

Ion Exchange Chromatography of Intact Brain Phosphoinositides on Diethylaminoethyl Cellulose by Gradient Salt Elution in a Mixed Solvent System*

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By a solvent fractionation procedure attributed to Folch (1), one can isolate readily the myo-inositol phospholipid of brain tissue. However, as is revealed by chromatography on the formaldehyde-treated paper system described by Horhammer, Wagner, and Richter (2), the product is a mixture of three inositides contaminated with phosphatidylserine.

Modified extraction and solvent fractionation procedures have been used by others to obtain the triphosphoinositide component pure, but the mono- and diphosphoinositides were lost in the process (3).

Rouser has described the use of chromatography on DEAE-cellulose as a method of fractionating phospholipids (4). For displacement, he has used varying mixtures of chloroform and methanol and has added acetic acid or ammonia, when it is required, to elute the more polar components.

We are reporting in this paper a procedure for gradient salt elution of phospholipids from DEAE-cellulose. Although used here to separate the components of the Folch brain inositide preparation (1), the method would seem to have wide applicability. The phospholipid mixture, in sodium salt form dissolved in chloroform-methanol-water (20:9:1), is adsorbed on a column of DEAE-cellulose prepared with the same solvent. Gradient elution of the phospholipids is then carried out with 0 to 0.6 M ammonium acetate in the chloroform-methanol-water mixture. All three inositides separate cleanly from each other and may be recovered from the eluent by evaporation of the organic solvents and dialysis of the residue to remove the salt.

EXPERIMENTAL PROCEDURE

Material—Fresh beef brain was used as the source of lipid for most of the studies. Human brain was obtained within 24 hours post-mortem, kept frozen, and extracted several weeks later. DEAE-cellulose (0.56 meq per g) and the chelating resin, Chelex 100 (Na⁺, 100 to 200 mesh), came from Bio-Rad Laboratories, Richmond, California. Anisil-S (a finely graded silicic acid with no binder, for thin layer chromatography) and Anakrom ABS (a siliconized support for gas-liquid chromatography) were obtained from Analytical Engineering Laboratories, Hamden, Connecticut.

Routine Analytical Methods—Phosphorus was determined by the method of Bartlett (5); glycerol, according to Renkonen (6);

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and fatty acid esters, by the method of Rapport and Alonzo (7). Myo-inositol was assayed by a microbiological procedure (8) with *Kloeckera brevis* as the test organism.

Thin Layer Chromatography—Thin layer chromatography of phosphatides was carried out with the use of Anisil-S as the adsorbent and a chloroform-methanol-water (65:25:4) solvent system in a lined jar. The components were revealed by placing the developed chromatograms in a jar saturated with iodine vapors.

Formaldehyde-treated Paper Chromatography—Formaldehyde-treated paper was prepared by a modification of Horhammer's procedure (2). Schleicher and Schuell No. 2043b filter paper was cut into strips 18 cm × 57 cm, which were rolled together loosely and placed in a large beaker. A mixture of 100 ml of 37% formaldehyde solution, 5 ml of glacial acetic acid, and 0.2 g of ammonium thiocyanate was poured over the filter paper until it was thoroughly saturated. The excess solution was then poured off. The beaker was covered with a large watch glass and autoclaved under 15 pounds of pressure at 123° for 4 to 6 hours. When cool, the papers were washed several times with water, unrolled, and washed overnight in running tap water. After drying, they were ready for use.

The amount of lipid applied is critical, since the paper is very easily overloaded. Inositol phosphatides (50 to 100 μg) were chromatographed with the upper phase of an *n*-butyl alcohol-acetic acid-water (4:1:5) mixture. Descending chromatography was carried out in a lined jar for 18 hours. As the paper aged, it became less effective; but chromatography could be improved then by mixing 4 parts of the *n*-butyl alcohol-acetic acid-water mixture with 1 part of diethyl ether and using the resulting upper phase as the chromatographic solvent.

After drying, the chromatogram was dipped repeatedly through a solution of 0.001% Rhodamine G6 in 0.25 M dipotassium hydrogen phosphate (9), washed with water, and viewed under ultraviolet light. The paper could then be counterstained by dipping it in 0.01% Nile blue and washing with water to remove the excess dye.

