The Quantitative Extraction and Analysis of Brain Polyphosphoinositides*

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ABSTRACT: The polyphosphoinositides in brain extracts were analyzed by the quantitative fractionation and determination of the phosphate diesters produced by deacylation of the lipids. A maximum yield of polyphosphoinositide was obtained by extraction with mixtures of chloroform–methanol followed by chloroform–methanol–HCl. The handling of brain under conditions which would be expected to be conducive to autolysis and extraction of brain with acetone leads to the hydrolysis of triphosphoinositide to diphosphoinositide and to some degradation of the latter lipid. The hydrolysis in acetone homogenates does not go to completion immediately but appears to involve only a specific portion of the triphosphoinositide.

Triphosphoinositide (TPI)[1] was originally isolated from extracts of brain obtained by two different methods (Dittmer and Dawson, 1961). The first procedure used by Folch (1949) in the isolation of diphosphoinositide (DPI) involves sequential extraction of fresh brain with acetone, ethanol, and petroleum ether. The second was an adaptation of a procedure first described by Folch (1952) by which a trypsin-resistant, chloroform–methanol-insoluble residue was extracted with chloroform–methanol mixtures acidified with HCl. The TPI and DPI were found in the petroleum ether (bp 30–60°) and the acidified chloroform–methanol extracts as shown by isolation of the intact lipids (Dittmer and Dawson, 1961; Hendrickson and Ballou, 1964) or inferred from studies of hydrolysis products (Brockerhoff and Ballou, 1961; Grado and Ballou, 1961; Dawson and Dittmer, 1961; Ellis *et al.*, 1963; Kerr *et al.*, 1964a). Apparently, some of the DPI may be extracted with neutral chloroform–methanol depending on the ratio of chloroform and methanol used (Eichberg and Dawson, 1964).

Quantitative data on the recovery of polyphosphoinositides are limited and no specific study of the recovery of polyphosphoinositides with different extraction techniques is available. However, the data reported by Dawson and Dittmer (1961) and Eichberg and Dawson (1964) indicate that extraction with chloroform–methanol solvent systems gives a higher yield of TPI than is obtained with petroleum ether and the ratio of DPI to TPI is higher in the latter extract. Similarly, Kerr *et al.* (1964b) found that when brain is allowed to stand prior to extraction the total yield of polyphosphoinositide is decreased and the relative amount of DPI is increased.

Further knowledge of the factors which effect the recovery of polyphosphoinositides from brain is important both for the quantitative analysis of these lipids and with regard to the interpretation of experiments on their metabolism. This paper describes an investigation of the yield of the polyphosphoinositides with various extraction procedures and the factors affecting the yield.

Experimental Methods

Materials. All solvents and other reagents were reagent grade and used with no further treatment. All solvents were mixed in proportions by volume. Rat brains were from male, Sprague–Dawley rats that weighed approximately 200 g. Purified phosphoinositides were prepared as described by Dittmer and Dawson (1961).

Extraction of Lipids. Rat brains were dissected and except where indicated were frozen in Dry Ice within 0.5 min of decapitation of unanesthetized animals. Extraction was begun within 10 min. In some experiments on the effect of storing the brain under various conditions before extraction, the appropriate details have been given with the results. Ox brains used in this study were obtained within 30 min of slaughter and transported to the laboratory in ice. The extraction was begun within another 30 min.

Extraction was carried out by one of two basic techniques. The first of these was a modification of the chloroform–methanol and acidified chloroform–methanol method described by Dittmer and Dawson (1961). The details of this procedure are given in Figure 1. This procedure, of all those investigated, consistently

* From the Department of Biochemistry, College of Medicine, University of Kentucky, Lexington, Ky. *Received June 10, 1965.* This work will form part of a dissertation to be submitted by M. A. W. to the University of Kentucky in partial fulfillment of the requirements for the degree of Doctor of Philosophy and was supported by a grant (AM-06008) of the Institute of Arthritis and Metabolic Diseases of the Public Health Service.

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[1] Abbreviations used in this work: TPI, triphosphoinositide; DPI, diphosphoinositide; MPI, monophosphoinositide.

