A simplified preparation of phosphatidyl inositol

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SUMMARY A method is described for the rapid isolation of phosphatidyl inositol from soybean phosphatides (Asolectin). The product is obtained pure as the crystalline sodium salt.

KEY WORDS phosphatidyl inositol · soybean phosphatides · cation exchange · monophosphoinositide.

THE NUMEROUS METHODS that have been described for the isolation of phosphatidyl inositol from both animal (1–4) and plant (4–7) sources are very laborious, and in some, the final product is not pure. We are reporting a convenient, reproducible method for obtaining phosphatidyl inositol from Asolectin, a commercially available, inexpensive source of soybean phosphatides. The product is pure as judged by chromatographic homogeneity and by analyses for phosphorus, ester, and inositol. The method represents several modifications introduced into the procedures of Okuhara and Nakayama (5, 8, 9) and of Rouser, Kritchevsky, Heller, and Lieber (4).

Analytical Methods. Analytical methods for phosphorus (10) and ester groups (11) have been described. Nitrogen was determined by the micro-Kjeldahl procedure. Inositol analysis was carried out by densitometry after paper chromatography of the hydrolysate according to Norton and Autilio (12), and silver oxidation of inositol according to a procedure developed by Dr. S. Samuels (private communication). Gas–liquid chromatography of the fatty acid methyl esters was carried out on a 6 ft column of 15% ethylene glycol succinate on 80–100 mesh Gas-Chrom CLZ, at 185°C with a Packard instrument and flame ionization detector.

Na and K were determined with a flame photometer (Baird-Atomic, Inc., Cambridge, Mass.) by the internal standard ($LiNO_3$) method. Ca and Mg were measured in a Perkin-Elmer (model 303) atomic absorption spectrophotometer. Optical rotation was determined in a Cary model 60 spectropolarimeter equipped with a cell that had a 1 cm light path.

Solvent Fractionation. 5 g of Asolectin (American Lecithin Co., Inc., Woodside, Long Island, N.Y.) is dissolved in 50 ml of ice-cold chloroform, and 120 ml of ice-cold methanol is added. The mixture is stirred very gently with a glass rod for not longer than 10 *seconds*; a white flocculent precipitate of crude phosphatidyl inositol forms and is collected immediately by centrifugation (2°C, 2000 rpm, 15 min, International Centrifuge, model P.R.2, International Equipment Co., Boston, Mass.). Longer or more vigorous stirring or temperatures above 5°C cause a brown oily precipitate to separate, in which case the preparation is discarded.

The supernatant solution is decanted, and the residue is dissolved in 25 ml of ice-cold chloroform and reprecipitated with 60 ml of cold methanol. The precipitate is collected by centrifugation as before and dissolved in 1 liter of cold chloroform in a 2 liter glass-stoppered cylinder. Cold methanol (500 ml) is added, and after mixing, the solution is shaken vigorously with 300 ml of cold water and allowed to stand at 5°C for 20 hr.

The clear lower (chloroform) phase is separated from both the clear upper phase and a 3–5 mm thick fluffy interphase layer, and evaporated to dryness in vacuo at 30°C. The residue is redissolved and taken to dryness four times with 100-ml portions of solvent, twice with chloroform–methanol 2:1, and twice with chloroform. The residue is finally dissolved in chloroform to give a concentration of 30–50 mg/ml and stored at 5°C. The clear amber solution contains about 850 mg of crude product of which the major contaminant is phosphatidyl ethanolamine. In practice, two 5 g batches of Asolectin are processed simultaneously.

Chromatography on Basic Silicic Acid. A column of adsorbent 3.3 × 30 cm is prepared at room temperature

Abbreviation: PI, phosphatidyl inositol. Fatty acids are designated by number of carbon atoms: number of double bonds.

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from a slurry of 100 g of Unisil silicic acid (200–325 mesh) in 250 ml of chloroform–methanol–concd NH_4OH 80:20:2. The column is washed with 500 ml of this solvent and then transferred to the cold room. A solution of 1 g of the water-washed lipid in 5 ml of this cold solvent is placed on the column and 3 liters of solvent are run through (overnight) to elute triglycerides, fatty acid, phosphatidyl ethanolamine, and phosphatidyl choline. The eluting solvent is then changed to absolute ethanol, and 20 fractions of 50 ml each are collected (3 ml/min).

