minireview

Lipids of *Paramecium*

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Abstract This review is the first on the composition and metabolism of *Paramecium* lipids. This ciliated protozoa is a useful system for studying the structure and function of biomembranes since it can be grown under chemically defined culture conditions in large numbers; much is known about its genetics, membrane electrophysiology, and ultrastructure; and mutants with defective membrane functions are available which are reported to have lipid alterations. Pure preparations of the cell surface ciliary membrane are readily isolated. The organism and its ciliary membrane contain a variety of polar lipids, sterols, and steryl esters. The polar lipids include substantial amounts of ether lipids, sphingolipids, and phosphonolipids. The biosyntheses of fatty acids and specific moieties of complex lipids in this organism are beginning to be examined with promises of elucidating biosynthetic mechanisms that are more difficult to study in other organisms. More information on lipid metabolism is required to identify the bases for the defects in putative lipid/membrane mutants.—**Kaneshiro, E. S.** Lipids of *Paramecium*. *J. Lipid Res.* 1987. **28:** 1241–1258.

Supplementary key words cilia • ether lipids • membranes • phosphonolipids • sphingolipids • ciliated protozoa • ion channels • mutants • thermal avoidance

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I. *Paramecium*, a eukaryotic protozoan used for the study of membranes

In the period from 1930 into the 1970s substantial progress was made on the understanding of nuclear divisions, mating, and genetics of *P. aurelia*, primarily by Sonneborn (1, 2) Preer (3), and their colleagues. During the same period, significant advances were made on the understanding of membrane electrophysiological events of *P. caudatum* by workers such as Kamada and Kinosita (4–6), and later by Naitoh, Eckert, and others (7–9). Being a larger cell than *P. aurelia*, *P. caudatum* was easier to manipulate for intracellular recordings of membrane electrical responses. Current technology enables similar studies on *P. aurelia* (see below). The avoidance reaction (10) in *Paramecium* was explained by the Ca^{2+} hypothesis (7–9). Depolarization of the surface membrane activates voltage-sensitive Ca^{2+} channels in the ciliary membrane (11, 12). The opening of these channels permits extracellular Ca^{2+} to move down its concentration gradient into the cilia. The increased intraciliary Ca^{2+} levels trigger the reversal of the effective stroke of ciliary beat resulting in

Abbreviations: AEP, aminoethylphosphonate; APnP, 2-amino-3-phosphonopropanate; ATP, adenosine triphosphate; CoA, coenzyme A; CL, cardiolipin; CMP, cytidine monophosphate; DPnE, N-acyl-sphinganine-1-phosphonoethanolamine, N-acyl-dihydrosphingosine-1-phosphonoethanolamine; DPsE, N-acyl-sphinganine-1-phosphoethanolamine, N-acyl-dihydrosphingosine-1-phosphoethanolamine; GTP, guanosine triphosphate; IP_3, inositol triphosphate; LCB, long-chain base; PA, phosphatidic acid; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine and 1-alkyl, 2-acyl-*sn*-glycero-3-phosphocholine; PEP, phosphoenolpyruvate; PI, 1,2-diacyl-*sn*-glycero-3-phosphoinositol and 1-alkyl-2-acyl-*sn*-glycero-3-phosphoinositol, phosphatidylinositol; PI-P, phosphatidylinositol phosphate; PI-P_2, phosphatidylinositol diphosphate; PnE, 1,2-diacyl-*sn*-glycero-3-(2-aminoethyl)phosphonate and 1-alkyl, 2-acyl-*sn*-glycero-3-(2-aminoethyl) phosphonate; 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; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine and 1-alkyl, 2-acyl-*sn*-glycero-3-phosphoserine; PsE, 1,2-diacyl-*sn*-glycero-3-(2-aminoethyl)phosphate and 1-alkyl, 2-acyl-*sn*-3-(2-amino-ethyl) phosphate; PUFA, polyunsaturated fatty acids; RSA, relative specific activity; SFA, saturated fatty acids; SPL, sphingophospholipids and sphingophosphonolipids; SPnE, N-acyl-sphingenine-1-phosphonoethanolamine, N-acyl-sphingosinephosphonoethanolamine; SPsE, N-acyl-sphingenine-1-phosphoethanolamine, N-acyl-sphingosine-1-phosphoethanolamine; UI, unsaturation index.

backward swimming. The channel is inactivated or closed by elevated intraciliary Ca^{2+} levels (13); and membrane ATPases (14, 15) pump Ca^{2+} out during the renormalization period during which the cell pivots around its posterior end (10). When intraciliary Ca^{2+} levels are sufficiently decreased, the cell swims in the normal forward direction. After Kung (16) isolated a membrane (behavioral) mutant of *P. tetraurelia* and, with Eckert and Naitoh (17, 18), demonstrated that the pawn mutant had defective membrane voltage-sensitive Ca^{2+} channels, the species of choice for most biochemical studies has been *P. tetraurelia.* Over 300 membrane mutant lines representing more than 25 complementation groups have since been isolated (19, 20). In many cases, the mutations have been correlated with altered membrane electrical properties.

Paramecium aurelia is not only a useful unicellular eukaryotic system for studying the structure and function of electrically excitable membranes, but this ciliate also serves as a model for exocytosis. The extrusion organelle, the trichocyst, is synthesized and assembled in the cytoplasm, moves to the cell surface, docks at predetermined sites at the cell surface where it attaches to the cell membrane, and the contents of the membrane-bound organelle are released upon fusion of trichocyst and cell surface membranes (21–25). Membrane fusion results from events triggered by an appropriate stimulus and involves an influx of Ca^{2+} (22, 23). Defects at different steps in trichocyst release in mutants have been correlated with altered intramembranous particle arrays suggesting specific functions for different arrays. The "ring" and "rosette" arrays are in the cell surface membrane and the "annulus" is in the trichocyst membrane (21, 23–25). Nondischarge mutants have been isolated that are either defective in the formation of trichocysts, fail to attach trichocysts to preformed sites at the cell membrane (abnormal "annulus" or "ring"), or fail to respond to stimuli even when properly docked (defective "rosette") (21, 23–25).

Paramecium tetraurelia has more recently been developed as a system for understanding chemoreception and the nature of receptor binding and signal transduction in chemotaxis (26, 27). Attractants such as folic acid have been shown to bind to surface membrane receptor sites. Binding of an attractant is correlated with hyperpolarization of the cell membrane and increased frequency of the beat of somatic cilia. The cell, therefore, swims faster toward an attractant. A chemotactic mutant, d_4534, that does not respond to folate fails to bind the attractant molecule (26, 27).

Ample information is now available on the ultrastructure, physiology, and genetics of *P. tetraurelia.* These cells can be axenically cultured under chemically defined conditions (28, 29) and subfractionation procedures have been developed (30–32). The bulk of analyses done on *Paramecium* lipids to date are on *P. tetraurelia.* Very little is currently known about the lipids of other species, thus, unless otherwise indicated, this review will be on the lipids of this species.

II. Lipid nutritional requirements for growth of *Paramecium*

The first ciliated protozoan that was found to require lipids for growth was *Paramecium.* This organism requires a C_{24} alkyl-substituted sterol with a double bond at the $\Delta 5$ or the $\Delta 7$ position, best fulfilled by stigmasterol or poriferasterol (33, 34). It also needs a fatty acid and/or complex lipids containing fatty acids. *Paramecium aurelia* has a stringent requirement for oleic acid, presumably being unable to desaturate stearate (34). The fatty acids required by other species such as *P. multimicronucleatum, P. calkinsi,* and *P. caudatum* may be different. The reader is referred to a review of earlier nutritional studies by Van Wagtendonk (34) who was the investigator primarily responsible for the establishment of axenic cultures and the eventual formulation of chemically defined media for the growth of *Paramecium* which made reliable biochemical studies possible utilizing this ciliated unicell. To date, a comprehensive review of the lipids of *Paramecium* has not been published.

Although studies of *P. tetraurelia* lipids have been done on cells grown in a grass (Cerophyl) or lettuce extract with bacteria, most of the lipids identified and characterized have been documented by analyses of lipids from axenically grown cells. This review focuses on the latter studies. A modified (35) enriched crude medium (34) that includes stigmasterol, phosphatidylethanolamine (PsE), and a mixture of fatty acids is commonly used. Peptones and crude RNA preparations in this medium are not usually lipid-free and often contain fatty acids and sterols. Also, some commercially available phospholipids contain sterol contaminants. In some studies a chemically defined medium containing the same lipid supplements as those included in the crude medium is used (29, 34, 35). In other studies that require stringent, defined conditions, stigmasterol and either oleate, monolein, or synthetic dioleoyl PsE are the only lipids included in the chemically defined medium (29, 34, 35).

III. Uptake of lipids

The degree to which *P. tetraurelia* (36) and *P. multimicronucleatum* (originally designated as *P. caudatum* and subsequently identified as *P. multimicronucleatum,* 37) (38) remove lipids from the culture medium indicates that these compounds are selectively and efficiently transported by the cells **(Fig. 1).** Stationary phase *P. tetraurelia* cells (2–3 ng of lipids/cell) transferred into fresh medium have high rates of lipid uptake and, consequently, cells in early culture growth (lag phase) have high concentrations of lipids (7–13 ng/cell) and are three times larger than stationary phase cells (35, 39). Lipid droplets

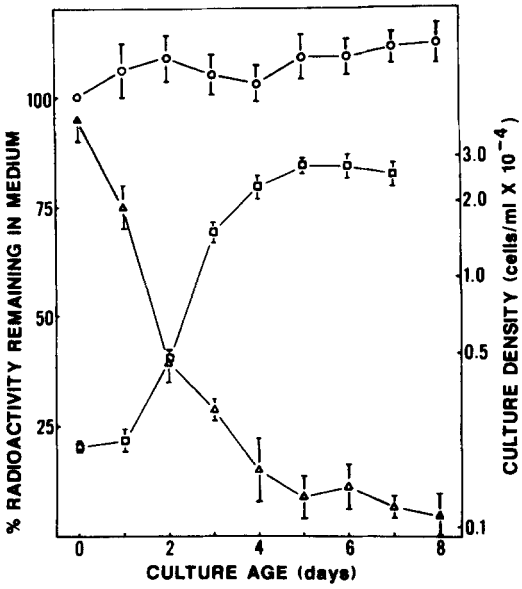

Fig. 1. The depletion of radiolabeled oleic acid and amino acids from the culture medium during culture growth. *Paramecium tetraurelia* 51s was grown with [U-^{14}C]oleic acid (Δ) (37) or [U-^{14}C]protein hydrolysate (○) (Reuter, S. F., S. J. Merkel, P. J. Soldano, and E. S. Kaneshiro, unpublished data). The radioactivity remaining in the medium was determined during culture growth (□) (49). Death phase occurred after day-8 and no live cells were seen after day-12. The cells rapidly removed the fatty acid from the medium whereas amino acids remained in high concentration in the medium. Similar observations were made on *P. multimicronucleatum* (38).

