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Characterizations of six ethanolamine sphingophospholipids from *Paramecium* cells and cilia

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Abstract Six ethanolamine sphingophospholipids from axenically cultured *Paramecium tetraurelia* were isolated from cells and purified ciliary fractions, and were characterized. The sphingolipids comprised 10.7% of whole cell and 32.5% of ciliary ethanolamine phospholipid fractions purified by ion exchange column chromatography. The individual sphingolipids were characterized by thin-layer chromatographic analyses of parent compounds and the polar head group and long chain base moieties, gas–liquid chromatography, and mass spectrometry of amide-linked fatty acids and long chain bases, and nuclear magnetic resonance of the compounds. Colorimetric assays of differential hydrolysis products and ^{31}P nuclear magnetic resonance were used to determine the nature of phosphorus linkages. The sphingolipids were identified as N-acyl-*trans*-4-hydroxysphinganine-1-phosphonoethanolamine, N-acyl-*trans*-4-hydroxysphinganine-1-phosphoethanolamine, N-acyl-sphingenine-1-phosphonoethanolamine, N-acyl-sphingenine-1-phosphoethanolamine, N-acyl-sphinganine-1-phosphonoethanolamine and N-acyl-sphinganine-1-phosphoethanolamine. All six had >90% saturated fatty acids. These sphingolipids were quantified by radioisotope methods and plate densitometry of thin-layer chromatograms. Changes in the relative amounts of each species were detected in cells grown in different culture media as well as in cells at different culture ages.—**Kaneshiro, E. S., D. F. Matesic, and K. Jayasimhulu.** Characterizations of six ethanolamine sphingophospholipids from *Paramecium* cells and cilia. *J. Lipid Res.* 1984. **25:** 369–377.

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Sphingolipids have been found in nearly all eukaryotes examined (1) and have been identified in a bacterium (2). The ciliated protozoan, *Tetrahymena*, has ceramide aminoethylphosphonate (Cer AEP) and ceramide-N-monomethylaminoethylphosphonate, both of which have predominantly branched chain sphingosine bases (3, 4). The ceramide AEP contains N-acylated fatty acids with or without α-hydroxyl groups and these subspecies can be separated by thin-layer chromatography (TLC) (5). In *Paramecium tetraurelia*, six ethanolamine sphingophospholipids were previously identified (6–8) and shown to be either concentrated in the cilia of these cells (7) or confined exclusively in the ciliary membrane (6, 8). These earlier reports identified all six as ethanolamine lipids, three with phosphono-P and three with phospho-P, and identified phytosphingosine in two of the species by TLC of hydrolysis products (6, 8). The long chain bases (LCB) of the rest were not characterized nor were the fatty acids; therefore the grounds for separation into six species by two-dimensional TLC was not explained.

In the present report, we provide information on the compositions and structure of all six sphingolipids and present data on the concentrations of these compounds in cells and cilia during culture aging. This information was essential for subsequent studies concerning the biosynthesis of each moiety of these lipids.

MATERIALS AND METHODS

Organisms

Paramecium tetraurelia, 51s, was grown axenically in a crude medium or a chemically defined medium with monolein as the only fatty acid source, as previously described (7, 9). Unless otherwise indicated, analyses were on cells from axenic-enriched, crude medium late log

Abbreviations: Cer AEP, ceramide aminoethylphosphonate; D, dimensional; DPnE, N-acyl-sphinganine-1-phosphonoethanolamine, N-acyl-dihydrosphingosine-1-phosphonoethanolamine; DPsE, N-acyl-sphinganine-1-phosphoethanolamine, N-acyl-dihydrosphingosine-1-phosphoethanolamine; FAME, fatty acid methyl ester; FID, flame ionization detector; GLC, gas–liquid chromatography; LCB, long chain base; MS, mass spectrometry; NMR, nuclear magnetic resonance; PPnE, N-acyl-*trans*-4-hydroxysphinganine-1-phosphonoethanolamine, N-acyl-phytosphingosine-1-phosphonoethanolamine; PPsE, N-acyl-*trans*-4-hydroxysphinganine-1-phosphoethanolamine, N-acyl-phytosphingosine-1-phosphoethanolamine; SPnE, N-acyl-sphingenine-1-phosphonoethanolamine, N-acyl-sphingosine-1-phosphonoethanolamine; SPsE, N-acyl-sphingenine-1-phosphoethanolamine, N-acyl-sphingosine-1-phosphoethanolamine; TEAE, triethylaminoethyl; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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cultures. For studies on the sphingolipid composition at different culture ages, cells were analyzed at mid log (3 days), late log (5 days), or stationary (7 days) phase of growth (10). In some studies, sphingolipids were radiolabeled with [U-^{14}C]L-serine (New England Nuclear, Boston, MA) by growing the cells with 0.1–0.6 μCi/ml of the labeled compound for 3 days (day 2 to day 5). In other experiments, phospholipids were radiolabeled with ^{32}P by addition of 500 μCi of [^{32}P]H_3PO_4 (Amersham Corp., Arlington Heights, IL) per ml of culture medium for 3–5 days. Cilia fractions were prepared as previously described (7).

