Biochemical studies of the excitable membrane of *Paramecium tetraurelia*. VII. Sterols and other neutral lipids of cells and cilia

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Abstract The neutral lipid content of cells and cilia of *Paramecium tetraurelia* was determined as a function of growth stage and of growth medium composition. The major sterol(s) of deciliated cells and of cilia were the sterol provided in the growth medium (e.g., stigmasterol) and its 7-dehydro derivative. Body sterol esters and triglycerides accumulated during exponential cell growth and became depleted during stationary phase. Isolated cilia contained neither sterol esters nor triglycerides. The ratio of phospholipid to free sterol in cilia was constant (approximately 5:1) throughout exponential growth in axenic or bacterized medium, but the relative proportions of free sterol and 7-dehydrosterol varied with growth phase and with growth medium composition. Cholesterol did not support the growth of *Paramecium*, but was taken up rapidly into cells and cilia as the free sterol and its 7-dehydro derivative. The fatty acids of sterol esters and of triglycerides were similar to those of membrane phospholipids.—**Hennessey, T. M., D. Andrews, and D. L. Nelson.** Biochemical studies of the excitable membrane of *Paramecium tetraurelia.* VII. Sterols and other neutral lipids of cells and cilia. *J. Lipid Res.* 1983. **24:** 575–587.

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The excitable ciliary membrane of *Paramecium tetraurelia* regulates the swimming behavior of this ciliated protozoan by coordinating the direction of the power stroke of the locomotory cilia that cover the cell's surface. A variety of stimuli produce membrane depolarization, which causes voltage-sensitive Ca^{2+} channels in the ciliary membrane to open, thus allowing the entry of extracellular Ca^{2+}. The influx of Ca^{2+} triggers a temporary reversal in the direction of ciliary beating, and thus of cell motion (1). The voltage-sensitive Ca^{2+} channels as well as other ion channels that function during excitation (2) are presumably membrane proteins that span the lipid bilayer of the surface membrane. It is reasonable to suppose that the interactions of lipids of the excitable membrane with these membrane proteins may influence their function as ion channels. In fact, we recently described a class of mutants of *Paramecium* with defects in membrane excitability and in ciliary membrane phospholipid composition (3). The physiological defects in these mutants were "cured" by growth in medium supplemented with stigmasterol.

The type and amount of sterol in a biological membrane affects both the activities of many membrane-localized enzymes and the physical state of the membrane bilayer (4, 5). Very little is known concerning the role of sterols in excitable membranes; it has not been easy to manipulate the sterol composition of neural tissue or of cultured excitable cells. *Paramecium* provides an excellent opportunity to explore the effects of membrane lipid composition upon excitability. The excitable membrane is readily separable from other cellular membranes (6). Mutants in both excitability and lipid composition exist, and excitable membrane function may be assayed sensitively by electrophysiological or behavioral tests (2).

Previous studies have established that the ciliary membrane of *Paramecium tetraurelia* is rich in sphingolipids, phosphonolipids, and ether-linked alkyl chains (7, 8). Ciliary phospholipids and fatty acid composition change with the stage of growth in axenic medium (8, 9) although ciliary lipid composition changes little with growth stage in bacterized cultures (7). The sterol composition of isolated ciliary membranes has not been described, nor is it known whether the sterol composition of cilia varies with growth stage or growth medium composition. We have therefore determined the kinds and amounts of sterols and other neutral lipids in cilia and deciliated bodies as a function of growth stage and growth medium, as a first step towards defining the relationship between lipid composition and excitable membrane function. We have also measured thermal avoidance behavior, a sensitive indicator of excitable

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; FAME, fatty acid methyl ester.

membrane function, as a function of growth stage and medium.

MATERIALS AND METHODS

Materials

Paramecium tetraurelia (syngen 4, stock 51s) were grown at 28–29°C in two media: bacterized Cerophyl (4) and the crude axenic medium of Soldo and Van Wagtendonk (10). Both media contained 5 mg/l stigmasterol (Sigma). Cerophyl powder was obtained from Cerophyl Laboratories, Inc., Kansas City, MO.

Growth, harvesting, and deciliation

Both axenic and bacterized Cerophyl cultures were started with a 10% (v/v) inoculum from stationary phase cultures. Five 800-ml bacterized Cerophyl cultures of *Paramecium* were harvested and deciliated after 17, 24, 28, 44, and 69 hr by the Ca^{2+} shock method described previously (6). We confirmed the finding of Brugerolle, Andrivon, and Bohatier (11) that catalase is associated with cell bodies and not with cilia, and we therefore routinely monitored catalase activity in cilia and ciliary supernatants. If more than 5% of the total cell catalase was released during deciliation, we assumed that enough cell lysis had occurred to cause contamination of the ciliary preparation with material released from cell bodies, and we discarded such preparations. Harvesting and deciliation methods for axenic cultures were similar to those used for bacterized cells except that 2–4 times the Ca^{2+} concentration was needed to deciliate the cells. Three 400-ml axenic cultures were harvested and deciliated after 1, 2, and 3 days. Another 400-ml culture was split, half was harvested and deciliated after 4 days and the rest after 5 days. Growth was monitored by counting an aliquot of cells after immobilizing them with 0.5 mM $NiCl_2$.

Lipid extraction

The deciliated body or ciliary pellets were extracted using a modified Bligh and Dyer procedure (12) as described by Andrews and Nelson (7). Butylated hydroxytoluene (Sigma) was added (final concentration 5 μg/ml) as an antioxidant. All organic solvents were redistilled before use.