are visible in these cells which may be sites of triglyceride stores (triglycerides increase tenfold from 145 to 1,500 pg/cell (39)). The lipid content per cell and lipid droplets decline throughout log phase (40). Onset of stationary phase follows the depletion of lipids from the medium (36, 38). Supplementation of cultures depleted of exogenous lipids, with a mixture of lipids equivalent to the concentrations in fresh media, increased culture densities and prolonged log phase (Reuter, S. F., S. J. Merkel, P. J. Soldano, and E. S. Kaneshiro, unpublished data). In stationary and death phases, and in clonally aged cells, membrane-bound lipofuscin-like vesicles are present in the cytoplasm (41, 42). These vesicles have not been isolated and direct analyses of their contents have not been done. Thus, whether or not they contain lipids that the cell is unable to metabolize or contain peroxidation products of cellular lipids, as described in lipofuscin from other organisms (43), is not known.

Phagocytotic rates (food vacuole formation) in *P. multimicronucleatum* were highest at early culture ages (38) which could account for the rapid increase in lipids in lag phase cells. However, in *P. tetraurelia,* food vacuole formation was highest during log phase and lowest in lag and death phases (Reuter, S. F., S. J. Merkel, P. J. Soldano, and E. S. Kaneshiro, unpublished data). Thus, the rate of lipid uptake in *P. tetraurelia* is not correlated with the rate of food vacuole formation, suggesting that bulk transport is not the major mechanism for uptake of these compounds.

Whether or not fatty acids were transported by simple diffusion was examined (Reuter, S. F., S. J. Merkel, P. J. Soldano, and E. S. Kaneshiro, unpublished studies). Uptake rates of straight chain saturated fatty acids of even carbon numbers, from 2:0 to 20:0, were not directly correlated with chain length (hydrophobicity). Thus it is unlikely that simple diffusion of fatty acids across the cell surface membrane is the major mechanism of transport. It was further demonstrated that the uptake rates of radiolabeled fatty acids were competitively inhibited by the presence of other (unlabeled) fatty acids **(Fig. 2)**; thus fatty acid transport appears to involve carrier-mediated mechanisms.

IV. Total lipids

The following lipid classes have been identified in *Paramecium* **(Table 1):** stigmasterol, 7-dehydrostigmasterol, cholesterol, 7-dehydrocholesterol, stigmasteryl esters, 7-dehydrostigmasteryl esters, cholesteryl esters, 7-dehydrocholesteryl esters, free fatty acids, free fatty alcohols, monoglycerides, diglycerides, triglycerides, two neutral ceramides, four glycosphingolipids, cardiolipin (CL), quinones, phosphatidylcholines (PC), choline sphingophospholipids (choline SPL), phosphatidylethanolamine (PsE) and its phosphono analog (PnE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylinositol phos-

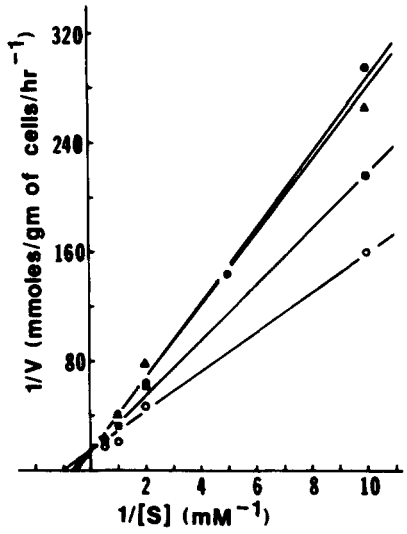

Fig. 2. Uptake of radiolabeled palmitic acid by *Paramecium tetraurelia* 51s. Cells were grown for 5 days in the enriched crude medium, then washed and equilibrated in a nonnutrient buffer solution for 1 hr at 25°C. The cell suspension was then incubated for 1 hr with either 0.1, 0.5, 1.0, or 2.0 mM palmitate and tracer amounts of [1-^{14}C]palmitate in the absence of an inhibitor (○, or in the presence of 4.0 mM) 4:0 (■), 12:0 (▲), or 18:0 (●). Double reciprocal plots indicating the crossing of lines at the y-axis suggest that fatty acid transport is carrier-mediated. Data are from Kaneshiro, E. S., and S. F. Reuter, unpublished results.

TABLE 1. Composition of *Paramecium tetraurelia* whole cell and cilia lipids

Lipids	% by Weight[a]	
	Cells[b]	Cilia[b]
Monoglycerides, diglycerides, and fatty alcohols	0.9 (39)	0.9 (39)
Sterols	14.8 (39)	18.7 (39)
Cholesterol	3.6 (39)	5.2 (39)
7-Dehydrocholesterol	0.5 (39)	1.0 (39)
Stigmasterol	9.7 (39)	10.3 (39)
7-Dehydrostigmasterol	1.0 (39)	2.2 (39)
Free fatty acids	2.3 (39)	0.0 (39)
Quinones	2.1 (39)	0.4 (39)
Triglycerides	12.8 (39)	3.6 (39)
Steryl esters	8.0 (39)	5.3 (39)
Cholesterol	0.9 (39)	2.0 (39)
7-Dehydrocholesterol	0.2 (39)	0.1 (39)
Stigmasterol	5.3 (39)	2.0 (39)
7-Dehydrostigmasterol	1.7 (39)	1.3 (39)
Neutral sphingolipids (sum of two ceramides and four glycosphingolipids)[c]	7.2	4.1
Phosphatidylcholine (PC)	14.2 (40)	7.1 (40)
Diacyl PC	7.7 (54)	4.9 (54)
Alkacyl PC	4.7 (54)	2.1 (54)
Phosphatidylethanolamine (PsE)	15.5 (40)	7.5 (40)
Diacyl PsE	12.0 (54)	5.9 (54)
Alkacyl PsE	1.7 (54)	1.6 (54)
Ethanolamine phosphonolipid (PnE)	11.8 (40)	26.4 (40)
Diacyl PnE	1.4 (54)	11.1 (54)
Alkacyl PnE	10.3 (54)	15.3 (54)
Phosphatidylserine (PS)	1.2 (40)	4.2 (40)
Diacyl PS	0.9 (54)	2.8 (54)
Alkacyl PS	0.3 (54)	1.4 (54)
Phosphatidylinositol (PI)	1.9 (40)	2.0 (40)
Diacyl PI	1.8 (54)	1.5 (54)
Alkacyl PI	0.2 (54)	0.5 (54)
Phosphatidylinositol-phosphate (PI-P)	0.2 (49)	ND
Phosphatidylinositol-diphosphate (PI-P_2)	0.3 (49)	ND
Choline sphingolipids[c]	2.1	2.5
Ethanolamine sphingolipids	3.8 (40)	15.5 (40)
Dihydrosphingosine, phosphoryl (DPsE)	0.2 (52)	0.9 (52)
Sphingosine, phosphoryl (SPsE)	0.6 (52)	1.6 (52)
Phytosphingosine, phosphoryl (PPsE)	0.5 (52)	1.8 (52)
Dihydrosphingosine, phosphonyl (DPnE)	0.2 (52)	1.5 (52)
Sphingosine, phosphonyl (SPnE)	1.1 (52)	4.4 (52)
Phytosphingosine, phosphonyl (PPnE)	1.3 (52)	5.2 (52)
Cardiolipin (CL)[d]	0.8 (40)	0.0 (40)
Lyso, PC, PsE, PnE[e]	0.4 (40)	2.2 (40)
Phosphatidic acid (PA)	0.1 (49)	ND
CDP-diacylglycerol (CDP-DAG)	0.1 (49)	ND
Total neutral lipids	48.1	32.9
Total phospholipids	51.9	67.2

[a]Calculations are based on lipids extracted with an acidified solvent system (49). Original data from the indicated references were recalculated and expressed as total weight %. Quantitations are for cells grown axenically in the crude medium for 5 days at 25°C. Identifications and quantitations of *Paramecium* lipids are also confirmed in references 35, 44–52, and 75.

[b]Numbers in parentheses indicate reference with original data.

[c]Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished data.

[d]Calculations based on 1/2 mole %.

[e]Calculations based on 2/3 mole %.

phate (PI-P), phosphatidylinositol diphosphate (PI-P_2), phosphatidic acid (PA), CDP-diacylglycerol (CDP-DAG), and six ethanolamine SPL (two with dihydrosphingosine, two with sphingosine, and two with phytosphingosine, and three with P in phosphoryl bonds and three in phosphonyl bonds, DPsE, DPnE, SPsE, SPnE, PPsE, PPnE). The glycerophospholipids include diacyl and alkyl acyl molecular species. Lyso forms of the major glycerolipids, PC, PsE, and PnE, have been detected (20, 39, 40, 44–52, and Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished studies).

Total lipids/cell decrease with culture age (see above) in

P. tetraurelia and *P. multimicronucleatum*. The neutral and polar lipid fractions increased significantly upon inoculation of stationary phase cells into fresh medium and both fractions decreased with culture age (38–40, 49, 50) **(Fig. 3)**. The high concentration of polar lipids in axenically grown *P. multimicronucleatum* cells during early culture age was correlated with the proliferation of intracellular membrane profiles (foci of tubular rough endoplasmic reticulum) during this stage as observed by electron microscopy (42, 53).

