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Note

Isolation of hens' egg phosphonolipids by thin-layer chromatography, their identification and silicic acid column chromatographic separation

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Many attempts have been made at the isolation and identification of hens' egg phospholipids[1], and a variety of extraction and chromatographic procedures have been employed for this purpose[1,2].

Earlier workers reported the occurrence of several components in egg yolk, such as β-carotene, cryptoxanthin, lutein, tocopherols, vitamins A, D and K, cholesterol, ergosterol, lecithin, cephalin and sphingomyelin[1–3]. To our knowledge, there is, however, no mention of the presence of phosphonolipids.

In this paper, we report the isolation of the total egg-yolk phospholipids by the procedure of Bligh-Dyer and the subsequent thin-layer chromatographic (TLC) separation of the phosphonolipids from the total phospholipids[4,5]. The presence of phosphonolipids has been confirmed by phosphono-phosphorus determinations on the various phospholipid and phosphonolipid fractions and extracts obtained[5,6] and by IR and NMR spectroscopy.

EXPERIMENTAL

Materials

All reagents used were pro-analysi grade (Merck) and were distilled before use. Silica gel G was also a Merck product. Fresh fertile eggs were purchased from Viofas S.A. (Schimatari, Viotias, Greece). Silicic acid for column chromatography was purchased from Sigma (St. Louis, MO, U.S.A.).

IR[7] spectra were recorded on a I97 Perkin-Elmer spectrometer, NMR spectra on a Varian 100-MHz Fourier-transform spectrometer.

Methods

The egg yolks from twenty hens' eggs, separated from the whites by a suitable procedure, were extracted according to the method of Wells and Hanahan[8,9]. After removal of the solvents *in vacuo* the phospholipid fraction was stored in a freezer at −30°C.

The phosphonolipids were separated from the other phospholipids by TLC with the solvent methanol–water (2:1, v/v) (system A)[4], using silica gel G plates of thickness 0.75 mm. The area on the plate with R_F 0.80–0.95 was eluted with chloroform–methanol (4:1, v/v). This fraction was subjected to phosphono-phospho-

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rus determination and the IR and NMR spectra were recorded. The solvent chloroform–methanol–water (65:25:4, v/v/v) (system B) was also used for identification of the respective phosphonolipids. Visualization of spots was effected by spraying with iodine, ammonium molybdate, α-naphthol–sulphuric acid or ninhydrin and by the Stillway–Harmon procedure[10].

IR spectra were recorded for thin films from dry chloroform. A glass column (35 × 1.6 cm I.D.) was used for the chromatographic separation of the isolated total phosphonolipids.

Phosphono-phosphorus was determined essentially by the following procedure[5,6]. The lipid fraction was subjected to rigorous acid hydrolysis with hydrochloric acid in tightly sealed vials (6 *M* HCl, 110°C, 48 h), which yielded the phospholipid phosphorus. This fraction was then treated with 72% perchloric acid, yielding the phosphono-phosphorus in the sample.

Procedure

The lipids from the homogenized egg yolks were extracted[8,9] and the solvents were evaporated under vacuum at a bath temperature of 35°C. The total lipids were then dissolved in chloroform and washed repeatedly with saline solution. The chloroform was evaporated and the residue extracted repeatedly with acetone to constant phospholipid weight. The total phospholipids were dried in a vacuum desiccator over phosphorus pentoxide for 24 h.

The total phospholipids were then dissolved in 250 ml of chloroform and part of this solution was subjected to preparative TLC in solvent system A. The band with R_F values 0.8–0.95 was scraped off and the phosphonolipids removed from the silica gel with chloroform. The phospholipids were similarly obtained from the origin on the TLC plates. The phosphonolipids were then subjected to TLC analysis to identify the lipids present, on glass plates coated with silica gel (0.25 mm thick) in solvent system B.

After initial identification, the phosphonolipids were fractionated on a silicic acid column as described previously[11,12]. Five grams of silicic acid were used and the column was leaded to a height of 5.5 cm and a total volume of 14 ml. The flow-rate maintained in the elution was 1.6–1.8 ml/min.

RESULTS

The IR spectrum of the total egg phosphonolipids is shown in Fig. 1. Chromatography of the isolated phosphonolipids in TLC system B provided evidence for the presence of two phosphonolipids, with R_F values of 0.34 and 0.65, corresponding to the phosphono analogues of phosphatidylcholine and ethanolamine respectively. The total phosphonolipids were then fractionated on a silicic acid column and their fractionation pattern is shown in Fig. 2. It was found that the phosphono analogue of phosphatidylethanolamine comprised 98.1% of the total phosphonolipids isolated.

