviolet radiation for several minutes according to the procedure of Bandurski and Axelrod (14), the phosphate esters appeared as blue regions on a light background. A tracing copy of this strip was made, as the treatment leaves the paper in a very fragile condition and exposure to daylight tends to turn the entire paper blue.

Separation of the components of the sample mixture was clear and distinct, as shown in Figure 8, and the migration distances closely corresponded to the standard values. There was some shading of the separation of ATP from myo-inositol-2-phosphate using the molybdate procedure; presumably this treatment is not sensitive enough to distinguish clearly between the two areas.

Inositol was used as a non-charged compound to detect solvent flow. This strip was sprayed with ammonical silver

nitrate (20) and heated to 90°C. in an oven until the dark brown area appeared, indicating inositol. This compound ran 4.2 cm. from the origin under the conditions stated above.

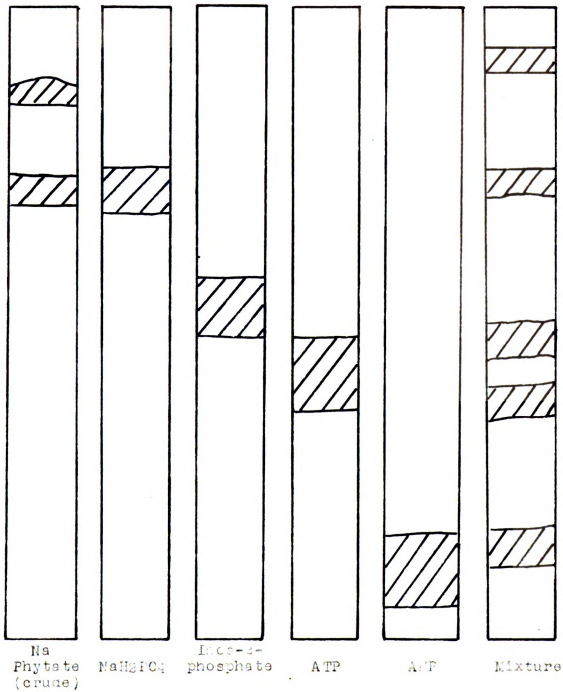


Figure VIII. Typical electrophoretic separation of five standard compounds and a mixture of the five.

ION-EXCHANGE SEPARATIONS

Two resins were used for the column separations of phosphorus-containing compounds. The ion-exchange resin Dowex 2 X 8 chloride form, (100-200 mesh), which was washed repeatedly with 3.0 M ammonium formate to convert the resin to the formate form, and N,N-diethylaminoethyl cellulose (Eastman 7392) which was used without further treatment.

A column 12 mm. in diameter was used in all cases. The elution procedure of Hubscher and Hawthorn (21) was followed, which consisted of stepwise increasing concentrations of formic acid and ammonium formate.

Typical separation attempts follow:

Column A. Five hundred micromoles of α-glycerol phosphate and five hundred micromoles of a crude preparation of sodium phytate were applied to a column, and two hundred milliliters of distilled water passed through to remove any free inositol. Three different eluting solvents were used, beginning with 0.01 M formic acid in 0.03 M ammonium formate. Two milliliters of the collected samples were used to run phosphorus determinations. (See page 30 for phosphate determination procedure). Approximately two hundred milliliters of this solvent was used, but all of the determinations for phosphorus, both hydrolyzed and unhydrolyzed were negative.

The eluting solvent was changed to 0.05 M formic acid

in 0.20 M ammonium formate, and another 200 ml. were collected. Every other tube was tested for phosphorus by means of the colorimetric method, again with negative results.

The final solvent was composed of 3.0 M formic acid in 1.0 M ammonium formate. Although inorganic phosphate was found in the first hundred milliliters of the eluant, the remaining organic phosphorus compounds were not released, and the column was discontinued after three hundred milliliters of this solvent had been used.

