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ISOLATION AND SEPARATION OF INOSITOL PHOSPHATES FROM HYDROLYSATES OF RAT TISSUES

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SUMMARY

1. An electrophoretic method for the rapid separation of the phosphate esters of inositol is presented.

2. It is used, together with ion-exchange chromatography, to establish the presence of inositol di- and triphosphates in hydrolysates of trichloroacetic acid residues of rat brain, liver, heart, kidney, and lung.

3. With the aid of a ^{32}P marker, inositol di- and triphosphates were also found in hydrolysates of rat erythrocyte stroma.

4. Glycerol diphosphate was found in hydrolysates of rat liver.

5. Inositol tetraphosphate was not found in the hydrolysates of any of the tissues studied.

6. Residues remaining after extraction of rat brain by established lipid extraction procedures retained substantial amounts of bound inositol di- and triphosphates.

INTRODUCTION

PhI (refs. 1, 2), PhIP (ref. 3), and $PhIP_2$ (refs. 4–6) have been identified in extracts of brain. Difficulties in extracting and purifying PhIP and $PhIP_2$ have been ascribed to their presence as insoluble calcium and magnesium salts and to ionic binding to protein[6,7,8]. It has been shown that preliminary extraction with acetone or the use of acidified solvents[6,9] facilitates the extraction of substances which appear to be PhIP and $PhIP_2$. Preliminary extraction with acetone[10], or lyophilization[11], in each case followed by Soxhlet extraction with ether and by chromatography, has been utilized by WAGNER *et al.*[11,12] to isolate from brain and other tissues three inositol-containing compounds which they assume to be PhI, PhIP, and $PhIP_2$. HENDRICKSON AND BALLOU[13] have recently separated PhIP from $PhIP_2$ and from a calcium-containing form of $PhIP_2$.

Interest in these lipids led us to study the completeness of extraction of the various described purification procedures by comparison with the amounts of IP_2 and

Abbreviations: PhI, phosphatidyl inositol; PhIP, phosphatidyl inositol phosphate (diphosphoinositide); $PhIP_2$, phosphatidyl inositol diphosphate (triphosphoinositide); IP, inositol monophosphate; IP_2, inositol diphosphate, etc.

IP_2 which could be released from tissue preparations by acid hydrolysis. It had been shown[14] that controlled acid hydrolyses of $PhIP_2$ give about 80% yields of IP_2. While such treatment can result in isomerization of the product, relatively little IP or P_i is found. IP_2 accounts for about 15% of the P.

In the experiments reported here, methods were developed for the isolation of IP_2 and IP_3 from acid hydrolysates of rat-tissue fractions. It is shown that solvent extraction procedures do not release sufficient PhIP and $PhIP_2$ to account for all of the inositol phosphates obtained by acid hydrolysis.

METHODS AND MATERIALS

Phytic acid was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. Myo-inositol 2-monophosphate, (−)inositol 3-monophosphate, and phytic acid were purchased from California Corporation for Biochemical Research. L-Myo-inositol 1-monophosphate was prepared from soybean lipid[15]. Glycerol 1,3-diphosphate and glycerol 1,2-diphosphate were the gift of Dr. C. BALLOU.

Rats were a Sprague–Dawley strain. ^{32}P was purchased from Iso/serve Inc., Cambridge, Mass. as Na_2PO_4.

Radioactive samples were counted with a gas-flow counter or in a liquid-scintillation counter. Electrophoresis was performed on a Model D Electrophorator (Gilson Medical Electronics, Middleton, Wisc.). Strips were counted in a Vanguard 880 strip scanner (Vanguard Instruments, LaGrange, Ill.). Inositol was determined by periodate consumption[16,17] and phosphate by the method of BARTLETT[18]. Paper chromatography of inositol phosphates was performed according to DESJOBERT AND PETEK[19]. Chromatograms and electrophoresis strips were developed with molybdate–perchlorate spray followed by ultraviolet irradiation[20].