Extraction of lipids and isolation of sphingolipids

Total lipids were extracted from cells and isolated cilia by a modification (7) of the method of Bligh and Dyer (11). The total lipid extract was fractionated on a triethylaminoethyl (TEAE) cellulose chromatographic column (Sigma Chemical Co., St. Louis, MO) according to the methods of Turner and Rouser (12). The fraction from TEAE columns containing the ethanolamine lipids was further resolved by preparative TLC on 0.5 mm Silica Gel G or H plates developed in chloroform–methanol–water 65:35:5 (v/v/v) (13). Iodine vapors or water spray were used to visualize the lipids on the plates. The fractions were scraped with a razor blade and eluted from the Silica Gel with chloroform–methanol 1:2 (v/v) and filtered through Whatman No. 1 filter paper. Radioautograms of radiolabeled lipids resolved by TLC were on Kodak NS-2T X-ray films (Eastman Kodak Co., Rochester, NY).

The six individual sphingolipid species were isolated by 2-D TLC. Sphingolipid samples isolated from other ethanolamine lipids by 1-D TLC were spotted on 0.25-mm pre-coated Silica Gel 60 plates (E. Merck, Darmstadt, Germany). The sphingolipids were separated in the first dimension using chloroform–methanol–water 65:35:5 (v/v/v) and in the second dimension using chloroform–acetic acid–water 65:35:5 (v/v/v), with 30 min of drying under a stream of N_2 before exposure to the second solvent system. In some analyses, plates were developed in the first dimension with chloroform–methanol–conc. ammonium hydroxide 65:35:5 (v/v/v) and in the second dimension either with chloroform–acetone–methanol–acetic acid–water 4:3:1:1:0.5 (by vol) (12) or with chloroform–acetic acid–water 65:35:5 (v/v/v). Ninhydrin reagent, iodine vapors, or a P-sensitive reagent (14) were used to detect and analyze the lipids. Silica Gel 60 Al-backed TLC plates with or without fluorescence indicator (Merck) and Adsorbosil plus Prekotes (Applied Sciences Laboratories, State College, PA) were also treated with a reagent containing 5% phosphomolybdate in ethanol. After staining with phosphomolybdate, the plates were exposed to ammonia vapors to whiten the background (15). Lipids separated by TLC and stained by spray reagents were quantified by plate densitometry (Kontes densitometer, Kontes, Vineland, NJ) in the visible wavelength range at a scan rate of 6 cm/min. The densitometer was equipped with a Varian A-25 strip chart recorder (Varian Associates, Palo Alto, CA) that was operated at 1 in/min.

As an alternative method for isolation of sphingolipids, the ethanolamine phospholipid fraction from TEAE cellulose columns was hydrolyzed in a mild alkali solution (16). The alkali-stable fraction, recovered by silicic acid column chromatography (Unisil, Clarkson Chemical Co., Williamsport, PA) (16), was subjected to 2-D TLC as above to separate sphingolipids from lyso-glyceryl ether lipids. The individual sphingolipids used for further analyses were separated by 2-D TLC, visualized by water spray, scraped, and eluted.

Acid hydrolysis

To release fatty acids and long chain bases (LCB), the sphingolipids were hydrolyzed in methanol–water–conc. HCl 11:2.6:1 (v/v/v) at 80°C overnight in sealed glass ampoules (17). After cooling to room temperature, the fatty acid methyl esters (FAME) were extracted three times with 5 ml of hexane or petroleum ether (40–60°C) and dried under N_2 for analysis by GLC or GLC–mass spectrometry (MS). The remaining fraction containing the LCB and water-soluble products was dried under N_2 and resuspended in a small vol of water. The pH was adjusted to 10.0–11.0 with 1 N NaOH and the LCB were extracted with chloroform. The remaining water phase containing the head groups was dried and resuspended in methanol–water 1:1 (v/v) for analysis by TLC. Alternatively, the sphingolipids were enzymatically digested with phospholipase C from *Bacillus cereus* (Sigma) (18) and the water-soluble head groups were recovered in the aqueous phase by partitioning according to Folch, Lees, and Sloane Stanley (19). Also, FAME released by acid hydrolysis were isolated for GLC analysis by elution from Unisil columns with chloroform to verify the completeness of hexane or petroleum ether extraction procedures described above.

Fatty acid analysis

The methyl ester derivatives of amide-linked fatty acids were separated and quantified on a Hewlett-Packard 5830A GC (Hewlett Packard, Palo Alto, CA) with a flame ionization detector (FID). The FAME were dissolved in petroleum ether and analyzed on a 6-ft glass column

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packed with 10% EGSS-X (100–200 mesh, Applied Sciences Lab., State College, PA) at 150°C for 30 min; then the temperature was increased to 220°C at a rate of 1°C/min. The N_2 carrier gas flow rate was 15 ml/min, the FID temperature was 250°C, and the injection port temperature was 270°C. The FAME were also analyzed by GLC–MS (Department of Biochemistry, Mass Spectrometry Facility, Michigan State University) to verify their structures.