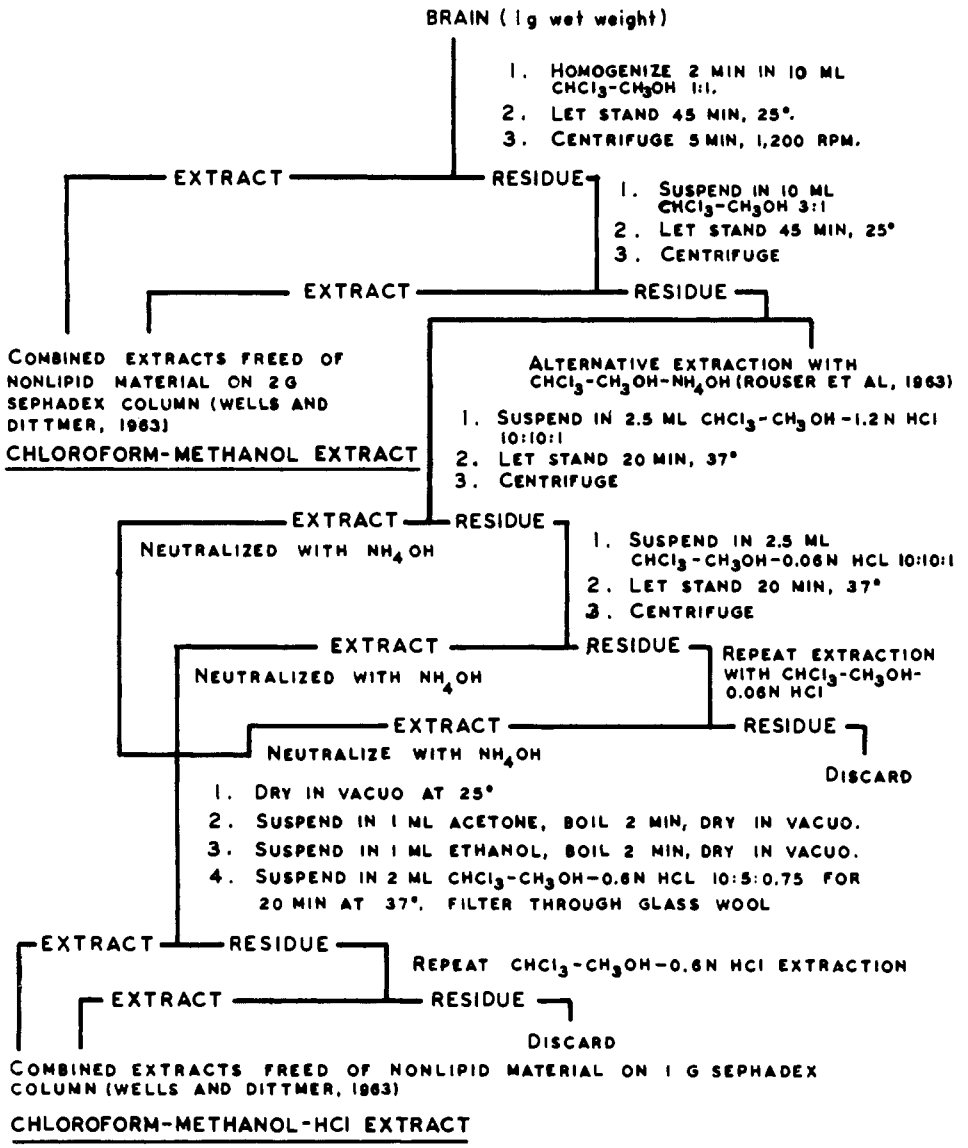

FIGURE 1: Chloroform–methanol and chloroform–methanol–HCl extraction of brain tissue.

gave the highest yield of both TPI and total polyphosphoinositide and was used as a control in the investigation of other extraction procedures. The second extraction procedure was investigated in some detail because it has been widely used. This method was that of Folch (1949), except that the extraction times for each solvent were standardized. Acetone extractions were carried out for 30 min, although in some experiments on the effect of this step on lipid recovery the extraction time was varied. In these experiments the exact time is noted in the description of the results. The ethanol and each of the petroleum ether extractions were carried out for 45 and 120 min, respectively. As an alternative procedure, the tissue was first extracted with acetone and then the extraction was continued with the chloroform–methanol and acidified chloroform–methanol extraction procedure given in Figure 1.

Quantitative Analysis of the Phosphoinositides. The quantitative analysis of the phosphoinositides in various brain lipid preparations was carried out by the selective methanolysis of the acyl esters of the lipids followed by fractionation of the phosphate diesters by the anion-exchange chromatographic procedure of Lester (1963). We are indebted to Dr. Lester for details of this procedure which have not yet been published in full.

The methanolysis conditions used were based on those described by Brockerhoff (1963). The lipid, containing up to 12 μmoles of phosphorus, was suspended in 1 ml of chloroform–methanol (1:4). To this was added 0.1 ml of 1.2 N NaOH in methanol–water (1:1). This mixture was incubated 15 min at 37° and then neutralized with acetic acid. The water- and lipid-soluble products were separated into two phases by adding 2 ml of chloroform–methanol (9:1), 1 ml of isobutyl alcohol,

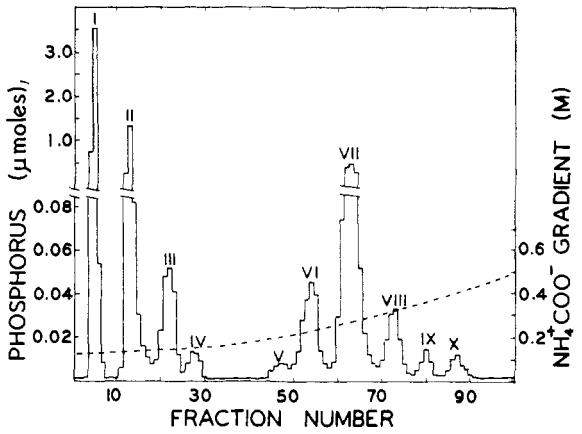

FIGURE 2: Fractionation of the deacylated lipids of a chloroform–methanol extract of brain. A sample containing 10 μmoles of phosphorus was deacylated and the water-soluble portion containing 7.0 μmoles of phosphorus was chromatographed on a 0.4 × 80 cm column of Bio-Rad AG-1, 200–400 mesh anion-exchange resin with the gradient indicated (Lester, 1963). Fractions (2 ml) were collected at a flow rate of 0.4 cc/min. Of immediate interest to this study, peak III is glycerophosphorylinositol and peak X is glycerophosphorylinositol phosphate. None of the other fractions contained inositol as shown by microbiological assay. Complete characterization of these other fractions will be given elsewhere.