The phosphonolipids thus fractionated were analysed for total nitrogen, phosphorus, phosphono-phosphorus and lipid nitrogen (Table I). The IR spectrum of the fractionated phosphono analogue of phosphatidylethanolamine is shown in Fig. 3. Tables II and III provide information relating to the silicic acid column chromatography of the isolated phosphonolipids. No sugars and/or amino acids were detected on the chromatoplates during the TLC analyses.

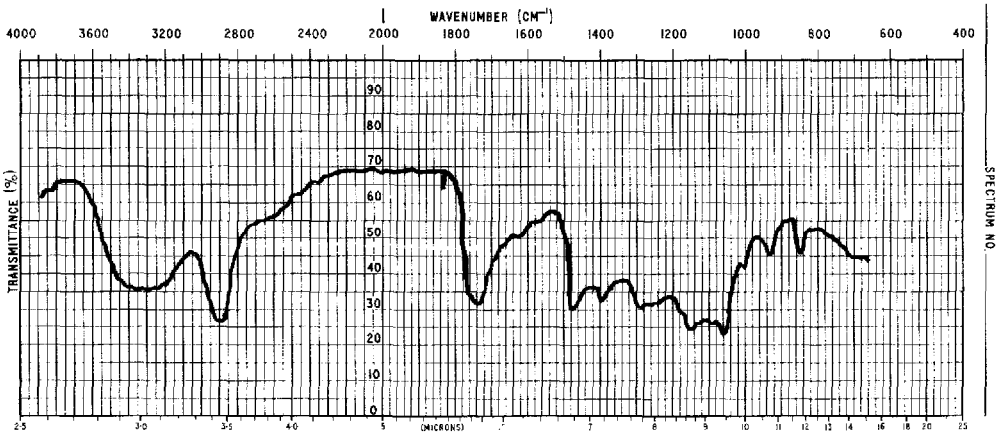

Fig. 1. IR spectrum of total egg yolk phosphonolipids, as a thin film from dry chloroform.

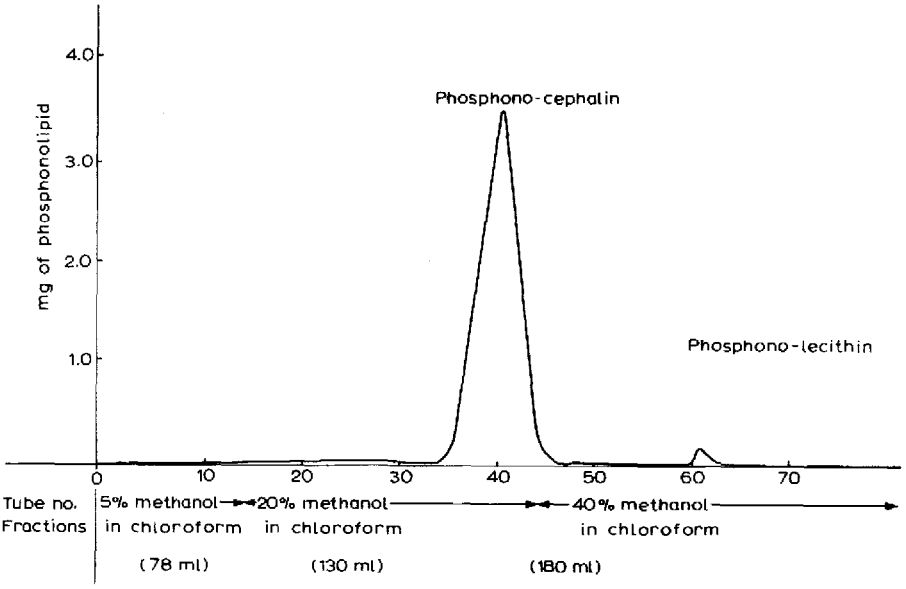

Fig. 2. Silicic acid column chromatographic elution pattern of the natural phosphonolipids isolated from hens' egg yolks. The solvents employed were various percentages of methanol in chloroform. The eluted fractions contained 8.829 mg phosphono analogue of phosphatidylethanolamine and 0.171 mg of the phosphono analogue of phosphatidylcholine. The phosphonolipids were applied to the column in 2.0 ml of chloroform.

