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BIOCHEMICAL STUDIES OF THE EXCITABLE MEMBRANE OF *PARAMECIUM TETRAURELIA*

II. PHOSPHOLIPIDS OF CILIARY AND OTHER MEMBRANES

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Summary

The phospholipids of cilia and deciliated bodies of *Paramecium tetraurelia* were isolated and characterized. 1-alkyl-2-acyl-*sn*-glycero-3-(2′-aminoethyl) phosphonate (GAEPL), phosphatidylethanolamine, and 1-alkyl-2-acyl-*sn*-glycero-3-phosphorylcholine (GPC) were the major lipids of *Paramecium*, and the minor lipids included phosphatidylinositol, cardiolipin, ceramide-(2-aminoethyl) phosphonate (CAEP), ceramide phosphorylethanolamine (COPE) and four sphingolipids whose identity was not established. The deciliated bodies contained 4% cardiolipin, 15% GAEPL, 41% phosphatidylethanolamine, 30% GPC and 3% each of CAEP and phosphatidylinositol; the cilia contained no cardiolipin, 24% GAEPL, 37% phosphatidylethanolamine, 15% GPC, 15% CAEP, 3% phosphatidylinositol, 2% COPE and small amounts (approx. 1%) of the four uncharacterized sphingolipids. No alteration in phospholipid composition was found among cells harvested in the various stages of growth. The phospholipids of six *Paramecium* mutants of three distinct phenotypes (pawn, paranoiac and fast) were also examined. Only one significant difference was found on comparison of the whole cell, deciliated body and cilia fraction of the mutants with the analogous fractions from wild type cells: the fast mutant, fA 97, had two extra, minor phospholipids (approx. 2%) in the deciliated body fraction that were tentatively identified as 1,2-diacyl-*sn*-glycero-3-(2′-aminoethyl) phosphonate (AEPL) and 1-alkyl-2-acyl-*sn*-glycero-3-phosphorylethanolamine (GPE).

Abbreviations: GAEPL, 1-alkyl-2-acyl-*sn*-glycero-3-(2′-aminoethyl) phosphonate; AEPL, 1,2-diacyl-*sn*-glycero-3-(2′-aminoethyl) phosphonate; GPE, 1-alkyl-2-acyl-*sn*-glycerol-3-phosphorylethanolamine; GPC, 1-alkyl-2-acyl-*sn*-glycero-3-phosphorylcholine; CAEP, ceramide-1-(2′-aminoethyl) phosphonate; COPE, ceramide-1-phosphorylethanolamine; LGAEPL, lyso GAEPL; LGPC, lyso GPC.

Introduction

In the ciliate protozoan *Paramecium*, an excitable membrane mediates the transfer of information from receptors for various stimuli to the surface cilia which propel the cell by their coordinated beating. When some stimulus, such as an obstacle in the protozoan's path, triggers membrane depolarization, the membrane's permeability to Ca increases. The resulting inward current of Ca^{2+} raises the intracellular Ca^{2+} level, which in turn activates some mechanism for reversing the direction of the ciliary beat. The consequence is an 'avoidance response', backward swimming which takes the protozoan away from the obstacle in its path. Extensive electrophysiological studies [1–5] have documented the existence of a gated Ca^{2+} channel which plays a role in this process analogous to that of the Na^{+} channel of nerve. Kung and his collaborators have begun to dissect the Ca^{2+} gating mechanism genetically [6—8], and we are interested in doing the same with biochemical techniques.

Dunlap and Eckert [9] and Ogura and Takahashi [10] have reported that deciliated *Paramecia* have lost their excitability, and that with the regeneration of cilia excitability returns. It seems likely from these observations that at least some of the components of the excitable membrane are located in the cilium, and we have therefore begun to characterize the components of the ciliary membrane.

Local anaesthetics and a variety of related compounds block depolarization of excitable membranes, apparently by interacting with membrane lipids [11]. Lee [12] has described a model in which membrane lipid fluidity is critical to the functioning of the gated channels of excitable membranes, and has suggested that anaesthetics act by altering lipid fluidity. Local anaesthetics also affect the swimming behavior, and perhaps the excitability of *Paramecium* [13], and we consider it possible that the lipids of the membranes of *Paramecium* play a significant role in membrane excitation.

In this paper we identify the phospholipids of the ciliary membrane of *Paramecium tetraurelia* and compare the phospholipid composition of cilia with that of deciliated cells and also with these fractions from six mutant strains with altered excitability. We find that several phospholipids are unique to the ciliary membrane. If the ciliary membrane is functionally specialized (for excitability) this distinctive lipid composition may be a reflection of that specialization.

Materials and Methods

Growth. P. tetraurelia was grown at 28—29°C in a cerophyl medium [14] inoculated with *Aerobacter cloacae* and supplemented with 5 mg/l of β sitosterol (Sigma). The medium was buffered to pH 7.0 with 0.5 g/l anhydrous Na_2HPO_4.

Strains. The wild type strain is syngen 4, stock 51s (non-Kappa-bearing). Mutants used here included four paranoiac strains (PaA, d4-90; PaA_1, d4-578; PaC, d4-150; fna^p, d4-149), a pawn (PwA, d4-95), and a fast (fA, d4-97), all derived from the wild type and selected for their altered swimming behavior. All strains were provided by Dr. C. Kung. Each mutant has a known defect in electrophysiological properties [8,15,16].

