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The Isolation of a New Lipid, Triphosphoinositide, and Monophosphoinositide from Ox Brain

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The classic work of Folch (1942) showed that inositol was a constituent of brain phospholipids and an inositol phosphatide fraction was isolated which was rich in these inositol lipids. This inositol phosphatide was subsequently subfractionated with solvents, and an inositol-containing phospholipid was isolated which was designated diphosphoinositide because it produced inositol diphosphate as a major product on acid hydrolysis (Folch, 1949). Also, a trypsin-resistant protein fraction was isolated from brain tissue. This fraction contained a phosphoinositide bound to it by a salt-like linkage so that it was not extracted with lipid solvents unless these were acidified (Folch, 1952; Lebaron & Folch, 1956).

The starting point of the present investigation was an attempt to prepare pure diphosphoinositide as a substrate for metabolic experiments. It was soon realized that the brain phosphoinositide fraction was heterogeneous and from it were isolated two other inositol-containing phospholipids, monophosphoinositide and a new lipid designated triphosphoinositide because it contains inositol and phosphate in the molar ratio 1:3. The fractionation of these inositol phospholipids was followed by examining ionophoretically the products they formed after deacylation by mild treatment with alkali (Dawson, 1960). This showed that only the

monophosphoinositide was extracted from brain tissue with neutral lipid solvents such as chloroform-methanol unless the tissue was pretreated with acetone. This has led to a convenient method for the isolation of triphosphoinositide by removing it with acidified lipid solvents from brain tissue previously extracted with neutral solvents. The protein-triphosphoinositide complex obtained is then disrupted to liberate the free lipid.

This paper is concerned with the isolation of triphosphoinositide from brain tissue and describes some properties of the new lipid. It also reports the isolation and analysis of monophosphoinositide from the same tissue. A preliminary account of this work has been given (Dittmer & Dawson, 1960). Confirmation of the existence of triphosphoinositide in brain has also been recently obtained independently by Grado & Ballou (1960, 1961), who isolated inositol triphosphate from acid hydrolysates of purified diphosphoinositide preparations.

EXPERIMENTAL

Materials

Ox brain was obtained from the animal as soon as possible after death and transported to the laboratory packed in ice. No attempt was made to separate white and grey matter for the fractionation. Guinea-pig brains were removed from the animals a few minutes after killing with chloroform and accumulated in solid CO₂.

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Isolation of monophosphoinositide and triphosphoinositide from acetone-pretreated brain tissue

The brain tissue was stripped of adhering blood clots and then treated with acetone and ethanol and extracted with light petroleum according to Folch (1942, 1949). The ethanol-insoluble phosphoinositide fraction was subjected to multiple precipitation from chloroform with methanol to obtain the 'diphosphoinositide' fraction (Folch, 1949). The soluble material from all these methanol precipitations was combined, dried *in vacuo* and dissolved in chloroform. Monophosphoinositide was isolated from this chloroform solution by chromatography on silicic acid columns as described by Hanahan, Dittmer & Warashina (1957) or on alumina columns followed by silicic acid chromatography (Dawson, 1958).

The insoluble 'diphosphoinositide' fraction obtained by the multiple-precipitation procedure described above was dissolved in chloroform-methanol (4:1, v/v) and transferred to a column of Amberlite IR-120 (H+) resin which had been previously washed with chloroform-methanol (4:1, v/v). Approximately 4 ml. of resin was used/mg. of organic P in the preparation. The phospholipid was washed through the column with chloroform-methanol (4:1, v/v) by using 4 ml./ml. of resin. This treatment effectively removes cations and also 90-95% of the inorganic P which is usually present to the extent of 50% of the total P. This may be due to a combination of the phosphate with bivalent cations such as Ca^{2+} or Mg^{2+} adsorbed on the sulphate groups of the resin.

The eluent from the ion-exchange resin was dried under vacuum and a countercurrent distribution carried out in a solvent system consisting of chloroform, ethanol, ether and water in the proportions of 3:2:5:2 by vol. Four transfers were usually sufficient to effect good separation of an inositol fraction in the upper layer although a little triphosphoinositide remained in the chloroform or bottom layer of the system. Emulsions formed in the course of the fractionation were broken by centrifuging; accordingly, it was found most convenient to carry out the fractionation in centrifuge tubes. Transfer of the top fractions was facilitated by drawing them off with a siphon bottle connected to a water aspirator. The top layer from this fractionation was dried *in vacuo* (acetone was added to prevent foaming) and the triphosphoinositide dissolved in moist chloroform.