In *P. tetraurelia,* the changes with culture age in the neutral lipid content per cell were larger than changes in polar lipids (39, 40, 51). All classes in the neutral lipid fraction (triglycerides, free sterols, steryl esters, free fatty acids) decreased with culture age (39). Since the decrease in the neutral lipid fraction was greater than that of the polar lipid fraction, the percentage of polar lipids increased with culture age (40). In the polar lipid fraction, the concentrations per cell of PsE, PC, and PS decreased with culture age (40). When the relative concentrations of different lipid classes as percent total lipids or percent total phospholipids were measured, PnE, CL and the neutral and ethanolamine SL fractions showed relative increases with age. This was reflected in the increase in the relative concentrations of total phosphonolipids, SPL, and ether lipids with culture age (40).

The lipids of isolated preparations of somatic cilia have been analyzed (Table 1 and **Table 2)**. The cilium is enriched in phosphonolipids (40, 45), SPL (40, 45, 52), ether lipids (40, 54), and polyunsaturated fatty acids (PUFA) (36) **(Table 3)**. Ciliary phospholipids increase in the percentage of phosphonolipids (40, 52) and SPL (40, 52) as cultures age. The relative amounts of PUFA among the ester-linked fatty acids of cilia increase (35), with the polar lipid fraction exhibiting a greater increase than the neutral lipid fraction (39, 40, 49). This increase in PUFA reflects the changes in the composition of glycerophospholipids (relative increase in PnE and decreases in PsE, PC, and PS). Only slight changes in the fatty acid compositions of individual glycerophospholipids occur (40, 49).

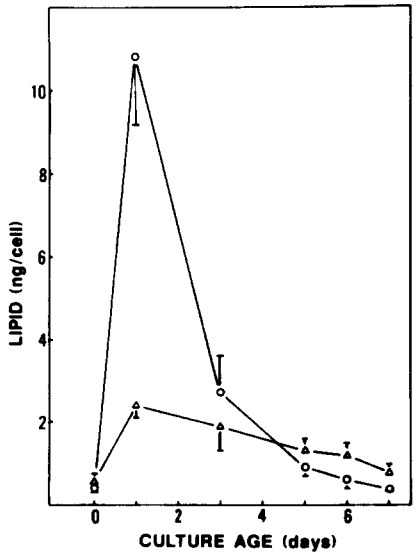

Fig. 3. Changes in the lipid content of *Paramecium tetraurelia* 51s cells. The neutral (○) and polar (△) lipid fractions of cellular lipids changed with culture age (40, 49). Changes in these two fractions also occurred in *P. multimicronucleatum* (38).

Only a few analyses have been done on lipids of membrane preparations subfractionated from cilia. This preparation represents about the purest obtainable membrane fraction from any cell type since cilia are initially isolated in the absence of cell lysis. Where membranes have been analyzed, it is not clear whether this represents the lipid composition of the entire ciliary membrane or microdomains. Using hypotonic lysis and sucrose gradient centrifugation, two membrane fractions separate. One membrane fraction frequently contains impurities and has been referred to as incompletely demembranated cilia (30, 31). Lysis of isolated cilia by hydrostatic pressure (French pressure cell) and separation by Percoll gradient centrifugation yields two membrane fractions and these are structurally and biochemically distinct (55, 56). The lighter fraction contains unilamellar vesicles and voltage-dependent Ca^{+} channels. The more rapidly sedimenting, broadly dispersed fraction contains multilamellar membrane structures and guanylate cyclase activity (55, 56). For the most part, isolated membrane preparations have been reported to contain the same lipids as those detected in cilia preparations. A possible exception is discussed below concerning membrane sterols.

The integrity of the cell surface membrane of *Paramecium* is sensitive to phospholipase C digestion. The phospholipids within the membrane covering the cell body may be more accessible to the enzyme than those which surround cilia. The cationic electron-opaque dye, ruthenium red, becomes entrapped only in the pellicular alveolar sacs underlying the cell membrane after cells were treated with the enzyme (57). Elsewhere along the surface membrane, the dye was observed only bound to the outside surfaces of the cell. The cell surface membrane is also readily perturbed by detergents (58) and hydrophobic drugs such as local anesthetics (59), presumably by their interactions with membrane lipids. Increased hydrophobicity of probe compounds increased their potency in eliciting ciliary reversals in *Paramecium* (59). The ciliary reversal triggered by these compounds may be the result of perturbation of membrane lipids resulting in depolarization and/or interactions of the compounds with lipids that affect ion gating mechanisms (59).

V. Fatty acids

The major fatty acids esterified to *Paramecium* lipids are palmitate and 18- and 20-carbon unsaturated acids (Table

TABLE 2. Fatty acid compositions of *Paramecium* cellular lipids

Fatty Acid	% by Weight[a]													
	Ester-Linked											Amide-Linked		
	Total	FFA	TG	SE	PC	PsE	PnE	PS	PI	PI-P	PI-P_2	NSL[b]	CSL[b]	ESL
	(35)[c]	(39)	(39)	(39)	(47)	(47)	(47)	(49)	(49)					(52)
14:0	1.1	2.2	1.6	1.6	0.3	0.6	0.2	1.0	4.4	5.8	2.2	1.5	0.3	0.3
14:1	[d]													
15:0	0.5	1.5			0.2	0.7	0.1		1.4	2.0	0.2		0.1	
16:0	19.1	33.5	23.6	14.3	8.0	26.5	1.6	32.0	27.5	16.8	18.1	1.5	2.3	82.8
iso-16:0	0.1													
16:1 (Δ7) + 16:1 (Δ9)	1.3	1.2	2.2	2.1		0.5		0.4	2.0	1.0	3.1			0.7
17:0	0.4	2.2	0.5	0.5	0.1	0.3						0.6	0.4	5.6
17:1 (Δ8) + 17:1 (Δ9)	0.5					0.3								
18:0	4.6	28.7	6.9	6.5	0.8	0.6		5.8	7.9	15.2	28.6	4.1	35.9	9.3
18:1 (Δ9) + 18:1 (Δ11)	22.9	17.9	40.5	34.7	9.1	9.3	0.4	6.8	12.8	16.1	20.5	1.4		1.3
18:2 (Δ9, 12)	17.1	2.1	5.1	4.7	25.4	30.6	1.5	7.6	18.3	14.8	8.5			
18:3 (Δ6, 9, 12)	9.9	0.8	2.4	3.8	15.4	20.4	1.3	4.8	14.9	10.4	6.4			
20:0							0.4					0.4	1.7	
20:1 (Δ11)	2.5	1.5	1.9	2.7	0.4	0.1		8.6	0.8	0.9	0.5	0.4	1.7	
20:2 (Δ8, 11)	0.7				0.1	0.6			1.4	4.4	3.4			
20:2 (Δ11, 14)	0.4								1.6	1.8	2.2			
20:3 (Δ5, 8, 11)	0.6				0.2		0.4							
20:3 (Δ8, 11, 14)	2.3	0.5	1.0	3.6	11.7	1.5	0.4	2.4	1.6	0.4				
20:4 (Δ5, 8, 11, 14)	11.9	0.9	2.4	6.7	26.4	7.3	89.6	27.1	2.7	3.0	1.1			
20:5 (Δ5, 8, 11, 14, 17)	2.4	0.6	1.2	3.2	1.7	0.4	4.5	2.6	0.2					
22:0 + OH-18:0												8.4	7.5	
22:1												2.5	2.0	
22:4 (Δ7, 10, 13, 16)	0.9													
22:6 (Δ4, 7, 10, 13, 16, 19)	0.9	0.1	1.1	0.8										
23:0												2.5	1.9	
23:1												0.9	1.9	
24:0 + OH-20:0												12.0	4.9	
24:1												26.2	33.5	
25:0												1.0	0.9	
25:1												3.0	2.3	
26:0 + OH-22:0												4.2	0.8	
26:1												8.5	2.9	
OH-23:0												2.3		
OH-24:0												6.7		
OH-24:1												7.8		

[a]Quantitations are for *Paramecium tetraurelia* 51s cells grown in modified enriched crude medium (35) for 5 days at 25°C. FFA, free fatty acids; TG, triglycerides; SE, steryl esters; PC, phosphatidylcholine; PsE, phosphatidylethanolamine; PnE, ethanolamine glycerophosphonolipid; PS, phosphatidylserine; PI, phosphatidylinositol; PI-P, phosphatidylinositol-phosphate; PI-P_2, phosphatidylinositol-diphosphate; NSL, total neutral sphingolipids; CSL, choline sphingolipids; ESL, total ethanolamine sphingolipids.

[b]Erwin, J. A., Jayasimhulu, and E. S. Kaneshiro, unpublished data.

[c]Numbers in parentheses indicate reference with original data.

[d]Blank spaces indicate fatty acids that were not detected or were less than 0.5% (by weight).

2). Minor fatty acids such as anteiso-16:0 are taken up by the cells from the crude medium and incorporated into cellular lipids. By growing cells in the chemically defined medium with either oleic acid, monolein, or dioleoyl PsE as the only fatty acid source, these minor fatty acids were distinguished from those that the organism synthesized, thus making it possible to examine biosynthetic pathways present in this organism (35) **(Fig. 4)**.

Palmitate is the major amide-linked fatty acid associated with the ethanolamine SPL (Table 2) (52). The dominant amide-linked fatty acids in choline SPL are 18:0 and 24:1 (Kaneshiro, E. S., unpublished data). Four glycosphingolipids have been identified in a neutral alkali-stable fraction. These contain high concentrations of long chain (C_{22} to C_{26}) saturated, monounsaturated, and hydroxy fatty acids (Table 2) (Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished results).

Incorporation patterns of exogenous radiolabeled 16:0, 18:0, and 18:1 indicate that the metabolism of saturated and unsaturated acids in *Paramecium* differs. Eighty five percent of the radioactivity from [1-^{14}C]16:0 or 18:0, but only 10% of the radioactivity from 18:1, that was incorporated into cellular lipids was found in the neutral lipid fraction after 5 days of growth with the labeled compound (39, 49).