Lipid separation

The total lipid fraction of body or cilia was separated into phospholipids and neutral lipids on a silicic acid column (Bio-Sil A, 100–200 mesh, from Bio-Rad, Richmond, CA) as described by Kates (13). The column was packed in a 5-3/4 inch Pasteur pipette plugged with glass wool. The final column volume was 1.5 ml. After packing with a methanol slurry of silicic acid, the column was washed with ten volumes of chloroform. The total lipid fraction was dissolved in chloroform and added to the column. The neutral lipids were eluted with ten volumes of chloroform and the phospholipids were eluted with ten volumes of methanol. Both fractions were stored under N_2 at −20°C.

The neutral lipids were further fractionated by two methods. The first employed a 2-ml silicic acid column washed with ten volumes of hexane, and loaded with the neutral lipids dissolved in hexane. Hydrocarbons were eluted with 5 ml of hexane, then sterol esters, triglycerides, fatty acids, and alcohols, and sterols were eluted with hexane–diethyl ether mixtures of 99:1, 95:5, 92:8, and 85:15, respectively. The second method used 4-mm thick preparative TLC plates (silica gel 60, EM Reagents). The solvent system was hexane—diethyl ether—acetic acid 80:20:1. Areas with retention times similar to iodine-stained standard spots were scraped, extracted with chloroform–methanol–water 30:50:20 and stored under N_2 at −20°C.

Analysis

Phospholipids were identified by comigration with standards on one-dimensional TLC with chloroform–acetone–methanol–acetic acid–water 6:8:2:2:1 as the solvent system. Spots were visualized with iodine and other stains as described by Andrews and Nelson (7). Phospholipid concentration was determined by measuring inorganic phosphate using the phosphate test of Chen, Toribara, and Warner (14).

Neutral lipids were separated by 0.25-mm-thick one-dimensional TLC (silica gel 60, EM Reagents) with hexane–diethyl ether–acetic acid 80:20:1. The identification of sterol esters was based on their hydrolysis (in NaOH) to sterols and free fatty acids as monitored by TLC, and on specific staining reactions for sterols on TLC plates. Charring with either H_2SO_4 or $FeCl_3$ was used to stain sterols and sterol esters (13). Triglycerides were identified by their co-chromatography with triglyceride standards in TLC and GLC, their sensitivity to hydrolysis in NaOH, and the release of fatty acid methyl esters upon treatment with methanolic-HCl. Free fatty acids were identified by co-chromatography with standards and by their staining on TLC plates with bromcresol green spray (13). Sterol identification was based on staining behavior, resistance to saponification, and co-chromatography with standards in several TLC systems and on GLC columns. The identification of the fatty alcohol fraction was tentative, and was based on position of elution on the silicic acid column described above and on co-chromatography (TLC) with fatty alcohol standards. Standards were cholesterol myristate,

tripalmitin, and stigmasterol as a representative sterol ester, triglyceride and sterol, respectively. Iodine vapor was also used as a general stain, but iodine-stained lipids were not used in chemical characterization studies. A representative iodine-stained TLC plate of the neutral lipids of deciliated bodies and cilia is shown in **Fig. 1.** On heavily loaded TLC plates, a yellowish spot was visible between the sterol and sterol ester region, where a ubiquinone marker is found. This spot, like ubiquinone, stained strongly with rhodamine under UV light; it is probably ubiquinone. The sterol ester and free sterol regions, as well as spots in the regions marked TG, FFA, and ALC fluoresced under long wavelength UV light.

Sterols and triglycerides were further analyzed by a Varian Gas Chromatograph Model 3700 with a CDS III integrator. Sterols were tentatively identified by comparison of their retention times with those of standards (cholesterol, 7-dehydrocholesterol, stigmasterol, and sitosterol) on a 3-ft glass column (2 mm id) packed with 3% OV-17 on 100/200 mesh Supelcoport. Carrier gas was N_2 at a flow rate of 20 cc/min. Injector temperature was 320°C and the ion detector temperature was 350°C. The column temperature was programmed to rise from 260–320°C at 5°C/min. Sterols were quantitated by comparison of their peak areas with the peak areas of standards of known concentration. Triglycerides were similarly analyzed with a 3-ft column packed with Dexsil 300 on 100/200 mesh Supelcoport and N_2 carrier gas at a flow rate of 40 cc/min. Column temperature was programmed for an 8°C/min rise from 330 to 360°C. Injector and ion detector temperatures were 350°C. Trimyristate, tripalmitin, and tristearin were used as standards.

Free fatty acids were generated from sterol esters and triglycerides by alkaline hydrolysis and analyzed by GLC. Samples were incubated in 5 ml of 3 N NaOH–methanol 1:9 at 70°C for 2 hr. Free sterols were removed by extracting with three volumes of hexane. Free fatty acids were recovered by acidification with 0.3 ml of 6 N HCl and extraction with three volumes of hexane. Free fatty acids were converted to fatty acid methyl esters (FAMEs) for GLC analysis by incubating in methanol–HCl 5:1 for 5 hr at 70°C. FAMEs were recovered by extracting with three volumes of hexane. Since sterol esters could not be identified on any of the columns used, the amount of sterol esters present was determined by the amount of free sterol generated by alkaline hydrolysis. This method converted 99% of cholesteryl myristate samples to cholesterol and caused all of the peaks identified by GLC as triglycerides to disappear. Individual sterol ester and triglyceride spots