Application of the same isolation procedure to fraction I described by Folch (1942) rather than to the purified 'diphosphoinositide' (8 ml. of resin/mg. of organic P) produced a preparation which contained in addition to triphosphoinositide a nitrogenous compound that has not yet been identified. This contaminant was, however, eliminated by the following procedure.

Fraction I was suspended in aqueous 50% (v/v) ethanol (2 ml. of 50% ethanol/mg. of lipid P) and saturated $\text{Ba}(\text{OH})_2$ solution was added until the mixture was alkaline to phenolphthalein. This mixture was extracted twice with equal volumes of chloroform. The combined chloroform extracts were washed twice with water and the volume was reduced to approx. one-tenth. Methanol (4 vol.) was added and the precipitate produced after standing at -18° for 0.5 hr. was collected by centrifuging and was then dissolved in chloroform-methanol (4:1, v/v). The final purification of triphosphoinositide was carried out as

described above starting with the passage of the chloroform-methanol solution through an Amberlite IR-120 column.

Preparation of triphosphoinositide from chloroform-methanol-extracted brain-tissue residue

Ox brain (200 g.) was freed from adhering blood clots and homogenized in a Waring Blendor in 2 l. of chloroform-methanol (1:1, v/v). After 10 min. the suspension was centrifuged, the insoluble matter was washed with 1 l. of chloroform-methanol (2:1, v/v) and again recovered by centrifuging. The residue was extracted three times with 400 ml. volumes of chloroform-methanol (2:1, v/v) each containing 1 ml. of conc. HCl. The extraction was carried out at 37° with stirring and for 20 min. periods. The triphosphoinositide was only slowly extracted under these conditions and hardly at all at room temperature. The combined acidified chloroform-methanol extracts containing a good deal of haem pigments were shaken with 0.2 vol. of 0.9% NaCl. After centrifuging, the upper aqueous phase was sucked away and discarded, the layer of interfacial white material penetrated and the lower chloroform phase removed by suction and collected. The interfacial material containing most of the triphosphoinositide was saved. The lower chloroform layer was shaken with 0.2 vol. of 0.9% NaCl, and, after centrifuging, a small amount of interfacial material was collected. The lower chloroform phase containing the haem pigments and traces of lipids not removed in the original extraction was discarded.

The combined interfacial material fractions were shaken with 400 ml. of chloroform-methanol (2:1, v/v) and 80 ml. of 0.9% NaCl, and, after centrifuging, the interfacial material was collected. To the interfacial material was added 80 ml. of acetone, the mixture was heated to boiling for 2 min. and then dried *in vacuo*. The residue was treated with 80 ml. of ethanol, which was evaporated off on a boiling-water bath and finally *in vacuo*. The residue was extracted twice with 200 ml. of chloroform-methanol (2:1, v/v) containing 0.1 ml. of conc. HCl. The mixture was initially homogenized in a blender to break up any lumps and the extraction carried out for 5 min. at 37° . The extracts were filtered through a pad of glass wool (filter paper often resulted in low yields and its use was avoided throughout the fractionation). The combined extracts were vigorously shaken with 0.2 vol. of N-HCl and, after centrifuging, the lower layer containing the crude triphosphoinositide was collected. To remove protein the chloroform layer was mixed with 0.5 vol. of methanol and shaken with 0.2 vol. of N-HCl. The lower layer was collected, any interfacial material was rejected, and 0.5 vol. of methanol added. The solution was shaken with 0.2 vol. of water and the lower layer collected after centrifuging. It contained crude triphosphoinositide corresponding to 3.17-9 mg. of P in various preparations.