Most of the dietary saturated fatty acids (SFA) such as palmitate and stearate are incorporated directly into cellular neutral lipids (39). Dietary oleic acid is primarily

TABLE 3. Fatty acid compositions of *Paramecium* cilia lipids

	% by Weight[a]								
		Ester-Linked							Amide-Linked
Fatty Acid	Total	TG	SE	PC	PsE	PnE	PS	PI	ESL
	(35)[b]	(39)	(39)	(40)	(40)	(40)	(49)	(49)	(52)
14:0	0.8	1.3	4.1	0.5	1.6	[c]	1.1	3.8	1.1
15:0	0.5	0.7	1.0		0.9				
16:0	12.3	29.0	25.9	14.9	42.8	2.8	33.8	23.5	65.8
16:1	0.5	0.7	3.4	0.6	1.3	0.1	0.5	1.5	2.4
17:0	0.2	0.8	0.7						6.5
18:0	2.6	26.3	9.9	3.5	5.8	1.7	6.8	5.5	19.6
18:1	6.7	16.6	36.2	8.7	11.9	0.4	7.7	11.7	4.7
18:2	8.6	5.9	24.0	25.6	0.3	6.6	23.9		
18:3	3.2	3.8	1.8	11.2	7.9	0.2	3.9	20.5	
20:1	1.0	1.2	1.4				8.7		
20:3	1.6	0.9	0.8	3.8		0.4	0.8	0.9	
20:4	53.3	5.6	2.0	25.2	2.1	82.6	22.8	2.5	
20:5	7.5	0.9	2.4			10.3	2.3	0.1	
22:6	0.1	1.6	0.8						

[a]Quantitations are of lipids from cilia isolated from *Paramecium tetraurelia* 51s grown in the modified enriched crude medium (35) for 5 days at 25°C; FFA, free fatty acids; TG, triglycerides; SE, steryl esters; PC, phosphatidylcholine; PsE, phosphatidylethanolamine; PnE, ethanolamine glycerophosphonolipid; PS, phosphatidylserine; PI, phosphatidylinositol; ESL, total ethanolamine sphingolipids.

[b]Numbers in parentheses indicate reference with original data.

[c]Blank spaces indicate fatty acids that were not detected or were less than 0.5% (by weight).

used for PUFA synthesis (36). The major end product of fatty acid synthesis that accumulates in high concentrations esterified to phospholipids is arachidonate (35).

Inhibition of culture growth by 2 μM triparanol (an inhibitor of cholesterol synthesis in mammals) was correlated by the specific inhibition of oleate desaturation to linoleate, creating a block in the synthesis of PUFA (36). Similar observations, the inhibition of fatty acid desaturation by this drug, were earlier made in another ciliate, *Tetrahymena* (60–62). The phospholipids of triparanol-inhibited paramecia had significantly less PUFA than control cells (36). The duration of the avoidance reaction in triparanol-treated cells was longer than that observed in control cells (36). The renormalization period was the component of the avoidance response that was most affected (36) suggesting that the efficiency of Ca^{2+} pumps and/or ion channel inactivation was diminished as a result of the alterations in the membrane lipids.

Oleate apparently undergoes β-oxidation and the resultant two carbon units are utilized for de novo synthesis of SFA, which explains the ability of the organism to grow on oleate alone and yet have the same fatty acid composition as those grown in a mixture of saturated and unsaturated acids (49). Furthermore, some radiolabel from oleate was incorporated into 14:0, 15:1, 16:0, 16:1, and 18:0 (36) presumably by randomization of the label following β-oxidation.

Inhibition of culture growth by cerulenin (an inhibitor of β-keto acyl carrier protein synthetase) was correlated with a suppression of fatty acid synthetase activity using a cell-free preparation and cerulenin (49). Inhibition by cerulenin was found not to alter the ability of wild type cells grown in bacterized cultures to extrude trichocysts. This was also true of the conditional mutant, nd9, which does not exocytose at the restrictive temperature of 27°C (24). The mutant lacks the ability to organize the "rosette" array of intramembranous particles of the cell surface membrane which has been found necessary for the regulation of membrane fusion during exocytosis (20, 23–25). Mutant cells first grown at the restrictive temperature (27°C), and then treated with cerulenin and transferred to the permissive temperature (15–18°C), failed to restore exocytotic activity as well as the assembly of "rosette" arrays. These observations indicated that compensatory changes in membrane lipid composition in response to the temperature shift were inhibited due to the effects of cerulenin. The drug presumably had an effect on fatty acid metabolism that sufficiently altered the membranes of nd9 preventing the cell's recovery to its phenotype at the permissive temperature (24).

Elongation of fatty acids in this ciliate may involve two separate enzyme systems, one for saturated and the other for unsaturated fatty acids, as observed in other cell types (63). Indirect evidence for this hypothesis includes the observation that cells grown with ample dietary oleate incorporate radioactivity from 8:0 into 16:0 and 20:4 (presumably as two carbon units following β-oxidation of 8:0). When cells were placed in a fatty acid-free medium, incorporation of radiolabel from tracer amounts of 8:0 into 16:0 was low but incorporation into 20:4 remained high

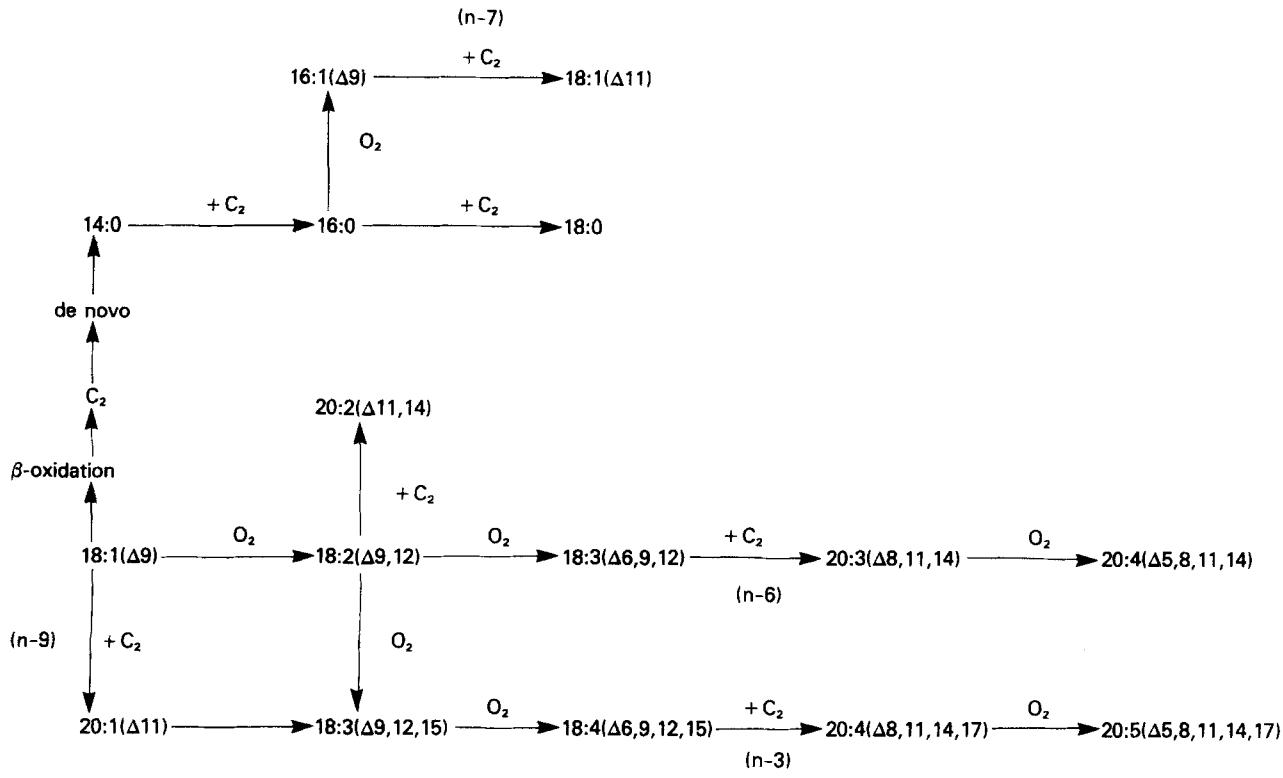

Fig. 4. Metabolism of oleic acid by *Paramecium tetraurelia* 51s. Radioactivity in fatty acids isolated by preparative gas-liquid chromatography was determined after cells were grown in either the crude or chemically defined medium with either [^{14}C]8:0, 16:0, 18:0 (Rhoads, D. E., O. Honer-Schmid, and E. S. Kaneshiro, unpublished data) or 18:1 (Δ9) (36, 49). The presence of these proposed pathways can explain the fatty acids identified in the organism's lipids when cells were grown in a chemically defined medium containing either oleate, monolein, or dioleoyl PsE as the only fatty acid source (35). The major pathway is the conversion of oleic acid to PUFA with arachidonate as the major end product of fatty acid biosynthesis (36). Radiolabel from 18:1 into shorter chain acids is probably by β-oxidation and randomization of radioactivity during the de novo synthesis of shorter chain acids (36, 49). Retroconversion of oleate cannot be ruled out to explain radiolabeling patterns observed. Although 18:3 (Δ6, 9, 12) is the only 18:3 fatty acid detected in *Paramecium* lipids, when cells were grown with 18:3 (Δ9, 12, 15), 85% of the label remained with the original radiocompound and 13% of the radioactivity was found in 18:4 (Δ6, 9, 12, 15) (n = 2) (Rhoads, D. E., O. Honer-Schmid, and E. S. Kaneshiro, unpublished data). The end product, 20:5 (Δ5, 8, 11, 14, 17) of the (n-3) pathway (which seems to be a minor pathway) has been identified in *Paramecium* lipids (35). Thus, *Paramecium* appears to have (n-3), (n-6), (n-7), and (n-9) pathways for fatty acid synthesis.

(49). Thus, in the fatty acid-free medium, the unsaturated fatty acid elongase appears more active than the saturated acid elongase. Also, in cells treated with cerulenin, radiolabel from 8:0 readily incorporated into 20:4 but incorporation into 16:0 was reduced. Thus the synthetase activity for de novo SFA synthesis was inhibited; the unsaturated fatty acid elongase appeared not to be affected by the drug (49).

Metabolic radiolabeling studies of ester-linked fatty acids indicate that complex pathways are present for fatty acid metabolism in this ciliate (Fig. 4). Biosyntheses of amide-linked fatty acids, such as C_{22-26} saturated, monounsaturated, and hydroxy fatty acids, have not yet been examined.