Long chain base analyses

The LCB were separated by TLC on 0.25 mm Silica Gel G prepared with 0.01 M sodium carbonate. The plates were developed in chloroform–methanol–water 100:42:6 (v/v/v) (17) and the LCB were visualized with ninhydrin.

The LCB were prepared for GLC or GLC–MS by acetylation with methanol and acetic anhydride (20), followed by conversion to their O-trimethylsilyl (TMS) derivatives by reaction with 0.1 ml of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Applied Sciences Labs.) for 15 min at room temperature. The samples were either analyzed directly, or first dried under N_2 and dissolved in hexane or dichloromethane. They were analyzed isothermally at 200°C by GLC on a 6-ft 3% OV-17 glass column. Authentic standards included dihydrosphingosine (Sigma or Miles Laboratories, Inc., Elkhardt, IN), sphingosine (Sigma), and phytosphingosine (Sigma or Calbiochem-Behring, San Diego, CA).

Electron impact mass spectra of N-acetylated, TMS-derivatized LCB were obtained on a Kratos MS-80 high resolution mass spectrometer (Kratos, Manchester, England). These data were continuously collected during MS runs and processed on a Data General NOVA/4C computer with a DS-55 data system. Exact mass data were obtained under the following operating conditions: ionizing current, 1×10^{-4} A; ionizing energy, 40 eV, accelerating voltage, 4 kV, scan range, 40–600 m/z; scan rate, 3 sec/decade at a resolution of 3000, and scan interval of 1 sec.

Samples were introduced either via a direct insertion probe or through a Carlo Erba Series 4160 GC interfaced with the MS. The gas chromatograph was equipped with an open tubular fused silica capillary column (30 m × 0.321 mm i.d.) coated with SE-54 (film thickness of 0.25 μm) or a 6 ft × 2 mm i.d. glass column packed with 3% OV-17 on 100–120 mesh Gas Chrom Q. The GLC injection port was maintained at 240°C for operation of the packed column; the oven temperature was increased from 230°C to 300°C at 10°/min. For analyses on the capillary column, samples were introduced directly onto the column at 60°C. After the solvent was pumped out, the temperature was increased to 230°C then further increased at 10°/min to 280°C.

Polar head group analyses

The water-soluble fractions obtained from acid hydrolysis or phospholipase C digestion of sphingolipids were analyzed by TLC on pre-coated cellulose plates (0.25 mm, Eastman Chemical Co.). The plates were developed with butanol–acetic acid–water 80:20:20 (v/v/v) (21) and visualized with ninhydrin. Authentic 2-aminoethanol (Sigma), 2-aminoethylphosphonic acid (Sigma), and 2-amino-3-phosphonopropionic acid (Calbiochem-Behring) were used as standards.

To determine the relative percent of phosphonyl and phosphoryl bonds of the polar head group attached to the ceramide component, both the total sphingolipid fraction and the acid-hydrolyzed, water-soluble products were analyzed according to the differential hydrolysis and colorimetric methods of Ferguson et al. (16). Phospho-P and phosphono-P were also identified and quantified on a sample of 2-D TLC-purified total ethanolamine sphingolipids from whole cells by ^{31}P NMR. The ^{31}P spectrum was recorded at 121.5 MHz on a Bruker WM 300 NMR spectrometer (Bruker Instruments, Inc., Billerica, MA) using phosphoric acid as the reference standard.

General methods

Organic solvents used in lipid analyses were distilled in glass before use. Butylated hydroxy toluene (BHT, Ionol, Shell Chemical Co., NY) was added to all solvents to minimize oxidation of lipids, except when samples were analyzed by GLC (22). Water used in all solutions was distilled twice, the second time in an all-glass and quartz system (Kontes).

RESULTS

Identification of ethanolamine sphingophospholipids

The ethanolamine sphingophospholipids from *Paramecium* were characterized by: *1*) their stability to mild alkali hydrolysis as shown by identical R_f values on TLC plates before and after hydrolysis; *2*) their positive reactions to ninhydrin (parent compounds as well as the water-soluble head groups and LCB following acid hydrolysis); *3*) their positive reactions to a P indicator reagent; *4*) their incorporation of [^{32}P] and [^{14}C]serine; and *5*) their elution with ethanolamine lipids from TEAE cellulose columns. Six species were resolved by 2-D TLC (**Fig. 1**). Densitometry of ninhydrin-stained plates, as well as of radioautograms of ^{32}P-labeled lipids, indicated that the sphingolipids comprised 10.7% of the ethanolamine lipid fraction from whole cell lipids and 32.5% from ciliary lipids. These were identified as N-acyl-*trans*-4-hydroxy-