RESULTS

Preparation of inositol phosphates

To obtain a mixture of inositol phosphates, phytic acid was partially hydrolyzed with acetate buffer (pH 5.2)[19]. A portion of the phytate hydrolysate was separated on a Dowex-1 X 8, Cl^-(200–400 mesh) column[21] and fractions (P analysis) tentatively designated as IP_2, IP_3, and IP_4 were collected. Analysis gave the following phosphate/inositol ratios: IP_2, 2.0:1; IP_3, 2.84:1; IP_4, 4.1:1.

The dried esters were stored at −15°.

Electrophoretic separation of inositol phosphates

Samples of known inositol phosphates and of phytate hydrolysate were applied to a strip of Whatman No. 1 paper, 57 cm in length. The samples were applied in bands less than 1 in wide to allow for the width of the strip scanner. As can be seen in Fig. 1, electrophoresis in 0.1 M sodium oxalate buffer (pH 1.5) yields satisfactory separations. Migration rates were calculated from the leading edge of each spot and are shown in Table I. Distinct bands are seen in the hydrolysate for P_i, IP, IP_2, IP_3, IP_4, IP_5, and IP_6. Faint bands are seen for isomers and these may be further resolved after preparative column chromatographic separation of the isomeric mixtures and longer electrophoresis.