Paramecium therefore seems capable of de novo fatty acid synthesis but lacks the ability to desaturate stearate to form long chain PUFA needed for normal functioning of the cell. However, the following observations remain puzzling. *1*) Radiolabel from exogenously supplied acetate, citrate, glucose, malonate, pyruvate, or butyrate does not incorporate into *Paramecium* fatty acids. These compounds are taken up by the cells at lower rates than are medium and longer chain fatty acids, and radioactivity from some of these compounds incorporates into lipids. However, after hydrolysis, none of the radioactivity was found to be associated with fatty acids (49). *2*) Lipid extracted from entire contents of *Paramecium* cultures (cells plus medium) was less than that in the uninoculated medium; therefore, net lipid synthesis did not occur during culture growth (40). *3*) The saponifiable fatty acid composition of total phospholipids in cells inhibited by cerulenin and cultured in a defined medium in which oleate was the only fatty acid present (as monolein) had relatively less longer chain, unsaturated, and PUFA compared to controls. However, these inhibited cells contained three times more total phospholipid esterified fatty acids as control cells at the same culture age (stationary phase) and about the same amount of these components as did day-1 cells (lag

phase). There were increases in saponifiable phospholipid 14:0, 16:0, 16:1, and 18:0 in the cells (49). These observations suggest that either de novo synthesis of these acids occurred in the presence of cerulenin at concentrations that completely inhibited culture growth, or retroconversion of dietary 18:1 occurred to form these shorter chain acids.

Radioactivity from dietary fatty acids was readily incorporated not only into cellular lipids, but also into cellular proteins and CO_2 (49, Reuter, S. F., S. J. Merkel, P. J. Soldano, and E. S. Kaneshiro, unpublished results). The observation that total lipids in cells plus medium after 5 days of culture growth was less than in the original medium (see above, 40) further indicates that fatty acids are *1*), used for energy production and are directly converted to CO_2; *2*), used as carbon sources for synthesis of other cellular compounds such as proteins; and *3*), metabolized into other cellular compounds that are subsequently metabolized to form CO_2.

VI. Neutral lipids

A. Sterols

Paramecium introduces Δ7 unsaturation into stigmasterol and cholesterol, forming the terminal products, 7-dehydrostigmasterol and 7-dehydrocholesterol (44). Cholesterol and 7-dehydrocholesterol fail to support culture growth and were found only in the free form in *P. octaurelia* (44) and *P. tetraurelia* (50). However, Kaneshiro, Meyer, and Reese (39) reported that all four of these sterols were found esterified in lipids from *P. tetraurelia* when the cells were grown in the crude medium in which cholesterol was present. Upon reexamining these discrepancies, both species were grown in the crude or chemically defined medium with [^{3}H]cholesterol. The neutral lipid fractions were analyzed by thin-layer chromatography and fluorography. Two distinct bands were present that had similar migrations as cholesterol and cholesteryl esters **(Fig. 5)**. The radioactivities in the two bands were in the same proportions as that earlier determined as the quantities of cholesterol plus 7-dehydrocholesterol in the free and esterified forms (39, Table 1).

Paramecium octaurelia growth for 5 days in a medium supplemented with 50 μM cholesterol exhibited avoidance reactions with longer backward swimming (49 sec) than did control cells (37 sec) in response to K^+ depolarization (64). Thus the incorporation of high levels of cholesterol into the ciliate's membranes may have altered mechanisms involved in the inactivation of ion channels and/or Ca^{2+} pump activity.

The neutral lipid fraction from sucrose gradient-purified ciliary membrane preparations contains mainly sterol esters, sterols, and free fatty acids (39). Up to 78% of the neutral lipids in the preparation were identified as steryl esters. This value could actually be higher since free fatty acids were not detected in cilia preparations (see above). Degradation of some steryl esters and the release of fatty acids and free sterols may have occurred during the purification procedure (39). Steryl esters have also been reported in the cell surface membrane of the parasitic flagellated protozoan, *Trypanosoma cruzi*. Of the total membrane sterols of this organism, 30–40% were esterified (65). Although steryl esters are clearly present as storage lipids in some cell types, these observations on isolated cell surface membranes from *Paramecium* and *T. cruzi* suggest the possibility that membranes from other cell types contain steryl esters and that perhaps the difficulty in detecting them may be due to degradation occurring during extensive procedures used for isolating most membranes.

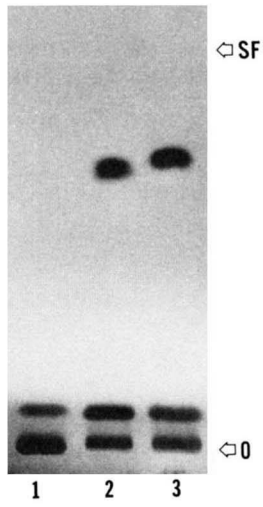

Fig. 5. Autoradiogram of a thin-layer chromatographic separation of the neutral lipid fraction of *Paramecium tetraurelia* 51s (lane 2) and *P. octaurelia* 299s (lane 3). Cells were grown for 5 days with [^{3}H]cholesterol (lane 1) in the enriched crude or chemically defined medium and total lipids were extracted. The neutral lipid fraction was isolated by silicic acid column chromatography and separated on an aluminum-backed Silica Gel 60 plate developed in petroleum ether–diethyl ether–glacial acidic acid 80:20:1 (v/v/v). The plates were dried and sprayed with EN^{3}HANCE (New England Nuclear, Boston) and subjected to autofluorography. The middle radioactive component in the neutral lipid fraction of both species had an *Rf* value similar to [^{3}H]cholesterol and the top radioactive component had an *Rf* value similar to that of steryl esters in this solvent system. The O marks the origin and SF indicates the solvent front. The radioactivity in the free sterol band was 56.8% in 299s and 74.2% in 51s and that in the steryl ester band was 43.4% in 299s and 25.9% in 51s. These observations support the report (39) that cholesterol and 7-dehydrocholesterol can be found in the esterified form in *P. tetraurelia* and that the radioactivity from [^{3}H]cholesterol that had been incorporated into the free and esterified sterol fractions was in the proportions reported for the quantitation of cholesterol and 7-dehydrocholesterol in the two fractions of *P. tetraurelia* 51s cells (39, Table 1). Data are from Kaneshiro, E. S., unpublished.

B. Fatty acids

The fatty acid compositions of the free fatty acid and triglyceride fractions of cellular lipids resemble that of the culture medium (39, 51). Experiments involving relatively

short periods for incorporation of radiolabeled fatty acids indicate that exogenous fatty acids are initially incorporated into triglyceride stores (higher relative specific activities, RSA) before they are used for acylation of sterols (lower RSA) (39).

C. Neutral sphingolipids

A neutral sphingolipid fraction has recently been characterized (Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished results). This fraction is the alkaline-stable material initially eluted from silicic acid columns (Unisil) with chloroform–methanol 95:5 (v/v) after the neutral lipid fraction is removed by chloroform elution. This fraction contains at least six components, four of which are glycosylated. Two minor components may represent free ceramide intermediates in choline and ethanolamine sphingolipid synthesis. The amide-linked fatty acid composition of the whole neutral sphingolipid fraction is high in C_{22-26} saturated, monounsaturated, and hydroxy fatty acids, and is unlike the total cellular free fatty acids, esterified fatty acids, or the amide-linked fatty acids of choline and ethanolamine SPL (Table 2). The long chain bases (LCB) detected in this fraction include three isomers of C_{18} sphingosines, C_{18} dihydrosphingosine, and another component containing a yet unidentified LCB (which is not phytosphingosine or its cyclized product, 52) **(Table 4).**

VII. Polar lipids

A. Phosphonolipids

Direct P-C bonds have been identified in ethanolamine glycerolipids and SPL of *Paramecium* (20, 40, 45, 47, 50, 52, 66). The absence of the oxygen, as found in phosphoryl bonds, significantly influences the efficiency of phospholipase action. Phospholipase C *(Bacillus cereus)* and phospholipase A_2 *(Crotalus adamanteus, C. atrox,* and porcine pancreas) hydrolyze PsE faster than PnE, presumably due to steric effects (47).

The amide-linked fatty acids and long chain bases (LCB) of the ethanolamine SPL are the same (52, 66); however the compositions of the ester-linked fatty acids of PsE and PnE are distinct (40, 47) (Table 2). Almost all of the fatty acids in PnE are arachidonate (40, 47). Also, almost all of the PnE are alkacyl glycerolipids whereas only low amounts of PsE contain glyceryl ethers (40, 47) (see below).

Currently, there is no general agreement on the biosynthetic pathways that lead to the formation of ethanolamine phosphonolipid head groups in any organism (67, 68). In the ciliate *Tetrahymena,* free aminoethylphosphonate (AEP) is formed by decarboxylation and amination of phosphoenolpyruvate (PEP); however, free AEP was found to be synthesized after lipid-bound AEP (69). It has been suggested that in *Tetrahymena,* PEP undergoes rearrangement to phosphonopyruvate (as CMP-AEP or phosphatidylphosphonopyruvate) and then is converted to lipid-bound 2-amino-3-phosphonopropanate (APnP) followed by decarboxylation to produce ethanolamine phosphonolipids (70). However, neither a lipid-bound APnP intermediate nor PS were detected in *Tetrahymena,* and the incorporation of serine into ethanolamine phosphonolipids was not demonstrated in this ciliate (67–71).

The incorporation of radiolabeled serine into the head groups of ethanolamine phosphonoglycerolipids and sphingolipids in *Paramecium* has been demonstrated (66) indicating that the formation of ethanolamine phosphonolipids involves serine glycerolipid and sphingolipid intermediates. Decarboxylation of lipid-bound APnP has been suggested as a reaction occurring in ethanolamine phosphonolipid head group synthesis in *Paramecium* (66). As observed in *Tetrahymena,* ethanolamine was not incorporated into *Paramecium* phosphonolipid head groups and PEP was not incorporated into ethanolamine phospholipid head groups (66). Suggested biosynthetic pathways for ethanolamine head groups in *Paramecium* are shown in **Fig. 6**.