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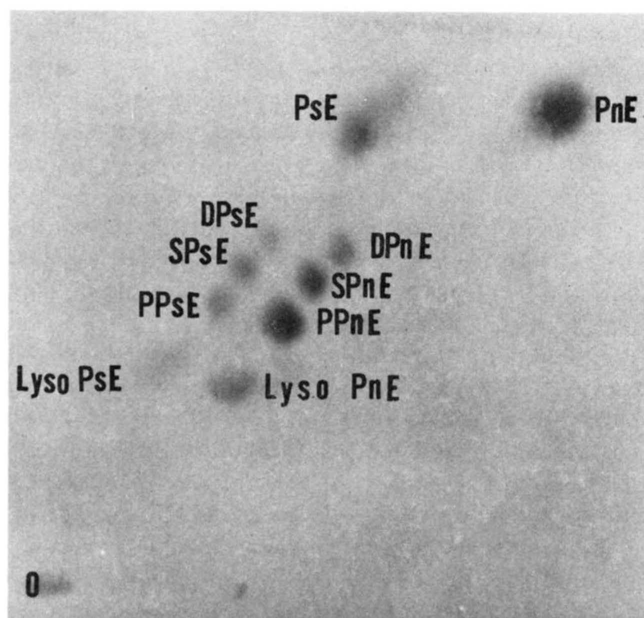

Fig. 1. A 2-D TLC separation of the ethanolamine phospholipids from *Paramecium* cilia stained with ninhydrin. The ethanolamine lipid fraction was isolated by TEAE cellulose column chromatography. Note the high concentrations of sphingolipids (PPnE, PPsE, SPnE, SPsE, DPnE and DPsE) compared to the ethanolamine glycerolipids (PnE and PsE) in cilia extracts. The ethanolamine lipid fractions from whole cell lipids have higher glycerolipid ratios (c.f. Fig. 3).

sphinganine-1-phosphonoethanolamine (N-acyl-phytosphingosine-1-phosphonoethanolamine, PPnE); N-acyl-sphingenine-1-phosphonoethanolamine (N-acyl-sphingosine-1-phosphonoethanolamine, SPnE), N-acyl-sphinganine-1-phosphonoethanolamine (N-acyl-dihydrosphingosine-1-phosphonoethanolamine, DPnE), N-acyl-*trans*-4-hydroxysphinganine-1-phosphoethanolamine (N-acyl-phytosphingosine-1-phosphoethanolamine, PPsE); N-acyl-sphingenine-1-phosphoethanolamine (N-acyl-sphingosine-1-phosphoethanolamine, SPsE) and N-acyl-sphinganine-1-phosphoethanolamine (N-acyl-dihydrosphingosine-1-phosphoethanolamine, DPsE).

Polar head groups

The nature and quantities of polar head group P were determined. A purified total sphingolipid fraction from whole cells from late log cultures analyzed by ^{31}P NMR indicated the presence of two peaks, one corresponding to the phosphoric acid standard, designated as 0 ppm, and the second at −22.7 ppm, which is characteristic of the chemical shift of alkylphosphonic acids and their esters (phosphonates). The relative amounts of each were determined by cutting and weighing the two peaks (**Table 1**). Individual sphingolipids resolved by 2-D TLC and quantified by plate densitometry of ninhydrin reaction products indicated that the sum of the species assigned as phosphono compounds compared to the sum of those assigned as phospho compounds agreed with the ^{31}P NMR data. Chemical analyses of P_i released from ester-linked P by mild acid hydrolysis compared with total P_i released by ashing further verified the above results and demonstrated that the total sphingolipid fraction contained about twice as much P–C as P–O–C bonds. These differential hydrolysis analyses were also done on individually isolated sphingolipids (Table 1) which showed that each contained only one type of P bond and that the separation of three species from the other three was based on the nature of the P bond. Those species containing phosphono P had lower R_f values than their phospho counterparts in basic or neutral solvent systems (first dimension) and greater R_f values in acid systems (second dimension) (Fig. 1).

Acid-hydrolyzed or enzyme-digested, water-soluble products from total sphingolipids showed two components by 1-D TLC. One had an R_f similar to that of authentic 2-aminoethanol and the other, an R_f similar to that of authentic 2-aminoethylphosphonic acid. Acid-hydrolyzed, water-soluble products of a mixture of PPnE, SPnE, and DPnE had only one component that comigrated with 2-aminoethylphosphonic acid by 1-D TLC, and the products of a mixture of PPsE, SPsE, and DPsE had only one component that comigrated with 2-aminoethanol, confirming the assignment of head group structures of each of the six sphingolipids (not shown).

Long chain bases

Three different species were detected by GLC of N-acetylated TMS derivatives of total sphingolipid LCB. One had a relative retention time similar to that of derivatized authentic sphingosine and another similar to

TABLE 1. Phospho-P and phosphono-P content of *Paramecium* ethanolamine spingophospholipids

Method of Quantitation	Relative %	
	Phosphono	Phospho
^{31}P NMR[a]	60.4	39.6
TLC plate densitometry[b]	65.3 ± 5.8	34.7 ± 1.9
Differential hydrolysis[c]		
Total sphingolipid fraction	68.0	32.0
PPnE	100.0	0.0
SPnE	94.3	5.7
DPnE	97.3	2.6
PPsE	3.5	96.5
SPsE	4.0	96.0
DPsE	0.0	100.0

[a] Values were obtained by peak areas on a ^{31}P NMR spectrum of a single sample of total cellular sphingolipids.
[b] Values are means ± SD of three determinations on 2-D TLC plates stained with ninhydrin.
[c] Values are means of triplicate determinations on single samples.