Deacylation of Lipids—Lipid, 10 mg, was dissolved in 0.65 ml of chloroform-methanol (2:1), and 0.4 ml of 0.5 N methanolic sodium hydroxide was added. After 10 minutes at room temperature, the reaction mixture was diluted with 10 ml of water and extracted twice with 10-ml portions of diethyl ether. The aqueous layer was immediately acidified with Dowex 50 (H⁺), filtered, and neutralized with cyclohexylamine. This solution was con-

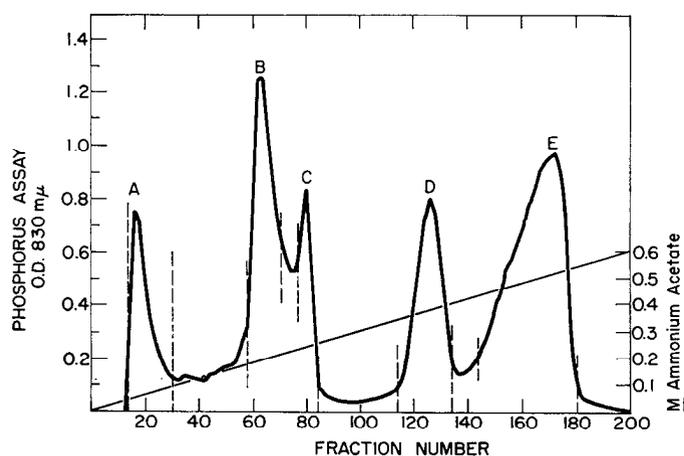


FIG. 1. DEAE-cellulose chromatography of beef brain inositol phosphatides.

centrated in a vacuum to a small volume (0.1 to 0.2 ml) for paper chromatography.

The ether extract was washed with water, dried over sodium sulfate, and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.3 ml of carbon disulfide for gas-liquid chromatography.

Paper Chromatography of Deacylated Lipids—The water-soluble portion of the deacylated lipids (10 to 30 μ g of P) was chromatographed on Whatman No. 1 paper in two systems: Solvent A, isopropyl alcohol-30% ammonia-water, 6:3:1 (descending for 24 hours); and Solvent B, phenol saturated with 0.1% aqueous ammonia, ascending (10). A molybdate spray (11) was used to locate phosphorus-containing components.

Ion Exchange Chromatography of Deacylated Lipids—The water-soluble portion of the deacylated lipids (20 to 30 mg of P) was chromatographed on a 45-ml Dowex 1 (Cl^-) column as described previously (12). Fractions of 5.5 ml were collected, and 2 drops were removed from every other tube for phosphorus analysis.

Gas-Liquid Chromatography of Fatty Acid Methyl Esters—An Aerograph model A-600-B gas chromatograph (Wilkins Instrument and Research, Inc., Walnut Creek, California) with a hydrogen flame ionization detector was used for the analyses. The column, 5 feet \times $\frac{1}{8}$ inch (outer diameter) stainless steel, contained 10% diethylene glycol succinate polyester coated on Anakrom ABS, 100 to 110 mesh, and was operated at 190° with a nitrogen flow rate of 22 ml per minute. A disk integrator (Disc Instruments, Inc., Santa Ana, California) was used to record peak areas.

Peaks were identified (a) by comparing the relative retention times with a semilogarithmic plot of relative retention time versus chain length and degree of unsaturation of known standards, and (b) by comparing hydrogenated and nonhydrogenated samples.

Isolation of Myo-inositol Lipid—Beef brain was extracted according to the method of Folch (1, 13). The crude inositide fraction was precipitated six times from chloroform with methanol (13), dialyzed against several changes of water, and lyophilized. About 1 g of the purified Folch inositide fraction was obtained from 850 g of brain. Human brain, 900 g, was extracted in the same manner and gave about 0.6 g of dialyzed Folch inositide fraction.

Removal of Calcium and Magnesium Ions—In order to chromatograph the inositol lipid on DEAE-cellulose, it was necessary to convert it to the sodium salt by the following procedure. Eighteen grams of the crude Folch inositide fraction were precipitated six times from chloroform with methanol (13). The resulting material was suspended in 100 ml of water containing 2 g of disodium ethylenediaminetetraacetate. The mixture was shaken vigorously, and the pH was adjusted to 7 with 1 N sodium hydroxide. A homogeneous emulsion was obtained that was dialyzed against several changes of water at 4°.