B. Ether lipids

Two glyceryl ethers, 1-hexadecyl glycerol (chimyl alcohol) and 1-octadec-11-enyl glycerol (paramecyl alcohol) have been identified in *P. tetraurelia and P. multimicronucleatum* (48). Both are present in all glycerolipids examined in *P. tetraurelia* (PsE, PnE, PC, PS, and PI), however, PnE is distinctly rich in glyceryl ethers (90%)

TABLE 4. Long chain bases in *Paramecium* sphingolipids

Long Chain Base	Neutral Sphingolipids[a,b]	Choline Sphingolipids[a]	Ethanolamine Sphingolipids[c]
C_{18} Dihydrosphingosine	+[d]	ND[e]	+
C_{18} Sphingosines	+ (3 isomers)	+ (2 isomers)	+
C_{18} Phytosphingosine	ND	ND	+

[a]Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished data.
[b]An additional unidentified LCB was also present.
[c]From reference 52.
[d]+, present.
[e]ND, not detected.

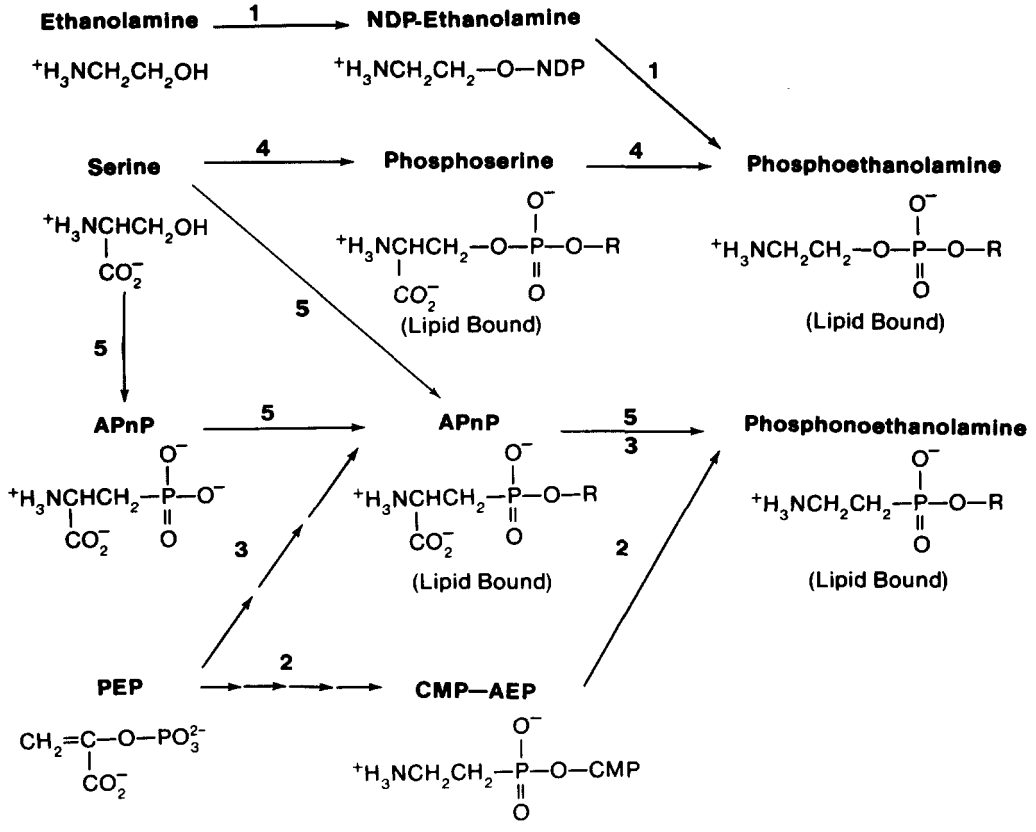

Fig. 6. Proposed pathways for the biosynthesis of ethanolamine lipid head groups in *Paramecium tetraurelia* 51s (redrawn from reference 66). Pathway 1 is the de novo pathway known for the synthesis of PsE from ethanolamine. *Paramecium* ethanolamine glycerophospholipids and sphingophospholipids can be synthesized by this pathway. Pathways 2 and 3 are those that have been proposed for the synthesis of *Tetrahymena* glycerophospholipids from phosphoenolpyruvate (PEP). One or both exist in *Paramecium*. Pathways 4 and 5 involve serine and include the decarboxylation of phosphatidylserine to PsE, as proposed for *Tetrahymena*. The proposed serine pathways leading to the formation of ethanolamine glycerophosphonolipids and sphingophosphonolipids involve the lipid-bound intermediates that contain APnP.

(40, 45, 47, 50, 54). The glyceryl ether concentrations of *Paramecium* PC, PnE, and PsE had been predicted by indirect methods such as quantitation of hydrolysis products (47) and the percent of alkaline-stable material (40, 49). The approximate values were: PnE, 90%; PsE, 10%; and PC, 80%. Direct analyses of glyceryl ethers confirmed the values for PnE and PsE but only 33% of the PC fraction contained glyceryl ethers. Diether glycerols were not detected (54). Recently, choline SPL were identified in the PC fraction (see below) which may explain the discrepancy between direct and indirect measurements, but preliminary results indicate that less than 20% of the cellular PC fraction are SPL (Kaneshiro, E. S., unpublished results). Therefore, the values calculated for the various choline lipid classes in Table 1 may require adjustments after more rigorous quantitations on choline SPL and detailed analyses of the choline lipid fraction have been completed.

The concentration of glyceryl ethers in total cellular phospholipids increased with culture age (54). Also, supplementation of the growth medium with 0.1 mg/ml of chimyl alcohol resulted in an increase in the concentration of this glyceryl ether in the phospholipids of cells (control, 33%; supplemented, 55%) and isolated cilia (control, 33%; supplemented, 63%) (49, 54).

As expected, radiolabel from the fatty alcohol, hexadecanol, was incorporated in vivo into chimyl alcohol, although paramecyl alcohol was also labeled (Kaneshiro, E. S., and K. B. Meyer, unpublished results). Radiolabeled octadecanol was readily incorporated into paramecyl alcohol suggesting the formation of a $\Delta 11$ C_{18} unsaturated fatty alcohol intermediate. Despite the inability to detect 1-octadecyl glycerol (batyl alcohol) in *Paramecium* lipids, radioactivity that had been incorporated from octadecanol into the cells' lipids was collected from a preparative gas-liquid chromatographic column at the elution time corresponding to batyl alcohol. Thus batyl alcohol may be an intermediate that is rapidly metabolized and hence not accumulated in the lipids of this organism. If batyl alcohol is an intermediate in paramecyl alcohol synthesis, this would require the presence of a desaturase capable of utilizing a glyceryl ether

as a substrate (Kaneshiro, E. S., and K. B. Meyer, unpublished results).

Radiolabel from palmitic acid was incorporated in vivo into paramecyl alcohol with a RSA that was twice that of chimyl alcohol (Kaneshiro, E. S., K. B. Meyer, and D. E. Rhoads, unpublished data). The probable fatty acid intermediates in the conversion of this fatty acid to paramecyl alcohol are 16:1 (Δ9) and 18:1 (Δ11). These fatty acids have been identified in the lipids of *Paramecium* (35). Radiolabel from stearic acid was not incorporated in vivo into either glyceryl ether, suggesting that the organism cannot convert this fatty acid to an alcohol (Kaneshiro, E. S., K. B. Meyer, and D. E. Rhoads, unpublished observations). These in vivo radiolabeling studies provide preliminary information on ether lipid metabolism in *Paramecium* **(Fig. 7)**. More work, including systematic analyses using in vitro assays, is needed to clarify the existence of these pathways.

C. Sphingolipids

The ethanolamine SPL of *Paramecium* have been characterized (52). Head group metabolism of ethanolamine phosphonolipids was discussed above. Recently, free ceramides, glycosphingolipids and choline SPL have been tentatively identified and characterized (Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished results). The identification of choline SPL explains the earlier observations of significant levels of incorporation of radioactivities from serine, [1-^{14}C]N-stearoyl sphingosine, [^{3}H] *erythro*-sphingosine, and [^{3}H]-*threo*-sphingosine into the choline lipid fraction (45, 66 and Matesic, D. F., and E. S. Kaneshiro, unpublished observations). Furthermore, the presence of serine sphingolipid intermediates has been predicted (66, see above). Thus, the concentrations of SPL in *Paramecium* cells and ciliary membranes appear far greater than earlier appreciated.