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dihydrosphingosine. High resolution GLC–MS of these LCB derivatives that were prepared from individually isolated sphingolipids showed that DPnE, DPsE, and a derivatized C-18 dihydrosphingosine standard gave mass spectra that were the same as published spectra (23). The molecular ion ($M^{+\cdot}$), at m/z 487 was not present, but

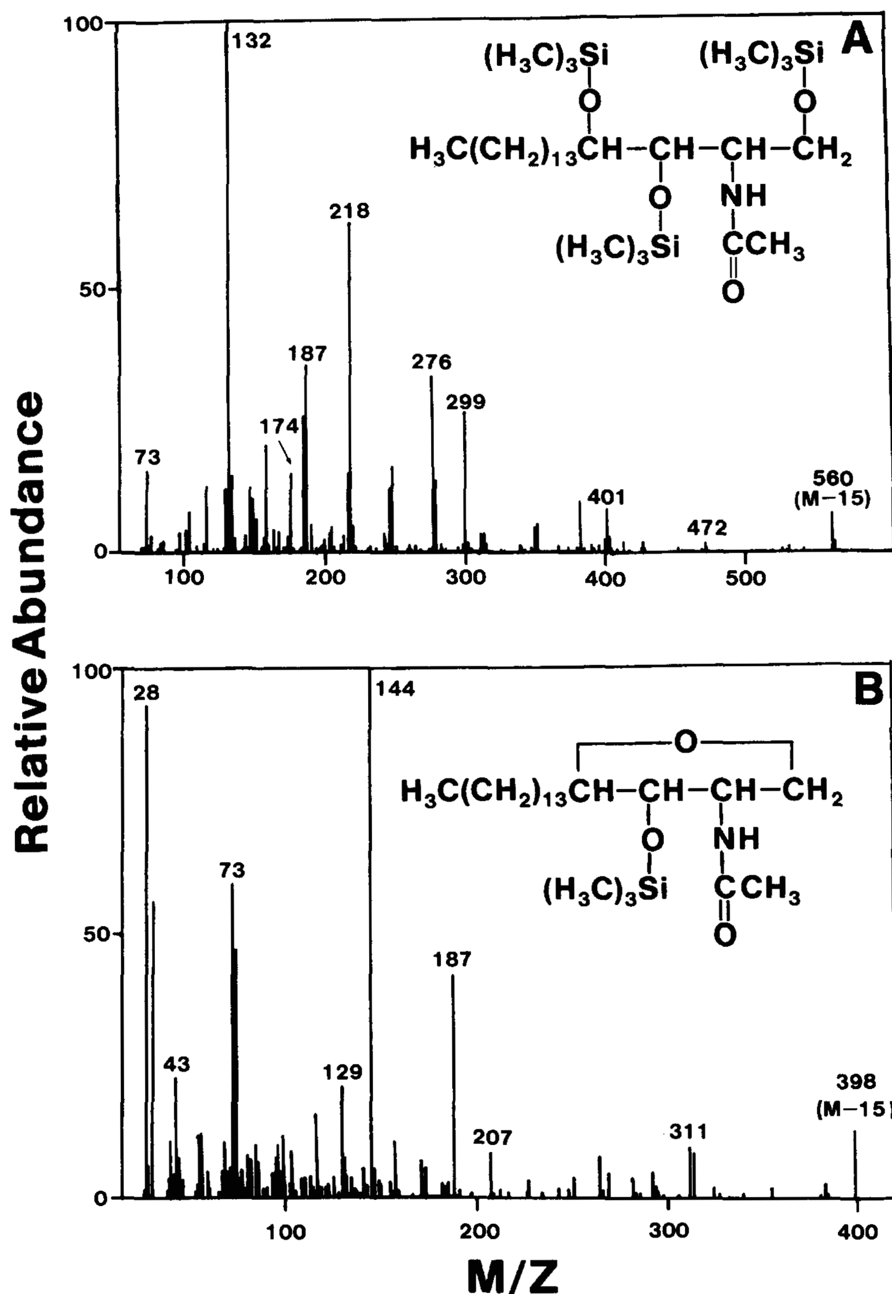

Fig. 2. Mass spectrum of N-acetylated, TMS-derivatized authentic phytosphingosine (A) and anhydrophytosphingosine from PPnE or PPsE (B). The spectrum for phytosphingosine and the exact elemental analyses of ion fragments by high resolution mass spectrometry indicate that the molecular ion ($M^{+\cdot}$ 575) which was not present has three TMS groups. The M-15 ion, typical of TMS derivatives at m/z 560 (loss of a TMS methyl group) was present. Also detected were the ions at m/z 472 (loss of C-1 with its TMS), m/z 401 (loss of C-1 and C-2 with the attached N-acetyl and the TMS), m/z 299 (C-4 through C-18, including the silylated hydroxyl group at C-4), m/z 276 (M-299), m/z 218 (loss of the amino acetyl group at C-2 from the m/z 276 ion), m/z 187 (loss of O-TMS from m/z 276 ion), m/z 174 (C-1 and C-2 with their derivatives), m/z 132 (C-1 with its TMS group and C-2 with an NH_2 group), and m/z 73 (TMS). (The cyclized compound, anhydrophytosphingosine, is a diagnostic product of phytosphingosine-containing lipids.) The M-15 fragment (m/z 398) and elemental compositions obtained from exact mass data of this derivatized LCB indicate that the molecular ion ($M^{+\cdot}$ 413) has only one TMS group. The probable structures of these compounds are indicated in the figures.