Isotopic labeling. 100 μCi/l of $H_3{}^{32}PO_4$ in 0.01 M HCl (New England Nuclear) was added to cerophyl medium buffered to pH 7.2 with Tris instead of phosphate.

Harvesting. The cells were harvested at mid-log phase (5000 cells/ml), by filtering the culture through four layers of cheesecloth and centrifuging at 200 × *g* for 1.75 min in pear-shaped oil centrifuge tubes. The cell pellet was transferred to Dryl's salt solution (250 ml/l of culture) with a Pasteur pipet, and washed twice by centrifugation. Dryl's salt solution contains 1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 2 mM Na_3 citrate and 1.5 mM $CaCl_2$.

Deciliation. After decanting the final Dryl's wash, the cell pellet was suspended in deciliating solution (0.15 M sucrose/15 mM Tris/2.5 mM Na_2EDTA/11% ethanol/30 mM KCl), (60 ml/l of culture), at 4°C and transferred to a 125 ml Erlenmeyer. 1.0 M $CaCl_2$ was immediately added (0.65 ml/60 ml of deciliating solution) and the solution was stirred for 5–10 min. The loss of cilia was monitored under a phase microscope, and after approximately 90% removal of the cilia the solution was centrifuged at 1500 × *g* for 3 min. The supernatant fluid was decanted and centrifuged at 10 000 × *g* for 20 min to pellet the cilia. The pellet, (deciliated bodies) from the 1500 × *g* spin was resuspended in cold Dryl's solution and centrifuged at 200 × *g* for 3 min.

Lipid extraction. The lipids were extracted using a modified Bligh and Dyer procedure [17]. The deciliated body or ciliary pellets were suspended in $CHCl_3$/MeOH/H_2O (1.25 : 2.5 : 1.0, v/v) and transferred to a 15 ml conical centrifuge tube. The centrifuge tube was closed with a serum stopper and flushed with nitrogen. After 20 min extraction on a vortex mixer, the solids were separated by centrifugation, and washed with another volume of the extracting solvent. The extract and wash were combined and treated with 2.5 ml each of $CHCl_3$ and H_2O. This solution was mixed and centrifuged at 1000 × *g* for 5 min. The chloroform phase containing the lipids was removed with a Pasteur pipet and concentrated on a rotary evaporator at 30°C. The lipids were redissolved in $CHCl_3$/MeOH (2 : 1, v/v) and for short-term storage were placed in tightly stoppered vials under an N_2 atmosphere.

Lipid separation. The phospholipids were separated on two-dimensional preparatory (750 μm) thin layer plates coated with Silica Gel G containing 13% $CaSO_4$ (Sigma). The solvent systems were: (a) $CHCl_3$/MeOH/NH_3 (aq) (65 : 25 : 5, v/v) and (b) $CHCl_3$/acetone/MeOH/HOAc/H_2O (6 : 8 : 2 : 2 : 1, v/v).

Quantitation. The individual phospholipids were detected using autoradiography and the corresponding locations on the thin layer chromatography (TLC) plates were marked and scraped into scintillation vials. The radioactivity was monitored using Patterson-Green scintillation solvent.

Analysis. Individual phospholipids were recovered from TLC plates after detection with autoradiography by scraping off and extracting the corresponding silica gel area twice with 4 ml $CHCl_3$/acetone/MeOH/H_2O (6 : 8 : 2 : 2). The $CHCl_3$ layer from the combined extracts separated upon the addition of H_2O and centrifugation, and was concentrated on a rotary evaporator. The dry lipid residue was taken up in $CHCl_3$/MeOH (2 : 1) and stored under N_2 at −20°C.

The migration of individual *Paramecium* lipids on TLC plates was compared

with that of authentic standards, including phosphatidylethanolamine, phosphatidylcholine, cardiolipin, sphingomyelin, *N,N*-dimethyl-phosphatidylethanolamine, phosphatidylinositol, lysophosphatidylethanolamine, lysophosphatidylcholine, *O*-phosphorylethanolamine, *O*-phosphorylcholine, aminoethyl phosphonate (all obtained through Sigma) and phosphatidylserine (extracted from bovine brain).

The solvent systems used to establish the identity and test the homogeneity of the $CHCl_3$-soluble lipids and their hydrolysis products were: (1) $CHCl_3$/MeOH/NH_3 (aq) (65 : 25 : 5), (2) $CHCl_3$/acetone/MeOH/HOAc/H_2O (6 : 8 : 2 : 2 : 1), (3) $CHCl_3$/MeOH/H_2O (65 : 25 : 4) and (4) $CHCl_3$/MeOH/HOAc/H_2O (25 : 15 : 4 : 2). Water-soluble hydrolysis products were separated and identified in three systems: (5) Phenol/H_2O (100 g/38 ml), (6) Phenol/H_2O)/EtOH/HOAc (50 : 5 : 6), and (7) isopropanol/H_2O/NH_3 (aq) (7 : 2 : 1).