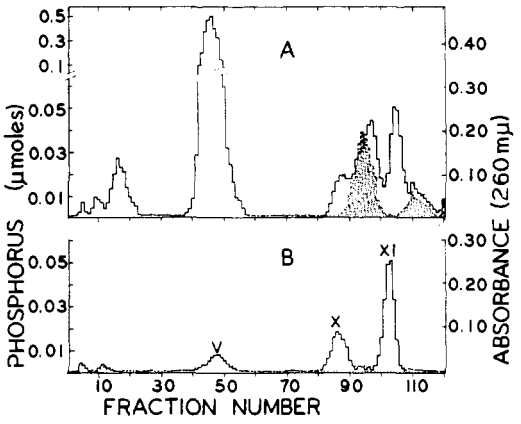

FIGURE 3: Fractionation of the deacylated lipids of a chloroform–methanol–HCl extract of brain before (A) and after (B) the removal of nonlipid material on Sephadex. A sample of extract before treatment with Sephadex containing 4.11 μmoles of phosphorus was deacylated and chromatographed as indicated in Figure 1. An equivalent sample of extract which after treatment with Sephadex contained 0.573 μmole of phosphorus was used in B. The solid lines indicate phosphorus and the dashed lines which have been shaded in gives the absorbance at 260 mμ. Peak V is inorganic phosphate and peaks X and XI are glycerophosphorylinositol mono- and diphosphate, respectively. The identification of the fractions showing absorbance at 260 mμ is given in the results.

and 2 ml of water, shaking the mixture, and then centrifuging at 1500 rpm for 10 min. The top water phase was transferred to a round-bottom flask and the bottom phase was re-extracted twice with 1 ml of methanol–water (1:2). The original water phase and washes were combined, 0.5 ml of isoamyl alcohol was added to prevent foaming, and it was then taken to dryness *in vacuo*. The residue was dissolved in 2 ml of 0.02 M ammonium borate which had been adjusted to pH 9.5 with NH_4OH and quantitatively transferred to an ion-exchange column for fractionation.

Analytical Techniques. Total phosphorus of ion-exchange chromatography fractions was assayed by the method of Bartlett (1959) after digestion of the dried fractions with 70% perchloric acid. Characterization of the mild alkaline hydrolysis products of the phosphoinositides isolated by ion-exchange chromatography included phosphorus analysis by the method of Fiske and Subbarow (1925) after digestion with perchloric acid and glycerol analysis by the method of Hanahan and Olley (1958) except the periodate oxidation was carried out directly on the 2 N HCl hydrolysates. In addition, inositol was assayed by either a microbiological method or by gas chromatography of the trimethylsilyl derivative. Hydrolysis was carried out as for glycerol but the HCl was removed by drying under an air jet. An inositol-requiring mutant of *Kloeckera brevis*[2] was used for the microbiological assay as described by Ridgeway and Douglas (1958). On the suggestion of R. L. Lester of this department, the media used in the microbiological assay was further buffered at pH 5 with sodium succinate in a final concentration of 0.025 M. This greatly improved the linearity of the growth response to inositol. Assay of the trimethylsilyl derivative of inositol was carried out by a modification of the method described by Wells *et al.* (1965). Samples containing 0.1–0.5 μmole of inositol were used. Borate from the buffer used in the ion-exchange chromatography interfered with this assay and was removed by adding 0.5 ml of 2% anhydrous HCl in methanol (w/v) and then boiling off the methanol at 100°. This procedure is repeated once. The trimethylsilyl derivative is prepared as described by Wells *et al.* (1965) using 0.3 ml of their chlorotrimethylsilane reagent. Instead of injecting the reaction mixture for gas chromatography, 0.5 ml of water and 0.5 ml of petroleum ether (bp 30–60°) were added, the sample was mixed, and a 5-mμl sample of the ether phase was injected. Samples duplicated within 5% and the response with a Barber-Colman Model 10 equipped with an ionization detector was linear over the range indicated.

[2] We are indebted to Dr. H. C. Douglas, University of Washington, for a culture of this yeast.

TABLE I: Chemical Characterization of Anion-Exchange Column Fractions.[a]

Fraction[b]	Molar ratios: Calcd Phosphorus	Calcd Glycerol	Calcd Inositol	Found Phosphorus	Found Glycerol	Found Inositol
III (Glycerophosphorylinositol)	1.00	1.00	1.00	0.99	1.00	1.04[c]
XI (Glycerophosphorylinositol phosphate)[d]	2.00	1.00	1.00	1.96	1.00	1.11[e]
XI (Glycerophosphorylinositol phosphate)[f]	2.00	1.00	1.00	1.99	1.00	1.02[c]
XII (Glycerophosphorylinositol diphosphate)[f]	3.00	1.00	1.00	3.08	1.00	0.97[c]
XII (Glycerophosphorylinositol diphosphate)[f]	3.00	1.00	1.00	3.04	1.00	1.09[e]

[a] The fractions were obtained from chromatographic runs using twice the volume of resin and eluting buffer as that described in Figure 2. Up to 500 μmoles of phosphorus was chromatographed with neutral chloroform–methanol extracts and up to 50 μmoles with acidified chloroform–methanol extract. [b] As designated in Figures 2 and 3. [c] Assayed as trimethylsilyl derivative by gas–liquid partition chromatography. [d] Isolated from neutral chloroform–methanol extract. [e] Assayed by microbiological method. [f] Isolated from acidified chloroform–methanol extract.

Nucleotide contaminants of fractions obtained from ion-exchange columns were detected by their absorption at 260 mμ as measured with a Zeiss PMQ II spectrophotometer. Spectra of these nucleotides for purposes of identification were obtained with a Cary 14 recording spectrophotometer.