Chelex 100 resin (Na^+ , 100 to 200 mesh) was washed with water to remove the fines. The pH was adjusted to 7 by repeated additions of hydrochloric acid followed by washings with water. The dialyzed lipid solution was passed through 80 ml of the resin in a column, and the column was washed with an additional 80 ml of water. When the column eluate was lyophilized, it yielded 2.2 g of white powder.

DEAE-cellulose Chromatography—DEAE-cellulose was suspended in water and allowed to settle for 30 minutes. The fines were decanted, and this process was repeated until the resin was free of fines. With the use of a sintered glass funnel, the resin was washed with 5% sodium hydroxide and then with water until the wash water was neutral. It was then washed first with 10% acetic acid, then with water until it was neutral, then with methanol, and finally with chloroform-methanol-water (20:9:1). The resin, in a slurry, was poured into a column, 3.6 cm \times 40 cm, with the chloroform-methanol-water mixture and packed to a constant height (33 cm) under pressure.

Sodium inositol phosphatide, 1 g, in the chloroform-methanol-water mixture was applied to the column. A linear gradient elution was begun with 1400 ml of chloroform-methanol-water (20:9:1) in the mixing chamber and 1400 ml of 0.6 M ammonium acetate in chloroform-methanol-water in the solvent reservoir. Fractions of 14 ml were collected, and a 0.1-ml aliquot was removed from every other tube for phosphorus analysis.

The chromatography of beef brain inositol phosphatides (Folch inositide fraction, sodium salt) is shown in Fig. 1. Each of the pooled fractions, 58 to 70, 77 to 84, 114 to 134, and 144 to 180, was concentrated in a vacuum to 0.5 volume. About 100 ml of water were added, and the volatile solvents were removed in a vacuum. The aqueous emulsion was dialyzed against several changes of water at 4° and lyophilized. White fluffy powders were obtained in the following amounts: Peak B (58 to 70), 217 mg; Peak C (77 to 84), 51 mg; Peak D (114 to 134), 117 mg; and Peak E (144 to 180), 198 mg. Peak A (13 to 30) was shaken with 0.2 volume of water. The lower phase was withdrawn, diluted with 0.5 volume of methanol, and shaken with 0.2 volume of water. The aqueous washings contained negligible lipid. The chloroform layer was evaporated to dryness, dissolved in benzene, and lyophilized; it gave 76 mg of light tan material.

RESULTS

Characterization of DEAE-cellulose Column Fractions—The analytic and chromatographic data for the DEAE-cellulose column fractions are given in Table I. Peak A was identical with triphosphoinositide by the chromatographic tests, although its higher ester and glycerol content suggests the presence of a nonpolar contaminant not detected by the chromatography. Its nonpolar character and insolubility in water indicate that

TABLE I
Characterization of DEAE-cellulose column fractions

Fraction and assigned composition	Group analyses				Chromatographic analyses			
	Phosphorus*	Glycerol	Myo-inositol	Ester	Thin layer chromatography	Formylated paper chromatography	Paper chromatography of deacylated lipids†	
							System A ($R_{GIP} = 1$)	System B
					R_F	R_F		R_F
A. Triphosphoinositide (Ca-Mg salt)	3	1.3	1.1	2.6	0.0	0.30	0.59	
B. Phosphatidylserine and phosphatidyl myo-inositol					0.42	0.67	1.0	0.17
C. Phosphatidylserine					0.49	0.53		0.08
D. Diphosphoinositide	2	1.2	0.9	2.1	0.42	0.67	1.0	0.17
E. Triphosphoinositide	3	1.1	0.9	2.2	0.0	0.30	0.74	
							0.59	

* Assumed integer values.

† Rates of migration of known standards: System A (R_{GIP}): glycerol myo-inositol phosphate (GIP) = 1; glycerol myo-inositol diphosphate = 0.74; glycerol myo-inositol triphosphate = 0.59. System B (R_F): glycerol myo-inositol phosphate = 0.08; glycerylphosphorylserine = 0.17.

Peak A is mainly a mixed calcium and magnesium chelate of triphosphoinositide (see below).