Mild alkaline hydrolysis was done according to Dawson et al. [18]. Drastic acid hydrolysis for the determination of phosphonolipids was done according to Rosenberg [19], and enzymatic hydrolysis with phospholipases A from bee venom, C from *Clostridium welchii* and D from cabbage, all obtained through Sigma, were done according to Wells and Hanahan [20], Ottolenghi [21], and Kates and Sastry [22], respectively. Selective mild acid hydrolysis for the identification of plasmalogens was done as described by Dittmer and Wells [23].

The methods employed in sphingolipid determination were methanolysis in 1 M HCl/MeOH (10 M H_2O) at 75—80°C for 16 h for long chain base analysis as described by Carter and Gaver [24], periodic oxidation in 0.055 M periodic acid in methanol according to Sweeley and Moscatelli [25] and the synthesis of ceramide *N,N*-dimethyl-phosphorylethanolamine from sphingomyelin and exhaustive methylation as reported by Stoffel et al. [26].

Staining techniques included: molybdate reagent for phosphorus as described by Hanes and Isherwood [27], commercial ninhydrin spray from Sigma for —NH- and —NH_2 groups, the periodate-Schiff reagent for vicinal OH's according to Baddiley [28] and Dragendorf and Na_2CO_3/dipicrylamine reagents for choline as described by Beiss [29] and Ambruster and Beiss [30], respectively, and iodine for all lipids.

A DEAE column was prepared according to Rouser for the isolation of acidic phospholipids [31]. Autoradiograms were made with Kodak X-Omat X-Ray film. Radioactivity was measured except where noted with Cherenkov radiation in a Nuclear Chicago Mark II scintillation counter. Inorganic phosphate concentrations were measured using the Chen phosphate test [32], and ^{32}P organic/^{32}P inorganic ratios after drastic hydrolysis were obtained using the method described by Sugino and Myoshi [33]. The fraction of ^{32}P in phosphoproteins and nucleic acids was determined by the method of Schneider [34].

Results

Incorporation of $^{32}P_i$ into whole cells during growth. The cerophyl medium contained about 0.22 μmol/ml of total phosphate, of which about 0.12 μmol/ml was P_i. In this medium, cell number increased exponentially to a cell density of about 7000 cells/ml, with a generation time of about 11 h (Fig. 1). Uptake

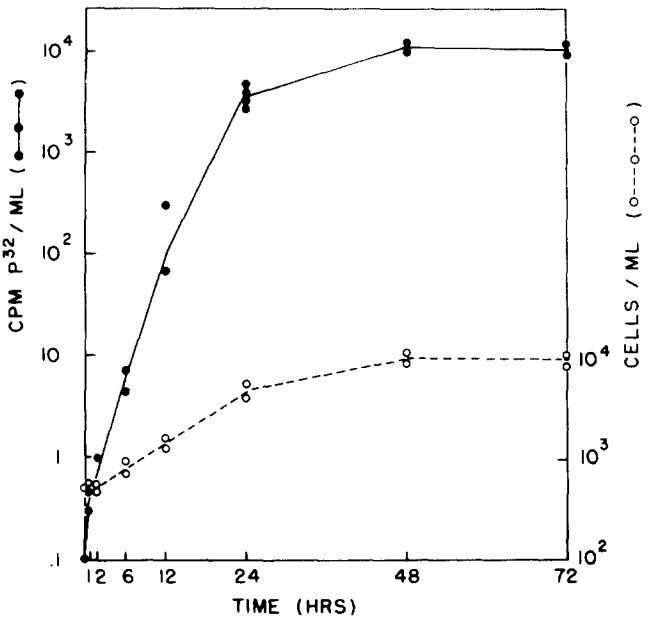


Fig. 1. Time course of ^{32}P incorporation into whole cells. Conditions for growth, labeling and harvesting of cells were as described in Materials and Methods. After the final wash in Dryl's solution, the cell pellet was suspended in cold, 5% trichloroacetic acid (5 vols. trichloroacetic acid per vol. packed cells). This suspension was filtered through Whatman GF-A glass fiber filters. The filters were washed with cold 5% trichloroacetic acid, dried, transferred to scintillation vials and counted in Patterson-Green scintillation solvent. Cell numbers were determined by direct visual count in the dissecting microscope of culture samples.

of $^{32}P_i$ from the medium increased with increased cell number, and when growth ceased, 2% of the $^{32}P_i$ from the medium had been incorporated into cellular material (Fig. 1). About 70% of the ^{32}P in cells at 48 h was in RNA, 24% was in lipids, 5% in DNA, and about 1% in phosphoproteins.

