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# PRODUCTION OF LIPID PREPARATIONS.

## II. ISOLATION OF NATURAL PHOSPHATIDYLETHANOLAMINES

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A description is given of the isolation of natural phosphatidylethanolamines from three sources — porcine kidneys, the marine ascidian *Halocynthia aurantium* and soybean phosphatides. The methods of isolating the phosphatidylethanolamines include the preparative fractionation of lipid extracts on alumina with the production of a fraction enriched with phosphatidylethanolamines and its subsequent further purification of a column with the mixed sorbent DEAE-cellulose—silica gel. The compositions of the fatty acids of the phosphatidylethanolamines from the sources used have been determined.

Phosphatidylethanolamines (PE), just like phosphatidylcholines are widely used in the investigation of biological membranes and lipid metabolism. PEs (together with phosphatidylserine) are potential anticoagulants in the blood-clotting system and, moreover, a number of membrane-bound enzymes exhibit an absolute specificity in relation to them [1].

The isolation of phosphatidylethanolamines in the chromatographically pure form is difficult, as a rule, because of their low stability resulting from their high degree of unsaturation [1-7]. Moreover, when they are isolated on silica gel columns, PEs proved to be contaminated with acidic phospholipids (phosphatidylserine, phosphatidylinositol), most frequently, phosphatidylserine [8-10]. This pattern is observed in the isolation of PEs from tissues in which the amount of acidic phospholipids is high. Consequently, egg yolk is more frequently used for the isolation of PEs [1, 11, 12] and, sometimes, bacteria [1] which contain none of these phospholipids or only trace amounts of them (bacteria).

However, our use as a sorbent for column chromatography of DEAE-cellulose mixed with silica gel and, as a preparative stage, alumina for obtaining fractions enriched with PEs has enabled us to obtain them also from sources in which the amount of acidic phospholipids is high — porcine kidneys (total amount of acidic phospholipids 17.5%; amount of PEs 22.4%), commercial soybean phosphatides (total amount of acidic phospholipids 20.8%; PEs together with N-acyl-PEs 29.5%); and the marine ascidian *Halocynthia aurantium* (sum of the acidic phospholipids 11.9%; PEs 18.6%).*

The use of alumina as a preparative step permitted the PE fraction to be obtained in approximately 1 h (since the elution of the lipids was carried out with the aid of vacuum, as described in the Experimental part) and with very small amounts of acidic phospholipids as impurities. The use of a silica gel column for the following purification of the PE fraction did not permit chromatographically pure PEs to be obtained because of the presence of impurities. However, a column with a mixed sorbent prepared as described in the Experimental part enabled these defects to be eliminated and a product of higher purity to be ob-

*The amounts of the individual classes of phospholipids are given in Table 1 of the preceding paper.

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TABLE 1. Composition of the Fatty Acids of the PEs

| Fatty acid | Percentage on the total fatty acids | | |
|---|---|---|---|
| | porcine kidneys | H. aurantium | soybean phosphatides* |
| 14:0 | 3.0 | 0,7 | |
| 14:1 | 2.5 | | |
| 15:0 | 2,1 | 0,1 | |
| 16:0 | 10,2 | 4,6 | 54,3 |
| 16:1 | 0.5 | 1,0 | |
| 18:0 | 20,0 | 10 0 | 0 8 |
| 18:1 | 18,2 | 10,0 | 2,2 |
| 18:2 | 11,7 | 1,5 | 38,7 |
| 18:3ω6 | 0,4 | 0,2 | |
| 18:3ω3 | | 0,2 | 4,0 |
| 18:4ω3 | | 0,4 | |
| 20:1 | | 1,5 | |
| 20:2 | | 0.2 | |
| 20:4ω6 | 26,4 | 8,0 | |
| 20:5ω3 | 5,0 | 51,0 | |
| 22:3 | | 0,3 | |
| 22:4ω6 | | 0,4 | |
| 22:5ω6 | | 0.4 | |
| 22:2ω3 | | 0,5 | |
| 22:6ω3 | | 9,0 | |
| $\Sigma_{saturated}$ | 35,3 | 15,4 | 55,1 |
| $\Sigma_{monoenoic}$ | 21,2 | 12,5 | 2,2 |
| $\Sigma_{polyenoic}$ | 43,5 | 72,0 | 42,7 |
| Molecular weight | 747 | 770 | 714 |

*PE together with N-acyl-PE.

tained, while, in addition, the elution of the PEs took place faster and in less polar mixtures of solvents than on similar columns with DEAE-cellulose along [13].