Peak B was shown to be a mixture of phosphatidyl myo-inositol and phosphatidylserine, and Peak C was shown to be pure phosphatidylserine by thin layer chromatography and paper chromatography of the deacylated lipids. Phosphatidyl myo-inositol could not be separated from phosphatidylserine by rechromatography of Peak B on DEAE-cellulose. Although the two lipids could be resolved by thin layer silicic acid chromatography, they could not be separated by silicic acid column chromatography. Hanahan and Olley (14) have reported the complete removal of nitrogenous impurities from beef liver phosphatidyl myo-inositol by silicic acid column chromatography. However, attempts by Carter *et al.* (15) to achieve similar results with an inositide preparation from flaxseed were unsuccessful. This difficulty may be due to differences in fatty acid composition (16, 17) or to multiple salt forms of the lipid (16, 18), or both.

Peaks D and E were shown to be diphosphoinositide and triphosphoinositide, respectively, by chromatography on formaldehyde-treated paper (Fig. 2), by paper chromatography of the deacylated lipids (Fig. 3), and by group analysis (Table I).

To confirm the identity of Peak A as a calcium and magnesium chelate of triphosphoinositide, Peak E triphosphoinositide was rechromatographed as a calcium and magnesium salt. Lipid from Peak E, 30 mg, was distributed between chloroform-methanol (2:1) and water (0.2 volume) containing 0.2% calcium chloride and 0.2% magnesium chloride. The lower chloroform layer was washed again with methanol-water. Of the lipid phosphorus, 83% remained in the chloroform layer, and this was chromatographed on DEAE-cellulose in the same manner as before. Two-thirds of the material was eluted at the same position as Peak A, and the remaining one-third was eluted at the position of Peak E.

Fatty Acid Composition—The fatty acid methyl ester fractions from the deacylation reactions were analyzed by gas-liquid chromatography to determine the fatty acid compositions of the lipids. The results are shown in Table II.

In order to obtain a pure sample of phosphatidyl myo-inositol

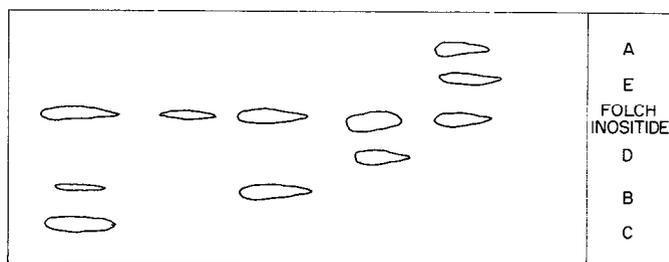


FIG. 2. Formaldehyde-treated paper chromatography of DEAE-cellulose column fractions.

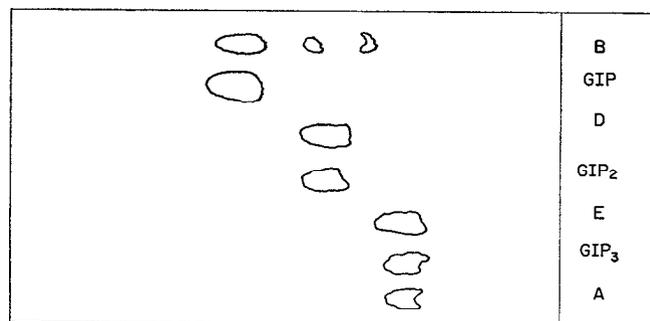


FIG. 3. Paper chromatography of deacylated lipids from DEAE-cellulose column fractions. *GIP*, Glycerol myo-inositol phosphate; *GIP*₂ and *GIP*₃, the diphosphate and triphosphate forms.

for fatty acid analysis, 2.5 mg of Peak B were chromatographed on a thin layer silicic acid plate, 20 × 20 cm. The zones were located by exposure to iodine vapor and scraped off, and the lipid was eluted from the silicic acid with water-saturated chloroform-methanol (2:1). Rechromatography of a portion of the zone corresponding to phosphatidyl myo-inositol gave a single spot, indicating that it was free of phosphatidylserine. The fatty acid methyl esters, obtained by refluxing the lipid 4 hours in 5% methanolic sulfuric acid, were analyzed by gas-liquid chromatography.