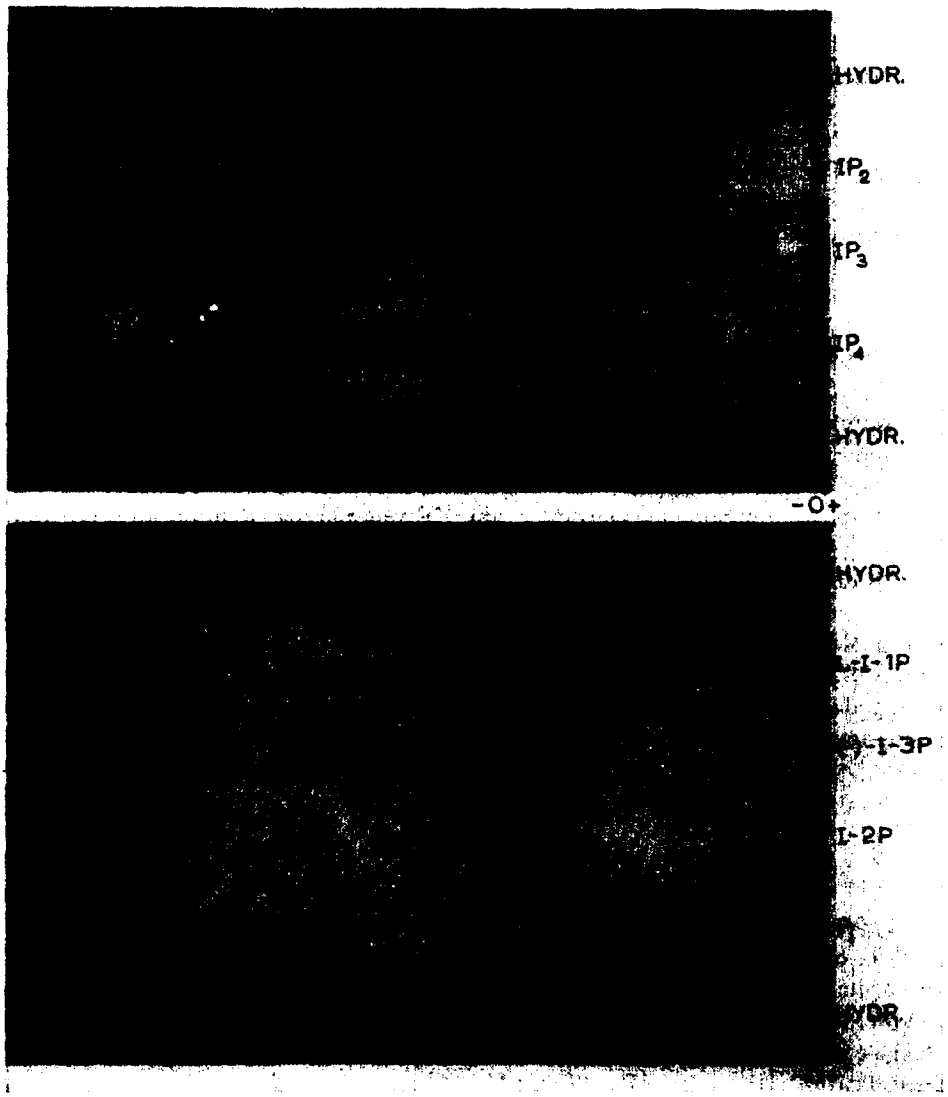

Fig. 1. Electrophoretic separation of known inositol esters and of products of a phytate hydrolysate. (A) IP_2, IP_3, and IP_4. Phytate hydrolysate (1.2 μmoles P) or 0.1 μmole of purified ester in 10 μl were applied to Whatman No. 1 paper. Strips were subjected to 80V/cm (4000 V) for 20 min. Isopar H was the coolant. Molybdate spray. The most rapidly moving spot in the hydrolysate ("IP_6") migrated 24.4 cm from the origin. (B) IP and P_i. A minor contaminant, presumably myo-inositol 2-monophosphate is present in the L-myo-inositol 1-monophosphate.

Acid hydrolysis and column separation of inositol phosphates

1 g of fresh tissue was homogenized in a Virtis blender for 1–2 min with 4 ml of 20% trichloroacetic acid. The residue obtained upon centrifugation was washed twice with 5% trichloroacetic acid. The trichloroacetic acid residue was taken up in 1 ml of 5 N HCl. When lipid fractions were taken for hydrolysis they were first lyo-

philized from benzene, then suspended in 1–2 ml of 5 NHCl. If necessary, the hydrolysis mixture was dispersed with a glass stirring rod. Hydrolyses were carried out in sealed tubes at 105° for 10 min in an oil bath. The tubes were cooled to 4° and the residue removed after centrifugation for 5 min at 2000 rev./min. The residue was washed twice with 1 ml of water. The combined clear supernatant solution (3–4 ml) was cooled to 4° and 0.5 ml of saturated $Ba(OH)_2$ and a drop of phenolphthalein indicator were added. Saturated LiOH was added for neutralization, followed by 4 vol. of ethanol. Precipitation of phosphates was judged complete by P determination and by counting of radioactive samples from ^{32}P-injected rats. The mixture was kept at 4° for 1 h. The precipitate was then collected by centrifugation and washed twice with 80% ethanol to remove LiCl. The residue was dissolved in about 1 ml of a slurry of

TABLE I

MIGRATION RATES OF PHOSPHATE ESTERS IN OXALATE BUFFER

Substance	*Rate relative to P_i (R_{Pi})*
Inorganic phosphate	1.0*
Myo-inositol monophosphate**	1.4
L-Myo-inositol 1-monophosphate	1.4
(−)Inositol 3-monophosphate	1.4
Myo-inositol 2-monophosphate	1.5
Myo-inositol diphosphates**	2.0–2.1
Myo-inositol triphosphates**	2.6, 2.8
Myo-inositol tetraphosphates**	3.0, 3.1, 3.3
"Myo-inositol pentaphosphate"	3.5
"Myo-inositol hexaphosphate"	3.7
α-Glycerophosphate	1.6
Band "X"	2.2

* Specific migration = 0.69 $cm^3 \cdot V^{-1} \cdot sec^{-1} \times 10^4$.
** Isolated from phytate hydrolysate. Major isomer underlined.

