ISOLATION OF N-ACYL PHOSPHATIDYLETHANOLAMINE FROM PEA SEEDS

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SUMMARY

The isolation and characterization of a new phospholipid from dry pea seeds is described. The phospholipid is unusual in that it contains three molecules of fatty acid per molecule of phosphorus, and one of the fatty acids is stable to mild alkaline methanolysis. Characterization of the chemical degradation products has shown that the phospholipid is N-acyl phosphatidylethanolamine.

During a recent investigation on the function of phosphatidylcholine phosphatidohydrolase (EC 3.1.4.4., Phospholipase D) in garden pea plants it was noticed that in the early stages of germination of the pea seed only one phospholipid, which constituted about 5% of the total lipid phosphorus, showed a marked decrease in concentration. From its behavior on thin-layer chromatography and chemical degradation this phospholipid could not be equated with any previously described (Quarles and Dawson, 1968). This communication describes its isolation and characterization as N-acyl phosphatidylethanolamine (APE).

EXPERIMENTAL

The lipids in 60 g of dry pea seeds were extracted with chloroform/methanol (2:1 v/v) and washed with 0.9% NaCl (Folch, Lees and Sloane-Stanley, 1957). The lipids in the lower phase were applied to a silicic

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acid column (25 cm long x 2.0 cm diameter) which had been poured in chloroform. The column was eluted with 500 ml of chloroform followed by 250 ml of chloroform/methanol 19:1 v/v. The APE was eluted in the latter fraction along with diphosphatidylglycerol, phosphatidic acid and neutral lipids. This mixture was fractionated by preparative thin-layer chromatography using a layer of silica gel H, 0.25 mm thick, a solvent of chloroform/methanol/6.5 N NH_3 solution (65:30:4.7 v/v) and a loading of 12 µg P/cm. The APE band located by spraying the edges of the plate with phosphorus reagent (Dittmer and Lester, 1964) ran faster (R_F, 0.70) than either diphosphatidylglycerol (R_F, 0.44) or phosphatidic acid which remained near the origin. The band was scraped from the plate and the APE recovered from the silicic acid by two elutions with ethanol/chloroform/H_2O (10:3:2 v/v) yielding about 1 mg of lipid phosphorus. The product was chromatographically homogeneous on thin-layer chromatography in several solvent systems.

IDENTIFICATION OF PHOSPHOLIPID

On acid hydrolysis (2 N HCl, 3 hr., 100°) APE liberated 2.86 mole. of titratable fatty acid per mole. of phosphate present. By gas-liquid chromatography using an internal standard of arachidic acid the ratio found was 2.9 mole. fatty acid per mole. of phosphate. Acetolysis of APE with acetic acid - acetic anhydride and examination of the lipoidal products formed by thin-layer chromatography showed the presence of diglyceride acetate (Renkonen, 1965).

Mild alkaline methanolysis (Dawson, Hemington and Davenport, 1962) for one hour at 37° yielded quantitatively methyl fatty acid esters and a deacylated phosphorus-containing product (*APE) which was still lipoidal in nature and distributed in the lower phase of chloroform/water mixtures. *APE was also prepared in quantity by the direct alkaline methanolysis of pea lipid extracts, and distribution of the methanolysis products between mixtures of equal volumes of petroleum ether and 50% aqueous ethanol. The lower phase (aqueous-ethanol) was

extracted with chloroform and the chloroform extracts fractionated on a silicic acid column. Successive stepwise elution of the lipids with increasing concentrations of methanol in chloroform eluted the ∝APE when the methanol content in the solvent reached 40% by volume.

Acid hydrolysis of ∝APE (2N HCl, 3 hr., 100°) liberated 0.96 mole. of titratable fatty acid per mole. phosphate, suggesting that the mild alkaline methanolysis had freed two molecules of fatty acid esterified to glycerol leaving a third in a combined form which was stable to the methanolysis. An infra red spectrum of ∝APE indicated strong amide adsorption bands at 1645 cm^{-1} and 1540 cm^{-1} which were also observed in a similar spectrum of sphingomyelin. This suggested the likelihood that the third fatty acid was in a fatty amide linkage.

On acid hydrolysis of ∝APE for short periods (2N HCl, 12 min.,100°) the molecule was broken down quantitatively into non-phosphorus containing lipoidal products and glycerophosphoric acid, the latter being identified by paper ionophoresis and chromatography. The lipoidal products were shown on thin-layer chromatography (silica gel H, chloroform/methanol/H_2O/NH_3) and I_2 vapor localization to consist predominantly of two products, a major one at R_F, 0.64 and a minor one which was ninhydrin-reacting and ran slightly faster at R_F, 0.67. They were more readily separated using a chloroform/methanol/H_2O/acetic acid solvent (130:50:4:1 by volume) but on isolation seemed to be readily interconvertible. On longer periods of acid hydrolysis (2N HCl, 1 hr., 100°) both liberated fatty acid and a water-soluble base which was identified by paper ionophoresis and chromatography as ethanolamine. Semiquantitative estimation of ethanolamine after running on an ion-exchange resin column in an amino acid analyzer indicated a ratio of 1.11 mole. ethanolamine to one of phosphate.

On alkaline hydrolysis of ∝APE (1N NaOH, 30 min., 100°) the molecule was broken down into glycerophosphate and the same major lipoidal product as was observed in the short term acid hydrolysis

(15 min.) of ^cAPE. This lipid was isolated by preparative thin-layer chromatography and identified as N-acyl ethanolamine by (1) liberation of ethanolamine and fatty acid on acid hydrolysis (2) co-chromatography with synthetic N-oleoyl ethanolamine (prepared by the method of Roe, Scanlan and Swern, 1948) on thin-layer chromatography (3) possession of an infra red spectrum identical to that of synthetic N-oleoyl ethanolamine.

It seemed possible that under the conditions of the acid hydrolysis of ^cAPE, some of the liberated N-acyl ethanolamine would undergo acyl migration forming O-acyl ethanolamine. This would explain why a ninhydrin-reacting lipid was formed as well as N-acyl ethanolamine in the acid hydrolysis. This acyl migration was in fact demonstrated in further experiments when synthetic N-oleoyl ethanolamine was treated for short periods with 2N HCl at 100°. This resulted in the formation of a ninhydrin-reacting lipid which ran to the same position on thin-layer chromatography as the ninhydrin-reacting lipid released by the short term acid hydrolysis of ^cAPE.

CONCLUSIONS

The above observations leave little doubt that the new phospholipid isolated from pea lipid extracts is N-acyl phosphatidylethanolamine (APE). This is the first demonstration of the occurrence of N-acyl phosphoglycerides in nature and it is conceivable that it could fulfill a function carried out by sphingomyelin in animal tissues. The reason for the rapid disappearance of the phospholipid from the pea seed during early germination and the enzyme involved are unknown.

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