TABLE II
Fatty acid composition of beef brain phosphatides*

Fatty acids†	Peak A (triphosphoinositide)	Peak B (phosphatidyl myo-inositol and serine)	Peak C (phosphatidyl-serine)	Peak D (diphosphoinositide)	Peak E (triphosphoinositide)	Phosphatidyl serine (Folch III)	Phosphatidyl myo-inositol from thin layer chromatography
16:0	8.0	3.3	2.8	6.7	6.7	0.8	8.9
16:1	0.8		0.4	0.5	0.5		2.3
17:0	1.3	0.7	0.8	1.2	1.5	0.6	2.6
18:0	39.8	41.0	41.8	35.9	35.2	58.6	47.7
18:1	18.1	20.6	42.2	14.7	15.6	34.7	8.4
18:2			1.1		1.2		
20:0			0.9			0.6	
20:1	1.0	2.9	6.4	0.8	1.4	4.6	0.6
20:2		0.9	0.8	1.0	0.9		0.6
20:3	3.2	1.5		3.4	3.8		1.9
20:4	21.9	21.2	2.0	26.4	26.4		21.8
22:0	3.6	2.1	0.8	5.0	4.3		2.1
22:4‡	2.3	2.9		4.2	2.4		0.8
22:5‡		3.1					1.4
Total saturated	52.7	47.1	47.1	48.8	47.7	60.6	61.3

* Composition is expressed as uncorrected area percentage.

† Ratio of carbon length to degree of unsaturation.

‡ Tentative assignment.

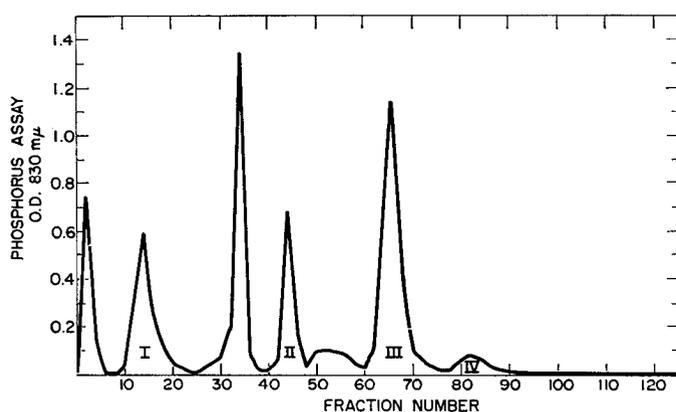


Fig. 4. Dowex 1 column chromatography of deacylated lipids from human brain inositol phosphatides.

TABLE III

Analysis of deacylated lipids from human brain inositol phosphatides

Fraction from Fig. 3	Phosphorus*	Myo-inositol	Glycerol
	<i>moles/mole of compound</i>		
I	1.0	0.58†	0.90
II	2.0	1.00	0.93
III	3.0	0.97	0.87
IV‡	3.0	0.22	0.20

* Assumed integer values for phosphorus content.

† Glycerol myo-inositol phosphate (58%) contaminated with a nitrogen-containing glycerol phosphate (12).

‡ Component IV had an ultraviolet spectrum identical with that of adenosine and an extinction relative to phosphate very similar to that of adenosine triphosphate. On paper chromatography, it had the same R_F value as ATP.

Analysis of Human Brain Myo-inositol Phosphatide—The Folch inositol fraction from human brain was deacylated and chromatographed on a Dowex 1 (Cl^-) column. The results are shown in Fig. 4. After conversion to the cyclohexylamine salts, the phosphates were identified by paper chromatography and group analysis (Table III). Peaks I, II, and III were identified as glycerol myo-inositol mono-, di-, and triphosphate, respectively. Fractions 30 to 36 contained glycerol phosphate and myo-inositol phosphate, and Fractions 48 to 60 contained myo-inositol diphosphate. Peak IV migrated on chromatography slightly behind glycerol myo-inositol triphosphate and had the same R_F value as adenosine triphosphate in System A. It had an ultraviolet absorption peak at $259 \text{ m}\mu$ (pH 7) with an extinction coefficient of 5.7×10^3 per mole of phosphorus, the 280:260 ratio being 0.23 and the 250:260 ratio being 0.79. Adenosine triphosphate has a maximum at $259 \text{ m}\mu$ (pH 7) with an extinction coefficient of 5.2×10^3 per mole of phosphorus and almost identical absorbance ratios. Very little glycerol and myo-inositol could be detected in this material.