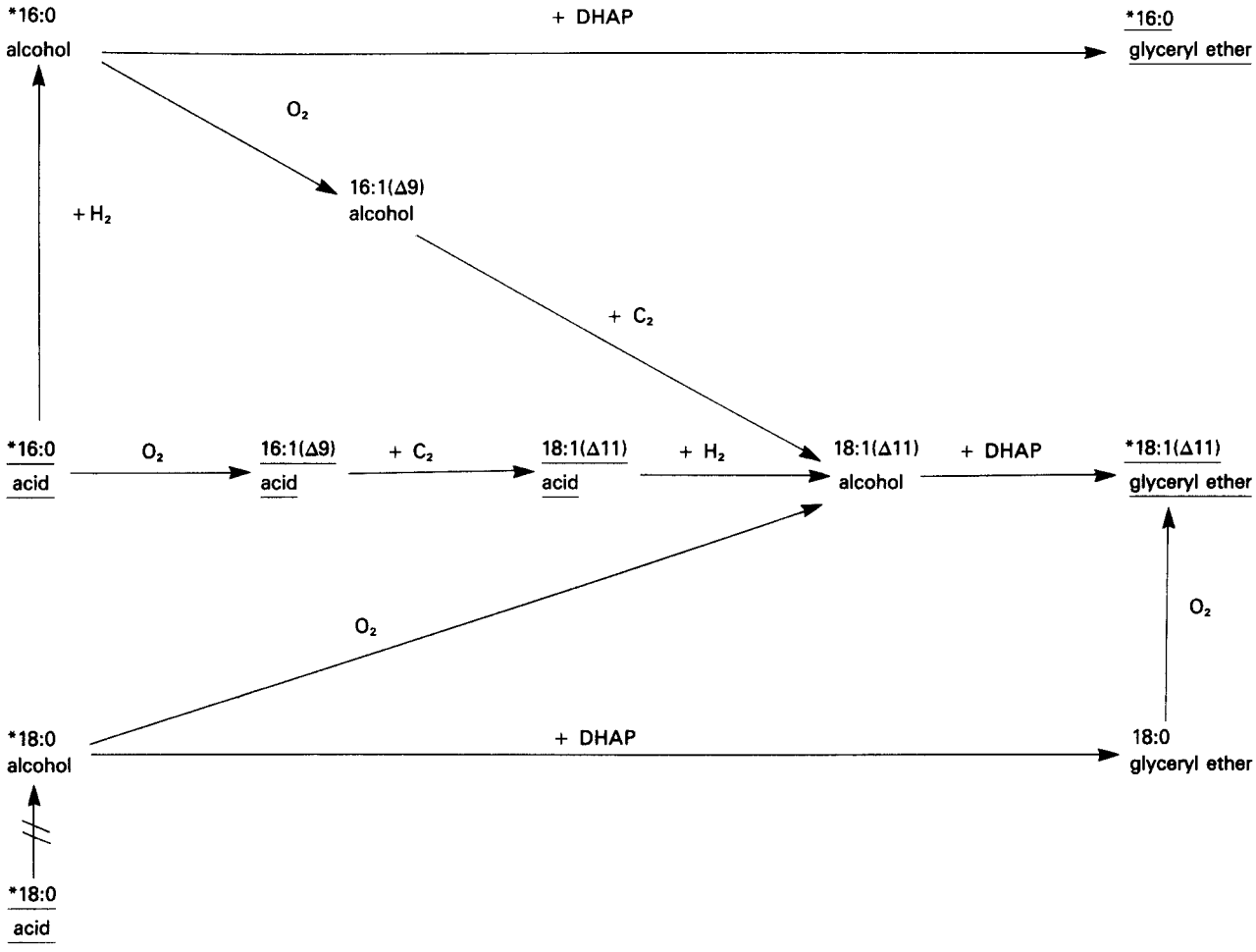

Fig. 7. Proposed possible pathways for the biosynthesis of glyceryl ethers in *Paramecium tetraurelia* 51s. The hypothesized pathways shown are based on studies of the incorporation in vitro of radiolabeled [1-^{3}H]hexadecanol and octadecanol, and [1-^{14}C]palmitate and stearate (indicated by asterisks). Underlined compounds have been identified in the lipids of the cell (35). It is likely that aldehyde intermediates are formed during the reduction of fatty acids to fatty alcohols and that dihydroxyacetone phosphate (DHAP) is involved in the initial reactions leading to the formation of acyl glycerols (data are from Kaneshiro, E. S., and K. B. Meyer, unpublished results).

The fatty acid compositions of the sphingolipids are different from those that are found esterified to glycerolipids (52). The choline, ethanolamine, and glycosphingolipids have their own characteristic fatty acid compositions (Table 2) (52 and Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished data). The LCB compositions of these three sphingolipid groups are also distinct (Table 4) (Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished data).

The total amounts of ethanolamine SPL increase in the cellular and ciliary polar lipids with culture age (40, 52). The relative concentrations of sphingosine- and dihydrosphingosine-containing ethanolamine SPL increase while those of the phytosphingosine-containing ethanolamine SPL decrease (52). Preliminary analyses on the effects of culture age on the relative concentrations of different LCB, including isomeric forms of sphingosine, in the choline and the neutral sphingolipid fractions indicate that these are not affected (Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished results).

Long chain base synthesis in *Paramecium* appears to involve the condensation of serine with palmitoyl-CoA forming 3-ketodihydrosphinganine as the initial reaction in C_{18} LCB synthesis (45, 66), similar to that occurring in other organisms (72). Reduction of the keto group in 3-ketodihydrosphinganine produces dihydrosphingosine which accumulates to detectable levels in *Paramecium* neutral ceramide and ethanolamine SPL fractions (52, 66, and Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished results). The RSA of ethanolamine SPL radiolabeled with serine were at least twice those of ethanolamine glycerolipids (66). Radioactivity from serine was incorporated only into the glycerolipid head groups whereas the radioactivities in SPL were in both the LCB and polar head group moieties (66). Radioactivity from palmitic acid was incorporated equally into the fatty acid and LCB moieties of ethanolamine SPL whereas radioactivity from stearic acid was not incorporated into LCB of ethanolamine SPL (Matesic, D. F., and E. S. Kaneshiro, unpublished results). Together, these observations indicate that palmitoyl-CoA and serine participated in the initial reaction in LCB synthesis in *Paramecium*.

The pathways for the conversion of dihydrosphingosine to the other LCB identified in *Paramecium* are not known. Dihydrosphingosine and sphingosine were detected in the neutral sphingolipid fraction suggesting that the syntheses of phosphosphingolipids and glycosphingolipids proceed through free ceramide intermediates (fatty acylation of the LCB). These intermediates are present in low concentrations in *Paramecium* (see above) (Erwin, J. A., K. Jayasimhulu, and E. S. Kaneshiro, unpublished observations). The inability to detect phytosphingosine in the neutral sphingolipid fraction (see above) indicates that the conversion of either dihydrosphingosine or sphingosine to phytosphingosine might occur after the addition of the head group. Further evidence supporting the notion of LCB modification after DPsE and DPnE are formed is the observation that, during the relatively short period of labeling of late log phase *Paramecium* cells (9 hr) with [^{32}P] phosphate, the RSA were highest for DPsE and DPnE and lowest for PPsE and PPnE (66). This sort of labeling pattern would be expected if the biosynthetic pathways present in this organism involved modification of the LCB moiety of the SPL since the radiolabel was on the phosphorus of the head group, not in the LCB. Also, it is currently not possible to rule out the possibility that head groups are first added to LCB before acylation occurs. Similar reactions have been observed in other organisms (73).

D. Inositol lipid metabolism

The metabolism of inositol lipids has been implicated in transmembrane signal transduction in a variety of cellular responses to stimulation. In many cases, activation of phosphodiesterase activity releasing the second messenger, inositol triphosphate (IP_3), has been observed (74).

Preliminary results on *Paramecium* indicate that the levels of the polyphosphoinositides, PI-P and PI-P_2, decreased in cells that were induced to swim backward by membrane depolarization (46). A concomitant increase in PI and no change in PA levels were noted suggesting that activation of monoesterase activities may be involved in the membrane events associated with the avoidance reaction. An in vitro system for analyses of kinase activity was employed utilizing soybean PI (similar to *Paramecium* PI with low 20:4) as substrate, [γ-^{32}P]ATP, and isolated *Paramecium* cilia. The formation of radioactive PI-P and PI-P_2 indicated that the cilia had relevant kinase activities (75). The kinase activity was further shown to be specifically associated with the membrane of this organelle. The activity was stimulated by Mg^{2+}, had a neutral pH optimum range, and preferred ATP over GTP as the phosphate donor. It has not yet been established whether separate enzymes exist for the phosphorylation of PI and PI-P. In these experiments, PI-P and PI-P_2 became radioactive during the incubations but the activity declined as incubation time progressed (75). This suggests that phosphatase (and/or other phospholipase) activity is also present in the cilia. More work on this group of minor lipids needs to be done. Further critical analyses including an evaluation of IP_3 levels and in vitro studies using purified ciliary membrane vesicles may help to clarify the physiological significance of inositol lipid metabolism with respect to the avoidance reaction and the regulation of ion translocations across this membrane.

VIII. Mutants

The availability of *Paramecium* behavioral mutants that

exhibit altered membrane electrical properties provides the possibility of identifying the functions of specific membrane molecules (19). Among the mutants thus far isolated, several are of interest with respect to the lipids of this organism.

The barium-shy mutant, baA, has been characterized as a lipid mutant by Forte et al. (20). This cell responds to lower concentrations of Ba^{2+} by swimming backward for long periods whereas wild type cells, at higher Ba^{2+} concentrations, typically exhibit short jerking avoidance reactions described as the "barium dance" (20). Barium ions are reported to enter cells through the ciliary voltage-sensitive Ca^{2+} channels and elicit all-or-none action potentials (76). Barium may have several effects on the cells. Wild type cells grown at an elevated temperature of 35°C are reported to lack a response to Ba^{2+} when tested at 15°C (77). The baA mutant not only exhibits greater sensitivity to Ba^{2+} but is also defective in thermal avoidance (50) (see below). The consequences of the baA mutation on the electrophysiological responses were described as decreases in the conductances of both the voltage-sensitive Ca^{2+} and K^+ channels (20). Analyses of cells grown in Cerophyl with $[^{32}P]H_3PO_4$ added prior to the addition of bacteria showed that differences in radioactivities in the ethanolamine SPL and phosphonolipids existed between wild type and baA cilia. Radioactivity incorporation into PPsE was dramatically lower in the mutant. The ratio of ethanolamine phosphonolipids to ethanolamine phospholipids was higher in the mutant. The Cerophyl plus bacteria culture system contains low levels of sterols. Upon supplementing these cultures with stigmasterol, it was reported that the mutant was cured, i. e., wild type cells were indistinguishable from baA cells with respect to lipid composition, electrophysiological and behavioral responses (20, 78).

Analyses of cellular lipids of the baA mutant and wild type cells grown axenically confirmed that there are: *1*) quantitative differences in their ethanolamine SPL during culture growth; *2*) the two cell types differ in their locomotory responses to stimulation solutions containing Ba^{2+}; and *3*) the presence or absence of stigmasterol in the growth medium influences both locomotory behavioral responses to Ba^{2+}-containing solutions and the relative amounts of the different ethanolamine SPL during culture aging (79). However, the specific differences observed in lipid composition, behavioral responses, and the effects of sterols were not consistent with the studies on cells grown in Cerophyl with bacteria. Quantitation of lipids in axenically grown cells was based on measurements of lipid masses, whereas the earlier studies of baA grown monoxenically (20) were on the incorporation of $[^{32}P]$ into different lipids. During axenic culture aging, the baA cells exhibited fewer changes in the relative amounts of the different ethanolamine SPL than did wild type cells. Analyses of the lipids of cilia or ciliary membranes from axenically grown mutant cells are yet to be done. The nature of the effect of baA mutation on membrane lipids and on the behavioral responses of cells remains an open question. The conclusion made by Forte et al. (20) and Adoutte et al. (78) that sterol supplementation "cured" the lipid, electrophysiological, and behavioral defects of the baA mutant must be reevaluated. Their results, in fact, show that sterol supplementation of the monoxenic cultures in the Cerophyl medium resulted in changes in the wild type cells, the consequences of which made them more like the mutants. The metabolism and biosynthetic pathways of phosphonolipids and sphingolipids in normal cells must be understood in greater detail. The specifics of the baA mutation will become more readily identified after such information becomes available.