Table 1 gives the compositions of the fatty acids of the PEs from the three sources used in this work.

The phosphorus contents of the PE preparations were 3.9% (kidneys), 4.2% (soybean phosphatides), and 3.8% (*H. aurantium*), which are close to the theoretical figures (4.1, 4.3, and 4.0%, respectively). The phosphorus:ester ratio was close to the theoretical value of 1:2.

The PE preparations were readily soluble in benzene, chloroform, and mixtures of chloroform and methanol. The PEs were stored at −20°C in chloroform with the addition of 0.05% of an antioxidant (on the weight of the lipid). Micro-TLC showed no appreciable changes in the course of two weeks.

## EXPERIMENTAL

Porcine kidneys were comminuted and extracted three times with acetone in a homogenizer, using a volume ratio of tissue to solvent of 1:5. The acetone extract was centrifuged, the acetone was discarded, and the residue of tissue was extracted by Folch's method [14]. The lipid extract from the marine ascidian, which had been trapped in Vityaz' Bay, Sea of Japan, was prepared as described in [15], after the removal of the tunic. The lipids of the soybean phosphatides were obtained as described previously [15]. Micro-TLC and the detection of the lipids were performed as described in the same paper [15]. The phospholipids were analyzed quantitatively and the phosphorus in the PE preparations was determined by the method of Vaskovsky et al. [16]. The fatty acid methyl esters were analyzed by a known method [17]. The determination of nitrogen, glycerol, and ester groups in the PE preparation was carried out as in Kates' handbook [18].

Preparation of the Columns. DEAE-Cellulose (bead polymer, anion-exchange resin, Hungary) was first covered with 1 N HCl and left to swell for 5 min and was then prepared by the method of Rouser et al. [13]. The DEAE-cellulose, converted into the acetic form and washed with methanol to a neutral pH, was mixed with an equal amount of silica gel 40/100 μ (Czechoslovakia) and with two volumes of methanol, and the suspension was carefully stirred

and was immediately transferred to a chromatographic column of suitable dimensions — the ratio of the diameter of the column to the height of the adsorption bed was 1:5-1:10. The excess of solvent was rapidly eliminated (in order to avoid the separation of the sorbent into layers), the sorbent being compacted with a glass plunger. Silica gel with a larger grain size (100/250 μ, Czechoslovakia) in methanol was deposited on the surface of the sorbent to form a 2-4 cm layer and was allowed to settle, and a paper filter with a diameter of the column was placed on its surface. The prepared column was left for about a day for the compaction of the bed of adsorbent, and then the ethanol was eliminated by washing the column with three column volumes of chloroform. A charge of 0.5-0.8 mg of lipid phosphorus per 1 g of mixed sorbent was used.

Isolation of PEs from Porcine Kidneys. The total kidney lipids (7.5 g, obtained from 250 g of tissue) were first fractionated on alumina in order to obtain a fraction enriched with PEs (PE fraction).

Isolation of the PE Fraction. A 100-g charge of alumina (Brockmann activity grade II, neutral, Hungary) was poured into a Büchner funnel of suitable diameter. A filter with the diameter of the funnel was placed on the surface of the sorbent. The sorbent was wetted with chloroform. A solution of the lipids in 100 ml of chloroform was poured onto the filter, and after it had soaked in, the sorbent was washed with 3 liters of chloroform—methanol (1:1 by volume), the eluate being sucked by means of a vacuum pump into a Bunsen flask. The eluate, which contained phosphatidylcholine, lysophosphatidylcholine, spingomyelin, and neutral lipids, was discarded.