DISCUSSION

The gradient elution procedure described above makes the isolation of the pure di- and triphosphoinositide components from the Folch brain inositol preparation simple and convenient. The phosphatidyl myo-inositol component, however, is contaminated with phosphatidylserine, and it must be purified by additional treatment.

DEAE-cellulose chromatography possesses advantages over silicic acid chromatography in that the lipids are eluted as sharp peaks with no tailing and the elution is not influenced as much by the type of fatty acid present. An important step in the preparation of lipids for DEAE-cellulose chromatography is the removal of heavy metal cations. The effect of magnesium and calcium on the elution position of triphosphoinositide is quite dramatic, their presence greatly decreasing the charge on this component, probably by forming a stable chelate. This com-

plex formation reveals itself also in the distribution of triphosphoinositide between water and chloroform. As the ammonium or sodium salt, it is appreciably soluble in the water layer; but in the presence of magnesium and calcium ions, the lipid passes almost completely into the organic layer.

Analysis of the fatty acid composition of each inositide reveals a striking similarity between the three components, which fact is consistent with the hypothesis that they are readily interconverted by phosphorylation and dephosphorylation of the myo-inositol ring with but little modification of the fatty acids (19).

All three phosphoinositides had arachidonic acid as the major unsaturated fatty acid. Phosphatidylserine differed in that it contained very little arachidonic acid, but more oleic acid. Folch Fraction III (13), obtained during the isolation of inositol phosphatides, gave only one spot on thin layer chromatography; this spot corresponded to phosphatidylserine and had a fatty acid composition similar to that of phosphatidylserine in Peak C. All the lipids had about an equal amount of saturated and unsaturated fatty acids.

Klenk and Hendricks (20) have reported the isolation of a complex inositol phospholipid from human brain. The analyses given for this material suggest that it probably is not a homogeneous substance, since it contained inositol, hexosamine, several hexoses, ethanolamine, glycerol, and phosphorus. Because the ratio of phosphorus to inositol was 4, a tetraphosphoinositide structure was proposed. The material was isolated from the "cephalin fraction" of human brain by chloroform-methanol precipitations, and we would expect the preparation of Klenk to contain the usual polyphosphoinositides (12) if such substances are present in human brain. To check this point, we have attempted to repeat the work of Klenk and Hendricks. Our results show that the "Folch inositide fraction" of human brain contains the same three phosphatidyl myo-inositol phosphates that are present in the brain of other animals. Although we have not accounted quantitatively for all of the inositol in the brain, we have obtained no evidence for the existence of a tetraphosphoinositide.

The possible existence of such a compound still requires substantiation. We have learned recently from Dr. Hokin¹ that the tetraphosphoinositide reported by him (21) to be present in beef brain turned out in fact to be the known triphosphoinositide. In their studies on the lipids of the canine adrenal, Chang and Sweeley (22) found an additional component following glyceryl-phosphoryl-myo-inositol diphosphate in its elution from an ion exchange column. They speculate that this component may be a deacylated tetraphosphoinositide. However, the ion exchange property of this material is similar to our Component IV (Table III) and indeed may be a nucleotide carried along in the lipid fraction.

SUMMARY

A procedure is described for ion exchange chromatography of intact brain phosphoinositides. The Folch inositide fraction

¹ L. E. Hokin, personal communication.

from beef brain was converted to the sodium form by treatment with ethylenediaminetetraacetic acid followed by passage through a chelating resin. The phospholipid mixture was then adsorbed on a diethylaminoethyl cellulose (acetate) column and eluted with a gradient of 0 to 0.6 M ammonium acetate in chloroform-methanol-water (20:9:1). Pure di- and triphosphoinositide and phosphatidylserine were isolated. The phosphatidyl myo-inositol component, however, was contaminated with phosphatidylserine and had to be purified by other means.

All three phosphoinositides had similar fatty acid compositions with arachidonic acid as the major unsaturated fatty acid. Phosphatidylserine differed in that it contained very little arachidonic acid, but more oleic acid.

The inositol phosphatide fraction of human brain was analyzed and was shown to contain the same three phosphoinositides that are present in the brain of other animals. There was no evidence for the existence of a tetraphosphoinositide.

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