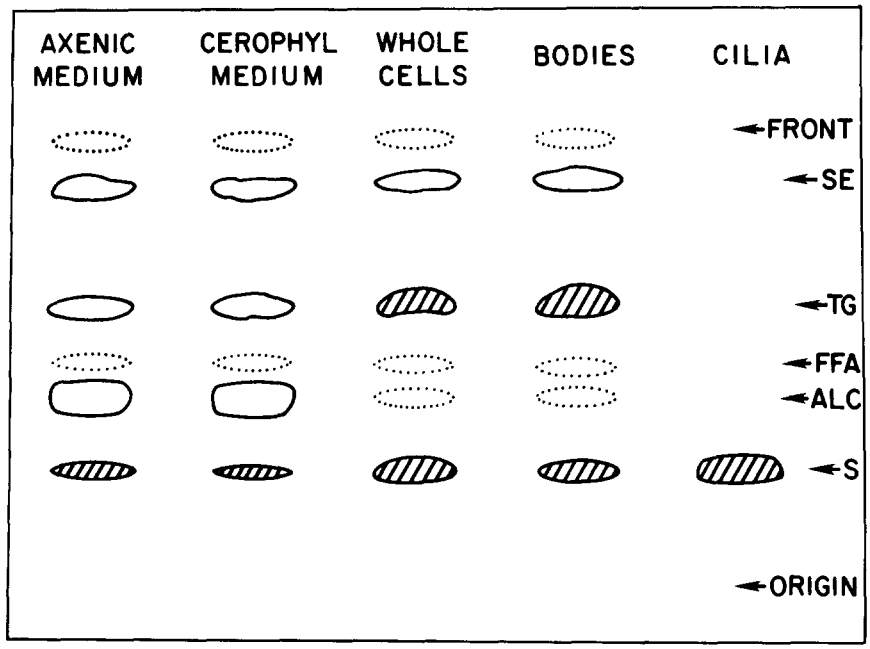

Fig. 1. Thin-layer chromatography of neutral lipids of media, cells, and cilia. The neutral lipids fraction was prepared as described in Materials and Methods from the Cerophyl and axenic media, and from whole cells, deciliated bodies, and isolated cilia. After thin-layer chromatography on silica gel (see Methods and Materials) the plates were stained with iodine and yielded the patterns drawn here. The cross-hatched regions were most dense, and the dotted regions were least dense. Not shown here is a yellow spot that ran just behind the sterol ester marker and with a ubiquinone marker. This spot was strongly stained when sprayed with rhodamine and viewed with UV light. SE, sterol esters; TG, triglycerides; FFA, free fatty acids; ALC, alcohols; S, sterols.

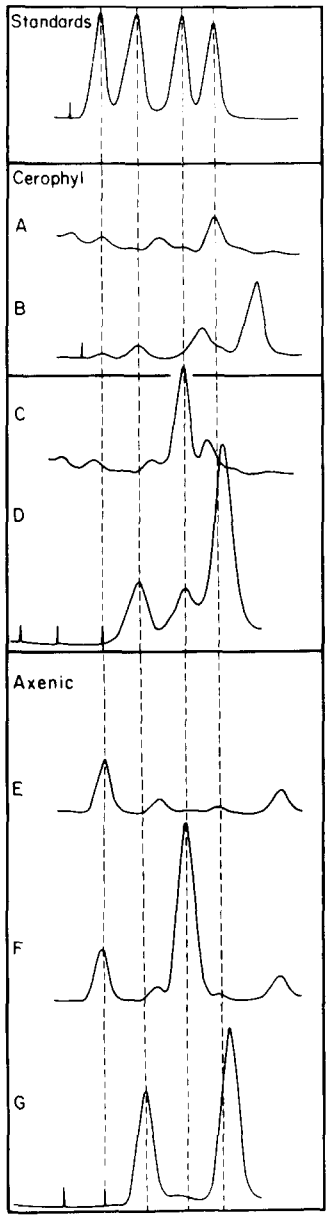

Fig. 2. Gas–liquid chromatographic elution profile of the sterol fractions of cilia and growth media. Sterol fractions were prepared and analyzed as described in Materials and Methods. The four sterol standards are (left to right) cholesterol, 7-dehydrocholesterol, stigmasterol, and β-sitosterol. Panel A, unsupplemented Cerophyl medium only; B, ciliary sterols from cells cultured in unsupplemented Cerophyl; C, Cerophyl medium supplemented with stigmasterol (5 mg/l final concentration); D, ciliary sterols from cells cultured in supplemented Cerophyl; E, axenic medium (only), before addition of stigmasterol; F, axenic medium containing added stigmasterol; G, ciliary sterols from cells cultured in axenic medium.

from preparative TLC plates were scraped, extracted, and subjected to alkaline hydrolysis to generate fatty acids representative of these fractions. The method for converting free fatty acids to FAMEs was also used to generate FAMEs from phospholipids. The FAMEs were analyzed on a 6-ft column packed with 10% SP-2330 on 100/120 mesh Supelcoport with a carrier gas (N_2) flow rate of 20 cc/min. Column temperature was programmed from 150°C to 200°C at 6° C/min following a 2-min delay. Injector temperature was 270°C and ion detector temperature was 300°C.

In some cases, sterols were analyzed with a GLC–mass spectrometer (Dupont 21-491B) interfaced with an AEI DS-50 data system. Ionizing voltage was 70 eV, 300 μa with source T at 290°C. The Varian 2740 GLC utilized a 6-ft column packed with 1% OV-17 on 100/120 mesh Varaport 30 with helium flow rate of 30 cc/min. The column was temperature programmed from 230°C to 270°C to 2°C/min. Injector temperature was 320°C and the ion detector temperature was 350°C.

Cholesteryl myristate, cholesterol, stigmasterol, sitosterol, 7-dehydrocholesterol, and cephalin were all obtained from Sigma Chemical Co., St. Louis, MO. Fatty acid methyl esters (GLC standards) were obtained from Supelco, Inc., Bellefonte, PA. Tripalmitin, trimyristin, and tristearin were from Eastman Kodak, Rochester, NY.

RESULTS

Neutral lipids of whole cells

Thin-layer chromatography of the neutral lipids fraction from whole cells revealed the presence of substantial quantities of triglycerides, sterol esters, and free sterols, and smaller quantities of material with the mobility of free fatty acids and fatty alcohols (Fig. 1).