Results

Phosphoinositide Assay. Ion-exchange chromatography of the phosphate diesters produced by deacylation of phospholipids (Lester, 1963) proved to be highly reproducible and gave good resolution when applied to brain lipids. Figures 2 and 3B show the fractionation of the deacylation products derived from chloroform–methanol and acidified chloroform–methanol extracts, respectively. Because our primary interest here is in the phosphoinositides, it was necessary to identify the fractions derived from these lipids. This was accomplished by chromatographing the methanolysis products of monophosphoinositide (MPI), TPI, and a mixture of DPI and TPI isolated from brain extracts (Dittmer and Dawson, 1961). It was evident from these chromatographic runs that only one product was obtained from each of these phospholipids and that peak III, Figure 2, was derived from MPI. Fraction X of both Figures 2 and 3B are derived from DPI and fraction XI of Figure 3B from TPI. The identification of fractions III, X, and XI as glycerophosphorylinositol, glycerophosphorylinositol phosphate, and glycerophosphorylinositol diphosphate was confirmed by analysis (Table I).

A check of a number of deacylation procedures using either $NaOCH_3$ or NaOH in mixtures of chloroform and methanol showed that all of these gave an essentially quantitative yield of the expected phosphate diesters from each of the phophoinositides. For this study, methanolysis conditions similar to those described by Brockerhoff (1963) in which the reaction mixture has a relatively high proportion of methanol were used in order to avoid artifacts formed from phospholipids other than the inositides. These artifacts, apparently cyclic glycerophosphate and glycerophosphorylmethanol, on chromatographic fractionation of the deacylated lipids, overlapped with glycerophosphorylinositol. A more detailed report on methanolyis conditions necessary for the quantitative analysis of all the phospholipids of brain will be given in a later paper. Of immediate importance, paper electrophoresis (Dittmer and Dawson, 1961) and ion-exchange chromatography showed that inositol diphosphate, inositol triphosphate, as well as other phosphate monoesters are present in only trace amounts when polyphosphoinositides preparations are deacylated by the method described here.

Clean-cut fractionation of the glycerophosphorylinositol phosphate and glycerophosphorylinositol diphosphate produced by methanolysis of DPI and TPI was obtained only after special precautions were taken to remove nucleotides from lipid extracts. Figure 3 shows the separation of the methanolysis products of an acidified chloroform–methanol extract before and after the use of Sephadex for the removal of nonlipid contaminants. The material eluted in fractions 86 through 101, Figure 3A, had absorbance maxima at pH 9.5 at 260 and at pH 1 at 256 mμ. The material eluted in fractions 106 through 119 had absorbance maxima at pH 9.5 at 258 and at pH 1 at 256 mμ. It was concluded that these are probably adenosine and guanosine nucleotides, respectively. If this identification is correct, calculations based on published molar extinction coefficients (Burton, 1959) and phosphorus analyses show both are present as monophosphonucleotides. Their 250/260, 280/260, and 290/260 mμ absorbancy ratios at both alkaline and acid pH agree with this identification. In addition to these nucleotides, the Sephadex also removes all but traces of the large amounts of inorganic phosphate found in acidified

TABLE II: Amount of Phosphoinositides in Various Extracts of Brain.

Extract	Phosphoinositide (μmoles/g wet weight of brain) MPI	DPI	TPI	Molar Ratio TPI/DPI
Rat brain[a]				
1. Acetone, ethanol, and petroleum ether (bp 30–60°) combined	...	0.150	0.064	0.43
2. Chloroform–methanol–HCl on residue from 1	...	0.102	0.041	0.42
Total of 1 + 2	...	0.252	0.105	0.42
3. Chloroform–methanol	1.95	0.079	0	0
4. Chloroform–methanol–HCl on residue from 3	0	0.122	0.256	2.10
Total of 3 + 4	1.95	0.201	0.256	1.28
Rat brain[b]				
5. Acetone, ethanol, and petroleum ether combined	1.78	0.238	0.096	0.41
6. Chloroform–methanol–HCl on residue from 5	0.27	0.060	0.027	0.45
Total of 5 + 6	2.05	0.298	0.123	0.42
7. Acetone, chloroform–methanol, and chloroform–methanol–HCl	2.09	0.267	0.125	0.47
8. Chloroform–methanol	2.04	0.070	0	0
9. Chloroform–methanol–HCl on residue from 8	0	0.116	0.230	1.98
Total of 8 + 9	2.04	0.186	0.230	1.24
Ox brain[c]				
10. Acetone, ethanol, petroleum ether combined	...	0.891	0.422	
11. Chloroform–methanol–HCl on residue from 10	...	0.150	0.083	
Total of 10 + 11	...	1.040	0.505	0.48
12. Chloroform methanol and chloroform methanol–HCl	...	0.753	0.784	1.04
13. Ethanol–ether[d]	...	Traces only		
14. Chloroform–methanol–HCl on residue from 13	...	0.670	0.510	0.762
15. Chloroform–methanol and chloroform–methanol–HCl	...	0.638	0.632	0.99

[a] Extracts 1 and 2 are from one half of a rat brain divided bilaterally and extracts 3 and 4 are from the other half. [b] Extracts 5 and 6 are from one half of an acetone homogenate of a single brain and extract 7 from the remaining half of the homogenate. Extracts 8 and 9 are from the brain of a litter mate of the rat used for 5, 6, and 7. [c] For comparison, 15-g samples were taken from opposite sides of a calf brain. Extracts 10 and 11 are from a sample paired with one from which extract 12 was prepared and extracts 13 and 14 from a sample paired with one from which 15 was prepared. [d] The tissue was homogenized for 2 min with ethanol–ether (3:1) (4 ml/g of brain) and allowed to stand 1 hr at room temperature. After filtering, the extraction was repeated twice with 5 ml of ethanol–ether (3:1)/g of brain.

chloroform–methanol extracts. Certain precautions were taken to avoid loss of lipid during the Sephadex treatment. If the acidified chloroform–methanol extracts are not subjected to the treatment with acetone and ethanol described, 30 to 70% of the TPI cannot be eluted from the Sephadex. Otherwise, on the basis of fatty acid and phosphorus recoveries, there was no loss of lipid on Sephadex.

