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Note

Unequivocal identification of sphingomyelin in the lipids of *Tetrahymena pyriformis*

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The occurrence of sphingolipid in *Tetrahymena pyriformis* W was first reported by Taketomi[1] who, on the basis of its stability towards mild acidic and alkaline hydrolysis, negative ninhydrin reaction and the presence of phosphorus and choline in the molecule, characterized it as sphingomyelin. Carter and Gaver[2], who studied the sphingolipids of *Tetrahymena pyriformis* W, detected a compound which possessed the same chromatographic mobility as sphingomyelin and which yielded a long-chain base on hydrolysis. Berger *et al.*[3] have reported the occasional occurrence of very small amounts of a "sphingomyelin-like" compound in this organism. While all these reports suggest strongly the occurrence of sphingomyelin, they did not provide unequivocal evidence. Such evidence is presented in this paper.

EXPERIMENTAL

Materials

Pre-coated Kieselgel F_{254} plates (E. Merck, Darmstadt, G.F.R.) were used for thin-layer chromatography (TLC) studies. Silica gel G (E. Merck), for TLC use, was used for the ascending dry-column chromatography (ADCC) studies.

Methods

The maintenance and culture of the organism *Tetrahymena pyriformis* E, and the procedures for the extraction and fractionation of lipids by either TLC or ADCC, has been described previously[4,5]. Acidic hydrolysis of lipids and the identification of the products has also been described[6]. Mild alkaline hydrolysis[2] of certain specific lipid fractions obtained by ADCC of Tetrahymena lipids was used for purifying the sphingolipids.

Specific group reagents used for the detection of lipids on the TLC plates were as follows; for amino lipids, 0.25% ninhydrin in acetone; for choline lipids, 0.25% *cis*-aconitic anhydride in acetic anhydride[7]; and for phospholipids in general, the molybdenum blue reagent of Dittmer and Lester[8].

Infrared spectra were determined on a liquid film using sodium chloride optics in a Unicam SP200 instrument.

High-resolution nuclear magnetic resonance (NMR) spectra were recorded

on a Varian HA-100 (100 MHz) spectrophotometer with a probe temperature of 34°, using a 10% solution of the compound in deuterochloroform. Tetramethylsilane was used as an internal standard. Chemical shifts are expressed in parts per million relative to the internal standard at 10 ppm (T scale)

RESULTS

A sample of lipids (5.0 g) extracted from *Tetrahymena pyriformis* E was fractionated by the ADCC technique using chloroform–methanol–concentrated ammonia solution (65:35:5) as the developing solvent. All the lipid fractions with mobility lower than that of phosphatidylcholine were pooled and subjected to mild alkaline hydrolysis[2]. The remaining lipids (ceramide monomethylaminoethylphosphonate, ceramide aminoethylphosphonate, the unknown phospholipid and neutral lipids) were fractionated by preparative TLC using the same alkaline solvent

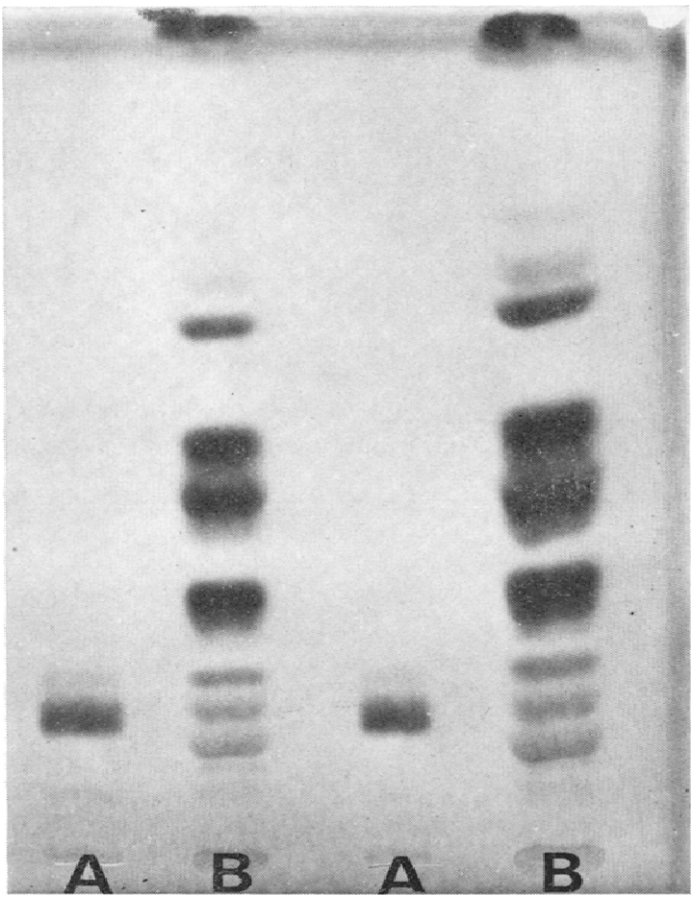

Fig. 1. TLC separation of lipids of *Tetrahymena pyriformis* E, showing the purity of sphingomyelin isolated from it. Adsorbent: Kieselgel F_{254}. Solvent: chloroform–methanol–conc. ammonia solution (65:35:5). Spray reagent: iodine vapour. Material spotted: (A) Sphingomyelin isolated from *Tetrahymena pyriformis* E; (B) Total lipids of *Tetrahymena pyriformis*.

system[6]. This yielded a ninhydrin-negative phospholipid which was still contaminated with ceramide aminoethylphosphonate (Cer-AEP). The new lipid was further purified in an acidic solvent system by preparative TLC[5,9]. The chromatographic mobility of this new lipid with reference to total lipids of Tetrahymena is shown in Fig. 1.