The pattern of neutral lipids in deciliated bodies was very similar, qualitatively and quantitatively, to that of whole cells (Fig. 1), as expected from the fact that more than 90% of the neutral lipids of whole cells remain in deciliated bodies. In contrast to this pattern, the only neutral lipids present in significant quantities in cilia were sterols (Fig. 1). The major classes of neutral lipids were resolved by chromatography on silicic acid columns for more detailed analysis of each class.

Neutral lipids of the growth media

The Cerophyl medium with no added stigmasterol contained four sterols (**Fig. 2**), the most prominent of which we have tentatively identified as sitosterol. The absolute amount of sterol in unsupplemented Cerophyl was different for different batches; the range of values for several batches was 0.05–0.6 mg/l. Sterol-supplemented Cerophyl contained 5 mg/l of added stigmasterol. Analysis of the free sterols of axenic medium (Fig. 2) revealed the presence not only of stigmasterol, but also of a sterol that co-migrated with cholesterol. On further analysis this component was found to be a con-

taminant of the animal lecithin used in the axenic medium and its concentration in the axenic medium was about 1.0 mg/l. Smaller amounts of other unidentified sterols were also present in the axenic medium; they originated in the proteose and trypticase peptones. The amount of stigmasterol added to axenic medium was also 5.0 mg/l, and it is therefore the major sterol of that medium.

Sterols and sterol esters of whole cells

The silicic acid column fraction containing free sterols of whole cells, grown in bacterized Cerophyl with added stigmasterol, contained two major sterol species and several minor species which were resolved by GLC (Fig. 2). One of the peaks co-migrated with authentic stigmasterol, and the other, slightly more polar, sterol was shown by GLC–mass spectrometry to have a parent ion of mass 410, as expected for 7-dehydrostigmasterol which Conner et al. (15) found to be a major sterol component of *Paramecium*.

The free sterols of cells grown in bacterized Cerophyl medium without added stigmasterol were different from those of cells grown with stigmasterol; one of the cellular sterols co-migrated with a sterol in the Cerophyl plant extract (Fig. 2), and another more polar sterol in cells was probably its 7-dehydro derivative (15). GLC–mass spectrometry of the less polar sterol yielded the same parent ion mass and the same fragmentation pattern as that from authentic sitosterol (data not shown). The axenic medium is supplemented with stigmasterol and also with lecithin and proteose-peptone of animal origin. Cells grown in axenic medium contained stigmasterol and 7-dehydrostigmasterol, but also two other sterols, probably cholesterol and its 7-dehydro derivative. Other sterols represented less than 5% of total sterol.

Upon saponification the sterol esters of whole cells yielded the same sterols that were detected in the free sterol fractions of cells grown on each medium except that no cholesteryl esters were detected in axenic cells (data not shown). In every case, the ratio of sterol to 7-dehydrosterol was the same in sterol esters as in the free sterol fraction; there was no preferential esterification of one or the other. The fatty acids released by saponification of sterol esters qualitatively resembled those present in unfractionated phospholipids of the same cells (**Table 1**).

Fatty acids of neutral lipids and phospholipids of whole cells

The absolute amount of triglyceride and sterol ester varied significantly with growth phase and growth medium (as described later). Exponentially growing cells contained primarily 16:0, 16;1, 18:0, and 18:1 in their sterol esters and triglycerides, and in stationary phase 18:3 also became prominent (Table 1). The fatty acids in phospholipids of growing cells were more unsaturated than those of the neutral lipids; 16:0, 16:1, and 18:1 were major components, but 16:1, 18:2, 18:3, and 20:4 were also prominent. The phospholipids of stationary phase cells had a similar distribution of fatty acids, except that the proportions of 20:1 and 20:4 were higher in stationary phase than in exponential phase (Table 1).

TABLE 1. Fatty acids of phospholipids, sterol esters, and triglycerides of whole cells grown in bacterized medium

	Sterol Esters		Triglycerides		Phospholipids	
Fatty Acid	Log	Stationary	Log	Stationary	Log	Stationary
14:0	1.2[a]	1.7	1.8	1.9	1.4	1.4
15:0	0.4	0.3	0.4	0.6	0.8	0.9
16:0	29.0	11.0	28.0	16.7	25.4	23.0
16:1	4.4	15.0	9.1	24.0	14.3	8.1
18:0	19.0	4.3	16.0	7.4	2.7	2.1
18:1	24.5	25.0	25.0	29.0	22.0	23.0
18:2	3.1	5.8	2.6	1.0	9.1	7.5
18:3	4.4	18.5	6.9	15.7	9.8	5.4
20:1	3.3	9.3	3.4	0.4	1.4	11.0
20:3	3.7	6.0	2.6	3.1	1.2	0.8
20:4	6.7	2.2	3.7	0.0	12.1	16.0
% Unsaturated	50.0	83.0	54.0	73.0	69.7	71.7
Index of unsaturation	90.0	143.0	86.0	112.0	137.0	140.0

The three lipid fractions were prepared and analyzed as described in Materials and Methods. Each value is from one determination. The cell density was 4000 cells/ml for the logarithmic phase culture, and the stationary phase culture, harvested 24 hr later, had 6000 cells/ml.

[a] All values are given as percentage of total fatty acids in sample.

TABLE 2. Distribution of neutral lipids after deciliation

	Whole Cells[a]		Deciliated Bodies	Isolated Cilia
	mg/10⁶ cells	*μgm/mg protein*	*μgm/mg protein*	*μgm/mg protein*
Protein	10	1000	1000 (97)[b]	1000 (2)
Phospholipid	1.10	110	99 (87)	720 (13)
Free sterol	0.077	7.7	5.8 (80)	77 (20)
Sterol ester	0.070	7.0	7.2 (>95)	<3[c] (<1)
Triglycerides	1.94	194	190 (>95)	<90[c] (<1)

Lipids were analyzed as described in Methods and Materials, and protein was determined by the method of Lowry et al. (39) with bovine serum albumin as standard.