Degradation of TPI during Acetone Extraction. The recoveries of phosphoinositides in various solvent fractions obtained by extracting rat and ox brain with the two basic methods described in this paper are given in Table II. There are several interesting features in this table. A comparison of the yield of the polyphosphoinositides obtained from matched halves of rat brain or samples of ox brain showed that extraction with acetone–ethanol–petroleum ether was less effective than extraction with chloroform–methanol and chloroform–methanol–HCl. This was confirmed by the extraction of additional phosphoinositide from the residue with acidified chloroform–methanol. Also of interest, there was a larger amount of TPI and less DPI extracted with the chloroform–methanol and acidified chloroform–methanol solvent system. This suggested that TPI may be degraded to DPI during extraction with the acetone–ethanol–petroleum ether solvent system. In an effort to verify this and to determine in what solvent the degradation occurs, the following experiment was carried out. Both halves of a rat brain were extracted with acetone and then one half was extracted with ethanol followed by petroleum ether and acidified chloroform–methanol. The other half was extracted with chloroform–methanol and chloroform–methanol–HCl. The combined extracts from each half were analyzed for TPI and DPI content (Table II). That there was no significant difference in the content

TABLE III: Hydrolysis of TPI Added to Acetone Homogenates of Rat Brain.[a]

Sample	Phosphoinositide (μmoles/g wet weight of brain) DPI	TPI
1. Control ($CHCl_3$–CH_3OH and $CHCl_3$–CH_3OH–HCl extract)	0.30	0.38
2. Acetone homogenate after incubation for 30 min	0.39	0.26
Change in concentration after 30 min in acetone	+0.09	−0.12
3. Control (acetone homogenate after 30 min; incubated additional 30 min with 0.05 ml of water)	0.40	0.23
4. Acetone homogenate after 30 min; incubated additional 30 min with added inositide	0.74	0.47
Change in concentration of added inositide after 30 min[b]	+0.21	−0.20
5. Same as sample 3 except incubated additional 120 min	0.45	0.18
6. Same as sample 4 except incubated additional 120 min	0.81	0.35
Change in concentration of added inositide after 120 min[c]	+0.23	−0.27

[a] Three rat brains were divided bilaterally and one set of halves was extracted with chloroform–methanol and chloroform–methanol–HCl for a control (sample 1). The other set of halves was homogenized for 2 min with acetone and allowed to stand for 30 min. A sample of this homogenate was analyzed for DPI and TPI (sample 2). Four additional samples were taken and to two of these 0.05 ml of water was added and then they were allowed to stand an additional 30 and 120 min, respectively (samples 3 and 5). To the other two samples, 0.05 ml of an emulsion of 0.44 μmole of TPI and 0.13 μmole of DPI was added, and the mixture was allowed to stand 30 and 120 min, respectively (samples 4 and 6). Chloroform–methanol and chloroform–methanol–HCl extracts of all of these samples were assayed for DPI and TPI. The entire experiment was carried out at room temperature. [b] Calculated as follows: value from sample 4 minus μmoles of inositide added and minus sample 3. This represents a decrease of 8% of the endogenous TPI and 45% of the added TPI. [c] Calculated as follows: value from sample 6 minus μmoles of inositide added and minus sample 5. This represents a decrease of 21% of the endogenous TPI and 61% of the added TPI.

of DPI and TPI was consistent with degradation occurring only during the acetone extraction.

In following up these observations, samples of an acetone homogenate of rat brain were taken at intervals over a 2-hr period and each sample then was subjected to further extraction with chloroform–methanol and chloroform–methanol–HCl. The combined extracts were then analyzed for phosphoinositide content. The data obtained are shown in Figure 4. It was quite clear that, during incubation of acetone homogenates of brain, TPI was hydrolyzed to give rise to DPI and it also appeared that DPI was hydrolyzed to a more limited extent. In this and other similar experiments, from 30 to 60% of the TPI was hydrolyzed within 30 min. After that time, hydrolysis proceeded at a much slower rate. Additional information on why only part of the TPI was immediately hydrolyzed under these conditions was obtained by adding a polyphosphoinositide preparation to an acetone homogenate of rat brain after the initial rapid breakdown of TPI was complete (Table III). Also, the extent of hydrolysis when the homogenate was disrupted further by homogenizing for longer times or treatment with ultrasonic vibration was determined (Figure 5).

