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Note

High-pressure chromatographic separation of glycerophosphorylcholine, phosphorylcholine, glycerophosphate and orthophosphate

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Certain data[1] have suggested that in septic bile ducts lecithins may be broken down to the final orthophosphate stage. In order to prove that this degradation process exists and to study it, it is necessary to isolate certain intermediate phosphorus products which may result from the hydrolysis of phosphatidylcholines, *viz.* glycerophosphorylcholine (GPC), phosphorylcholine (PC), glycerophosphate (GP) and orthophosphate (P_i).

However, methods previously described for the separation of these compounds are incomplete, for they make it possible to separate only two compounds from each other in a single chromatographic cycle, for instance, GPC from PC[2–4] or GP from P_i[4–7]. This has led us to develop a chromatographic technique by means of which the separation of all of the components in a mixture of GPC, PC, GP and P_i can be achieved.

MATERIAL AND METHODS

L-α-Glycerophosphorylcholine (cadmium chloride complex) was purchased from N.B.C., Cleveland, Ohio, U.S.A. PC (calcium salt) and α-GP (disodium salt) were obtained from Fluka, Buchs, Switzerland. P_i (A-grade) was supplied by Prolabo, Paris, France.

High-pressure columns, 15 cm long × 0.6 cm I.D. and 1.6 cm O.D. are filled with the anion-exchange resin Dowex 1-X4 (Cl^-), −400 mesh (Bio-Rad Labs, Richmond, Calif., U.S.A.). The chromatographic separation is effected under high pressure[8]; the eluent is drained from the flask using a Dosapro Milton Roy pump (Type R-90260) and carried to the column through high-pressure polyethylene tubing (I.D. 1/24 in., O.D. 1/8 in.). The connections are made with Swagelok tube fittings. The column has a plunger at the top which is tightened to the walls of the column by an O-ring joint. After packing the resin, the columns are washed with 100 ml of hydrochloric acid.

Phosphorus species eluted from the columns are measured by the procedure described below. The collected fractions (5 ml) are evaporated to dryness at 250°; after digestion with 9.45 *N* perchloric acid, the phosphorus content of the fractions is determined by an automatic phosphorus analysis method which has been described previously[9].

RESULTS AND DISCUSSION

The chromatographic separation of GPC, PC, GP and P_i is carried out by a two-step elution. In the first step, a linear ionic strength gradient in ammonium formate is produced by connecting two similar flasks filled with 100 ml of solution. The eluent is pumped from the flask containing a $5 \cdot 10^{-3}$ *M* ammonium formate–$5 \cdot 10^{-3}$ *M* sodium tetraborate solution at pH 8.5 into which flows, with constant mixing, a $4 \cdot 10^{-1}$ *M* ammonium formate–$5 \cdot 10^{-3}$ *M* sodium tetraborate solution at pH 8.5 to maintain equal solution volumes in the two flasks. When 35 5-ml fractions have been collected (175 ml), the second step consists in pumping in 100 ml of 1 *N* hydrochloric acid. This second step, which yields 15 5-ml fractions, results in the separation of the phosphorus species and regenerates the resin.

After regeneration of the resin and before each run, the columns are equilibrated with the solution of lowest ionic strength until the pH of the effluent has reached a value of 8.5 (*ca.* 100 ml of $5 \cdot 10^{-3}$ *M* ammonium formate–sodium tetraborate solution).

Elution is performed at a constant flow-rate of 1 ml/min, while the column pressure rises with the eluent ionic strength from 100 to 215 p.s.i. and rapidly falls to 70 p.s.i. with 1 *N* hydrochloric acid.

A first set of experiments consists in individually subjecting each of the four compounds to be separated to chromatography, so that the specific elution volume of each compound can be accurately determined. With the exception[9] of P_i, we do not have any means of characteristic identification for the phosphorus products studied. A 0.4–1 μmole amount of each product is introduced into the column in 0.5 ml of the original buffer. Each compound is eluted in a maximum of four fractions in a single symmetrical peak. The mean elution volumes obtained during various experiments are 17.5 ± 2.5 ml for GPC, 50 ± 5 ml for PC, 130 ± 5 ml for GP and 202.5 ± 2.5 ml for P_i. It can be seen that the elution volumes of the various products do not have a scatter of greater than two collected fractions (10 ml). The mean chromatographic yield is 95%.