[a] Cells for this typical experiment were from an axenic culture at mid-logarithmic growth phase.

[b] Numbers in parentheses are percentages of each component of whole cells found in deciliated bodies and in cilia.

[c] These values are upper limits; the amounts in cilia were below the limits of detection.

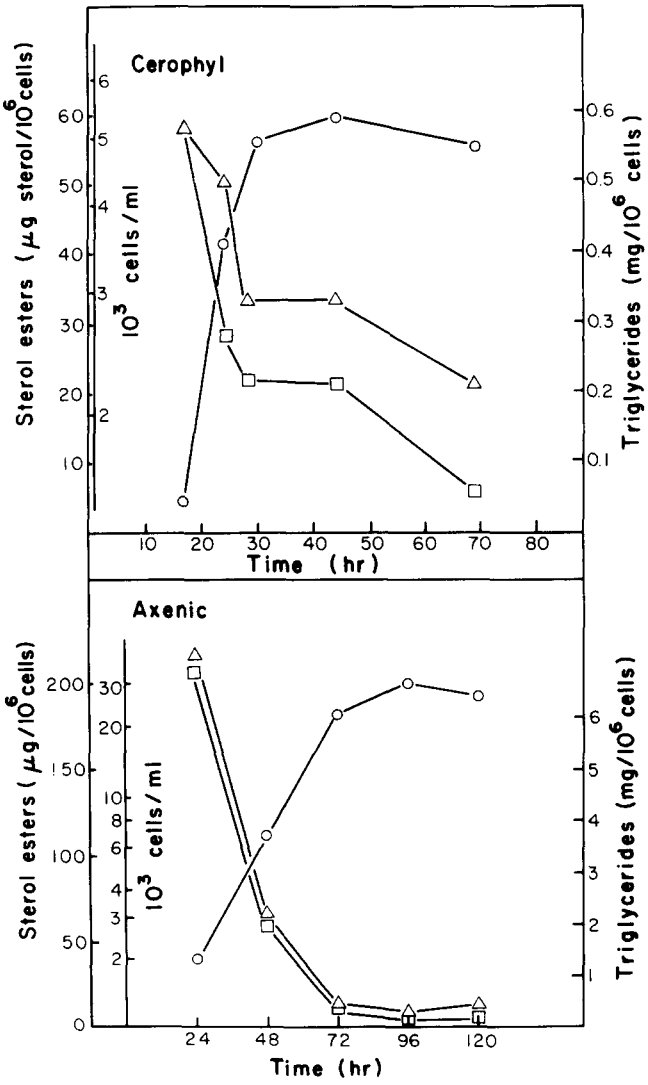

Fig. 3. Variation in sterol esters and triglycerides with growth stage. Cells cultured in bacterized Cerophyl or axenic medium were harvested, washed, and deciliated at the indicated times, and the cellular content of triglyceride and sterol ester of deciliated bodies was determined as described in Materials and Methods (cilia contained neither triglyceride nor sterol ester; see text). Each point represents the average from two completely independent experiments. The sterol ester content is expressed as mg of sterol generated from sterol ester by alkaline hydrolysis. Lines represent number of cells per ml (○ —— ○), sterol esters (△ —— △), and triglycerides (□ —— □).

Distribution of neutral lipids within deciliated cells and cilia

Cells were deciliated by the STEN-$CaCl_2$ method (6). Washed cells were resuspended in a 1:1 mixture of Dryl's solution and STEN (0.5 M sucrose, 20 mM Tris-chloride, 2 mM EDTA, 6 mM NaCl, pH 7.5) for 10 min. Cilia were detached by adding concentrated $CaCl_2$ and KCl to a final concentration of 10 mM caicium and 30 mM potassium. The resulting deciliated bodies (which represent ~97% of total cell protein) contained virtually the same amount of triglycerides and of sterol esters as intact cells (**Table 2**). Isolated cilia contained 10–15% of the phospholipid of whole cells and substantial quantities of free sterol, but in most experiments the ciliary fraction contained no detectable triglycerides or sterol esters. Occasionally, when deciliation resulted in the lysis of a small fraction of the cells, triglycerides and sterol esters were recovered in the ciliary fraction, presumably as contaminants. To monitor for cell lysis we measured the activity of catalase (an enzyme found in cell bodies) (12) in the supernatant fraction after deciliation and low speed centrifugation to remove cell bodies. Normally, less than 3% of the total cellular catalase activity was recovered in this fraction, but occasionally up to 10% of the catalase was released. Only in those cases in which catalase release indicated that lysis had occurred during deciliation did we detect triglycerides or sterol esters in the isolated cilia fraction.