Extraction of Polyphosphoinositides with Basic Solvents. Rouser *et al.* (1963) described an extraction procedure by which the residue of beef brain left after extracting with chloroform–methanol (2:1) was dried and the TPI then was extracted with chloroform–methanol (7:1) saturated with ammonium hydroxide. They further describe a procedure by which TPI was isolated from this chloroform–methanol–NH_4OH extract by chromatography on DEAE-cellulose. Although these authors claim that they achieve total recovery of the TPI, we were unable to confirm these results with either rat or ox brain. The yield of TPI from one of the paired halves of rat brain extracted by this procedure, as compared with the yield obtained by extraction with chloroform–methanol and chloroform–methanol–HCl, was variable, and the TPI left in the residue after the chloroform–methanol–NH_4OH extraction was recovered by extracting with acidified chloroform–methanol (Table IV). In order to establish that our failure to obtain a quantitative yield was not due to a species difference between the rat and ox, ox brain was also subjected to extraction in succession with chloroform–methanol (2:1) and chloroform–methanol–NH_4OH as described by Rouser, followed by extraction with chloroform–methanol–HCl. No TPI was detected in the chloroform–methanol (2:1) ex-

TABLE IV: Recoveries of Polyphosphoinositides with Chloroform–Methanol–NH_4OH Extraction.[a]

Extract	Phosphoinositide (μmoles/g wet weight of brain) DPI	TPI
Rat brain		
1. Chloroform–methanol–HCl	0.135	0.205
2. Chloroform–methanol–NH_4OH	0.059	0.053
3. Chloroform–methanol–HCl on residue from 2	0.079	0.158
Total of 2 + 3	0.138	0.211
Ox brain		
1. Chloroform–methanol–HCl	0.039	0.135
2. Chloroform–methanol–NH_4OH	0.024	0.064
3. Chloroform–methanol–HCl on residue from 2	0.012	0.070
Total of 2 + 3	0.036	0.134

[a] Brains were extracted with chloroform–methanol and the residue was dried as described by Rouser *et al.* (1963). The residue was then divided and part was extracted with chloroform–methanol–NH_4OH as described by Rouser *et al.* (1963). The residue left after extraction with chloroform–methanol–NH_4OH and another sample of the original residue was extracted with chloroform–methanol–HCl as described in Figure 1.

TABLE V: Effect of Treatment of Rat Brain before Extraction on Phosphoinositide Recovery.[a]

Treatment before Extraction	Phosphoinositide (μmoles/g wet weight of brain) DPI	TPI	Molar Ratio TPI/DPI
1. Iced for 5 min	0.175	0.236	1.35
2. Room temperature for 30 min	0.258	0.127	0.49
3. Frozen in Dry Ice for 5 min	0.256	0.350	1.37
4. Frozen in Dry Ice for 35 min	0.265	0.356	1.34
5. Frozen in Dry Ice for 5 min	0.259	0.349	1.35
6. Frozen in liquid nitrogen for 5 min	0.255	0.350	1.37
7. Frozen in Dry Ice for 5 min	0.242	0.316	1.32
8. Iced for 30 min	0.307	0.237	0.77

[a] Rat brains were dissected out within 30 sec after decapitation and subjected to the treatment indicated before being extracted with chloroform–methanol and chloroform–methanol–HCl. Except for samples 1 and 2, every two samples are separate halves of brains divided bilaterally.

tract. Of the TPI recovered, 45% was extracted with chloroform–methanol–NH_4OH and 55% with chloroform–methanol–HCl (Table IV).

Degradation of Polyphosphoinositides in Brain before Extraction. In addition to the effect of acetone described above, data on the recovery of DPI and TPI from brains which had been subjected to different treatments before they were extracted are given in Table V. When brains were handled under conditions conducive to autolysis, TPI was degraded to DPI and under the most rigorous conditions the level of both TPI and DPI dropped significantly.

Discussion

In the quantitative analysis of the phosphoinositides described here, the difficulty arising from hydrolysis of the phosphate diester bonds experienced with MPI by Dawson (1960) and with TPI and DPI by Dawson and Dittmer (1961) was eliminated by methanolysis with NaOH or $NaOCH_3$ in mixtures of methanol and chloroform. Of similar methods previously described (Hübscher *et al.*, 1960; Marinetti, 1962; Brockerhoff, 1963), one based on that of Brockerhoff's was found best because no artifacts from other phospholipids are formed. Fractionation of the deacylation products from relatively complex mixtures of phospholipids has been accomplished by paper chromatography and electrophoresis (Dawson, 1960; Dawson *et al.*, 1962) or anion-exchange chromatography (Hawthorne and Hübscher, 1959; Hübscher *et al.*, 1960; Lester, 1963; Chang and Sweeley, 1963). Anion-exchange chromatography

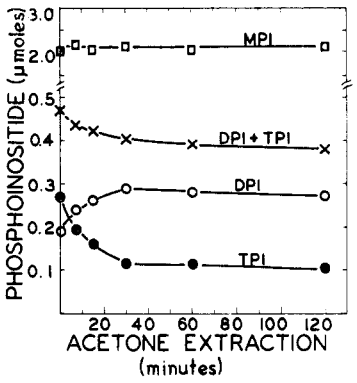

FIGURE 4: The hydrolysis of TPI and DPI during acetone extraction. The brains from five rats (8.54 g) were divided bilaterally and frozen in Dry Ice. One set of halves was extracted with chloroform–methanol and chloroform–methanol–HCl as a zero time control. The other set of halves was homogenized for 2 min with 12 ml of acetone. Five 2.0-ml samples of this homogenate were each mixed with an additional 2 ml of acetone. These samples were allowed to stand an additional length of time so that the total time from the initial homogenization equalled that given in the abscissa. The sample was then centrifuged and the residue was extracted with chloroform–methanol and chloroform–methanol–HCl. The combined acetone and chloroform–methanol extracts were analyzed for phosphoinositide content.