The crude triphosphoinositide was now purified via its sodium or ammonium salt. The lower chloroform layer was dried *in vacuo* below 35° and dissolved in 3 ml. of chloroform. In some preparations a clear solution was not obtained. This could often be remedied by adding an equal volume of methanol, drying *in vacuo* and redissolving in the same amount of chloroform. Any solid matter remaining was removed by centrifuging. To the clear brownish solution 20 ml. of methanol was added. After 2 hr. at -15° any precipitate was centrifuged off and rejected. It usually

contained only a small percentage of the total P and analysis showed it to be largely crude triphosphoinositide. To the supernatant, cooled in an ice bath, was slowly added methanolic NaOH (0.1 N) until a pH of 6.5-7 was reached and precipitation of the sodium salt was complete. Care had to be taken to avoid excess of alkali or deacylation resulted. In this respect preparation of the ammonium salt is preferable and this was simply done by passing dry NH_3 gas into the supernatant until a slight excess (pH 8) was present. After several hours at -10° the sodium or ammonium triphosphoinositide was centrifuged off and a small extra amount could be obtained by working up the mother liquors. The compound was then reprecipitated from chloroform solution with methanol (or acetone), centrifuged off and dried or stored in chloroform solution.

Analyses

Glycerol. This was assayed according to the procedure of Hanahan & Olley (1958). The lipid was hydrolysed with 2N-HCl in a sealed tube for 48-72 hr. at 105° to prevent loss of glycerol by volatilization or decomposition. Assays were done in triplicate and a standard curve was run with each set of determinations.

Inositol. This was determined microbiologically with *Kloeckera brevis*, by the method of Campling & Nixon (1954), as described by Dawson & Freinkel (1961). Lipid samples were hydrolysed for 30-48 hr. in 5N-HCl at 105° , the acid was removed by evaporation to dryness *in vacuo*, and the residue dissolved in water and adjusted to pH 5 with NaOH for assay.

Phosphorus. This was estimated by the method of Fiske & Subbarow (1925) after digestion with HClO_4 .

Acyl ester groups. These were determined by the method of Stern & Shapiro (1953) scaled down so that the colour was developed in 1.0 ml. total final volume and read in a microcell (1 cm. path) of the Unicam SP. 500 spectrophotometer. A standard curve with 0.0-5 μ -equiv. of triacetin was determined with each set of analyses. Pure ovolecithin gave the theoretical P:acyl ester ratio.

Fatty acid. The lipid residue was hydrolysed for 1 hr. at 100° in 1 ml. of N-KOH and after cooling 1.5 ml. of N-HCl was added. The mixture was shaken with 3 ml. of light petroleum (b.p. $60-80^\circ$) and centrifuged, and a 2 ml. sample of the upper layer was carefully removed without touching the walls of the tube. To the solution of fatty acids was added 3.5 ml. of acetone and cresol red indicator. The acid was titrated with methanolic 0.005N-NaOH with a stream of N_2 passing through the solution. A standard curve was prepared with stearic acid.

Nitrogen. This was estimated by the procedure of Lang (1958).

Sodium. This was determined by using a flame photometer (EEL).

Acid and alkaline hydrolysis of lipids. The technique used and the separation of the hydrolysis products by paper chromatography and ionophoresis and column chromatography is described in the next paper (Dawson & Dittmer, 1961).

RESULTS

Examination of the isolated monophosphoinositide. On analysis the lipid contained 3.45 % of P, 10.2 % of glycerol, 20.6 % of inositol and 0.08 % of N.

The acyl ester : P ratio was 2.06 : 1 and no reaction for plasmalogen was given. The values agree closely with the theoretical values for distearoyl-phosphatidylinositol, which contains 3.58 % of P, 10.6 % of glycerol, 20.8 % of inositol, no nitrogen and an acyl ester : P ratio of 2 : 1.

The structure was more firmly established by an analysis of the products obtained after mild hydrolysis with alkali and acid. With the former (5 min. at 37° in 0.25N-NaOH) the major phosphorus-containing product was glycerylphosphorylinositol. Of the total phosphorus of the hydrolysate 85 % was present as glycerylphosphorylinositol, an additional 4 % was found as inositol monophosphate and 0.7 and 4 % was present as glycerophosphate and cyclic glycerophosphate respectively. On longer hydrolysis (20 min.), less glycerylphosphorylinositol (62 %) and correspondingly larger amounts of inositol monophosphate (21 %) and cyclic glycerophosphate (18 %) were detected. Mild acid hydrolysis of brain monophosphoinositide produced 77 % of inositol monophosphate, 11 % of glycerophosphate plus traces of inorganic phosphate. Additional glycerol was isolated as monoglyceride (42 %) and diglyceride (28 %). The hydrolysis products obtained by both acid and alkaline treatment suggest, and are consistent with, a structure for the monophosphoinositide of brain that corresponds to that found for liver monophosphoinositide, that is phosphatidylinositol (diacylglycerylphosphorylinositol).