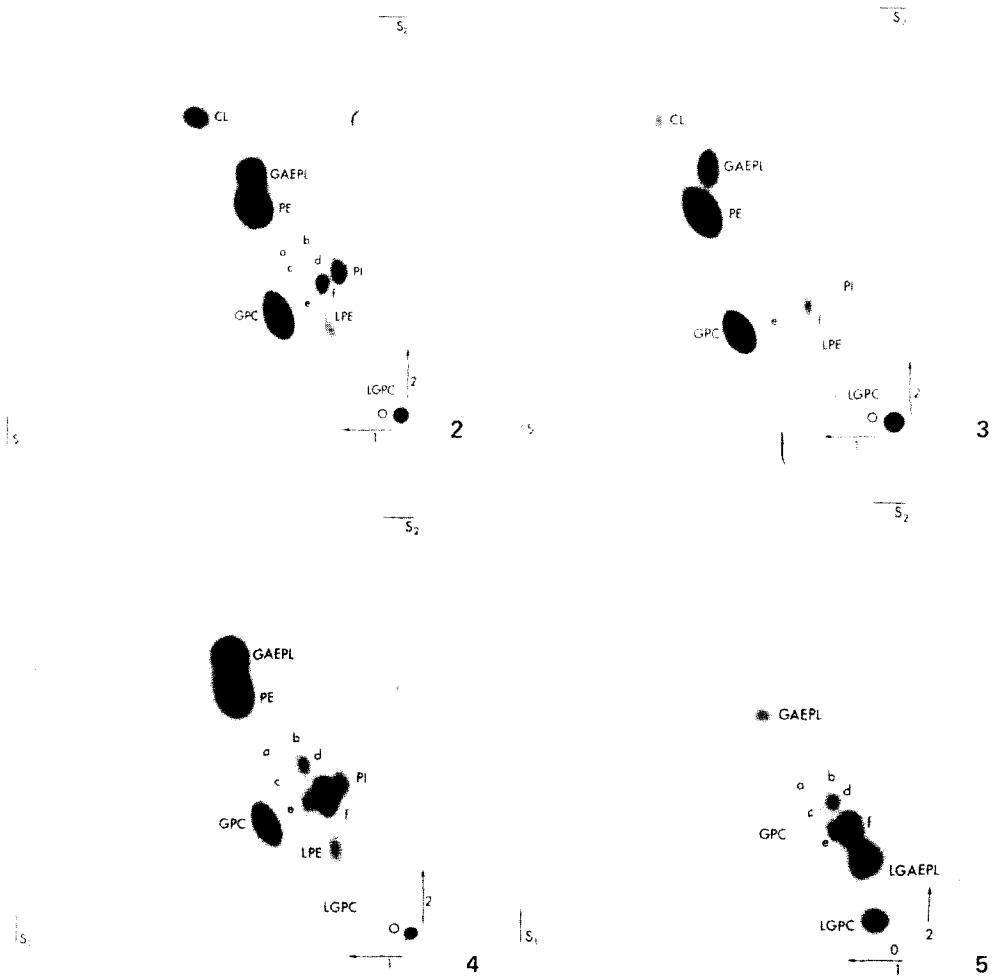
^{32}P in isolated cilia. Deciliation by the Ca-ethanol treatment yielded deciliated bodies and isolated cilia. The cilia contained 4% of the ^{32}P of intact cells, and 13 ± 2% (six measurements) of total cell ^{32}P lipid. The rest of the ^{32}P lipid remained associated with deciliated bodies. The absence of cardiolipin (a major mitochondrial lipid which was always found in deciliated bodies) in the isolated cilia suggested that cilia were relatively free of contamination by deciliated bodies or fragments of broken cells, and light microscopy confirmed the absence of bodies or large fragments.

Assessment of bacterial contamination. The washing procedure described above was intended to remove contaminating bacteria, and examination of washed paramecia and cilia by light and electron microscopy revealed no gross contamination of either fraction by bacteria. Differential centrifugation readily separated paramecia from the much smaller bacteria, but isolated cilia and bacteria were not so easily separated. We therefore assayed viable bacteria in our ciliary suspension by plating on nutrient agar or Cerophyl medium agar, and found less than one viable bacterium per 1000 cilia, representing no more than 0.01% contamination by mass.

The phospholipids of *Aerobacter aerogenes* consisted of phosphatidyl-

ethanolamine (85% of total) and about equal amounts of cardiolipin and phosphatidylglycerol (data not shown); no phosphonolipids were present. The absence of phosphatidylglycerol in our whole paramecia and isolated cilia, and the presence of large amounts of phosphonolipids in these fractions (see below) argues against major contamination of our *Paramecium* fractions by bacterial lipids. The fatty acid composition of *Aerobacter* lipids and *Paramecium* lipids was also distinctly different (data not shown).

Identification of the major ^{32}P-containing lipids. Labeled lipids, extracted from cilia or deciliated bodies with chloroform/methanol/H_2O, were separated by two-dimensional TLC, and detected by autoradiography (for 1—2 days, (Figs. 2—5). Individual labeled species were eluted and characterized as



Figs. 2—5. Two-dimensional TLC of ^{32}P-lipids. Cells were labeled, harvested, deciliated and extracted as in Materials and Methods, and phospholipids were separated by TLC. Labeled regions were detected by autoradiography. Fig. 2, ^{32}P-lipids of whole cells; Fig. 3, ^{32}P-lipids of deciliated bodies; Fig. 4, ^{32}P-lipids of cilia; Fig. 5, ^{32}P-lipids of cilia after mild alkaline hydrolysis. CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LPE, lysophosphatidylethanolamine; other abbreviations as in text.

described below. With the limited quantities of ciliary lipids available, certain types of structural analysis (e.g. P : N : fatty acid ratios) were impossible. We could, nevertheless identify the major labeled species.