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the M-15 ion (characteristic of TMS derivatives and due to loss of a TMS methyl group) at m/z 472 was evident. Also, an abundant ion at m/z 313 (M-174, loss of the TMS- and N-acetyl-linked polar end of the molecule) was present.

Derivatized authentic sphingosine and LCB from SPnE and SPsE had spectra identical to those published (23). Due to the presence of a double bond, the molecular ion, and the M-15 and M-174 fragments were two mass units smaller than those of dihydrosphingosine. Elemental compositions obtained from exact mass data of molecular and fragment ions confirmed the identification of dihydrosphingosine and sphingosine as the LCB of *Paramecium* sphingolipids.

The LCB from PPnE or PPsE analyzed by 1-D TLC or by GLC did not show identity with phytosphingosine standards. Mass spectra of LCB derivatives prepared from isolated PPnE or PPsE did not correspond to the spectrum of derivatized authentic C-18 phytosphingosine or sphingadiene. The M-15 ion (**Fig. 2**) indicated that the molecular ion ($M^{+\bullet}$ 413) had a greatly reduced mass, and the elemental analysis indicated that only one TMS group was present, accounting for this mass. The major ion fragments, however, did agree with those of derivatized anhydrophytosphingosine, which is a common cyclized product formed during hydrolytic release of phytosphingosine from the parent compound (24). Since the cyclization involves an ether linkage between C-1 and C-4, the only TMS group that could be added was at the oxygen at C-3.

These analyses of LCB showed that individual sphingolipid species resolved by 2-D TLC had only one type of LCB and that the separation of the six species was based on the difference in polarity of the LCB moiety, in addition to the nature of the P linkage (see above).

Fatty acid composition

The amide-linked fatty acids of the total sphingolipid fraction from late log cells were identified and quantified by GLC (**Table 2**). Co-chromatography with authentic standards and GLC–MS analyses established the identification of these acids. No branched chain, hydroxyl, or polyunsaturated acids were detected. About 95% of the sphingolipid fatty acids were saturated. The fatty acids present in individual sphingolipids were the same as those in the total sphingolipid fraction. Subtle differences in the fatty acid compositions of phosphono compounds as compared to phospho compounds were noted. Sphingolipids with phytosphingosine, sphingosine, or dihydrosphingosine also showed minor differences with respect to fatty acid compositions (Table 2). The amide-linked fatty acid composition of sphingolipids from cells did not show noticeable changes with culture age between mid log and stationary phase of growth (data not shown).

Relative changes of sphingolipid classes with culture age

Individual sphingolipid species at different culture ages were quantified by densitometry of 2-D TLC of cellular ethanolamine phospholipids (**Table 3**). The species identified as DPsE was undetectable at mid log phase of growth, after which, this and DPnE as well as SPnE and SPsE increased with age. Relative decreases were noted in PPnE and PPsE (% of sphingolipids) with culture aging. The major species within the ethanolamine sphingophospholipids were PPnE and SPnE, consistently comprising over 60% of the group. Since total sphingolipids in *Paramecium* increase with culture age with respect to total cellular phospholipids (7), the percentage of each sphingolipid was compared to total phospholipids (Table 3). This showed that all species, except PPsE, increased with culture age.

Growth in the monolein-defined medium

Cells grown for 5 days in the defined medium that had monolein as the sole source of fatty acids had an altered sphingolipid composition. Only trace amounts of DPnE or DPsE were detected by ninhydrin or ^{32}P incorporation (**Fig. 3A**).

TABLE 2. Fatty acid composition of total cellular sphingolipids and of individual sphingolipids isolated from cilia[a]

Fatty Acid	Percent of Total Fatty Acids							
	Cellular Sphingolipids	Cilia Sphingolipids						
	Total[b]	Total[b]	PPnE	SPnE	DPnE	PPsE	SPsE	DPsE
14:0	0.3 ± 0.4	1.1 ± 0.8	trace	trace	trace	trace	trace	trace
16:0	82.8 ± 5.9	65.8 ± 9.8	78.2 ± 2.2	75.8 ± 4.4	54.8 ± 4.3	65.6 ± 3.4	69.4 ± 2.4	54.0 ± 5.6
16:1	0.7 ± 0.8	2.4 ± 1.4	1.7 ± 1.2	2.8 ± 2.5	7.5 ± 3.1	5.6 ± 2.0	4.1 ± 1.5	12.3 ± 4.8
17:0	5.6 ± 1.3	6.5 ± 2.5	6.3 ± 1.0	7.1 ± 1.4	5.3 ± 1.0	5.3 ± 0.8	5.6 ± 1.0	4.5 ± 0.7
18:0	9.3 ± 4.3	19.6 ± 6.1	10.8 ± 4.2	12.3 ± 4.3	25.1 ± 4.4	14.9 ± 2.9	13.0 ± 1.0	20.1 ± 3.9
18:1	1.3 ± 0.9	4.7 ± 3.3	2.1 ± 1.4	2.3 ± 1.9	5.9 ± 4.0	3.6 ± 3.0	3.2 ± 1.1	8.3 ± 4.1