Column B. Ten mature peas which had been previously soaked in water overnight were homogenized with ice-cold 5% perchloric acid in a Vertis blender. The homogenate was spun down in a clinical centrifuge, and then 5 N KOH added to a pH of about 6. This solution was allowed to stand for one hour in the refrigerator, after which time it was filtered through Whatman # 50 paper.

The clear solution (35 ml.) was placed on a 10 cm. column, and two hundred milliliters of distilled water was run through. Inorganic phosphate was not found in this water wash. One hundred milliliter fractions were collected, treated with IR 120 (H) and flash evaporated. The first solvent, 0.5 M formic acid in 0.2 M ammonium formate released inorganic phosphate as determined by the phosphorus determination, and as evidenced on a paper chromatogram of these samples. A total volume of 500 ml. of this solvent

was used. The concentration of eluting solvent was increased as in Column A to 3.0 M formic acid in 1.0 M ammonium formate, but no other phosphate-containing materials could be located.

Column C. One gram of Eastman N,N-diethylaminoethyl cellulose was packed into a column 10 mm. in diameter, to a height of seven cm. Fifty milliliters of water was used to wash this column, and then 50 mg. of crude sodium phytate was applied to the column. Fifty milliliters each of distilled water, 0.1 M formic acid, 0.5 M formic acid, 1.0 M formic acid, and 3.0 M formic acid in 1.0 M ammonium formate were used, in that order, to elute the column. The samples were collected in fifty milliliter fractions, and evaporated to a volume of two milliliters. One milliliter each of these solutions were used for the acid hydrolysis procedure which would indicate total phosphorus, while the remainder was saved for inorganic phosphorus determinations and for chromatography. The results of the colorimetric determinations are shown in Table III.

Table III. Micromoles of phosphorus released from Column C.

Sample	Inorg. Phos.	Total Phos.
Water wash	6.66 uM	5.76 uM
0.1 M formic acid	0.67 uM	0.94 uM
0.5 M formic acid	0.56 uM	0.87 uM
1.0 M formic acid	0.00 uM	0.70 uM
3 M formic acid in 1 M amm. formate	0.00 uM	0.38 uM

The above fractions were chromatographed and run in two solvent systems, t-Butanol/ TCA/ water (80:4g:20) and Methanol/ formic acid/ water (80:15:5). The reproduction of the

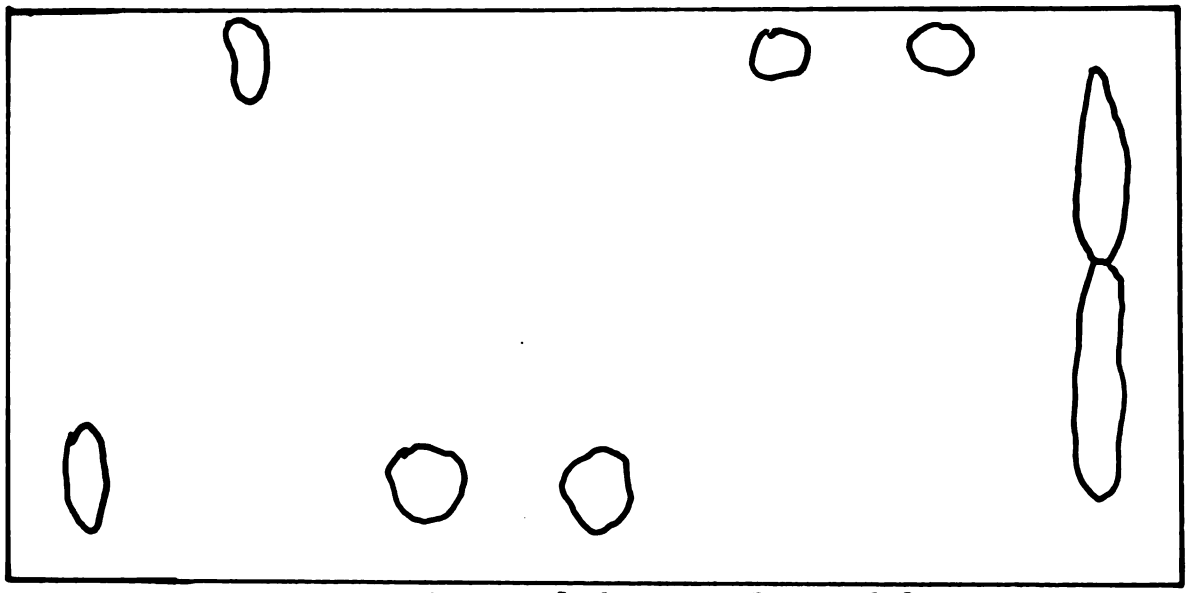
chromatogram run in the latter solvent is shown in Figure IX.

