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On the nature of dialyzable phosphate associated with the di- and tri-phosphoinositides of brain phospholipids

While preparing a concentrate of the phosphoinositides from calf brain, FOLCH[1] noted the presence of large amounts of P_I. He reported, however, that removal of the latter by dialysis interfered with the purification of the "diphosphoinositide", which was accomplished by multiple reprecipitation of the methanol-insoluble phospholipids (FF-I) from chloroform. More recently this product has been shown to contain TPI and DPI (see refs. 2, 3) as well as MPI (see ref. 4). The possibility that P_I might be attached to the PP-I was suggested by a number of observations made during the preparation of TPI in this laboratory.

Although P_I may be removed by prolonged dialysis against water, very little is released by a short period of shaking with water or dilute trichloroacetic acid. It is released within 3 min by 0.1 N H_2SO_4 at 100°, and within a few seconds by alkaline methanolysis (0.3 N methanolic KOH at 37°), and either of these procedures may be used for its quantitative determination.

The PP-I secured by counter-current distribution of FF-I between light petroleum and 96% methanol[4] was found to contain P in excess of that required for TPI. The amount of P_I released by acid hydrolysis or by alkaline methanolysis was nearly identical, and approximated 2 moles of P_I per 3 moles of inositol-bound P, or 40% of the total P. Similar results were obtained when FF-I was distributed between 96% methanol and a 1:1 mixture of CCl_4 and light petroleum[5]. The distribution of P_I and P_O was such that P_I represented 40% of the total P in the non-polar phase, whereas in the starting material P_I was 30–32% of the total P.

PP-I in the form of its magnesium or calcium salt shows strongly hydrophobic properties which are reversed once the metal ions and P_I are removed. Thus the ratio of P concentration in the two phases of COLE's solvent system[5] (non-polar–polar) was found to be 24 after FF-I had been equilibrated 15 times with the polar phase. In the system light petroleum–96% methanol[4] it was 31. This affinity for the non-

Abbreviations: P_O, organic phosphate; FF-I, FOLCH Fractions I and II (see ref. 1); TPI, DPI and MPI, tri-, di-, and monophosphoinositides; PP-I, mixed TPI and DPI.

polar phase is reversed when PP-I is converted to the free acid by treatment with cation-exchange resin.

These observations indicated the need to investigate the nature of the attachment of the dialyzable P to PP-I before undertaking the isolation of the latter in its natural state. An experiment was designed to test whether the "bound" P_i could exchange freely with the P_i pool of brain tissue. ^{32}P-labeled sodium phosphate was mixed with homogenized brain, producing a labeled P_i "pool". A comparison was then made of the specific activities of P_i isolated from a protein-free lipid-free filtrate and the P_i liberated from PP-I in the lipid fraction.

100 g of fresh sheep brain (chilled in ice at the slaughter house) was homogenized with 100 ml of ice-cold 0.14 M NaCl. Approx. 2 mC of ^{32}P in the form of sodium phosphate was added to the mixture, which was again homogenized for 1 min. After an interval of 5 min for equilibration of the added ^{32}P with the P_i pool of the tissue, the mixture was separated into 2 portions: Fraction A, representing approx. 10 g of brain for isolation of orthophosphoric acid, and Fraction B for isolation of PP-I. During this period the homogenized tissue was kept immersed in an ice-bath.

Fraction A was treated with 100 ml of 10% trichloroacetic acid. The filtrate was neutralized to pH 8.5 and P_i was precipitated as the calcium salt. The precipitate was dissolved in HCl and freed of calcium by means of Amberlite IR-120. A 1-ml aliquot of the resin filtrate containing 1.157 μmoles of P_i per ml was mixed with 10 μmoles of orthophosphate as carrier and treated with magnesia mixture. The precipitate was collected on a filter and dried. Its activity was determined in a gas-flow counter, and the P_i content of the precipitate was redetermined. The activity was found to be 63300 counts/min/μmole of P_i.

From the larger Fraction B the alcohol-insoluble glyceryl phospholipids were isolated by the method of DEBUCH[6]. This material was then precipitated from chloroform, once by ethanol at 60% and once by methanol at 65% concentration, thus yielding FF-1 (see ref. 1). From this the PP-I was isolated by distribution between 96% methanol and a 1:1 mixture of CCl_4 and light petroleum[5], the lower phase being washed 10 times with the upper phase. The PP-I, in which some 40% of the total P is the "bound" P_i, was subjected to alkaline methanolysis. P_i in the water-soluble hydrolysate was isolated as the magnesium salt and its activity, determined as described above, was found to be 61700 counts/min/μmole of P_i.

Thus the P_i liberated from PP-I by alkaline methanolysis had the same activity, within experimental error, as that of the acid-soluble P_i of the brain.

Although the experiment described above indicates that the P_i associated with PP-I exchanges freely with the acid-soluble P of brain tissue, the possibility remains that it may be loosely attached by coordinate bonds to PP-I through calcium or magnesium which form its salts.

The molar ratio of the "bound" P to the sum of calcium and magnesium was determined in a PP-I preparation in which the "bound" P represented 39.9% of the total P. A sample containing 144.6 μmoles of total P, and 57.6 μmoles of "bound" P (hence 87.0 μmoles of lipid P_o) was ashed in a platinum dish. Calcium was precipitated from the dissolved residue as oxalate at pH 6 and determined by titration with permanganate. Magnesium was precipitated as magnesium ammonium phosphate in the filtrate from calcium oxalate, and estimated through its content of P.

The results may be summarized as follows: calcium, 18.1 μequiv; magnesium,

44.6 μequiv; total calcium plus magnesium, 62.7 μequiv; "bound" P, 57.6 μmoles; P_o, 87.0 μmoles.

These results, showing molar ratios of 0.92 for "bound" P to the sum of calcium and magnesium, and 1.97:3 for "bound" P to P_o, suggest the existence of a complex in which the coordinate bonds of the alkaline earth metals bind P_i to the monoester phosphate groups of the inositide. DITTMER AND DAWSON[2] found it possible to remove P_i from PP-I by means of a cation-exchange resin and suggested that this was "due to a combination of the phosphate with bivalent cations such as Ca^{2+} or Mg^{2+} adsorbed on the sulfonate groups of the resin".

FOLCH's observation[1] that purification of PP-I by multiple reprecipitation with methanol from chloroform was ineffective when the P_i had first been removed by dialysis is explained by the change in polarity mentioned earlier. Whereas the alkaline earth salt of PP-I together with its "bound" P_i moves to the non-polar phase when distributed in a biphasic solvent system, the free acid and the sodium salt have the opposite character. This phenomenon may be attributed to the strongly chelating character of PP-I, through its OH groups, on the cations Ca^{2+} and Mg^{2+}. The formation of mono- and especially poly-nuclear coordination complexes would be favored, the non-polar character of the complex becoming more pronounced as more OH groups become bound in coordination. The removal of P_i (*e.g.*, by a cation-exchange resin) would liberate the simpler molecules of TPI and DPI with their free OH groups and polar character.

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