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## Note

# Separation of phospholipids and phosphonolipids of *Tetrahymena* by high-performance liquid chromatography

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Because of the heterogeneous and varied nature of the polar head groups of phospholipids, most studies on the separation of classes of phospholipids by high-performance liquid chromatography (HPLC) have tended to restrict themselves to those phospholipids whose distribution is most widespread, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, sphingomyelin, etc. [1]. There are many other phospholipids which are of biological interest even though their distribution is more limited in nature. Such lipids are the phosphonolipids** — phospholipids containing the direct C–P bond — which are found primarily in marine invertebrates and protozoa but which have also been detected in vertebrates, including man [2].

In studying the phospholipid metabolism of the ciliate protozoan *Tetrahymena thermophila* [3–5], it has become desirable to develop an efficient HPLC method for the separation and recovery of the phospholipids and phosphonolipids. Several recent publications have reported other approaches to separating phospholipids and phosphonolipids [6,7]. However, Moschidis and Andrikopoulos [7] only used synthetic lipids, which had identical fatty acid components, and they did not deal with the problem of lipid separation in the presence of fatty acid heterogeneity. We report a procedure which is complementary to those methods and which is more effective for the particular phospholipid complement of *Tetrahymena* [3–5] and related organisms, such as *Paramecium* [8].

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**The phosphonolipids of *Tetrahymena* consist of 2-aminoethylphosphonate esterified either to ceramide (AEP-ceramide) or glyceride (AEP-glyceride). The glyceride contains both diacylglycerol and 1-O-alkyl-2-O-acylglycerol.

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## EXPERIMENTAL

### *Materials*

*Tetrahymena* phospholipids were isolated from cell cultures as previously described [4]. Standard phospholipids (egg phosphatidylcholine, sphingomyelin and phosphatidylethanolamine; soybean lysophosphatidylcholine and bovine heart cardiolipin) were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade solvents were obtained from Fisher Scientific (Medford, MA, U.S.A.).

### *Methods*

HPLC was performed on a Rainin Instruments (Woburn, MA, U.S.A.) gradient system with 5-ml HP pumps. The Rainin Dynamax™ controller and data collection software were run by an Apple® Macintosh™ microcomputer. Elution of phospholipids was monitored with a Gilson Instruments (Middleton, WI, U.S.A.) Holochrome™ UV–Vis variable-wavelength detector set at 202 nm. The column was a 25 cm×4.6 mm 10-$\mu$m silica Resolvex-SIL™ (Fisher Scientific) with a guard column (Brownlee Scientific, SS-GU, 3 cm×4.6 mm , 5-$\mu$m silica, supplied by Rainin Instruments). Elution procedures were as described in the figure and table. When necessary, the column was washed with methanol and reequilibrated to the appropriate conditions with a blank gradient. The acetonitrile–methanol–water systems developed here were derived from those described by Gross and Sobel [9].

Phospholipids were dissolved in chloroform at a concentration of 20 mg/ml and filtered through a 0.22-$\mu$m filter before injection into the system. Up to 1 mg total phospholipid could be separated under the conditions described. The effluent from the detector was collected manually into different peak fractions. Several drops of 10% ammonium hydroxide in methanol were added to each tube and the solvent was evaporated under nitrogen at 40°C. The lipids were redried from absolute ethanol when necessary to remove traces of water. The lipids were dissolved in chloroform–methanol (2:1) and separated by thin-layer chromatography (TLC) on plates of Silica Gel G (Brinkman Instruments, Westbury, NY, U.S.A.) in chloroform–methanol–acetic acid–water (75:25:5:2.2) [10] for identification of the individual lipids and estimation of their purity. Quantitation of the phospholipids was performed as previously described [4,5].

## RESULTS AND DISCUSSION

The separation of *Tetrahymena* phospholipids is shown in Fig. 1. Standard lysophosphatidylcholine and sphingomyelin were added to the *Tetrahymena* lipid samples (Fig. 1A) for the purposes of detection. Although different strains (E and W) of *Tetrahymena* have been reported to contain these lipids [11,12], we have been unable to detect them in the strain of the organism (WH-14) employed in these studies. In fact, the presence of lysophosphatidylcholine is indicative of lipolysis during the isolation procedure [13].

