Isolation of ceramide-monomethylaminoethylphosphonate from the lipids of *Tetrahymena pyriformis W*

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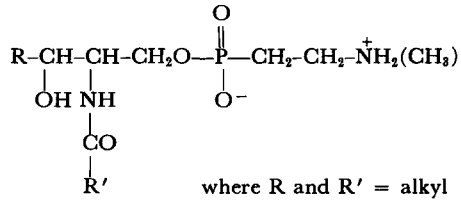
Abstract Ceramide-monomethylaminoethylphosphonate has been isolated for the first time from the lipids of *Tetrahymena pyriformis W* and characterized on the basis of its chromatographic mobility, chemical analysis, and infrared and nuclear magnetic resonance properties.

Supplementary key words sphingolipid · phosphonate · *N*-methyl · ascending dry-column chromatography

THE OCCURRENCE of a sphingolipid in *Tetrahymena pyriformis W* was first reported by Taketomi (1). The lipid was characterized as sphingomyelin on the basis of its stability towards mild acid and alkaline hydrolysis, a negative ninhydrin reaction, and the presence of phosphorus and choline in the molecule. Carter and Gaver (2) isolated and characterized two other sphingolipids, ceramide and ceramide-aminoethylphosphonate (ceramide-AEP), from the same organism. They also detected in this organism another minor sphingolipid, which was tentatively characterized as sphingomyelin on the basis of its chromatographic mobility and hydrolytic products. The presence of ceramide-AEP in *Tetrahymena pyriformis W* has been confirmed by Berger, Jones, and Hanahan (3) and in the present work. Jonah and Erwin (4) reported the occurrence of six ceramide-like lipids in *Tetrahymena pyriformis WH14*. All these as yet uncharacterized sphingolipids gave no reaction with ninhydrin and only one of them contained phosphorus. Recently, Ferguson et al. (5) reported the occurrence of hydroxy fatty acids in the sphingolipids of *Tetrahymena pyriformis W* that was grown on a proteose–peptone medium. However, when grown on a chemically defined medium, this organism failed to synthesize the hydroxy fatty acids. On the other hand, Thompson (6) and Smith and Law (7) detected no sphingolipids in the total lipids of *Tetrahymena pyriformis W* and *WH14*, respectively.

In the present work, the application of a large-scale fractionation procedure (8) to the lipids of *Tetrahymena pyriformis W* led to the discovery of a new sphingolipid in this organism. It was characterized as ceramide-monomethylaminoethylphosphonate (ceramide-MMAEP) on the basis of its chromatographic mobility, chemical analysis, and infrared and nuclear magnetic resonance properties.



Ceramide-MMAEP

MATERIALS AND METHODS

2-Aminoethylphosphonic acid and its *N*-methyl derivatives were gifts of Dr. A. S. Isbell. Normal and hydroxy fatty acid methyl esters were prepared from brain lipids. Sphingosine and phytosphingosine were gifts of Professor H. E. Carter. Silica gel G (E. Merck, for TLC use) was used for ADCC studies. Glass plates precoated with silica gel F254 and cellulose (E. Merck) were used for TLC studies. All chemicals used, including solvents, were of analytical grade and were not further purified.

Tetrahymena pyriformis was grown axenically at 28°C in a 2-l flask containing 700 ml of medium consisting of 2%

Abbreviations: TLC, thin-layer chromatography; ADCC, ascending dry-column chromatography; GPC lipids, glycerylphosphorylcholine lipids; AEP, 2-aminoethylphosphonic acid; MMAEP, monomethylaminoethylphosphonic acid.