Notes on the preparation of triphosphoinositide. During the development of procedures for the isolation of triphosphoinositide the fractionation was continually followed by acid and alkaline hydrolysis of the lipid fractions and the examination of the hydrolysis products by high-voltage ionophoresis. Brief acid hydrolysis (7 min. at 100° in 5N-HCl) was generally most useful and, as is described in the next paper (Dawson & Dittmer, 1961), yielded inositol triphosphate as the predominant phosphate ester.

The isolation of triphosphoinositide from the protein complex insoluble in neutral lipid solvents is probably the most useful for routine preparation. Over a dozen preparations have now been made in this way and a reasonable yield was obtained on each occasion. The other lipids, including a troublesome nitrogen-containing non-phospholipid contaminant of the preparation made from a diphosphoinositide fraction, are effectively removed in the initial lipid extraction with neutral solvents. In the preparation, protein can be a contaminant but the vigorous shaking with N-HCl of the chloroform-methanol solution of triphosphoinositide reduces this to very low limits. The most troublesome contaminant, however, appears to be neutral lipid material, consisting largely of diglyceride, which

Table 1. *Analysis of typical preparations of triphosphoinositide*

Species	Yield (mg./kg. of brain P)	Salt isolated	Acyl ester	Total fatty acid	Inositol	Glycerol
			(molecules/3 atoms of P)			
Guinea-pig	24.0	Na	—	—	1.1	1.13
Ox	—	Na	2.26	—	0.95	1.05
Ox	39.5	Na	2.02	1.97	0.98	0.93
Ox	38.7	Na	2.30	2.31	0.97	1.30
Ox	45.7	NH ₄	2.10	2.11	1.13	1.07
Ox	—	NH ₄	2.11	2.19	1.22	1.09
Ox	—	Free acid	1.92	—	1.05	0.94
Ox	30.7	Free acid	1.86	—	0.91	1.02

probably arises by breakdown of triphosphoinositide itself during the course of the preparation. This appears to form a complex with the triphosphoinositide so that under certain circumstances of precipitation of the sodium salt the acyl ester:P ratio is almost exactly 1:1. At first it was thought that this complex was another form of triphosphoinositide (triphosphoinositide B; Dittmer & Dawson, 1960), but certain evidence now suggests this is not so. Thus material rich in acyl ester and glycerol but containing no P can be eluted with chloroform when such preparations of triphosphoinositide are placed on a silicic acid column, and material rich in fatty acid and low in P is concentrated in the supernatant on precipitation with acetone. When the substance eluted from a silicic acid column with chloroform was subfractionated on a similar column with ether-light petroleum mixtures (Dawson & Dittmer, 1961), diglyceride was the predominant fraction emerging (acyl ester: glycerol ratio 2.01:1; no P or inositol).

Properties of isolated triphosphoinositide. The sodium salt of triphosphoinositide is a snow-white amorphous solid, which has not yet been crystallized. It is readily soluble in water, forming a clear solution with detergent-like properties. It is less readily soluble in wet chloroform, and is virtually insoluble in methanol, acetone and ether. The free acid is likewise soluble in water and chloroform and differs in being completely soluble in methanol and ethanol. The sodium salt showed no evidence of instability when stored in moist chloroform solution at -18° , whereas the free acid slowly decomposed, presumably by hydrolysis as the products were similar to those observed on acid hydrolysis of the lipid (inositol triphosphate is the main P-containing decomposition product).

Analysis of triphosphoinositide fractions. The sodium triphosphoinositide preparations obtained contained no detectable choline (Appleton, La Du, Levy, Steele & Brodie, 1953), sphingosine or chloroform-soluble long-chain bases (McKibbin & Taylor, 1949), ninhydrin-reacting material or galactose. The N:P atomic ratio varied between 0 and 0.08:1. Only traces of sulphur were present. The