[a] *Paramecium tetraurelia*, 51s, grown in the axenic medium to day 5 of culture age (late log). Values are means ± SD. Cells, n = 7; cilia, n = 5.

[b] Mixed model analysis of variance (ANOVA) showed that the fatty acid compositions of whole cell and ciliary sphingolipids were significantly different; $P < 0.0001$.

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TABLE 3. *Paramecium* ethanolamine sphingophospholipids: relative compositional changes with culture age

	% of Total Sphingolipids[a]				% of Total Cellular Phospholipids[b]		
	Cilia	Cells					
Sphingolipid	Late Log[c]	Mid Log	Late Log[c]	Stationary	Mid Log	Late Log	Stationary
PPnE	33.7 ± 4.5	45.6 ± 6.4	33.0 ± 4.6	31.7 ± 4.1	1.41	1.40	1.55
SPnE	28.4 ± 4.1	27.7 ± 1.3	27.9 ± 6.4	33.6 ± 3.0	0.86	1.33	3.65
DPnE	9.7 ± 6.9	1.5 ± 0.9	5.6 ± 3.9	9.6 ± 1.2	0.05	0.21	0.47
PPsE	11.7 ± 5.5	21.9 ± 5.2	12.6 ± 5.0	7.9 ± 2.7	0.68	0.59	0.39
SPsE	10.8 ± 5.7	3.3 ± 1.0	14.5 ± 3.8	10.5 ± 0.7	0.10	0.74	0.51
DPsE	5.7 ± 1.4	N.D.[c]	6.5 ± 3.1	6.7 ± 2.4	N.D.	0.24	0.33

[a] Values were obtained by densitometry of 2-D TLC plates stained with ninhydrin; they represent means ± SD of three to seven determinations.
[b] Values were calculated from values of total spingolipids and other phospholipids from ref. 7.
[c] Mixed model analysis of variance (ANOVA) showed no significant differences between whole cell and ciliary sphingolipid compositions at day 5 of culture growth; $P = 0.648$.

Staining of sphingolipids with phosphomolybdic acid

During quantitative analyses of these sphingolipids, phosphomolydbate staining of TLC plates was tested. Only SPnE and SPsE reacted to this stain (Fig. 3B). The differential staining of sphingolipids by phosphomolybdate is not understood, but this observation indicates that this commonly used general lipid stain is not reliable for quantitative analyses of all sphingolipid species.

DISCUSSION

Characterization of sphingolipids

All components of each of the six ethanolamine sphingolipids of *Paramecium* were identified in this study. These studies showed that the reasons for separation of these compounds by 2-D TLC is due to the nature of the phosphorus bond (P–C or P–O–C) and the polarity of the LCB (dihydrosphingosine, sphingosine, or phytosphingosine). The amide-linked fatty acid compositions were only slightly different in different species. The fatty acids were mainly saturated acids; no hydroxyl acids were detected, as were found in Cer AEP from *Tetrahymena* (5). Therefore, separation was not due to the fatty acyl group.

Although the ciliary membrane is greatly enriched in these sphingolipids, we consider it unlikely that these compounds are solely restricted to this membrane. Andrews and Nelson (6) were unable to detect sphingolipids in deciliated cell body fractions. However, they did not unambiguously establish that these fractions were devoid of cilia. It is known that ciliates, deciliated by a variety of methods, retain oral ciliature (10, 25–27) and that cilia are brought down into the cell body pellet during differential centrifugation.[2] We detected these sphingolipids in the crude cell body fraction obtained by low speed centrifugation (unpublished observations). The results obtained by Andrews and Nelson (6) could be explained by a low sensitivity of the procedures they used. The conclusion that these compounds are restricted to the ciliary membrane (6, 8) was shown to be inaccurate by our fatty acid composition studies on cellular and ciliary sphingolipids (Table 2). If these lipids were entirely in somatic cilia, then it would be expected that whole cell and isolated cilia sphingolipid fatty acid compositions would be identical at any given culture age. Our results demonstrated that they are significantly different indicating that sphingolipids may reside in other compartments of the cell. Furthermore, it would seem likely that other parts of the cell have these compounds, as predicted by present concepts of membrane biogenesis and biosynthesis of lipids. That is, sphingolipids and their intermediates are probably not synthesized in their entirety in situ in the ciliary membrane.