Dowex-50W-H^+ (50–100 mesh). The resin was washed twice with water, the combined supernatant fractions were taken to dryness, and treated again with an equal amount of Dowex-50-H^+ to remove traces of cationic contaminants. For larger amounts of hydrolysate, a Dowex-50 column was used instead of the slurry. The clear supernatant solution or eluate contains the free phosphate esters, and may be evaporated to dryness without detectable hydrolysis.

Column separation of inositol phosphates

Dowex-1X8 (200–400 mesh) was washed with 10% HCl. Fines were discarded. The resin was washed with distilled water and then with 2 N sodium formate in a column 1 cm (internal diameter) × 4 cm. It was then washed with water until formate was no longer detected. The solution of free phosphate esters (under 30 μmoles) was diluted to 50 ml and applied to the column. This was followed sequentially by 200 ml of water, then 400 ml each of 2.5 N formic acid, 0.05 N HCl, and 0.1 N HCl.

When phytate hydrolysate was applied to this column, no P was eluted with water. The formic acid eluate contained all of the P_i and inositol monophosphate. 8 N formic acid did not elute additional substances. The 0.05 N HCl eluted only IP_2.

0.1 N HCl eluted only IP_3. IP_4, IP_5, and Ip_6 were eluted without degradation by 0.75–1.0 N HCl.

Thus this column appears to separate completely the various hydrolysis products of interest to us. In subsequent analyses with tissues described below, a negligible fraction of the P recovered was found in the water wash or upon stripping the column with 1 N HCl after elution of the 0.1 N HCl fraction. Authentic α-glycerophosphate was eluted in the formic acid fraction.

^{32}P was used as a marker for the electrophoretic identification of P-containing compounds. Three young male rats (70–75 g) were each injected with 60 μC of carrier-free ^{32}P ($6 \cdot 10^7$ counts/min) intraperitoneally and were sacrificed by CO_2 asphyxia. Organs were removed and immediately treated with trichloroacetic acid as described above. Following hydrolysis of the residue, barium precipitation, and Dowex-50 and Dowex-1 treatment, the column fractions were subjected to electrophoresis. In general, the radioactive peaks were found to coincide with P_i, α-glycerophosphate, and IP in the formic acid eluate, and with IP_2 and IP_3 in the 0.05 and 0.1 N HCl eluates. The radioactive peaks were discrete and no bands other than those described were observed in hydrolysates from rats which were sacrificed 2h–32h following injection. In the case of liver, there was a band which gave a faint color with molybdate spray, but contained no detectable radioactivity at 3 h. It moved ($R_{Pi} = 2.2$) slightly ahead of inositol diphosphate. It was termed band "X" and is further described below.

Separation of P_i, α-glycerophosphate, and IP peaks was not complete in 20 min when large amounts of P_i were present. Fractions from bone and intestine gave poor separations on the column apparently due to large amounts of P-free contaminants in the extract applied to the Dowex-1. Nevertheless, radioactive bands for IP_2 and IP_3 were seen. In all of these experiments KOH had been used for neutralization of residual HCl in the hydrolysate resulting in a large KCl precipitate. The later use of LiOH permitted removal of LiCl through its solubility in the ethanol washes of the Ba precipitate.

Molybdate spray of the 0.05 and 0.1 N HCl eluates of rat tissues after electrophoresis revealed visible spots for IP_2 and IP_3 (except for IP_3 from heart). Small amounts of IP_2 and IP_3 could be detected by radioscanning in hydrolysates of erythrocyte stroma of ^{32}P-injected rats.

Comparison of different extraction procedures

The amounts of IP_2 and IP_3 recovered (Table II) were 2.4–27-fold larger than expected from reported values of PhIP and $PhIP_2$ in ether extracts of tissues[11]. In order to investigate this apparent discrepancy, the combined ether and methanol extract of lyophilized brain[11] and the resulting residue were subjected to acid hydrolysis and separation of esters on Dowex-1 and electrophoresis.