Variation of cell body lipid composition with growth phase

The growth rate of *Paramecium* in the bacterized Cerophyl medium was about twice that in axenic medium, but the axenic medium supported growth to a

TABLE 3. Sterol and phospholipid content of cell bodies and cilia as a function of growth stage

Growth Stage[a]	Cell Bodies: Axenic Phospholipid	Cell Bodies: Axenic Free Sterol	Cell Bodies: Axenic Phospholipid/Sterol Ratio	Cell Bodies: Bacterized Phospholipid/Sterol Ratio	Cilia: Axenic Phospholipid/Sterol Ratio	Cilia: Bacterized Phospholipid/Sterol Ratio
	mg/10⁶ cells					
1 Early logarithmic	1.48	0.093	16 ± 2.7	14 ± 2.0	13 ± 2.0	5.8 ± 1.9
2 Mid-logarithmic	1.00	0.061	16 ± 1.7	14 ± 2.3	6.1 ± 1.4	3.9 ± 0.2
3 Late logarithmic	0.33	0.019	17 ± 0.2	16 ± 3.3	5.2 ± 1.6	4.0 ± 0.5
4 Early stationary	0.27	0.020	14 ± 0.05	13 ± 0.6	5.1 ± 0.30	3.2 ± 1.3
5 Late stationary	0.18	0.009	20.0	17 ± 4.8	12 ± 6.7	4.4 ± 0.05

[a] These growth stages correspond to the five points on Fig. 3 for axenic cultures; data in this table and in Fig. 3 are from the same experiments. The free sterol and phospholipid compositions were determined as described in Materials and Methods. Each value represents the mean ± SD (n = 2). Absolute values are not given for cilia because the recovery of cilia after deciliation was variable.

higher final cell density than did the bacterized Cerophyl (**Fig. 3**). Samples of each culture were removed for lipid analysis at early, mid-, and late logarithmic phase, and at early and late times during stationary phase. The amount of each major lipid class per cell, as well as the relative proportion of the several lipid classes, varied systematically with growth phase. The total lipid per cell decreased as cells approached stationary phase, as expected; Kaneshiro et al. (9) have reported that cell size decreases twofold (in axenic medium) during this period. The phospholipid per cell decreased twofold in bacterized Cerophyl medium and more than sixfold in axenic medium, indicating that there is a reduction not merely in neutral lipid stores, but in amount of membrane lipids.

Cells in both bacterized Cerophyl and axenic medium contained relatively large amounts of triglycerides early in exponential phase of growth. These lipids were progressively depleted as cells approached stationary phase (Fig. 3). When the apparent reduction in cell size and phospholipid content in stationary phase is taken into account, the depletion of triglycerides was at least 70% in bacterized cultures, and more than 90% in axenic cultures. Exponentially growing cells in both media accumulated sterol esters in addition to triglycerides, although in much smaller amounts. In axenic medium, similar large reductions in sterol esters also occurred in late exponential phase (Fig. 3). The depletion of sterol esters during growth was not as striking in bacterized Cerophyl medium as in axenic medium. In contrast with these changes, the ratio of free sterol to phospholipid in cell bodies was nearly constant throughout growth, and was virtually the same in axenic and bacterized cultures (**Table 3**).

Throughout growth stigmasterol and 7-dehydrostigmasterol were the principal free sterols of deciliated bodies from both axenic and bacterized Cerophyl cultures. The relative proportions of the two sterols varied with growth phase; during early log phase, approximately equal amounts of both were present, but as cultures approached stationary phase, a progressive increase in the proportion of 7-dehydrostigmasterol occurred, so that by late stationary phase, 7-dehydrostigmasterol represented 80–95% of the free sterol of deciliated bodies (**Table 4**).

The fatty acid composition of sterol esters and triglycerides was also determined at each growth phase (**Table 5**). The major species present in both fractions were palmitic (16:0), palmitoleic (16:1), oleic (18:1), and linolenic (18:3) acids, the relative proportions of which

TABLE 4. Ratio of stigmasterol/7-dehydrostigmasterol in deciliated bodies and cilia

Growth Stage	Cell Bodies: Bacterized	Cell Bodies: Axenic	Cilia[a]: Bacterized	Cilia[a]: Axenic
Early logarithmic	0.56 ± 0.04	0.54 ± 0.04	0.41	0.06
Middle logarithmic	0.53 ± 0.02	0.26 ± 0.02	0.68	0.10
Late logarithmic	0.30 ± 0.02	0.16 ± 0.004	0.78	0.03
Early stationary	0.28 ± 0.03	0.10 ± 0.01	0.18	0.08
Late stationary	0.20 ± 0.005	0.06 ± 0.01	0.21	0.08

Stigmasterol/7-dehydrostigmasterol ratios of deciliated bodies and cilia from bacterized and axenic cells as a function of growth stage. Sterol determinations were made as described in Materials and Methods. Total sterol was assayed for all samples. Each value represents the mean ± SD (n = 2).

[a] Only one determination.

TABLE 5. Fatty acids of neutral lipids (triglycerides and sterol esters) and of phospholipids of deciliated bodies at different growth stages in Cerophyl medium supplemented with stigmasterol

	Growth Stage									
	Early Log		Mid-Log		Late Log		Early Stationary		Late Stationary	
Fatty Acid	NL	PL	NL	PL	NL	PL	NL	PL	NL	PL
14:0	1.8	1.8	1.4	1.4	1.5	1.3	1.5	1.9	1.5	1.4
15:0	0.2		0.4	0.8		0.7	0.5	1.1	0.5	0.9
16:0	26.7	30.4	26.6	25.6	23.2	25.2	21.4	24.6	19.3	24.2
16:1	12.1	11.7	13.3	14.5	14.7	13.7	12.6	11.0	11.7	8.2
18:0	6.6	3.3	4.3	2.8	5.1	2.7	6.1	2.6	5.5	2.1
18:1	24.3	19.9	22.9	22.2	28.1	20.6	27.7	19.9	29.6	23.0
18:2	7.3	8.1	7.6	9.2	7.2	7.1	7.3	8.0	7.2	7.6
γ18:3	3.2	7.2	4.7	9.9	3.2	8.1	3.3	6.6	4.6	5.4
18:3	12.1	11.7	11.8	1.4	11.5	10.3	11.7	11.5	11.7	11.1
20:4	5.7	6.1	6.9	12.2	5.5	10.2	7.8	12.9	8.1	16.1
Percent unsaturated	64.7	64.7	67.2	69.4	70.2	70.0	70.4	69.9	72.9	71.4
Cells/ml	1,500		3,600		5,000		5,300		4,500	

Fatty acids of neutral lipids (NL) and of phospholipids (PL) of deciliated bodies as a function of growth phase. Cells were grown in bacterized medium. Each value represents one determination.

remained nearly constant throughout growth. There was a slight increase in the proportion of unsaturated fatty acids (from 65% to 72%) as cells entered stationary phase. The fatty acid composition of neutral lipids was similar to those of the phospholipids extracted from the same cells (Table 5), except that in the stationary phase, arachidonic acid (20:4) became more prominent in phospholipids but not in neutral lipids.