Column D. Twenty mature peas and 100 mg. of calcium phytate were homogenized with ice-cold 5% perchloric acid in a Vertis blender for several minutes. The homogenate was spun down in a small clinical centrifuge, and the clear supernatant filtered through Whatman # 50 paper after standing for one hour in the cold.

The clear solution was applied to a five cm. Dowex 2 X 8 formate column, which was then washed with 50 ml. of distilled water. Increasing concentrations of formic acid were used to elute the column; 0.1 M formic acid, 0.5 M formic acid, 1.0 M formic acid, and 3.0 M formic acid in 1.0 M ammonium formate. Fifty milliliters of each of these solvents were used with the exception of the 3.0 M formic in 1.0 M ammonium formate. One hundred milliliters of this solvent was used, in two fractions. The collected samples were evaporated to approximately two milliliters, and used for the phosphorus determination. Table IV shows the results of this column.

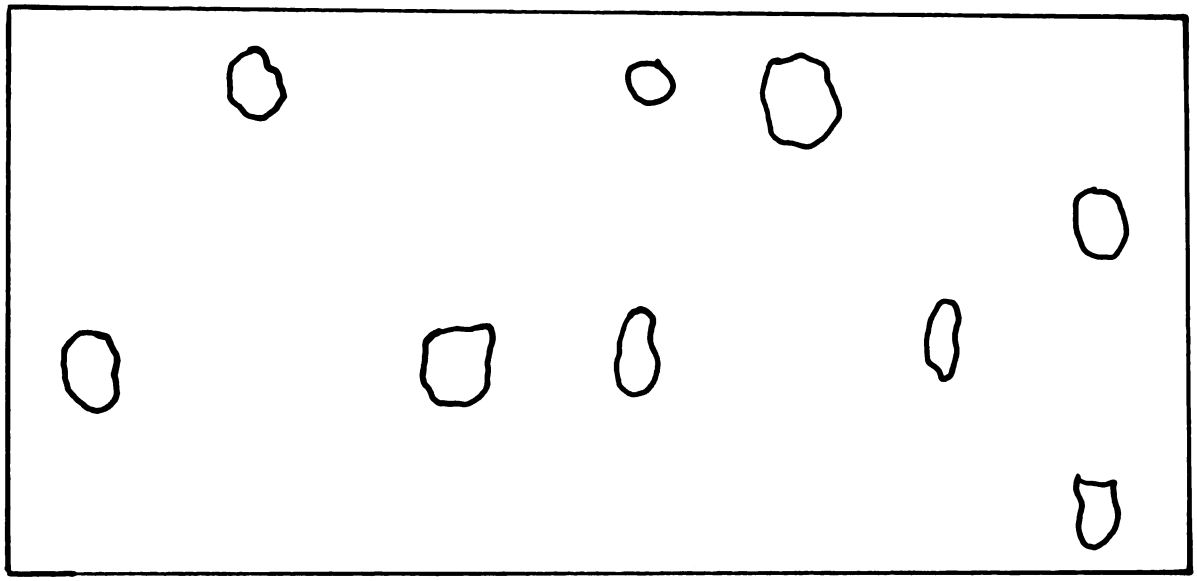
Table IV. Micromoles of phosphorus released in each fraction from Column D.