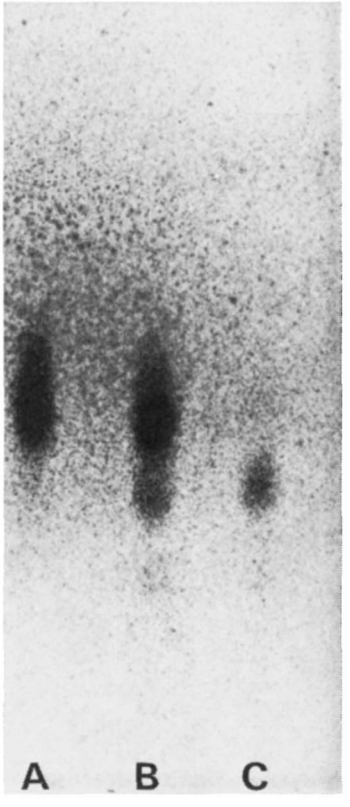


FIG. 1. TLC separation of ceramide-MMAEP and ceramide-AEP. Adsorbent, silica gel F254; solvent, chloroform–methanol–acetic acid–water 100:20:12:5 (by vol); spray reagent, 50% aqueous sulfuric acid followed by charring. Material spotted: *A*, ceramide-MMAEP; *C*, ceramide-AEP; *B*, *A* + *C*.

proteose–peptone (Difco), 0.2% yeast extract, 0.5% glucose, and 0.1 mM Fe–EDTA complex. The flask was shaken on a gyrotory shaker at 160 oscillations/min. The cells were harvested at late log phase of growth (about 2 × 10^6 cells/ml) and washed once with 0.9% saline. Details of the lipid extraction and preparative fractionation are given elsewhere (8).

Methanolysis of sphingolipids was carried out in screw-capped (Teflon-lined) tubes for 18 hr at 80°C with aqueous methanolic hydrochloric acid (8.9 ml of 12 N acid and 9.4 ml of water diluted to 100 ml with absolute methanol) (9). The fatty acid methyl esters and sphingosine bases were separated by the extraction procedure of Kates (10). Alternatively, the sphingolipids were also hydrolyzed with 6 N aqueous HCl at 110°C for 48 hr. The fatty acids and sphingosine bases were removed by extraction with chloroform, and the aqueous layer, containing water-soluble products, was concentrated for characterization by TLC. Sphingosine bases were separated by TLC on silica gel F254. The TLC plates were developed in chloroform–methanol–2.5 N ammonia 40:10:1 (by vol), and the sphingosine bases were detected by spraying the plates with ninhydrin or with 50% aqueous sulfuric acid followed by charring (11). Normal and hydroxy fatty acid methyl esters were separated from each other on silica gel F254 plates developed in toluene. The plates were sprayed with 50% aqueous sulfuric acid and then charred to detect these compounds. The water-soluble products were separated and characterized by TLC on cellulose, using either butanol–acetic acid–water 12:3:5 (by vol) or 88% phenol–water 4:1 (by vol) as the developing solvents (12). The water-soluble phosphorus compounds were detected according to the procedure of Bandurski and Axelrod (13) after spraying the plates with the Hanes and Isherwood (14) reagent.

Infrared spectra were determined in a liquid film using a Pye Unicam SP200 with sodium chloride optics.

High resolution NMR spectra were recorded on a Perkin-Elmer 60-MHz spectrophotometer with a probe temperature of 33°C, using 10% solutions of the compounds in deuterochloroform; tetramethylsilane (TMS) was used as the internal standard. Chemical shifts are expressed in parts per million relative to internal standard at 10 ppm (T scale).

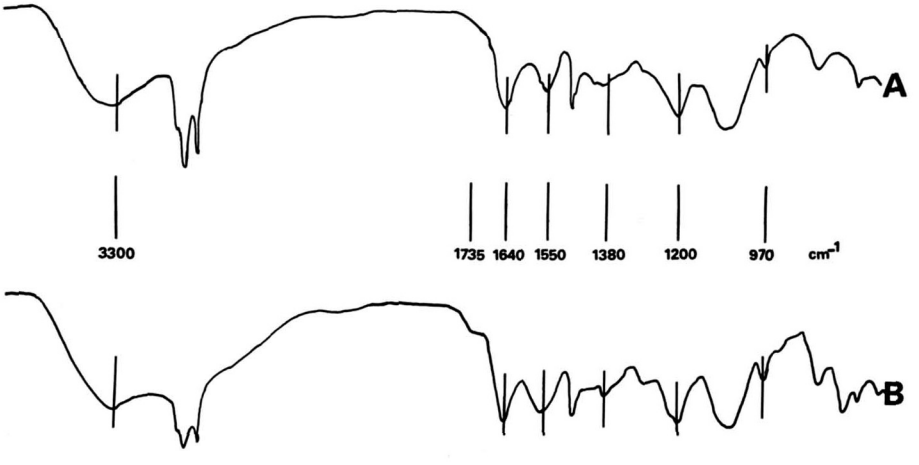


FIG. 2. Infrared spectra of sphingolipids. *A*, ceramide-AEP; *B*, ceramide-MMAEP.