A 160-g male rat was injected with 570 μC of ^{32}P intraperitoneally and sacrificed 32 h later. The brain was weighed, homogenized in 2 vol. of water, and lyophilized. The dried tissue was extracted 6 h with ether, and 18 h with methanol. The extracts were combined. In Table III, Expt. 2, results for both the extract and the residue are presented. It can be seen that much apparent IP_2 and IP_3 remain after extraction. This was verified by radioscanning following electrophoresis.

In another experiment, 3 rats were each injected with 960 μC of ^{32}P and sacri-

ficed after 8 h. The brains were extracted according to LEBARON[7,8]. Table III, Expt. 3, shows the P recovered for combined chloroform-methanol (2:1, v/v), acetone, and chloroform–methanol–HCl (200:100:1, v/v) wash, as well as for the residue. Total P is similar in each instance. The acid extraction appears incomplete but somewhat better than that obtained by Wagner and Hörhammer. The chloroform–methanol–HCl (200:100:1, v/v) fraction hydrolyzed separately in another experiment yielded P in all 3 elution fractions.

TABLE II

PHOSPHATE CONTENT OF COLUMN FRACTIONS FROM HYDROLYSATE OF TRICHLOROACETIC ACID RESIDUES

	μmoles P per g wet wt.				
Fraction	*Brain*	*Liver*	*Kidney*	*Heart*	*Lung*
Whole eluate	26.6	24.2	18.7	9.98	15.8
2.5 N formic acid	21.3	21.0	16.0	8.27	12.7
0.05 N HCl	1.4	1.46	2.24	1.63	1.45
0.10 N HCl	0.89	0.26	0.46	0.17	0.34

TABLE III

PRODUCTS OF ACID HYDROLYSIS OF LIPID EXTRACTS OF RAT BRAIN

Experiment	*Solvent*	*Fraction*	*μmoles P per g wet wt.*		
			Extract	*Residue*	*Total*
1	Trichloroacetic acid residue	0.05 N HCl	—	—	1.40
		0.10 N HCl	—	—	0.89
2	Ether, methanol	0.05 N HCl	0.65 (0.57)	0.82	1.47
		0.10 N HCl	0.54 (0.30)	0.28	0.82
3	Chloroform–methanol, acetone, chloroform–methanol–HCl	0.05 N HCl	1.10	0.31	1.40
		0.10 N HCl	0.50	0.17	0.67

After extraction by the technique of WAGNER *et al.*[11] or of LEBARON[8], the residue and combined extracts are hydrolyzed and fractionated as described in the text. Numbers in parentheses are calculated from reported values of WAGNER *et al.*[11].

Identification of band "X"

Band "X", first seen in hydrolysates of trichloroacetic acid residues of liver, was also noted in chloroform–methanol (2:1, v/v) and ether extracts of liver obtained from the animals described above. 32 h after ^{32}P administration, band "X" was labeled and could be isolated by eluting the radioactive band ahead of IP_2 after electrophoresis of the 0.05 N HCl fraction from rat liver. This material contained no inositol. Since the migration rate suggested a diphosphate, glycerol diphosphate seemed a likely possibility. Authentic glycerol 1,2-diphosphate and glycerol 1,3-diphosphate were subjected to electrophoresis. Glycerol 1,2-diphosphate had an R_{P_1} of 2.2, identical with band "X". Glycerol 1,3-diphosphate had an R_{P_1} of 2.15, slightly ahead of inositol diphosphate and behind band "X". Complete separation of both glycerol diphosphate isomers from the isomers of inositol diphosphate is readily achieved with electrophoresis for 20–30 min. When authentic glycerol 1,3-diphosphate was subjected to the conditions of acid hydrolysis, two spots were seen on electrophoresis: a major spot for glycerol 1,2-diphosphate, and a smaller one for glycerol 1,3-diphosphate, indicating that isomerization had taken place under conditions of the hydrolysis. No P_1 or glycerophosphate was seen. When radioactive band "X" was subjected to des-

cending paper chromatography for 60 h, a radioactive spot was found slightly ahead of the most rapidly moving isomer of inositol diphosphate, and corresponded to the R_F obtained with glycerol 1,2-diphosphate and glycerol 1,3-diphosphate. The isomers did not separate.