only polyol-reacting substances detected on chromatography of hydrolysates were inositol and glycerol. Table 1 shows the analysis of a number of typical preparations in terms of the molar ratio of phosphorus to the other components and indicates that the lipid contains phosphate, inositol, glycerol and fatty acid in the molar ratios of 3:1:1:2. The preparations isolated as the free acid (Table 1) were obtained by the fractionation of the lipids extracted from acetone- and ethanol-pretreated brain tissue with light petroleum (Folch, 1949). In 11 preparations the mean phosphate:inositol molar ratio was $3:1.02 \pm 0.11$ (s.d.). (The microbiological assay of inositol is subject to error of about $\pm 6\%$.) All of the fatty acid is combined through a carboxylic acid ester linkage. Analysis of a preparation for Na showed the atomic ratio Na:P to be 3.83:3, which suggests that the lipid is probably isolated as its tetrasodium salt. If it is assumed that the basic unit of triphosphoinositide contains three phosphate groups, one each of inositol and glycerol, two fatty acids (stearic acid) and four Na atoms, then the dry-weight analysis agrees well with the calculated theoretical (e.g. Found: P, 8.6; inositol, 16.4; glycerol, 7.9. Required: P, 8.4; inositol, 16.2; glycerol, 8.3%). Such dry-weight analyses indicate that all the components of the complex molecule have been accounted for, but owing to uncertainty of the mean molecular weight of the fatty acid components this evidence by itself is not entirely satisfactory.

DISCUSSION

The present results and those in the next paper (Dawson & Dittmer, 1961) clearly show that the phosphoinositide fraction of brain tissue is a complex fraction that probably contains at least three individual phospholipids. From it have been isolated monophosphoinositide and a new lipid which we have designated triphosphoinositide (Dittmer & Dawson, 1960). Additional evidence, which will be presented in the accompanying paper, suggests that a third lipid corresponding to the diphosphoinositide described by Folch (1949) may also be present. The

results therefore help to explain the recent observations of Hörhammer, Wagner & Hölzl (1960), who showed that 'diphosphoinositide' preparations split into three spots on chromatography on formaldehyde-treated paper.

There seems little doubt that the new lipid isolated, triphosphoinositide, is a chemical entity that is distinct from the diphosphoinositide described by Folch (1949). In 11 preparations the mean inositol : phosphate molar ratio was 3 : 1.02 and it is shown in the accompanying paper that inositol triphosphate is formed in 80 % yield on acid hydrolysis. In agreement with this, Grado & Ballou (1961) have described the isolation of inositol triphosphate from the acid hydrolysates of crude brain diphosphoinositide preparations.

Monophosphoinositide is a phospholipid that has been chemically characterized as a component of certain mammalian tissues such as liver (McKibbin, 1956; Dawson, 1958; Hanahan & Olley, 1958) and heart muscle (Faure & Morelec-Coulon, 1954, 1958; Gray & Macfarlane, 1958). Hörhammer *et al.* (1958, 1960) obtained paper-chromatographic evidence for the presence of monophosphoinositide in brain and also identified inositol monophosphate as a hydrolysis product. The present isolation and chemical characterization removes any remaining doubt as to the presence of this phospholipid in nervous tissue. Moreover, its acid and alkaline hydrolysis products (diglyceride, glycerylphosphorylinositol, inositol monophosphate, glycerophosphate) indicate that it also has the phosphatidylinositol structure (diacylglycerolphosphorylinositol), which is the same as the monophosphoinositide of other tissues.

Monophosphoinositide in brain tissue differs from the other phosphoinositides present in that it can be readily extracted by neutral chloroform-methanol solvent. Triphosphoinositide appears to be much more strongly bound to a protein-like component but it can be partially extracted either by pretreating the tissue with acetone or by acidifying the extracting solvent. Presumably acid breaks the linkage between the phospholipid and the protein by displacement whereas acetone may break it by denaturation of the protein. It seems likely that such a complex may be a component of the trypsin-resistant protein fraction containing combined inositol (phosphatido-peptide) studied by Folch (1952) and Lebaron & Folch (1956). Such a fraction, which is probably related to classical neurokeratin, was found to yield on hydrolysis fatty acid and a phosphate ester with a P : inositol ratio of 2.6 : 1.

It seems likely that triphosphoinositide may play an important metabolic role in the economy of nervous tissue since work with radioactive phosphate has demonstrated that the protein-bound P

fraction (which is probably mainly this phospholipid) has a high exchange rate (e.g. Findlay, Strickland & Rossiter, 1954). No clue as to such a function is as yet available except perhaps the fact that the lipid is only present in significant concentrations in the nervous system.