Variation of ciliary lipid composition with growth phase

In the bacterized Cerophyl medium the ratio of sterol to phospholipid in cilia was nearly the same at all stages of growth: sterols comprised 20–25% (by mass) of the ciliary lipids, and the rest was phospholipid (Table 3). During exponential growth, the cilia of cells grown in axenic medium had the same proportion of sterol to phospholipid; sterols made up 18–20% by weight. However, early in exponential phase and late in stationary phase the cilia from axenically grown cells contained relatively less sterol, 7–10% by mass. In both media, stigmasterol and 7-dehydrostigmasterol were the principal sterols of cilia. The axenic medium contained, in addition to stigmasterol, various amounts of cholesterol introduced as a contaminant of the lecithin. This cholesterol was taken up and incorporated into the body and ciliary membranes (as cholesterol and 7-dehydrocholesterol) to an extent that varied with the amount of cholesterol in the axenic medium. When cholesterol is the major sterol of the medium, cholesterol and its 7-dehydro derivative can represent up to half of the total sterol of cilia (see below).

Cilia from bacterized Cerophyl cultures contained relatively less 7-dehydrostigmasterol than cilia from axenically grown cells (Table 4), in which 7-dehydrostigmasterol comprised 90–95% of the total ciliary sterol. In both media, the ratio of stigmasterol to its 7-dehydro derivative generally decreased with growth stage (Table 4).

Dependence of growth and lipid composition upon sterol content of medium

Paramecium cannot synthesize its own sterols, but depends upon the medium to supply its sterol needs (10, 15). The hot-water extract of cereal grass (Cerophyl medium) provides enough sterol to support growth, but we found that the sterol content of different batches of Cerophyl medium ranged from below 0.05 mg/l to above 0.6 mg/l. To determine the concentration of sterol needed for optimal growth in the Cerophyl medium, we cultured cells in Cerophyl medium diluted fourfold from the usual composition to reduce the sterol content and supplemented with stigmasterol to give final concentrations between 0.03 and 10.0 mg/l. At low sterol concentrations, the growth rate was substantially less than that in high-sterol medium; the lowest stigmasterol concentration that supported optimal growth was 2.0 mg/l. Cells cultured in low-sterol (unsupplemented) bacterized Cerophyl medium contained significantly less free sterol and sterol ester than cells cultured in sterol-supplemented Cerophyl (data not shown).

When stationary phase cells from axenic medium were transferred to fresh axenic medium containing no added sterol, they underwent about three divisions be-

TABLE 6. Effect of incubation in low-sterol medium upon the free sterol content of axenic whole cells

	With Added Stigmasterol			Without Added Stigmasterol[a]		
	0 Day	1 Day	2 Days	0 Day	1 Day	2 Days
Cells ($\times 10^6$)	0.20	0.20	5.0	0.20	0.20	1.0
P-lipid (mg)	0.30	0.60	3.5	0.30	0.50	0.90
Sterol (μg)	20.0	35.0	270.0	20.0	11.5	8.4
Sterol (μg/10^6 cells)	95.0	144.0	54.0	95.0	76.0	8.5
P-lipid (mg/10^6 cells)	1.4	2.6	0.70	1.4	3.1	1.0
P-lipid/sterol (mg/mg)	15.2	18.0	13.0	15.2	41.0	112.0

[a] Axenic medium contains about 1 mg/l of cholesterol before addition of stigmasterol. Stigmasterol was added to a concentration of 5 mg/ml. Each value represents one determination. Culture volume was 200 ml.

fore their growth ceased, presumably due to sterol limitations. In comparison, axenic cells in stationary phase transferred to axenic medium *with* sterol went through four or five divisions in the same period (3 days). The sterol content of control cells three days after transfer to fresh medium was as expected for exponentially growing cells: the phospholipid/sterol ratio was about 13:1. In contrast, cells transferred to low sterol medium were measurably depleted of sterol after 1 day (phospholipid/sterol ratio was 41:1) and very severely depleted by 3 days (phospholipid/sterol ratio was 112:1) (**Table 6**). These sterol-depleted cells were smaller than the control cells, but appeared otherwise normal and were not grossly defective in either motility or Ba^{2+}-stimulated avoiding reactions (data not shown).

Uptake and metabolism of cholesterol

Although cholesterol does not satisfy the growth requirement for sterol (15), it was taken up rapidly from the growth medium and incorporated into ciliary membranes. Cells cultured in axenic medium, then washed and transferred to fresh medium containing cholesterol as the only added sterol, underwent one or two divisions before their growth stopped. They remained viable and motile for at least 24 hr in the cholesterol-substituted medium. Within 1 hr after the transfer to cholesterol medium, both cells and cilia contained significant amounts of cholesterol and its 7-dehydro derivative (**Table 7**), and by 4 hr cholesterol and 7-dehydrocholesterol made up 69% of the free sterol of deciliated bodies and 47% of ciliary sterols. The sterol which appeared in cells after 1–4 hr was primarily unaltered cholesterol, but by 24 hr, most of the cholesterol of cells and cilia had been converted to the 7-dehydro derivative (Table 7).