The residue in the funnel was washed with 4 liters of chloroform—methanol—water (10:10:3). The eluate was the PE fraction (containing, in addition to PEs, as impurities acidic phospholipids, neutral lipids, and lyso-PEs). Distilled water (20% of the volume of the fraction) was added to the eluate and the mixture was stirred and was left in the cold for complete separation into two phases. The upper aqueous methanolic layer was discarded. The lower chloroform phase was evaporated to dryness at 40-45°C in a rotary evaporator. The yield of the PE fraction was 1.4 g.

Final Purification of the PE Fraction on a Column with the Mixed Sorbent DEAE-Cellulose—Silica Gel. A 2.9 × 29 cm column was charged with 120 g of the mixed sorbent as described above. A solution of 1.4 g of the PE fraction in 30 ml of chloroform was transferred to the column and, after the lipids had soaked in, elution was performed successivly with 0.8 liter of chloroform and with mixtures of chloroform and methanol in ratios of 95:5 (1 liter) and 90:10 (1.5 liters). These eluates were discarded. Then elution was carried out with chloroform—methanol (80:20) (2 liters). The eluate was collected in 50-ml fractions with the course of separation being monitored by micro-TLC. The fractions containing the PEs were combined and evaporated. The yield of PEs was 0.6 g, their purity being 99%. The recovery of the PEs after all the procedures was 34.3% on its initial amount in the extract (Tables 1 and 2, pp. 178, 180).

Isolation of PEs from Commercial Soybean Phosphatides. Isolation of the PE Fraction. A solution of the lipids is 10.5 g,(obtained from 22 g of phosphatides) in 200 ml of chloroform was poured into a Bückner funnel containing 170 g of alumina (Brockmann activity grade II, neutral, Hungary). The lipids were washed with 1 liter of chloroform—methanol (1:1), and the eluate was discarded. The residue in the funnel — a PE-containing fraction — was eluted with 2.6 liters of chloroform—methanol—water (10:10:3) and was freed from water and evaporated. The yield of PE fraction was 2.2 g.

Final Purification of the PE Fraction on a Column with the Mixed Sorbent DEAE-Cellulose—Silica Gel. A 2.6 × 32 cm column containing 100 g of the mixed sorbent was prepared by the method described above. A solution of 2.2 g of the PE fraction in 30 ml of chloroform was added to the column. Then it was washed successively with 0.45 liters of chloroform and 1.4 liters of chloroform—methanol (90:10). The eluates were discarded. Subsequent elution was performed with chloroform—methanol (80:20) (2.5 liters), 50-ml fractions being collected. The fractions were analyzed by micro-TLC, and those containing PEs were combined and evaporated. The yield of PEs was 0.8 g (under the given conditions of chromatography, the PEs are eluted together with N-acyl-PEs; the percentage of N-acyl-PEs in the PE preparation was not determined). The recovery of the PEs was 44.4% on the initial amount in the extract ([15], Table 2, p. 180).

Isolation of PE from the Ascydian *H. aurantium*. A 3.5 × 20-cm column containing 80 g of the mixed sorbent was prepared in the manner described above. A solution of the total lipids of the ascidian (3 g, obtained from 180 g of tissues) in 15 ml of chloroform was transferred to the column and, after the lipids had soaked in, elution was carried out with 0.3 liters of chloroform, 0.1 liters of chloroform–acetone (1:1) and 0.6 liters of chloroform–methanol (90:10). The last eluate was collected in 50-ml fractions and these were analyzed by micro-TLC. The fractions containing PEs were combined and evaporated. The yield of chromatographically pure PEs was 0.27 g. The recovery of the PEs from the column was 53.4% of their initial amount in the lipid extract of the ascidian (Tables 1 and 2, pp. 178, 180).

## SUMMARY

1. Methods have been developed for isolating natural phosphatidylethanolamines from three sources — porcine kidneys, a marine ascidian *H. aurantium*, and soybean phosphatides.

2. The compositions of the fatty acids of the phosphatidylethanolamines from these sources have been determined.

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