When GPC and PC are subjected to chromatography, a second phosphorus peak emerges at the elution volume of P_i, representing 5% of the total phosphorus in the effluent. This second peak must correspond to contamination of the product by P_i.

Once the characteristic elution pattern of each compound has been determined, mixtures of two and three compounds are subjected to chromatography. The elution volumes of the various products are unchanged.

Finally, a mixture of the four compounds to be separated is subjected to chromatography. Fig. 1 shows the separation obtained from a mixture containing 0.48 μmole of GPC, 0.58 μmole of PC, 0.48 μmole of GP and 0.64 μmole of P_i. The four compounds are eluted in four completely separate peaks.

Essentially, the chromatographic separation is based on the difference in ionization of the phosphoryl radical in the four compounds studied. The dissociation constants of phosphoric acid are $pK_1 = 2.12$, $pK_2 = 7.21$ and $pK_3 = 12.67$ (ref. 10). At pH 8.5, between the second and third p*K* values, the difference in the degree of substitution of the phosphoryl radical in the different phosphorus products make it possible to effect their separation. Thus GPC is not held on the resin, PC and GP are

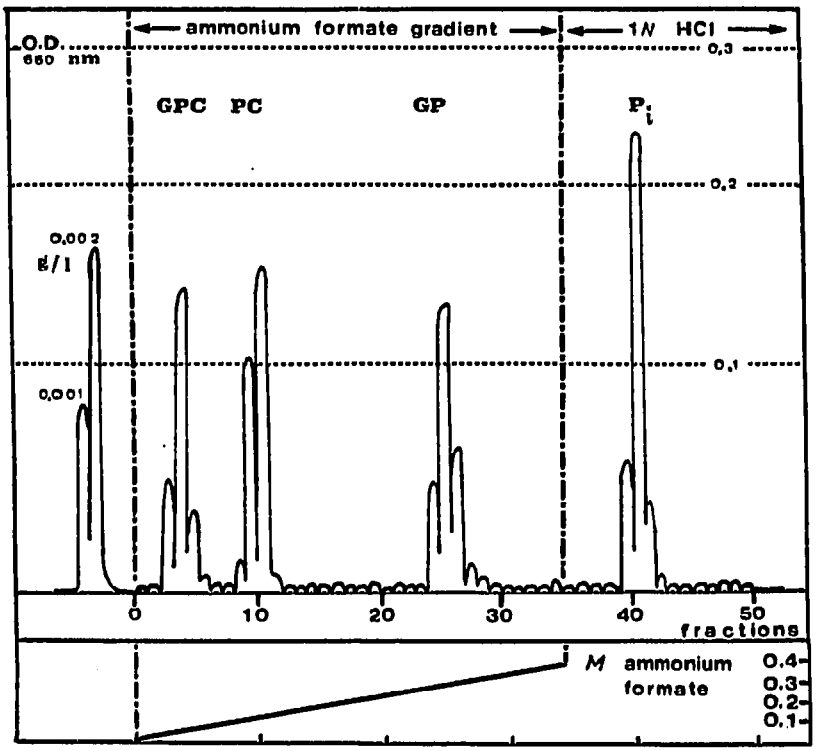

Fig. 1. Chromatographic separation of glycerophosphorylcholine (GPC), phosphorylcholine (PC) glycerophosphate (GP) and orthophosphate (P_i). Automatic phosphorus analysis of collected fractions, after digestion with perchloric acid. On the left side of the figure, the optical densities (O.D. 660 nm) for 0.001 and 0.002 g/l phosphorus concentrations are given.

separated by the ionic strength gradient and P_i, which is tightly held, is eluted only with a high concentration of hydrochloric acid.

The good separation achieved seems to be due largely to the presence of tetraborate in the eluent. In agreement with the investigations of Khym and Cohn[5] and Hübscher and Hawthorne[6], tetraborate provides a better separation than do ordinary buffers, owing to the complexes that it forms with the compounds to be separated. Moreover, the use of high pressure saves a great deal of time.

A study of the degradation of bile lecithins by this method will be published separately[11].

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