Another group of mutants that may have lipid alterations are those that are resistant to polyene antibiotics (80). These compounds are known to bind to membrane sterols and cause increased permeabilities of the cell membrane. Nystatin, filipin, and amphotericin B in micromolar concentrations kill wild type cells (39). A polyene antibiotic-resistant mutant, 1'(1), was reported to have decreased voltage-sensitive Ca^{2+} channel activity. All polyene antibiotic-resistant lines isolated were resistant to both amphotericin B and nystatin regardless of which drug was used for their selection. Thus, the sterols of polyene antibiotic-resistant mutants probably have been qualitatively or quantitatively altered (80). The lipids of these mutants, especially membrane sterols, are yet to be analyzed. All polyene antibiotic-resistant mutants, like the Ba^{2+}-shy mutants, respond to Ba^{2+}-containing solutions by continuous backward swimming at Ba^{2+} concentrations that elicit the "barium dance" in wild type cells. The barium-shy and polyene antibiotic-resistant strains, however, map at difference genetic loci (80).

The fast mutant, $d_4$97, fast-1A, swims in the forward direction faster than wild type cells when disturbed (19). This strain may have greater concentrations of alkacyl PsE and diacyl PnE (44). Andrews and Nelson (45) did not detect these lipids in wild type, a pawn ($d_4$95) strain, and four paranoiac strains grown in bacterized cultures but were able to detect these relatively minor lipids in the fast mutant. The lipids of this fast mutant require reexamination under axenic conditions since these lipids have been detected and characterized in axenically grown wild type (47) and the pawn mutant, $d_4$95 (54).

IX. Culture age and temperature effects

As indicated above, several changes occur in the lipid composition of cellular and cilia lipids with culture age (35, 39, 40, 50–52). Changes in behavioral responses (36) and responses to amphipathic drugs (59) that are correlated with culture age have been observed indicating that specific culture age-related lipid alterations may be relevant to these changes in membrane function. Cilia

from mid-log phase (day 3) contain ester-linked phospholipid fatty acids with an unsaturation index (UI) of 143 (49, 81). Ester-linked fatty acids of phospholipids in cilia from stationary phase cells (day 7) had a UI of 205, which by itself would suggest an increase in membrane fluidity of significant magnitude. However, there are high concentrations of lipids with aliphatic chains other than ester-linked fatty acids in *P. tetraurelia,* and the relative amounts of ether lipids and ethanolamine SPL increase as phosphoglycerides decrease during culture aging. When a balance sheet was made taking into account the amounts and compositions of ester-linked fatty acids, amide-linked fatty acids of ethanolamine SPL, ether-linked alcohols, and ethanolamine SPL long chain bases, phospholipids from day-3 cilia had a UI of 104 and day-7 cilia had a UI of 114 (49, 81). Thus it appears that UI per se does not change with culture age.

The magnitude of the membrane resting potential of wild type cells grown at 25°C is similar to that of cells grown at the suboptimal temperature of 15°C, but is greater than that of cells grown at the elevated temperature of 35°C (82). Heat causes graded, slow membrane depolarizations analogous to receptor potentials produced by mechanical stimulation to the anterior regions of the cell (83, 84). The effect of temperature on the membrane potential is thought to be the result of membrane lipid changes (50, 77, 82).

It is well documented that compensatory mechanisms present in poikilothermic organisms bring about changes in the aliphatic chains in membranes. Optimal membrane function is maintained by altering the fluidity of the bilayer. A change in the relative amounts of unsaturated acids or in the UI commonly occurs in these organisms, thus a temperature downshift is accompanied by an increase in the UI. In *Paramecium,* changes in the ester-linked fatty acids in cellular and cilia lipids occur at different growth temperatures. Saponifiable fatty acids in total phospholipids of cells grown at 15°C had a UI of 150 whereas cells grown at 26°C had a UI of 182, which is consistent with observations in other organisms (Kaneshiro, E. S., unpublished results). Hennessey and Nelson (50) observed that sustained growth of cells at an elevated temperature (35°C) resulted in a decrease in ester-linked 18:3, a decrease in the 16:0/18:0 sphingolipid fatty acid ratio and an increase in chimyl alcohol, all consistent with increased bilayer packing by either decreasing unsaturation or increasing chain length and hydrophobic interactions to compensate for the increased melting due to increased kinetic energy at higher temperatures. However, no changes in the total percent unsaturation and the UI of ester-linked fatty acids, or in the total acyl- and amide-linked fatty acids and ether-linked alcohols occurred. Total unsaturation was 51% (25°C) and 52% (35°C) and the UI was 158 at 25°C and 170 at 35°C. Thus, it appears that percent unsaturation and the UI of the aliphatic chains in the ciliary membrane of *Paramecium* are maintained at remarkably constant values regardless of temperature or culture age. Similar constancy of the esterified fatty acid UI was observed in *T. thermophila* in response to a temperature downshift (35° to 15°C), although a decrease in SFA and an increase in unsaturated fatty acids occurred (85). On the other hand, changes in the UI as well as membrane fluidity of various strains and species of *Tetrahymena* have been measured (86, 87). In *Paramecium,* changes in molecular bulky groups such as OH groups in SPL long chain bases and neutral sphingolipid fatty acids, and the lengths of aliphatic chains that affect hydrophobic interactions in the bilayer have not been sufficiently examined to determine their importance in maintaining membrane fluidity of the ciliary membrane or as possible thermosensory receptors (50). It is not known whether membrane fluidity is, in fact, maintained at different culture ages or growth temperatures since direct physical measurements on fluidity have not been made on membranes of this organism.

Paramecium cells grown at different temperatures exhibit different thresholds for avoiding higher temperature zones in a T-maze tube apparatus similar to that used for assaying chemotactic responses (50, 77). Thermotaxis involves avoidance reactions with a net movement away from the high temperature zone analogous to responses of the organism to repellent compounds (77). Cells grown in bacterized cultures at 15°C avoided temperatures that were lower than that avoided by cells grown at 28°C, and those cultured at 28°C avoided temperatures that were lower than that avoided by cells cultured at 35°C (77). Cells grown axenically for two generations at 25°C had a threshold response to avoiding 45°C that was lower than the threshold seen in cells grown at 28°C in bacterized cultures. These axenically cultured cells exhibited a reversible loss in their avoidance of 42°C when shifted to 35°C.

Reacquisition of the avoidance to 42°C in cells returned to 25°C was achieved only after 4 hr at the new temperature (50) **(Fig. 8)**. Thermal avoidance is not only influenced by culture temperature, but also depends on culture age. The greatest responses were observed in cells at late log phase (50).

If membrane lipid fluidity or other membrane properties change with growth temperature and culture age, membrane itself may contain the "thermosensory mechanism." More specifically, it is thought that the lipid composition may determine the absolute threshold temperatures for thermal avoidance. If so, the altered lipid composition, lipid-lipid or lipid-protein interactions may alter *1*) the membrane potential, *2*) the threshold for depolarization, and *3*) activation of Ca^{2+} channels in the ciliary membrane, since thermotaxis involves ciliary reversals. For example, the loss and reappearance of thermal avoidance were observed after several hours at the

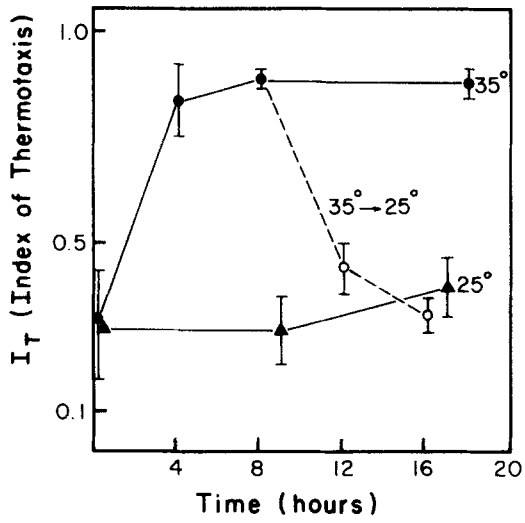

Fig. 8. Thermal avoidance of 42°C as a function of time at 25° and 35°C (50). *Paramecium tetraurelia* 51s cells were washed and tested in a solution containing 1 mM MOPS, 1 mM $Ca(OH)_2$, 0.35 mM citrate, and adjusted to pH 7.0. The I_T represents the percentage of cells entering the heated arm of a three-way stopcock; as thermal avoidance decreased, I_T increased. Cells were grown for two generations at 25°C then either kept at 25°C (▲) or transferred to 35°C (●). After 8 hr at 35°C, some cells were returned to 25°C (○). From Ref. 50, Fig. 2; Hennessey, T. M., and D. L. Nelson. 1983. Biochemical studies of the excitable membrane of *Paramecium tetraurelia*. VIII. Temperature-induced changes in lipid composition and in thermal avoidance behavior. *Biochim. Biophys. Acta.* **728:** 145–158. Reprinted by permission.

new temperature. If the fluidity of the membrane were the trigger, one would expect that an upshift in temperature would make the membrane "melt" and a downshift should make it more rigid. Thus, the effect on the avoidance reaction would be expected as an immediate consequence of temperature shifts. However, several hours are required to presumably bring about specific changes in the composition and/or molecular interactions in membranes, after which ion channel functions are altered.

X. Conclusions

Within the last 10 years, the lipids of *Paramecium tetraurelia* have been examined and characterized. Studies on lipid metabolism in this ciliate are beginning. Initially, it seemed that the lipids and the metabolism of lipids in this organism would be similar to those of *Tetrahymena,* even though *Paramecium* is a lipid auxotroph and some *Tetrahymena* strains do not have a lipid requirement. Recently, large differences in lipids and proteins of various species and strains of *Tetrahymena* have been found and thus it is not surprising to find that *Paramecium tetraurelia* has its own distinctive lipid composition and metabolic pathways. *Paramecium* lends itself to the elucidation of important questions in the understanding of lipid metabolism. For example, unlike the case with *Tetrahymena,* serine was found to be incorporated into glycero- and sphingoethanolamine phospholipid and phosphonolipid head groups of *Paramecium,* suggesting that serine lipid intermediates may be important in sphingolipid and phosphonolipid biosyntheses. Also, the mutants with putative defects in lipid metabolism are potentially useful for determining the importance of lipids in regulating ion channel gating mechanisms.

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