SUMMARY

1. A new phospholipid designated triphosphoinositide has been isolated from ox and guinea-pig brain.

2. Analysis shows that this compound contains inositol, phosphate, glycerol and fatty acid in the molar ratios of 1 : 3 : 1 : 2. All of the fatty acid is acyl ester-linked. The lipid contains no nitrogen as an integral part of its molecule.

3. Evidence is presented that triphosphoinositide is tightly attached to brain protein. Such a complex is probably a constituent of the phosphatido-peptide fraction described by Folch (1952). Pretreatment of the brain tissue with acetone partially breaks the linkage in the complex, and triphosphoinositide then becomes a component of the 'diphosphoinositide' fraction of brain tissue.

4. Monophosphoinositide has been isolated from brain tissue, and chemically characterized. Its degradation products on acid and alkaline hydrolysis indicate that it has the phosphatidyl structure (diacylglycerolphosphorylinositol).

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Evidence for the Structure of Brain Triphosphoinositide from Hydrolytic Degradation Studies

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In the preceding paper (Dittmer & Dawson, 1961) we have described the isolation from brain tissue of a new lipid, triphosphoinositide, which contains inositol, phosphate, glycerol and fatty acid in the molar proportions 1:3:1:2. In this paper a study of the chemical hydrolysis products of triphosphoinositide is reported, from which it can be deduced that the phospholipid is a diacylglycerolphosphorylinositol diphosphate. Similar observations have also been made upon a 'diphosphoinositide' fraction obtained from ox brain by the method of Folch (1949), which suggests that it may contain, as well as triphosphoinositide, a lipid that corresponds to a true diphosphoinositide. Preliminary results in this study have already been reported (Dawson & Dittmer, 1960).

EXPERIMENTAL

Methods. Triphosphoinositide and other brain-phospholipid preparations were prepared as described by Dittmer & Dawson (1961). These preparations were usually stored in chloroform at -18° . Since under these conditions preparations of the free acid were found to undergo slow hydrolysis over a period of several weeks, the triphosphoinositide was usually freshly prepared for each experiment and used within a few days or alternatively it was stored as the sodium salt.

The term fraction I is used here to designate the phosphoinositide-rich precipitate obtained by the addition of ethanol to a chloroform solution of brain kephalin as described by Folch (1949). The term Folch 'diphospho-

inositide' refers to the insoluble fraction obtained from fraction I after a sequence of precipitations from chloroform with methanol (Folch, 1949). Although no evidence was obtained for decomposition of these fractions when stored in chloroform at -18° , freshly prepared fractions were used for the hydrolysis studies described.

Analysis. The methods used for the estimation of glycerol, inositol, phosphorus and acyl ester were those described by Dittmer & Dawson (1961).

Acid hydrolysis of phospholipids. A sample of a chloroform solution of triphosphoinositide containing 250–500 μ g. of P was dried *in vacuo* and hydrolysed for 5–180 min. with 2 ml. of N- or 5N-HCl at 105° . After cooling, chloroform (4 ml.) was added and, after shaking and centrifuging, the upper aqueous layer was withdrawn and dried *in vacuo*. The lower chloroform layer was reserved when necessary for examination of the lipid degradation products. In some experiments these were removed by extracting twice with ether instead of chloroform. Even after short hydrolysis periods the P was almost all in the aqueous layer.

Mild alkaline hydrolysis of phospholipids. Mild alkaline hydrolysis was carried out by the procedure described by Dawson (1960) or alternatively a sample of the lipid, containing approx. 500 μ g. of lipid P, was suspended in 8–10 ml. of aqueous 80% (v/v) ethanol, 0.25 ml. of aqueous N-NaOH was added and the mixture incubated for 5–20 min. at 37° . Neutralization of the hydrolysate and subsequent preparation of the phosphate esters for chromatography was carried out as described by Dawson (1960). However, the use of Amberlite IRC-50 was preferable to neutralization with ethyl formate if the hydrolysate was to be examined directly by paper ionophoresis. Excess of sodium formate in hydrolysates that were spotted directly for paper ionophoresis resulted in poor resolution of the phosphate esters. No such effect was observed if a prior chromatographic run was made with a solvent system as in the two-dimensional systems described below.

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