Chromatographically homogeneous phosphatidyl inositol (thin-layer plates) is generally found in fractions 7–17; the preceding fractions contain sphingomyelin and phosphatidyl serine. The fractions containing phosphatidyl inositol are combined, the solvent is removed in vacuo, and the residue is dissolved and evaporated to dryness four times, twice with chloroform–methanol 2:1, and twice with chloroform. The product is stored in chloroform solution at 10–20 mg/ml at 5°C, under which conditions it remains stable for at least 6 months. From 5 g of Asolectin, 200 mg of pure phosphatidyl inositol are obtained as the ammonium salt. Analysis gave P, 3.68%. Molar ratios: ester/P, 2.00; inositol/P, 0.99; NH_4^+/P, 0.86. Fatty acid content (mole %): $C_{16:0}$, 54.6; $C_{18:0}$, 3.8; $C_{18:1}$, 2.9; $C_{18:2}$, 36.4; others, 2.3. No metals (Na, K, Mg, Ca) were found. Measurements of the specific rotation at 27°C of a solution in chloroform at a concentration of 0.83% were as follows: 589 mμ, + 6.0°; 550 mμ, + 6.4°; 500 mμ, + 7.4°; 450 mμ, + 9.0°; 400 mμ, + 11.7°; 350 mμ, + 16.0°; and 300 mμ, + 25.4°.

Purity. No reducing sugars were detectable (<0.5 μg) on paper chromatograms of 2 mg of NH_4-PI or its acid hydrolysate. Ninhydrin reaction on such chromatograms was also negative with 2 mg of NH_4-PI, but two spots were detectable in the hydrolysate corresponding in R_f to serine and a long-chain base (7) (phytosphingosine?), with intensities corresponding to 0.03% serine (0.3% phosphatidyl serine) and 0.2% sphingosine. These impurities were eliminated in the conversion to the Na salt.

Conversion to Na Salt. The Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.) ion exchange column described by Carter and Weber (13) produced some decomposition of the product. This did not happen with the procedure described by Pangborn and McKinney (14). To a solution containing 100 mg of NH_4-PI per ml of chloroform, 16 volumes of methanol–water 1:1 are added and mixed gently. 0.01 volume of saturated aqueous NaCl is added to the clear solution. The mixture is shaken for 20 sec, and then 0.2 volume of chloroform–methanol–water 86:14:1 is added. The mixture is shaken, the upper phase is separated and shaken again with this solvent, and the two lower phases are then combined and evaporated to dryness under a stream of N_2. Recovery of Na-PI is almost quantitative and no sign of decomposition is detectable by thin-layer chromatography.

The sodium salt is recrystallized from a 50 mg/ml solution in chloroform–methanol–water 86:14:1 by adding an equal volume of methanol, warming the solution to 40°C (higher temperatures cause breakdown), and allowing it to cool to room temperature before chilling overnight at 5°C. The clusters of rosettes of colorless, small needles are collected by centrifugation and washed with ice-cold methanol. After a second crystallization from chloroform–methanol 1:2, the material is stored under vacuum at room temperature or as crystals under methanol at 5°C. Analyses of two preparations gave: N, <0.05%; P, 3.69% (3.64%); molar ratios, ester/P 2.01 (2.00) and Na/P 0.98 (1.00).

Thin-Layer Chromatography. NH_4-PI and Na-PI behaved identically on plates of Silica Gel H (E. Merck) in two solvent systems: chloroform–methanol–water–concd NH_4OH 70:30:4:1 and chloroform–methanol–acetic acid–water 50:25:8:4. In the latter, the R_f was between those of phosphatidyl serine and phosphatidyl choline (15).

Discussion. The most important factors governing the quality of the final product are the removal of water-soluble impurities from the crude lipid mixture, the water content of the silicic acid adsorbent, and the ratio of lipid to silicic acid (loading factor). A number of variations of the methods described led to inferior products as judged by phosphorus and ester analysis, despite their homogeneity on thin-layer chromatograms.

Preparations which were either not washed or washed by the original Folch technique (16) had low P content (3.20 to 3.40%) and low values for the molar ratio of ester/P (1.50 to 1.70). Poor results were also obtained when the Unisil adsorbent was dried (17) before use (with removal of 7–10% moisture).

The stability of NH_4-PI is markedly affected by the nature of the solvent. A good preparation is stored best in chloroform (at 2°C), in which it is stable for 6 months. Preparations in which the molar ratio of ester/P was below 1.90 were less stable. The NH_4-PI cannot be stored well as the dry powder. In contrast, the Na salt is stable when stored dry (as crystals).

The method described is simple and rapid, as it requires chromatography on only one column. Removal of phosphatidyl ethanolamine is readily accomplished by use of the basic silicic acid column (4). The NH_4^+ can be readily replaced by other cations if necessary. This offers an advantage over PI preparations that contain appreciable amounts of divalent cations (Ca^{++}, Mg^{++}) where ion-exchange resins must be used; these resins may cause some decomposition.

Addendum. Trevelyan has described (18) a method for preparing Na-PI from yeast. This preparation contained an appreciable quantity of a sphingolipid impurity. Very recently Trevelyan reported that the impurity could be removed by means of chromatography on alumina (19).

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