Fraction	Inorg. Phos.	Total Phos.
Water wash	0.00 uM	0.30 uM
0.1 M formic acid	0.00 uM	0.16 uM
0.5 M formic acid	0.00 uM	0.27 uM
1.0 M formic acid	3.30 uM	3.46 uM
3 M formic in 1 M ammonium formate	2.08 uM	3.72 uM
3 M formic in 1 M ammonium formate	0.63 uM	0.74 uM



Na Phytate	NaH_2PO_4	3 M form. 1 M Amm. formate	1.0 M formic	0.5 M formic	0.1 M formic	Water wash

Figure IX. Chromatogram of the DEAE column fractions, and the standards sodium phytate and sodium dihydrogen phosphate. Solvent: Methanol/ formic acid/ water (80:15:5)



Na Phytate	NaH_2PO_4	3 M form. 1 M Amm. formate	3 M form. 1 M Amm. formate	1.0 M formic	Water wash	First fract.

Figure X. Chromatogram of the Dowex 2 X 8 formate column fractions, omitting the 0.1 M and 0.5 M formic acid fractions. Solvent: Methanol/ formic/ water (80:15:5)

A chromatogram of these samples (Figure X), which was run in the Methanol/ formic acid/ water (80:15:5) system, indicated the presence of phytic acid in both of the 3.0 M formic in 1.0 M ammonium formate fractions, and in the water wash fraction. Two areas appeared in the eluant of the pea extract which have not been identified.

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Phosphorus Determination. Inorganic phosphate and total phosphate were determined colorimetrically according to the procedure outlined in Experimental Biochemistry (22). Inorganic phosphorus was determined as follows: to a known volume of sample was added one milliliter of 2.5 % molybdate reagent (in 3 N sulfuric acid), one milliliter of reducing reagent (3% sodium bisulfite - 1% p-methylaminophenol sulfate), and water to make a total volume of ten milliliters. The samples were allowed to stand twenty minutes and were then read at 660 mu using the Spectronic 20 colorimeter.

Total phosphorus was obtained by heating one milliliter of the sample with one milliliter of 5 N HCl for two hours in a sand bath at 130°C. Four tenths of a milliliter of the hydrolysate was then used for the colorimetric procedure outlined above with one exception: the commercial 2.5 % molybdate reagent was replaced with a 2.5% ammonium molybdate solution in distilled water.

PREPARATION OF SODIUM PHYTATE

Purified samples of sodium phytate were obtained by treating solid barium phytate, which had been prepared as a laboratory preparation from wheat bran according to the procedure of Anderson (23), with IR 120 (H). The resin removed the barium yielding soluble phytic acid. This resulting solution was immediately neutralized with 5 N sodium hydroxide, and the sodium phytate chromatographed in a long band on Whatman # 3 mm paper and run in the Methanol/ formic acid/ water (80:15:5) solvent system. This system may be used to purify phytic acid when the only other constituent present is inorganic phosphate. Since inorganic phosphate separates well from the phytate, the phytate band can be cut from the chromatogram and the strip eluted with water.

DISCUSSION

The methods to be used for isolation and purification of inositol polyphosphates, clearly depend upon the particular separation desired, and the amount of material to be separated. In cases where the mixture is a complex one, as in the case of the pea extracts, and where a large amount of material is to be separated, a preliminary fractionation by an ion-exchange resin column may be useful. In most cases, the paper chromatograms were not effective in the presence of large amount of impurities. On the other hand, the ion-exchange columns did not give complete separations in many cases. A combination of the two procedures should provide a clean separation of even the most impure extracts.

Paper ionophoresis provides a useful tool for identification of the components in the mixtures tested. It is less useful for isolation purposes mainly because of the large amounts of buffer eluted from the electrophoresis strips along with the compounds.

The best procedure to use for a given separation can be selected by reference to the results shown in the Experimental Section.

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