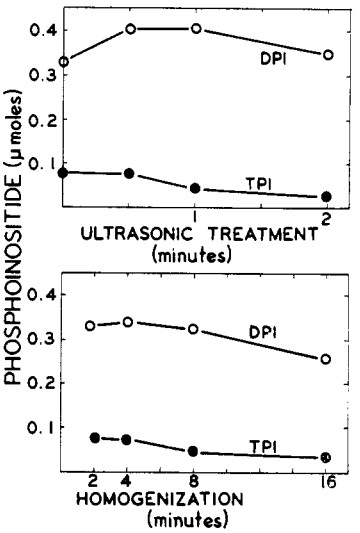

FIGURE 5: The effect of the degree of disruption of brain on the hydrolysis of phosphoinositides in acetone. Brains from four rats (7.38 g) were homogenized for 2 min with 15 ml of acetone. Four 1.0-ml samples of this homogenate were cooled in an ethylene glycol–Dry Ice mixture plus an additional 1 ml of acetone and then subjected to 0.5, 1.0, 1.5, and 2.0 min, respectively, ultrasonic vibration at 20 kcps with a Bronson S-75 sonifier set at intensity 4. The remainder of the homogenate was further homogenized with a VirTis micro homogenizer set to one-half maximum speed. Samples (1 ml) were taken after 0, 2, 6, and 14 min, respectively, to give total homogenization times of 2, 4, 8, and 16 min. An additional 1 ml of acetone was added to each of these samples. All of the samples were maintained at room temperature until 45 min after the initial homogenization. They were then centrifuged, the residue was extracted with chloroform–methanol and chloroform–methanol–HCl, and the combined extracts were assayed for polyphosphoinositides.

has also been used specifically for the separation of the phosphate diesters obtained from brain phosphoinositides (Brockerhoff and Ballou, 1961; Ellis *et al.*, 1963). Anion-exchange chromatography was used in this study because it permits greater flexibility in the amount of material which can be analyzed and was thereby useful in assaying relatively minor components in extracts containing large amounts of other lipids, for example, DPI in chloroform–methanol extracts of brain. The simple highly reproducible gradient anion-exchange fractionation procedure of Lester (1963) was particularly well adapted to this application.

Analysis of various extracts of brain shows that TPI and to a much lesser extent DPI are subject to degradation during isolation. This may occur as was noted by Kerr *et al.* (1964b) in handling the brain prior to extraction and/or during extraction with acetone as in the widely used procedure of Folch (1949). In either circumstance the ratio of DPI to TPI was much higher and the concentration of TPI was lower than was found under optimum conditions. The maximum yield of polyphosphoinositide was obtained only by extracting with acidified solvents. Although Rouser *et al.* (1963) claimed to obtain quantitative isolation and purification of TPI from ox brain with chloroform–methanol–NH_4OH, we found that additional TPI and DPI was extracted from the residue with acidified chloroform–methanol. We also found DPI in the chloroform–methanol–NH_4OH extract while Rouser did not account for this lipid in his quantitative fractionation of total brain lipids. It is perhaps significant that their purified TPI seems to have been characterized only by weighing, and no data supporting their claim of quantitative isolation were given.

When brain was homogenized with acetone, from 30 to 60% of the TPI was hydrolyzed to DPI within 30 min and the remainder appeared to be resistant over long periods. Several explanations may be considered. For example, the enzyme responsible for the hydrolysis was inactivated or subject to product inhibition. Alternatively, TPI may occur as two or more isomers, perhaps with respect to the position of the phosphates on the inositol, and the phosphatase was specific for only one of these. Finally, it was possible that part of the TPI was not accessible for hydrolysis because it occurred as part of the cellular structure or a complex not disrupted during homogenization. That the hy-

drolysis of TPI which was added to homogenates after the initial rapid hydrolysis of endogenous TPI was completed clearly rules out the possibility that the enzyme was inactivated. This was supported by the observation that additional endogenous TPI was hydrolyzed after further disruption of the homogenate. This latter observation also suggested that neither product inhibition nor the specific hydrolysis of an isomer was involved. The most probable explanation was that part of the endogenous TPI was not hydrolyzed readily because it occurred in a form not suitable as a substrate. How it was bound and whether metabolically distinctive forms exist require further investigation. It was quite possible that the peptide- or protein-bound inositide originally described by Folch (1952) was involved. The data obtained in this rather crude system must be interpreted cautiously; however, it appeared that the phosphatase showed a definite specificity for TPI. This was interesting in relation to the phosphatase activity described by Thompson and Dawson (1964; Dawson and Thompson, 1964). They found that an extract of an acetone powder of brain hydrolyzed both TPI and DPI at essentially equal rates and the final product was MPI. They also described a phosphodiesterase which hydrolyzed TPI and DPI to yield diglyceride and inositol di- and triphosphate, respectively. It appeared that this enzyme was not active in acetone homogenates because DPI appeared as rapidly as TPI disappeared. However, the slight decrease in total polyphosphoinositide may be due to phosphodiesterase activity. This disappearance could also be explained by hydrolysis of DPI to MPI. Solvent activation of phospholipases and the possible effect which solvents may have on specificity may be important; for example, Thompson and Dawson found that diethyl ether activated the phosphodiesterase and decreased the activity of the phosphatase.

The hydrolysis of TPI to DPI during extraction of brain with acetone has far-reaching consequences relative to previous studies on brain inositides. Folch (1949) probably failed to detect triphosphoinositide in his earlier fractionation of brain extracts because, depending on how the brain was handled prior to extraction, how finely it was dispersed, and how long it was extracted in acetone, all of the TPI may have been degraded to DPI. The implications with respect to studies recently reported on the metabolism of the phosphoinositides of brain (Brockerhoff and Ballou, 1962; Wagner *et al.*, 1962; Ellis and Hawthorne, 1962) is of more interest. These studies purport to show, on the basis of either the incorporation of $^{32}P_i$, ^{14}C-glycerol, or ^{3}H-inositol into the mono-, di-, and triphosphoinositide, that these lipids are interrelated by the following reaction sequence: MPI → DPI → TPI. In each of these studies, extraction with acetone was used in the isolation of the phosphoinositides. Consequently, the DPI examined for radioactivity represents, at best, a mixture which may have been derived in part by phosphorylation of MPI but also, in all probability, included DPI formed by hydrolysis of TPI.