RESULTS

The extraction of the lipids from stationary-phase cells and the large-scale fractionation of crude lipids by the technique of ADCC have been described elsewhere (8). In the course of this procedure, a number of fractions, representing about 5% of the total lipid, were found to contain what was termed "minor lipids." They consisted of four unidentified lipids and were contaminated with GPC lipids. As the GPC lipids migrated faster than the four uncharacterized minor lipids in the alkaline solvent system chloroform–methanol–concentrated ammonia 65:35:5 (by vol), preparative TLC was used to remove the GPC contaminant.

Preparative TLC in the neutral solvent system chloroform–methanol–water 70:30:5 (by vol) resulted in the isolation of four pure lipids. Their chromatographic mobilities in three different solvent systems, their staining properties, and their yields as pure products are given in Table 1. The two fastest-moving compounds in the neutral solvent system were shown to be chromatographically pure in another solvent system (Fig. 1) and were of special interest because of their infrared characteristics (Fig. 2). Both lipids showed: (*a*) a typical phosphonate absorption around 1200 cm^{-1} (8); (*b*) typical amide I and amide II absorption around 1640 cm^{-1} and 1550 cm^{-1}, respectively; (*c*) absence of ester carbonyl absorption around 1735 cm^{-1}; and (*d*) a strong absorption around 3300 cm^{-1}, characteristic of a free hydroxyl. As these characteristics suggested a sphingophosphonolipid nature of the two compounds, acid hydrolysis studies were carried out (9). The TLC characterization of hydrolysis products indicated the presence of almost equal amounts of two components having identical mobilities and staining properties of sphinganine and sphingenine, respectively (with notable absence of 1,3,4-trihydroxy-2-amino-alkane) (11), and the presence of normal fatty acid methyl esters (with notable absence of hydroxy fatty acid methyl esters) in both compounds. TLC of water-soluble products (Fig. 3) indicated the presence of MMAEP in the faster-moving sphingolipid and of AEP in the sphingolipid with a lower R_F value (12).

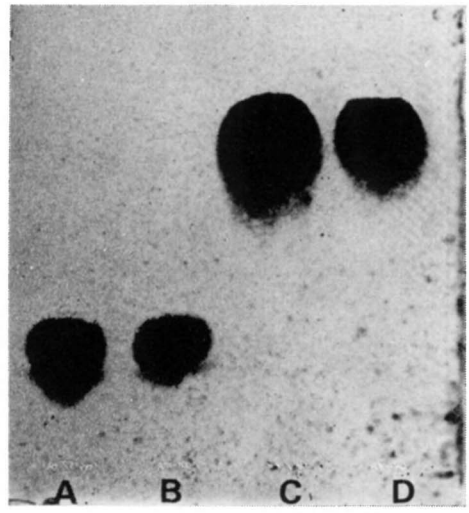


FIG. 3. TLC separation of MMAEP and AEP. Support, cellulose on glass; solvent, 88% phenol–water 4:1 (by vol); spray reagent, Hanes and Isherwood (14). Material spotted: *A*, synthetic AEP; *B*, AEP isolated from ceramide-AEP; *C*, synthetic MMAEP; *D*, MMAEP isolated from ceramide-MMAEP.

Examination of the intact lipids by NMR (Fig. 4) showed that the fast-moving lipid had a sharp single peak at 6.6 ppm (15), typical of *N*-methyl protons, which