1-Alkyl-2-acyl-sn-glycero-3-(2-aminoethyl) phosphonate (GAEPL). On TLC plates, this compound was ninhydrin positive. Mild alkaline hydrolysis yielded a labeled product still soluble in chloroform, with R_F values in TLC systems 1 and 2 very similar to those of lysophosphatidylethanolamine. This product was unaltered by the acid conditions normally used to cleave vinyl ether linkages (see Materials and Methods) and was not susceptible to hydrolysis by phospholiphase A from bee venom. Phospholipase A treatment of the original compound showed complete conversion to a (presumably lyso) derivative, and phospholipase C from *C. welchii* released a labeled, water-soluble product that co-migrated with authentic aminoethyl phosphonate in TLC systems 5 and 6. Phospholipase A from bee venom cleaves 2-acyl linkages specifically [35] establishing the acid- and alkali-insensitive linkage in position 1 of the glycerol moiety. The exact nature of this linkage was shown by comigration of the $CHCl_3$-soluble compound released on drastic acid hydrolysis with glyceryl-1-hexadecyl ether in light petroleum/diethyl ether/HOAc (70 : 30 : 1). Phospholipase D did not attack the compound, but phospholipase C released 2-aminoethyl phosphonate. Drastic acid hydrolysis of the original compound released no $^{32}P_i$, indicating the presence of a direct P-C bond in the compound.

Phosphatidylethanolamine. This ninhydrin-positive compound co-migrated with phosphatidylethanolamine in TLC systems 1—4. Mild alkaline hydrolysis converted the ^{32}P into a water-soluble form, and drastic acid hydrolysis quantitatively converted the ^{32}P to $^{32}P_i$, indicating the absence of a P-C bond. Treatment of the original compound with phospholipase C yielded a product that co-migrated with *O*-phosphorylethanolamine in TLC systems 5 and 6.

1-Alkyl-2-acyl-sn-glycero-3-phosphorylcholine (GPC). The third major phospholipid contained choline as established by Dragendorf and Na_2CO_3/dipicrylamine staining. This compound migrated slightly ahead of phosphorylcholine in systems 1—4 but the water-soluble phospholipase C hydrolysis product co-migrated with *O*-phosphorylcholine in systems 5 and 6. The mild acid-insensitive, $CHCl_3$-soluble product from mild alkaline and phospholipase A hydrolysis closely migrated with lysophosphorylcholine. The position and nature of the linkage in the 'lyso compound' was established by phospholipase A specificity and co-migration with glyceryl-1-hexadecyl ether as done with GAEPL. Drastic hydrolysis of the original labeled compound released all the phosphorus as P_i.

Phosphatidylinositol. This compound reacted positively to the Schiff-periodate stain and was isolated with the acidic lipids from a DEAE column. Co-migration with phosphatidylinositol was seen in the systems 1—4, and co-migration of the water-soluble phosphate after phospholipase C digestion was seen with the phospholipase C product of phosphatidylinositol in system 7. The lipid was sensitive to mild alkaline hydrolysis and the phosphorus was in the inorganic form after drastic acid hydrolysis.

Cardiolipin. Authentic cardiolipin comigrated with this mild alkali-sensitive phospholipid in systems 1—4. The acidic nature of this lipid was further confirmed by its behavior on a DEAE column.

Lysophosphatidylethanolamine. This compound was ninhydrin-positive staining, susceptible to mild alkaline treatment and it co-migrated with synthetic lysophosphatidylethanolamine in systems 1—4.

Lyso GAEPL. Though also ninhydrin staining, this compound was not susceptible to mild alkaline treatment. It co-migrated with the $CHCl_3$-soluble phospholipase A product of GAEPL in systems 1—4, and it was clearly separable from lysophosphatidylethanolamine in system 1.

Lyso GPC. This compound was choline-positive staining, not susceptible to alkaline hydrolysis, and it co-migrated with the $CHCl_3$-soluble phospholipase A product of GPC in systems 1—4.

Minor components a—f

All six components are probably sphingolipids. The relatively minor species labeled a—f (Fig. 4) were all unaltered by treatment with mild alkali or mild acid (Fig. 5), indicating the absence of ester-linked fatty acids. Stronger acid hydrolysis (sufficient to break fatty amide linkages) converted compounds e and f into products that co-migrated with authentic 4D-hydroxysphinganine (phytosphingosine) in $CHCl_3$/MeOH/2 M NH_3 (40 : 10 : 1). Compounds a, b, c and d were also altered by this moderate acid treatment, but the products could not be conclusively identified.

All of the compounds a—e were labeled when [3-^{14}C]serine (a biosynthetic precursor of sphingosine) was added to the medium during growth, and the ratio $^{14}C/^{32}P$ was greater in the six minor species than in GAEPL, phosphatidylethanolamine, or GPC (Table I), as expected if the minor components are sphingolipids.

Exhaustive methylation in chloroform/methanol/CH_3I at 37°C converted all six compounds into products that resembled sphingomyelin on two-dimensional TLC. Ceramide *N,N*-dimethylphosphorylethanolamine, which was clearly separable from the compounds a to f by two-dimensional TLC, was converted into sphingomyelin by the same methylation procedure.