In conclusion, the extraction and quantitative analysis of brain polyphosphoinositides is extremely sensitive to autolysis in the tissue prior to extraction and enzymatic degradation during extraction. Maximum recoveries were obtained only after extraction with acidified solvents. Whether the maximum yields obtained here represent the true content of these lipids in brain is not certain. They at least appear to represent a good first approach to the problem.

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Biosynthesis of Uridine Diphosphate D-Xylose. I. Uridine Diphosphate Glucuronate Carboxy-lyase of Wheat Germ*

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ABSTRACT: Uridine disphosphate D-glucuronate carboxy-lyase from wheat germ has been purified 350-fold. The only products of enzyme action are uridine diphosphate D-xylose and CO_2. The enzyme has a pH optimum between 6.8 and 7.0. K_m for uridine disphosphate D-glucuronate is about 3×10^{-4} M.

Wheat germ carboxy-lyase is neither activated by nicotine-adenine dinucleotide (NAD) nor inhibited by reduced NAD ($NADH_2$), in contrast to the analogous enzyme from *Cryptococcus laurentii*. However, NAD is released by wheat germ enzyme upon heat denaturation.

Polymers of D-xylose abound in higher plants. It was long suspected that the metabolic pathway in plants leading from hexose to pentose involved C-6 decarboxylation. This was substantiated by studies of many workers using either intact plants (Altermatt and Neish, 1956; Neish, 1958; Loewus *et al.*, 1958) or plant slices (Slater and Beevers, 1958). In these experiments the tissue was supplied with a suitably labeled hexose or uronic acid and the distribution of label in the pertinent metabolic products was determined. Involvement of sugar nucleotides in this conversion was suggested by the isolation of a mixture of uridine 5′-(α-D-xylopyranosyl pyrophosphate), uridine 5′-(β-L-arabinopyranosyl pyrophosphate), uridine 5′-(α-D-glucopyranosyl pyrophosphate), and uridine 5′-(α-D-galactopyranosyl pyrophosphate) (UDPXyl, UDPAra, UDPG, and UDPGal, respectively)[1] from mung bean seedlings by Ginsburg *et al.* (1956); uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate) (UDPGA) was isolated from the same source by Solms and Hassid (1957). Subsequently it was shown that UDPXyl was formed by the decarboxylation of UDPGA. This was demonstrated by use of partially purified extracts from mung beans (Feingold *et al.* 1960) and other plants. These preparations were of low activity and were always contaminated by UDPAra-4-epimerase.

Recently we have demonstrated and partially purified the UDPGA carboxy-lyase of *Cryptococcus laurentii* (Ankel and Feingold, 1964). This enzyme has an absolute requirement for catalytic quantities of nicotinamide-adenine dinucleotide (NAD) and is inhibited by reduced NAD ($NADH_2$). We have now reinvestigated the plant enzyme in order to compare it with the carboxy-lyase from *Cryptococcus laurentii*. In this paper we describe the partial purification and properties of the UDPGA carboxy-lyase of wheat germ.

* From the Microbiology Section, Department of Biology, University of Pittsburgh, Pittsburgh, Pa. *Received June 8, 1965; revised August 11, 1965.* Supported by a grant from the United States Public Health Service (GM-08820). Some of the results presented here were reported in a preliminary communication (Ankel and Feingold, 1965).

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[1] The following abbreviations are used: nicotinamide-adenine dinucleotide, NAD; reduced NAD, $NADH_2$; uridine monophosphate, UMP; uridine diphosphate, UDP; uridine triphosphate, UTP; thymidine diphosphate, TDP; uridine, adenine, cytosine, and guanine 5′-(α-D-glucopyranosyl pyrophosphate), UDPG, ADPG, CDPG, and GDPG, respectively; uridine 5′-(α-D-xylopyranosyl pyrophosphate), UDPXyl; uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate), UDPGA; uridine 5′-(α-D-galactopyranosyluronic acid pyrophosphate), UDPGalA; uridine 5′-(β-L-arabinopyranosyl pyrophosphate), UDPAra; uridine 5′-(α-D-galactopyranosyl pyrophosphate), UDPGal; uridine 5′-(α-D-*xylo*-hexopyranosyluronic acid-4-ulose pyrophosphate), UDP-4-keto-GA; uridine 5′-(β-L-*threo*-pentopyranosyl-4-ulose pyrophosphate), UDP-4-keto-Xyl. The following trivial names are used for enzymes which have been assigned systematic names by the Commission on Enzymes of the International Union of Biochemistry, 1961: β-D-glucose:O_2 oxidoreductase (EC 1.1.3.4), glucose oxidase; D-glyceraldehyde 3-phosphate:NAD oxidoreductase (phosphorylating) (EC 1.2.1.12), GAP dehydrogenase; UTP:α-D-xylose 1-phosphate uridylyl transferase (EC 2.7.7.11), UDPXyl pyrophosphorylase; NAD glycohydrolase (EC 3.2.2.5), NADase; orthophosphoric diester phosphohydrolase (EC 3.1.4.4), phosphodiesterase. *p*-Mercuribenzoate is abbreviated PCMB.