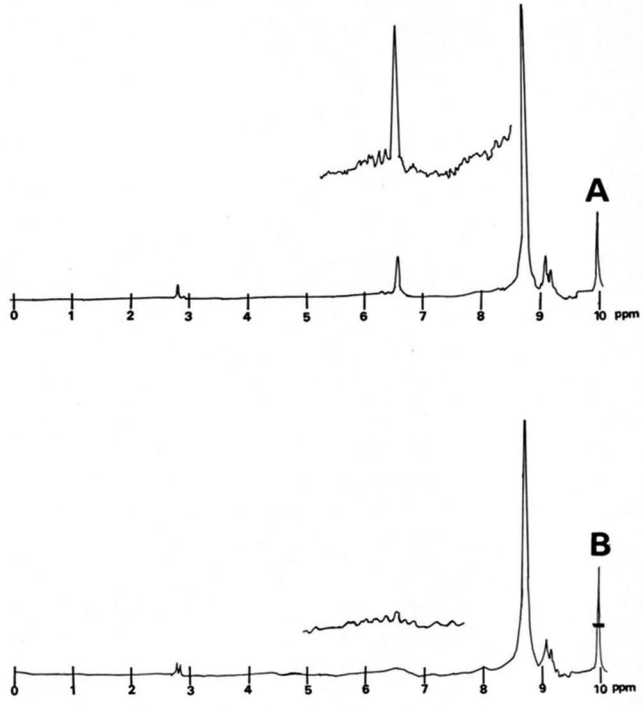


FIG. 4. NMR spectra of sphingolipids. *A*, ceramide-MMAEP; *B*, ceramide-AEP. Insets: spectra run at a higher sensitivity in the range 8–5 ppm.

TABLE 1. R_F values, staining properties, and yields of four minor lipids isolated from 6 g of total lipids of *Tetrahymena pyriformis W*

Lipid	R_F Values in Solvents[a]			Staining Properties: Reagent for		Yields
	A	B	C	P	NH_2	
						mg
1	0.25	0.22	0.26	+	+	60
2	0.20	0.18	0.17	+	+	50
3	0.15	0.16	0.37	+	+	80
4	0.10	0.14	0.08	+	−	40

[a] A, chloroform–methanol–water 70:30:5 (by vol); B, chloroform–methanol–concd ammonia 65:35:5 (by vol); C, chloroform–methanol–acetic acid–water 75:5:25:1.5 (by vol).

was absent from the NMR spectrum of the slower-moving sphingolipid. However, both the sphingolipids showed a typical sharp doublet at 9.15 ppm, with greater intensity for the lower-field segment, suggesting the presence of a terminal isopropyl group (16). It is known that nearly 90% of the sphingosine bases and 70% of the fatty acids in the sphingolipids of *Tetrahymena pyriformis* are branched (2, 3). This would indicate a theoretical ratio of 1:1.9 and 1:1.7 between the *N*-methyl peak and the terminal methyl and/or isopropyl peak of sphingosine bases and fatty acids, respectively. This yields a calculated ratio in the faster-moving sphingolipid of 1:3.6, which is close to the observed ratio of 1:3.4, confirming a monomethyl substitution at the NH_2 group in the fast-moving sphingolipid, which agrees with the results of hydrolysis studies. The more prominent absorption at 970 cm^{-1} and 1380 cm^{-1} in the infrared spectrum of the faster-moving sphingolipid (Fig. 2*B*), compared with the infrared spectrum of the slow-moving sphingolipid (Fig. 2*A*), also suggested a methyl substitution at the NH_2 group in the fast-moving sphingolipid (17). These results are consistent with the weak reaction with ninhydrin of the native fast-moving sphingolipid and of its acid hydrolysis product which, on the other hand, reacts strongly with the phosphorus reagent (14). The relative chromatographic mobilities of the two sphingolipids in the system of Hori, Sugita, and Itasaka (17) (Fig. 1) and complete identity of our infrared spectra with those published by Hori et al. (17) for ceramide-MMAEP and ceramide-AEP confirmed our assignment of ceramide-MMAEP to the fast-moving sphingolipid and of ceramide-AEP to the slow-moving sphingolipid.

DISCUSSION

The occurrence of ceramide-MMAEP in nature is well documented (17, 18). However, to our knowledge this is the first finding of this compound in the lipids of *Tetrahymena pyriformis*. This differs from the findings of Thompson (6) and of Smith and Law (19), who reported the absence of both sphingolipids and *N*-methylated AEP in this organism. Although this difference may be due to strain differences as well as to differences in growth conditions, it is significant that other workers (1–5) detected sphingolipids in several strains of *Tetrahymena*. The detection of a phospholipid-*N*-methylating system in this organism by Smith and Law (19) and the simultaneous detection of ceramide-AEP and ceramide-MMAEP in the same organism, in the present work, suggests possible occurrence of a sphingophosphono-*N*-methylating system.

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