Lipid compositional changes with culture age

Earlier reports on the acyl-linked fatty acids of phospholipids from *Paramecium* indicated a relative increase in polyunsaturated acids with culture age (7, 10). Such observations suggested that an increased unsaturation index in the hydrocarbon moieties of membrane lipids could be reflected in a more fluid membrane in stationary phase cells. However, since the ciliary membrane contains a high percentage of amide-linked fatty acids and LCB as parts of sphingolipids, and ether-linked fatty alcohols in glycerolipids, these components must all be taken into account in order to accurately predict the nature of the bilayer of this membrane. The present study has provided additional information with respect to the hydrophobic moieties of sphingolipids. These data, with data on the quantitation of ether lipids,[3] should now provide a more

[2] Kaneshiro, E. S., D. F. Matesic, and K. Jayasimhulu, Unpublished observations.

[3] Kaneshiro, E. S., K. B. Meyer, and D. E. Rhoads. Unpublished observations.

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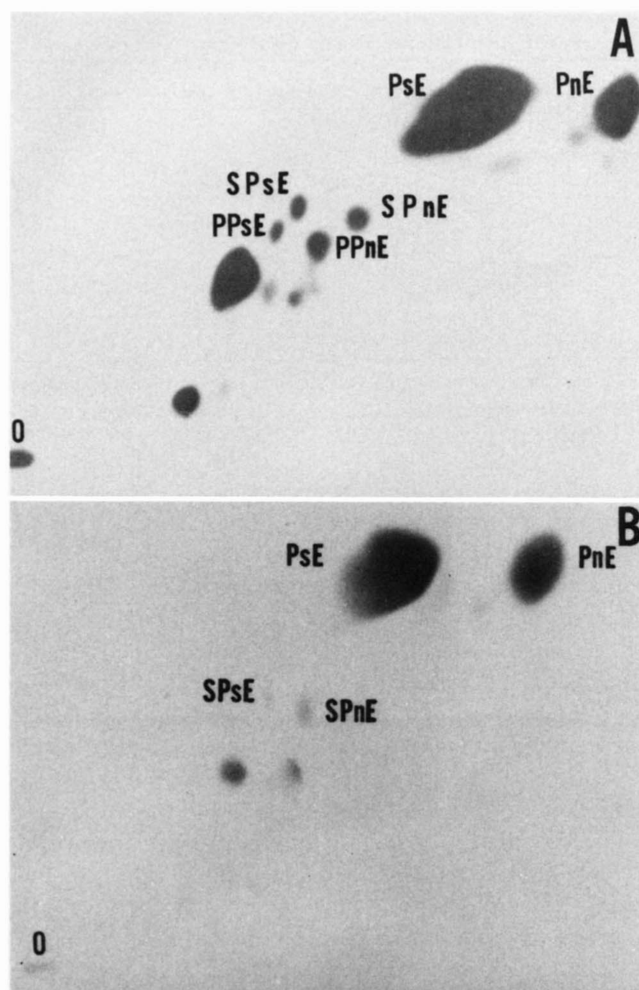

Fig. 3. Real and apparent alterations in sphingolipid compositions of ethanolamine sphingolipids and glycerolipids of *Paramecium tetraurelia,* 51s. The relative amounts of the different shingolipids changed when cells were grown in the chemically defined monolein medium as shown in the radioautogram of ^{32}P incorporation after 5 days of growth (A). This medium contains monolein as the sole source of fatty acids and stigmasterol as the only other lipid. The absence of DPnE and DPsE was striking. Ninhydrin staining of this TLC plate verified the absence of these two sphingolipids (not shown). Fig. 3B demonstrates that the concentrations of sphingolipids from day 5 cells grown in the axenic crude, enriched medium appeared different when the ethanolamine lipids were separated on aluminum-backed Silica Gel 60 TLC plates and were sprayed with phosphomolybdate. This reagent stained only SPnE and SPsE and the glycerolipids, but not DPnE, DPsE, PPnE or PPsE. All six sphingolipids were present in this sample as verified by ninhydrin-staining of another TLC analysis of this sample (not shown). Components in these figures of TLC analyses that are not identified are lyso derivatives of the major ethanolamine glycerolipids, or serine lipids that were prematurely eluted from TEAE cellulose columns.

comprehensive perspective on the nature of compensatory changes occurring in the bilayer of the ciliary membrane of *Paramecium.*

Nutritional effects

Changes in the growth medium and nutritional state (culture age) altered the composition of sphingolipids in *Paramecium.* The monolein-defined medium lacks fatty acids except oleate. The low levels of DPnE and DPsE in cells grown in this medium are interpreted as the result of a limited amount of available palmitate and/or stearate. Palmitate is utilized in LCB synthesis (28) and stearic acid and palmitic acid are acylated to all sphingolipids in this ciliate. Sphingosine and phytosphingosine are derived from dihydrosphingosine.[4] In cells grown in this stringent medium, conversion of dihydrosphingosine to the other LCB was apparently greater than its synthesis. These studies indicate that nutrition as well as mutation (8) can affect the turnover or metabolic rates of reactions in the biosynthesis of various moieties of sphingolipids in *Paramecium.*

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