Preparation of brain polyphosphoinositides

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SUMMARY A description is given of the preparation of mixed tri- and diphosphoinositides of brain by a sequence of three procedures, no one of which alone effects complete purification. About 7% of the phosphorus in the product represents diphosphoinositide when ox brain is obtained within an hour of slaughter.

AMONG THE PROCEDURES recently presented for the preparation of brain PP-I[1] (1–3), only the one described by Dittmer and Dawson (3) based on the extraction of protein-bound inositide has yielded in our hands a pure product. Analysis of the phosphoinositide preparation FF-1 of Folch (4) shows the presence of large amounts of phosphatidyl serine and other contaminants even after 13 precipitations from chloroform by methanol.[2] However, pure PP-I may be prepared from FF-1 by a sequence of procedures, no one of which alone effects complete puri-

[1] Abbreviations: TPI, DPI, and MPI, tri-, di-, and monophosphoinositides; PP-I, polyphosphoinositide (i.e., either DPI, TPI, or a mixture of these); FF-1, Folch Fractions I and II (see reference 4); C–M, chloroform–methanol mixture.

[2] The studies by Grado and Ballou (2) on PP-I structure were made on FF-1 after only six reprecipitations.

fication: (*a*) partition in a biphasic solvent system which separates DPI and TPI from MPI, phosphatidyl serine and cardiolipin; (*b*) removal of the remaining phosphatidyl serine, cations, and inorganic P by Dowex-50 W (H^+ form) resin; and (*c*) precipitation of a sugar-containing nitrogenous contaminant by methanol from a solution of the free acid in chloroform. These procedures are described in detail below, together with an outline of the method we have found to be the simplest for the preparation of FF-1.

The method of preparation presented below permits the storage in bulk of the stable FF-1, from which pure PP-I may be prepared rapidly in batches of any size.

Preparation of FF-1. The alcohol-insoluble phospholipids are first obtained by Debuch's procedure (5), with minor modifications. Several kilograms of ox brain are freed from water and cholesterol by 3–4 extractions with acetone. The glyceryl phospholipids are then dissolved by three extractions with ethyl ether. Stepwise concentration of the ether solution to volumes representing 1.5, 0.7, and 0.35 liters/kg of brain, with overnight chilling at each step, permits the elimination of protagon without the difficulties encountered in sedimenting this material by the Folch procedure (4). The phospholipids are precipitated from ether solution twice by excess acetone, and twice by ethanol (2.5 volumes). The crude "cephalin" thus obtained is fractionated by Folch's procedure (4) to eliminate the remaining lecithin, phosphatidyl ethanolamine, and part of phosphatidyl serine, FF-1 being secured by precipitation from ice-cold solution in chloroform (7 ml/mmole P), once by ethanol at 60%, and once by methanol at 65% final concentration. The FF-1 thus prepared is again dissolved in chloroform (10 ml/mmole P) and reprecipitated by methanol as before. The precipitate is dissolved in the lower phase of the solvent system C described by Cole et al. (6), this being carbon tetrachloride–petroleum ether(bp 40–60) 1:1, equilibrated with 0.6 volume of 92% methanol. Solution of the methanol precipitate in 100 ml of solvent per kg of brain gives a concentration of about 0.1 M phosphorus. This stock solution of FF-1 is analyzed for its content of total P (7) and inorganic P (8) and stored in the refrigerator. The yield thus obtained was found to be 10.3 mmoles of total P, or 6.9 mmoles of organic P (average for 5 preparations) per kg of brain.

Partition in Biphasic Solvent System. Preliminary experiments demonstrated that PP-I is concentrated in the lower nonpolar phase of the first few tubes during countercurrent distribution of FF-1 with Cole's solvent system C (6) while MPI, phosphatidyl serine, and cardiolipin pass into the polar phase. It was further noted that 46 transfers in the countercurrent apparatus effected no greater removal of nitrogenous contaminants than 15 transfers of fresh upper phase over three lower phases. At this stage the removal of organic phosphate and nitrogen reaches a plateau, the N:P atomic ratio in the lower phase being 0.18 after 15 transfers, and 0.16 after 46 transfers. Of the PP-I trapped in the lower phase the first tube contained 99.0% and the second tube 1.0% of the total P, while the third contained no P. Thus it is evident that 99% recovery of PP-I may be secured without use of the countercurrent apparatus by simply washing the FF-1, dissolved in the lower phase, 15 times with the upper phase.

A sample of the FF-1 stock solution containing 5 mmoles of total P, and diluted to 100 ml with the lower phase of Cole's solvent, is transferred to a glass-stoppered 250 ml cylinder and shaken with an equal volume of the upper phase for 3 min. After the phases separate, the upper layer is aspirated into a flask. The volume of the lower phase is restored to 100 ml by further addition of the solvent, and the procedure is repeated until the lipid sample has been equilibrated 15 times with fresh upper phase.

The recovery of organic P in the lower phase was 2.5 mmoles/kg of brain (average for 6 experiments), or 39% of that in FF-1. The atomic ratio of N to total P varied from 0.13 to 0.18.