The 0.05 N HCl fraction from a chloroform-methanol (2:1, v/v) extract of rat liver was concentrated, neutralized and treated with wheat phosphatase, and the polyols recovered by the use of a mixed-bed ion-exchange column[16]. After chromatography with butanol–acetic acid–H_2O (4:1:5, v/v)[23] and staining with $AgNO_3$ (ref. 24), distinct spots for inositol and glycerol were found.

Purity of IP_2 and IP_3 fractions

Although single radioactive peaks were seen in the 0.05 N HCl and 0.1 N HCl eluates of brain for IP_2 and IP_3, respectively, it remained possible that non-radioactive P-containing contaminants were present *e.g.*, band "X" in liver shortly after injection. Corresponding HCl eluates of both extract and residue from Expt. 3 (Table III) were combined and aliquots were taken for electrophoresis and for P determination. The liver of the same rat was treated identically. The radioactive bands were cut out and total P was determined. By comparison with the amount of P applied, the following purity of the inositol phosphates was obtained: Brain: IP_2, 76%; IP_3, 92%. Liver : IP_2, 49%; IP_3, 94%.

DISCUSSION

The electrophoretic method described separates not only the various phosphate esters of inositol in 20 min, but appears to offer the possibility of separating isomers of each ester as can be seen in Fig. 1. The method is superior to previously described electrophoretic separations of inositol phosphates[25,26]. Dowex-1-Cl^- appears not to separate isomers.

The acid hydrolytic method employed here gives minimal values for $PhIP_2$, since about 20% is reportedly destroyed[14]. The IP_3 obtained in 0.1 N HCl appears quite pure. The IP_2 values might be increased by a contribution from acid hydrolysis of $PhIP_2$. In liver, the 0.05 N HCl eluate is known to contain glycerol diphosphate. The 0.05 N HCl eluate P value then represents the maximal amount of PhIP present.

Despite the hydrolytic losses, the results obtained by us on hydrolysis of an ether and methanol extract indicate a larger amount of IP_3 than expected from reported amounts[11] of $PhIP_2$. Considerable additional amounts of IP_2 and IP_3 remain combined with the residue after extraction. A smaller amount remains after acidic extraction. It is possible that these additional bound inositol phosphates are not present as PhIP or $PhIP_2$.

In this regard it is also worthy of note that the hydrolysate of "phosphatido-peptide" (chloroform–methanol–HCl (200:100:1, v/v) extract) did not yield primarily IP_2 as might be expected[22]. It is possible that the $PhIP_2$ recovered by chloroform–methanol (2:1, v/v) extraction of brain[3,5] differs from the inositol lipid in "phosphatido-peptide".

The presence of IP_2 and IP_3 was detected in hydrolysates of erythrocyte stroma. It is of interest in that PhIP and $PhIP_2$ have been implicated in ion transport[27,28] in liver mitochrondria.

Glycerol 1,2-diphosphate results from isomerization of glycerol 1,3-diphosphate during acid hydrolysis. Glycerol 1,3-diphosphate is probably a degradation product of polyglycerylphosphates such as cardiolipin or phosphatidylglycerylphosphate and may be related to the unlabeled material found by MICHELL *et al.*[29] in alkaline hydrolysates of liver mitochondria.

IP_4 which might be expected in hydrolysates if $PhIP_3$ is present in tissues[30], was not found.

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Note added in proof. Since this paper was submitted, studies have been reported on the occurrence and turnover of polyphosphoinositides in various tissues[31], the incorporation of ^{32}P into polyphosphoinositides by erythrocyte ghosts[32], and the isolation and partial separation of IP_2 and GP_2 from hydrolysates of liver and pancreatic lipid extracts[33].

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