The native lipid gave a typical purple spot on the TLC plate when sprayed with the specific choline reagent developed by Vaskovsky and Suppes[7]. Methanolysis[10] and controlled acidic hydrolysis[6] yielded sphingosine bases[6], fatty acids[6] and choline[1].

The identity of this lipid was established by infrared spectroscopy (Fig. 2). The following absorption characteristics suggested it to be sphingomyelin and compared well with the spectrum of brain sphingomyelin[11]: (1) typical amide I and amide II absorption around 1640 and 1550 cm^{-1} (ref. 6); (2) typical choline and *trans*-absorption around 980 cm^{-1} (10.3 μm)[11]; (3) a strong absorption at 3300 cm^{-1} characteristic of a free hydroxyl group[6]; (4) the stretching vibrational frequency due to P=O around 1230 cm^{-1} characteristic of a phosphate rather than a phosphonate[4]; and (5) absence of carboxyl ester absorption around 1735 cm^{-1} (ref. 6).

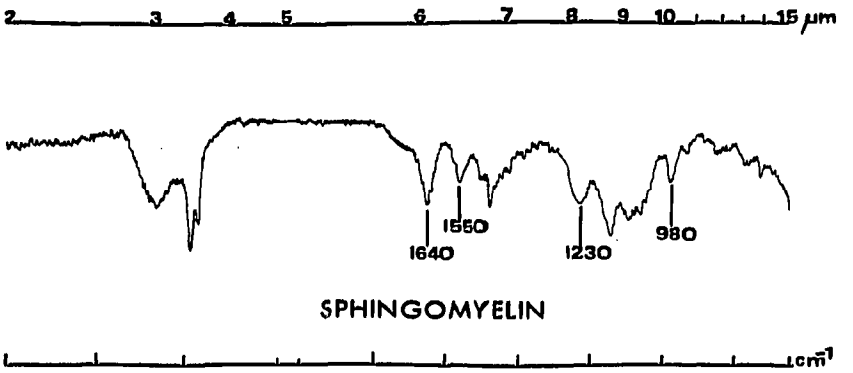

Fig. 2. Infrared spectrum of sphingomyelin isolated from *Tetrahymena pyriformis* E.

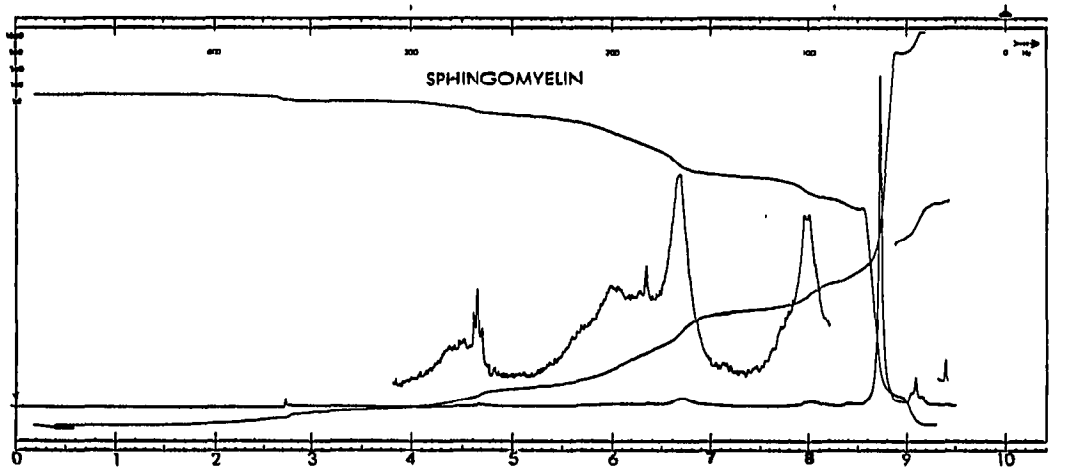

Fig. 3. Nuclear magnetic resonance spectrum of sphingomyelin isolated from *Tetrahymena pyriformis* E.

Further support for the identity of sphingomyelin was established by NMR spectroscopy (Fig. 3)[12]. The spectrum showed an absorption at 6.7 ppm characteristic of trimethylammonium protons, a broad band in the 5.6–6.2 ppm region characteristic of $-CH_2OP$ and $-CH_2N^+$ protons, and absorptions in the regions around 4.6 and 7.9 ppm indicating unsaturation in the molecule.

DISCUSSION

These results leave no doubt about the presence of small amounts of sphingomyelin in *Tetrahymena pyriformis*. While previous reports gave strong indications of the occurrence of sphingomyelin in this organism, the proof was not unequivocal. For instance, the alkali- and acid-resistant, choline containing and ninhydrin-negative phospholipid described by Taketomi[1] may have been 1-alkoxy-*sn*-glycero-3-phosphorylcholine. In the present work, phosphonoglyceride and phosphatidylcholine were removed from the total lipids of *Tetrahymena pyriformis*, before subjecting them to mild alkaline hydrolysis, for the isolation of sphingolipids. But for this, the 1-alkoxy-*sn*-glycero-3-phosphorylcholine and 1-alkoxy-*sn*-glycero-3-(2-aminoethylphosphonate) which would be formed from phosphatidylcholine and phosphonoglyceride, respectively, would have interfered in the preparative TLC isolation of sphingomyelin. Furthermore, the positive identification of sphingosine in the hydrolysis products, and the spectroscopic evidence, unequivocally identified the lipid as sphingomyelin.

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