TABLE I

ANALYTICAL DATA FOR THE ISOLATED PHOSPHONOLIPIDS AFTER COLUMN CHROMATOGRAPHIC SEPARATION

Phosphono-lipid	R_F *in system B*	*% Nitrogen found*	*% Phosphorus found*	*% Abundance in total phosphonolipid*
Analogue of cephalin	0.65	2.53	4.70	98.10
Analogue of lecithin	0.34	2.10	4.35	1.90

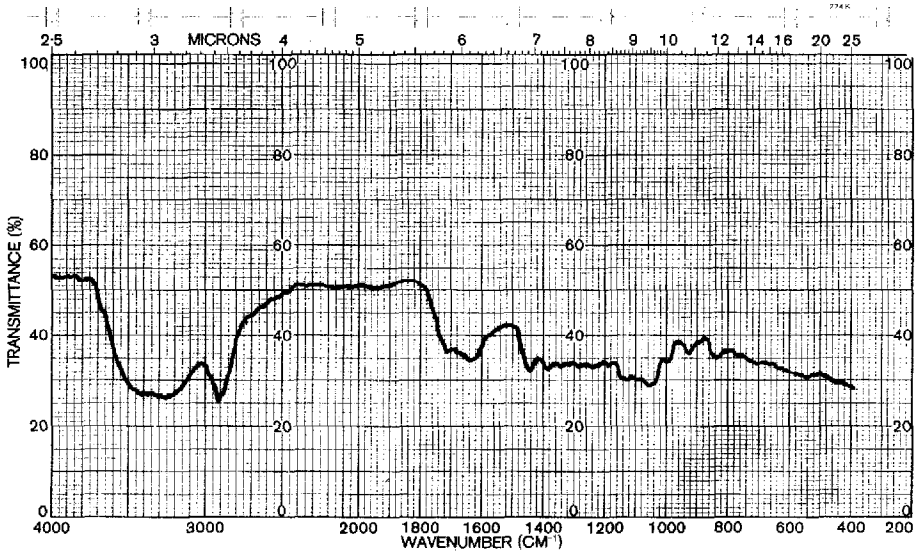

Fig. 3. IR spectrum of the phospho analogue of phosphatidylethanolamine fractionated on a silicic acid column from the total egg yolk phosphonolipids.

TABLE II

SILICIC ACID COLUMN CHROMATOGRAPHY OF TOTAL PHOSPHONOLIPIDS

Column: 35 × 1.6 cm I.D., loaded with 5.0 g of silicic acid to a height of 5.50 cm; volume 14.0 ml. Flow-rate: 1.6–1.8 ml/min. Fractions of *ca.* 5.0 ml were collected.

% Methanol in chloroform	*Column volumes*	*Total volume of solvent (ml)*	*Fractions collected*
5	3	78	1–16
20	5	130	17–43
40	7	180	44–81

TABLE III

TLC OF FRACTIONS OBTAINED FROM SILICIC ACID COLUMN CHROMATOGRAPHY OF NATURAL PHOSPHONOLIPIDS ISOLATED FROM EGG YOLKS

Recovery was quantitative.

Solvent	*Fractions collected*	*TLC*		*Component identified from IR spectral data*
		System A	*System B*	
5% Methanol in chloroform	–	–	–	–
20% Methanol in chloroform	36–47	0.86	0.65	Phosphono analogue of phosphatidylethanolamine
40% Methanol in chloroform	59–63	0.83	0.34	Phosphono analogue of phosphatidylcholine

DISCUSSION

The apparent lack of any evidence regarding the occurrence of phosphonolipids in animal and plant species is a result of the absence of efficient techniques for the separation of phosphonolipids from their phospholipid analogues.. Additional evidence in support of the efficiency of the chromatographic system solvent methanol–water (2:1, v/v), reported earlier is provided by its successful application in this work.

The phosphonolipid fraction when analysed for phosphono-phosphorus gave positive results, whilst use of the same procedure for the phospholipid fraction at the origin indicated the absence of phosphono-phosphorus. It was thus shown that phosphonolipids comprised 1.0% of the total phospholipid fraction.

The fractionation pattern of the two natural phosphonolipids closely resembles that of their synthetic counterparts[11,12]. Thus, the experimental data indicates the presence of phosphonolipids in hens eggs. The phosphonolipids present in the egg yolk were then separated by preparative TLC and were identified by IR and NMR spectroscopy. TLC analysis of the isolated phosphonolipids as well as spectral evidence indicate that the phosphonolipids present are largely analogues of phosphatidylethanolamine (98.1%) and to a lesser extent of phosphatidylcholine.

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