Purification by Resin. All but a trace of the nitrogenous contaminants together with Ca, Mg, inorganic P, and some inositol diphosphate are removed by the cation-exchange resin Dowex-50 W × 8, H^+ form, 100–200 mesh.[3] The resin is first freed from water and some colored material by two washings with methanol and is then transferred in methanol to the column. For each millimole of total P used as starting material a 10 cc resin bed (6 g if air-dried) is required, with a column not less than 25 cm high. A plug of glass wool is inserted on top of the resin, which tends later to float, and the methanol is displaced by 1 bed-volume of C–M 4:1.

Before passing the lipid preparation through the resin, petroleum ether is removed by evaporating the solvent to half volume at reduced pressure. An equal volume of C–M 4:1 is then added, and the mixture is passed slowly through the resin column, followed by 2 bed-volumes of C–M 4:1. The rate of flow should not exceed 1.5 ml/min.

The colorless filtrate and washings contain no inorganic P, and only traces of N, the N:P ratio being

[3] Dittmer and Dawson (3) removed inorganic P by means of Amberlite IR-120, H^+ form. We substituted Dowex-50 W × 8, 100–200 mesh (H^+ form), finding that it also adsorbs most of the nitrogenous contaminants.

The resin may be recovered by the following sequence: (*a*) removal of adsorbed phosphates by shaking with water; (*b*) conversion to the sodium form by addition of 25% NaOH until alkaline, liberating most of the adsorbed N; (*c*) reconversion to the H^+ form by treatment with hot 4 N HCl; (*d*) removal of acid by washing with water, and finally dehydration with methanol and drying in air.

TABLE 1 COMPOSITION OF OX BRAIN POLYPHOSPHOINOSITIDE

	Molar Ratio
P in anhydrous Na salt	7.66%
Glycerol by Blix method (9):P	0.35
Myoinositol by bioassay (10):P	0.33
Carboxylic ester (11):P	0.66
N by microKjeldahl:P	0.01
Carbohydrate (anthrone reaction)	None

0.01–0.05. The recovery of lipid P at this stage was 1.62 (average of 8 experiments) mmoles/kg of ox brain (23.5% of the organic P in FF-1).

Removal of Carbohydrate-Containing Nitrogenous Impurity. After complete removal of the solvent in a flash evaporator the residue is taken up in 1.6 ml of chloroform per millimole of FF-1 P used for the preparation. Any turbidity at this point disappears on addition of methanol up to one-eighth volume. Excess methanol (7 volumes) is then added, the mixture is chilled thoroughly, and the precipitate is removed by centrifugation. The loss of lipid P does not exceed 1% of the starting material. The methanol-soluble material is practically all PP-I as the free acid, the yield being 1.56 mmoles of P per kg of brain.

Preparation of the Sodium Salt. This is accomplished as described by Dittmer and Dawson (3), the chilled mixture being brought to pH 6.8–7.0 with 0.1 N methanolic NaOH. Recovery of 70% of the PP-I as sodium salt may be obtained by reducing the volume of the neutralized mixture in the flash evaporator to approximately one-fourth volume, and again chilling. The sodium salt thus recovered from the 5 mmoles of P used as starting material approximates 0.6 mmole of P (1.25 mmole of P per kg of brain).

Analysis of a typical preparation gave the results presented in Table 1. The data suggest that the material is the diacyl ester of TPI, but analysis of the fractions eluted from Dowex-2 × 8 (chloride form) after mild deacylation (0.1 N methanolic KOH at 20° for 15 min) showed 81.5% of the phosphorus to be glycerophosphoryl inositol diphosphate, 7.4% as glycerophosphoryl inositol monophosphate, and 6.7% as inositol diphosphate. The remaining phosphate was eluted at the salt concentration which characterizes glycerophosphate.

In other preparations chromatographic analysis of the deacylated lipid showed 85% of the P to be glycerophosphoryl inositol diphosphate and 7.1% glycerophosphoryl inositol monophosphate (average of five experiments). The absence of glycerophosphoryl inositol indicates the absence of MPI. Since no inorganic P was found it must be concluded that the glycerophosphoryl inositol phosphate is formed by deacylation of DPI, and that at least 7% of the P in our PP-I represents DPI. The inositol diphosphate and glycerophosphate probably represent products of decomposition of TPI during deacylation.

A similar analysis of PP-I prepared by extraction of protein-bound phosphoinositide with acid C–M (3) showed that DPI represented 5.3% of the total P.

On the basis of the content of fatty acids reported elsewhere (12) the molecular weight of the free acid of TPI was calculated to be 1040. The tetrasodium salt of TPI would then have a molecular weight of 1128, and the disodium salt of DPI, 1004. The theoretical content of phosphorus in a mixture of the sodium salts of TPI and DPI (81.5% of the P being TPI and the remainder DPI) is calculated to be 7.77%, whereas analysis showed 7.66% (Table 1).

This investigation was supported by Grant No. 296 from the National Multiple Sclerosis Society.

Manuscript received October 15, 1963; accepted January 22, 1964.

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