Under the conditions shown in Fig. 1, the lipids eluted in the following order:

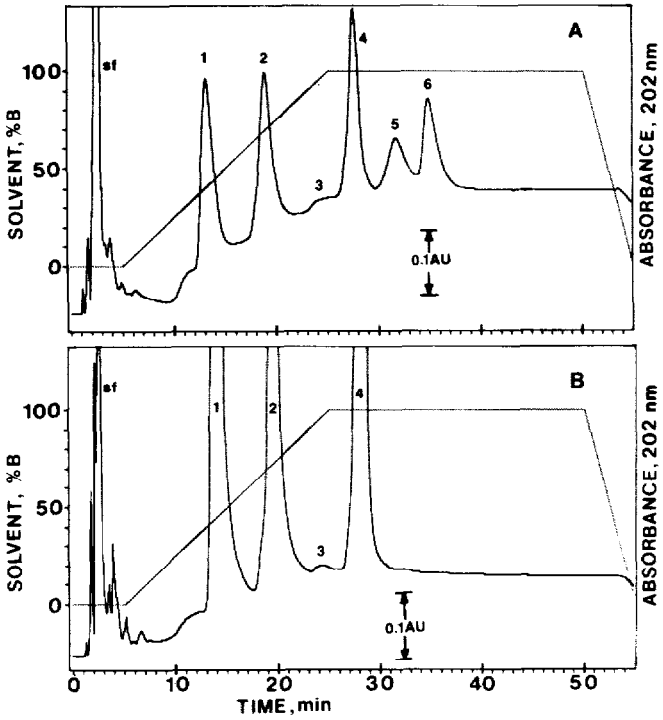


Fig. 1. (A) Separation of *Tetrahymena* phospholipids with the addition of sphingomyelin and lysophosphatidylcholine. A 400-$\mu$g amount of *Tetrahymena* phospholipid plus 100 $\mu$g each of sphingomyelin and lysophosphatidylcholine were injected in 20 $\mu$l chloroform. The elution procedure shown here is method II of Table I. (B) A 1-mg amount of *Tetrahymena* phospholipid, without additions, chromatographed under the same conditions as A. Peaks: sf = solvent front plus cardiolipin; 1 = phosphatidylethanolamine; 2 = AEP-glyceride; 3 = AEP-ceramide; 4 = phosphatidylcholine; 5 = sphingomyelin; 6 = lysophosphatidylcholine.

cardiolipin (with the solvent front), phosphatidylethanolamine, AEP-glyceride, AEP-ceramide, phosphatidylcholine, sphingomyelin and lysophosphatidylcholine. The phospholipids were identified by comparison with standards on TLC [10]. This same order of elution was obtained with other elution conditions in which the water content of the solvents was varied (Table I). If the methanol content was kept low, then peaks after AEP-glyceride did not elute. We also found that the acetonitrile–methanol–water combination was required to separate phosphatidylethanolamine from AEP-glyceride. These two lipids were not separated by the commonly used hexane–isopropanol–water systems (e.g. ref. 14) in our hands.

There was no visible cross-contamination of the HPLC fractions as monitored by TLC. Within experimental error, the phospholipid composition of *Tetrahymena* determined using the HPLC-separated lipids (30.1% phosphatidylcholine, 8.0% AEP-ceramide, 33.3% phosphatidylethanolamine, 26.2% AEP-glyceride and 3.6% cardiolipin) was the same as that determined from TLC-separated samples [5].

TABLE I

ELUTION TIMES OF *TETRAHYMENA* PHOSPHOLIPIDS AND PHOSPHONOLIPIDS WITH ADDED SPHINGOMYELIN AND LYSOPHOSPHATIDYLCHOLINE UNDER VARYING CHROMATOGRAPHIC CONDITIONS

Each method involved elution with solvent A for 5 min, a linear gradient to 100% solvent B from 5 to 25 min, followed by 100% solvent B from 25 to 50 min with return to solvent A from 50 to 55 min and 15 min reequilibration in solvent A. Data collection was stopped at 55 min.

Method I: solvent A = acetonitrile–methanol–water (100:5:17), solvent B = acetonitrile–methanol–water (100:50:17).
Method II: solvent A = acetonitrile–methanol–water (100:5:8.5), solvent B = acetonitrile–methanol–water (100:50:17).
Method III: solvent A = acetonitrile–methanol–water (100:5:1), solvent B = acetonitrile–methanol–water (100:50:17).
Method IV: solvent A = acetonitrile–methanol–water (100:5:8.5), solvent B = acetonitrile–methanol–water (100:50:8.5).

| Phospholipid | Elution time (min) | | | |
|---|---|---|---|---|
| | I | II* | III | IV |
| Cardiolipin | sf** | sf | 11.0 | sf |
| Phosphatidylethanolamine | 6.4 | 13.0 | 20.8 | 12.7 |
| AEP-glyceride | 11.9 | 18.7 | 24.3 | 20.5 |
| AEP-ceramide | 18.1 | 24.8 | 27.8 | 28.0 |
| Phosphatidylcholine | 22.2 | 27.4 | 30.8 | 32.4 |
| Sphingomyelin | 27.5 | 31.6 | 34.4 | 39.1 |
| Lysophosphatidylcholine | 30.1 | 34.8 | 37.0 | 44.3 |

*From Fig. 1.
**Solvent front.

The ability to rapidly and efficiently separate phosphonolipids and phospholipids by HPLC promises to be of great utility in future studies.

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