All six compounds are probably ethanolamine derivatives. Fluorescamine spray converted all six compounds into fluorescent derivatives, indicating the presence of primary amines. Under the same conditions a sphingomyelin standard gave no reaction. None of these compounds was found in the acidic phospholipid fractions eluted from a DEAE-cellulose column, indicating that none

TABLE I

DISTRIBUTION OF ^{14}C AND ^{32}P IN *P*-LIPIDS OF *PARAMECIUM* FOLLOWING GROWTH IN [3-^{14}C]SERINE AND $^{32}P_i$

	Compound (Fig. 2)								
	GAEPL	PE	GPL	a	b	c	d	e (COPE)	f (CAEP)
Ratio: $^{14}C/^{32}P$	0.12	0.30	0.66	0.80	0.74	0.88	0.71	0.90	0.72

Cells for the double label experiment were grown in 11 l cerophyl medium buffered with Tris (instead of phosphate) to pH 7.2 and supplemented with 50 μCi ^{32}P and 20 μCi L-[3-^{14}C]serine. Harvesting, lipid extraction, TLC, extraction of separated lipids, and quantitation were exactly as described in Materials and Methods. PE, phosphatidylethanolamine.

contained an acidic head group. Phospholipase C released a water-soluble product from compund f which co-migrated with authentic aminoethylphosphonate in the TLC systems 5 and 6.

Compounds b, c and f are phosphonolipids. Drastic acid hydrolysis yielded P_i from compounds a, c and e (indicating the presence of normal phosphate esters), but no P_i was released from b, d or f, suggesting that they were phosphonates.

Compounds e and f are ethanolamine-phosphate and 2-aminoethyl phosphonate derivatives of 4D-hydroxysphinganine (phytosphingosine). Treatment of the deacylated ciliary lipids (containing unaltered compounds a–f, Fig. 5) with periodic acid under conditions which allow oxidation of vicinal hydroxyls caused the disappearance of compounds e and f, and the appearance of labeled material at the origin. Treatment of isolated compounds e and f yielded the same results, suggesting that these compounds are 4D-hydroxysphinganine derivatives.

After moderate acid hydrolysis of compound f, both ether and water phases contained primary amines, determined by reaction with fluorescamine, and all of the phosphate after this treatment was in the aqueous phase. As noted above, exhaustive methylation of f yielded a product very similar to sphingomyelin, phospholipase C released aminoethyl phosphonate, and periodate cleaved compound F as expected for 4D-hydroxysphinganine derivatives. We conclude that compound f is *N*-acyl-1-*O*-(2′-aminoethyl) phosphonyl-4D-hydroxysphinganine (CAEP).

Results with compound e were similar, except that strong acid hydrolysis released P_i, and we conclude that this compound is *N*-acyl-1-*O*-phosphoryl-ethanolamine-4D-hydroxysphinganine (COPE).

P-lipid composition of whole cells, cilia, and deciliated bodies. The two cell fractions examined from *Paramecium* differed in composition (Table II). Cardiolipin was found only in the deciliated bodies which also had a slight enrichment of phosphatidylethanolamine (41% of the lipid phosphorus compared with 37% in the cilia). Cilia were enriched with two phosphonolipids, GAEPL and CAEP, representing 28 and 15% of the lipid phosphorus in the cilia but only 15 and 3% of the lipid phosphorus in the deciliated bodies, respectively. Phosphatidylinositol represented 3% of the lipid *P* in both fractions.

P-lipid composition at different stages of growth. The pattern of ^{32}P-labeled lipids obtained from cells in late exponential phase (48 h, Fig. 1) and stationary phase (72 h, Fig. 1) was virtually indistinguishable from that seen with cells harvested early in exponential phase (24 h, Fig. 1). The same major lipids were present in the same proportions (Fig. 6) at all three stages of growth, as were the minor lipids (sphingolipids).

P-lipid composition of behavioral mutants. The growth curves of the several behavioral mutants were not identical (data not shown) with that for the wild type; some of the mutant strains began to die and to lyse near the end of the exponential phase of growth. We therefore studied the lipid composition of mutant strains only in the early exponential phase, when cells appeared normal and no lysis was occurring. We examined six mutant strains with three different behavioral phenotypes (pawn, paranoiac, and fast), and found their *P*-lipid composition to be indistinguishable from that of wild type cells (Table III),

TABLE II

DISTRIBUTION OF PHOSPHOLIPIDS IN *PARAMECIUM* AND *TETRAHYMENA*

The values for *Paramecium* are the average of 3 experiments. For the major lipids representing >10%, none of the values differed by more than 3% from the average. All values for the minor lipids were within 1% of the average. The high values for the lyso lipids in the deciliated body fraction are due to breakdown during isolation. This breakdown was not seen in either the whole cell or ciliary fractions. Results are expressed as percent of total ^{32}P-lipid.

	Paramecium			*Tetrahymena* *		
	Whole Cells	Cilia	Deciliated Bodies	Whole Cells	Cilia	Deciliated Bodies,
Cardiolipin	4	—	4	5	1	4
GAEPL	16	30	13	23	47	21
GPE				37	11	33
Phosphatidylethanolamine	41	37	37			
GPC	30	11	30	33 **	28 **	31 **
CAEP	3	15	1			
Phosphatidylinositol	3	2	3			
LGAEP	<1	<1	2			
LPE	1	<1	4			
LGPC	<1	<1	3	2	1	3
Minor lipids	<1	2	—			

* Data from Nozawa and Thompson [38].

** May also contain lysophosphatidylethanolamine and LGAEPL.

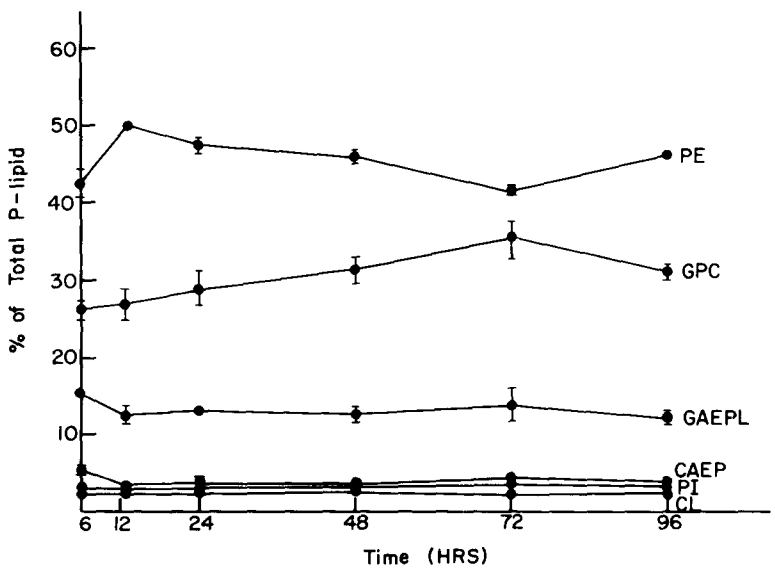


Fig. 6. Phospholipid composition of whole cells as a function of growth stage. Cells cultured and labeled as in Fig. 1 were harvested at the indicated times, and phospholipid composition determined as described in Fig. 1 and Materials and Methods. Two separate and independent cultures were analyzed, and the average of the two values is plotted here, with bars to indicate the range. PE, phosphatidylethanolamine; CL, cardiolipin; PI, phosphatidylinositol.

TABLE III

PHOSPHOLIPID COMPOSITION OF WHOLE CELLS, DECILIATED BODIES, AND CILIA OF WILD TYPE *PARAMECIUM* AND OF 6 MUTANT STRAINS

Two separate cultures of each strain were labeled by growth in $^{32}P_i$ and independently analyzed for phospholipid composition exactly as described in Materials and Methods, except that for the mutant strains, cultures were harvested in early exponential phase of growth. Results are expressed as percent of total ^{32}P-lipid.

	PWA		fA97		fna^{P}		PaA		PaA$_1$		PaC		WT
	a. Intact cells												
Cardiolipin	4	5	4	4	5	6	4	5	7	6	6	6	4
GAEPL	12	13	14	13	15	15	15	15	14	14	13	14	16
Phosphatidylethanolamine	40	39	37	34	42	41	42	40	41	39	40	39	41
GPC	32	31	33	30	30	28	28	29	28	29	31	31	30
Phosphatidylinositol	4	4	3	2	3	2	3	3	2	2	3	3	3
CAEP (f)	3	4	4	4	4	4	3	3	3	6	4	3	2
COPE (e)	1	1	1	1			1				1	1	1
Lysophosphatidylethanolamine	1	1	1	1			3	3	1	1		2	1
LGPC	1			1									
AEPL			1	2									
GPE			2	3									
	b. Deciliated bodies												
Cardiolipin	5	5	4	5	6	7	6	4	6	6	5	6	4
GAEPL	10	12	9	11	12	13	10	14	16	12	12	12	13
Phosphatidylethanolamine	39	39	35	34	40	40	38	40	26	35	40	40	37
GPC	37	36	39	36	30	30	32	34	36	32	31	36	30
Phosphatidylinositol	3	3	3	3	2	2	4	3	4	3	3	3	3
CAEP (f)	3	3	2	1	2	3	1	1	3	3	2	1	1
Lysophosphatidylethanolamine	2	3	3	5	2	3	6	4	3	3	3	4	4
LGPC	2	2	2	1	2	2	2	3	2	1	2	2	2
AEPL			1										
GPE			2										
	c. Cilia												
GAEPL	30	24	26	31	33	35	31	32	29	25	28	31	30
Phosphatidylethanolamine	36	35	30	31	30	30	30	31	31	36	33	32	37
GPC	14	18	14	12	10	8	11	11	10	16	12	10	11
Phosphatidylinositol	2	2	3	2	2	2	2	2	2	2	2	2	2
CAEP (f)	11	16	18	14	20	17	15	16	20	11	18	16	15
COPE (e)	3	2	3	3	3	2	2	2	3	2	3	1	2
d	1	1	1	1	1	1	1	1	1	1	1	1	1
c	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
b	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
a	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Lysophosphatidylethanolamine	2	2	2	5			5	5		5	2	5	
LGPC													

with one exception: the 'fast' mutant fA 97 contained two *P*-lipids not present in wild type or the other mutants. These lipids have been tentatively identified, on the basis of R_F values, reaction with specific spray reagents, and sensitivity to alkaline hydrolysis, as AEPL and GPE. They are not major components (approx. 2%) of the mutants, but their levels are much higher in fA 97 than in the wild type, where they are undetectable by our methods.

Discussion

The ciliary membrane of *Paramecium* contains a collection of lipids both qualitatively and quantitatively different from the membranes of deciliated cells. Deciliated cells are relatively rich in cardiolipin, phosphatidylethanolamine, and GPC, and cilia contain relatively large amounts of GAEPL, CAEP, COPE, and four other phosphosphingolipids.

We have established several chemical properties of the four minor phosphosphingolipids but have not conclusively identified them. Catalytic hydrogenation using 10% palladium on carbon had no effect on the R_F values of these four lipids. Therefore, the differences of R_F values seen on the initial TLC plates are not exclusively due to their long chain bases being either spingosine or sphinganine (dihydrosphingosine). Also, treatment of e and f under dehydrating conditions caused no conversion of these lipids into a and c, and b and d, respectively. The differences among these four lipids may well lie in the composition of the fatty acids. In *Tetrahymena* there are reports of α-hydroxylated fatty acids in amide linkages with the long chain bases of the sphingolipids [36] and it seems likely that compounds with α OH fatty acids would migrate differently from the non-hydroxylated analog.

Very similar conclusions regarding the distribution of the major and the identified minor lipids in *Paramecium* were independently reached by Rhoads and Kaneshiro [37]. They found enrichment of cardiolipin and phosphatidylinositol in deciliated bodies, and of phosphonolipids in the cilia. This phospholipid composition of *Paramecium* is also similar to that of the related ciliate *Tetrahymena pyriformis* [38], the major lipids of which are AEPL, GPC, GPE, and phosphatidylethanolamine (Table I). Though the ciliary phospholipids of *Tetrahymena* are enriched in phosphonates and are different from those of the pellicle membrane, the pellicle fraction characterized by Thompson and co-workers [38] consists of two bilayers, the alveolar membrane and the surface membrane. The suggestion has been made [38] that the outer pellicle membrane is probably identical with that of the cilia, and the compositional difference seen between the cilia and the pellicle results from the contribution of the alveolar membrane to the pellicle fraction.

We have not succeeded in fractionating the membranes of deciliated cells with sufficient resolution to study their compositions directly. Our quantitative results do suggest, however, the the ciliary membranes are unique. We found that approximately 13% of the total cellular phospholipids were in the cilia. Since ciliary membranes represent about 66% of the total cell surface in *Paramecium*, the outer membrane of the pellicle, representing 33% of the surface area must contain about 7% of the total cellular lipids or about 10% of the lipids of deciliated cells. Therefore the absence of minor sphingolipids in the

deciliated bodies coupled with their appearance in the extracts from whole cells strongly suggests that these sphingolipids are located exclusively in the ciliary membrane. These results need not be different from those in *Tetrahymena* since only the distribution of the major lipids was reported in *Tetrahymena* [36,38,39]. In any case, the relative abundance of phosphonolipids and sphingolipids in the ciliary membrane of *Paramecium* may reflect a functional specialization.

The phospholipid composition found for *Paramecium* is practically invariant with differences in growth stage, implying that *P*-lipid synthesis and turnover are tightly regulated. Even the minor sphingolipids, representing 1 or a few percent of the total *P*-lipids, are present in constant proportions. In *Tetrahymena* there is one report of a change in *P*-lipid content occurring with changes in the growth stage [39] and the changes known to occur in the fatty acid composition with growth may be associated with the triglyceride fraction and not with the fatty acid composition of the *P*-lipids [40,41]. The constancy of composition in wild type cells allowed us to make comparisons with mutants, even though their growth characteristics were not identical with those of the wild type. Within the limits of our experimental methods, there were no differences among the phospholipids of wild type, four paranoiac strains and one pawn strain. The fast strain showed a small but reproducible difference in *P*-lipid pattern, the present of two novel species (AEPL and GPE) representing about 4% of the total *P*-lipid. These compounds were localized primarily in the deciliated body fraction, not in cilia. Rhoads and Kaneshiro (personal communication) have detected a small amount of GPE in wild type *Paramecium* (in the same strain we used) grown under different, axenic conditions and both AEPL and GPE have been found in *Tetrahymena pyriformis* [38]. The reports of the sensitivity of the *P*-lipid content of *Tetrahymena* to the lipids in the growth media may explain the fluctuaction of GPE in wild type *Paramecium*, but it is difficult to assess the significance of AEPL and GPE in the fast strain.

We examined the phospholipids of behavioral mutants to test the hypothesis that the mutant phenotypes were the result of alterations in phospholipids which interact with and influence the function of ion gates in the excitable membrane. Our findings exclude the possibility of gross differences in *P*-lipid head groups in the mutants we examined, but leave open the possibility that the lesions are due to some alteration in lipid composition such as altered fatty acid or sterol content, or changes in head groups for a small fraction of the total lipid, which interacts specifically with the ion gates.

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