Thermal avoidance behavior varies with growth stage

The avoiding response given by paramecia as they enter a too-warm region can be quantitated with a simple T-maze assay in which one compares the number of cells that enter a control arm with the number that enter a heated arm. We have shown (16) that for cells at a given growth stage the thermal avoidance behavior is a function of both the growth temperature and of the temperature in the heated arm of the T-maze. Inexcit-

TABLE 7. Uptake of cholesterol into cell bodies and cilia

	Hours after Transfer to Cholesterol Medium							
	Bodies				Cilia			
Sterol	0	1	4	24	0	1	4	24
	μg sterol/mg P-lipid							
Stigmasterol	2.5	1.6	0.5	0.11	6.5	0	0	0
7-Dehydrostigmasterol	34.5	24.4	17.3	31.9	161.0	71.5	71.5	120
Cholesterol	0.6	33.5	33.8	6.2	0	54.1	42.3	1.4
7-Dehydrocholesterol	3.8	4.8	5.2	11.5	33.1	16	16.7	66.5
Total of all sterols	41.4	64.3	56.8	49.7	200	142	131	188
Ratio: $\frac{\text{cholesterol + 7-dehydrocholesterol}}{\text{stigmasterol + 7-dehydrostigmasterol}}$	0.12	1.5	2.2	0.55	0.20	0.98	0.83	0.57

Uptake of cholesterol into cell bodies and cilia following transfer to axenic medium containing 5 μg/ml cholesterol and no added stigmasterol. Each value represents one determination.

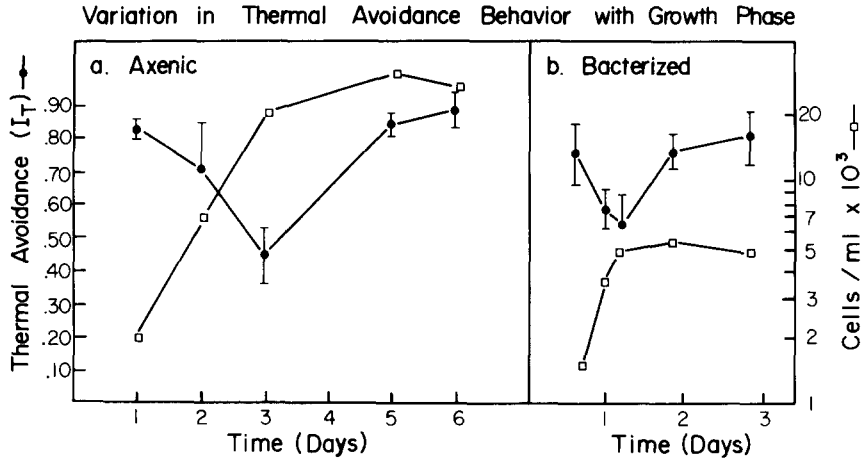

Fig. 4. Thermal avoidance and growth of axenic (a) and bacterized (b) cells. Test temperatures were 40°C for bacterized and 42°C for axenic cells. Both were tested in control solution (1 mM MOPS, 1 mM $Ca(OH)_2$, 0.35 mM citric acid, pH 7.0).

able mutants were incapable of thermal avoidance. To determine whether thermal avoidance also varied with growth stage, we assayed it for each of the growth stages for which we had determined lipid composition. For these studies, the growth temperature (28°C) and the temperature of the heated arm (40°C for bacterized cultures, 42°C for axenic) were held constant. In either bacterized Cerophyl medium (**Fig. 4b**) or axenic medium (Fig. 4a), thermal avoidance was greatest (I_T was smallest) late in exponential phase. Thermal avoidance was significantly poorer early in exponential phase and in stationary phase; the value of I_T at these times was only slightly smaller than the value (0.9) obtained with inexcitable (pawn) mutants, which show virtually no thermal avoidance (16). The increase in thermal avoidance during the late exponential phase of growth was not due merely to a change in the *motility* of cells with growth stage; the index of motility (see reference 16) did not vary significantly with growth stage (data not shown).

Cells grown in the axenic medium were generally less sensitive to the thermal stimulus than those grown in bacterized culture medium; at each growth stage, the temperature needed to elicit a given avoidance response from axenic cells was about 2°C higher than that needed to get the same response from cells cultured in bacterized Cerophyl.

DISCUSSION

Utilization of sterol esters and triglycerides during growth

There are at least three plausible explanations for the striking decrease in cellular triglycerides and sterol esters as cultures pass from the early exponential phase into the stationary phase of growth. *1*) Fatty acids of these compounds may be utilized for energy production. The decrease in these lipids is not due solely to cell division and consequent dilution; there is a net decrease in cellular triglycerides and sterol esters in the whole culture. Growing paramecia contain structures that have been identified as fat droplets by light and electron microscopy. We have observed that the size and number of these droplets decreased as cells enter stationary phase (data not shown). *2*) *Tetrahymena* has the enzymes of the glyoxalate cycle (17), and is therefore capable of using fatty acids for the synthesis of amino acids and carbohydrates. If *Paramecium* utilizes the glyoxylate cycle, some of the fatty acids stored in triglycerides and sterol esters may be used for biosynthesis. *3*) Borowitz and Blum (18) have shown that the triglycerides of *Tetrahymena* turn over rapidly during growth, and that much of the turnover is the result of triglyceride utilization for phospholipid synthesis. *Paramecium* may also use triglycerides for phospholipid synthesis.

Our analyses of the fatty acid composition of triglycerides and sterol esters are consistent with a biosynthetic role for these compounds; the kinds of fatty acids present in neutral lipids are similar to those in phospholipids (Table 1). Our results do not allow us to determine which of the three pathways is responsible for the disappearance of neutral lipids from cells during growth. The question might be resolved by prelabeling triglycerides with radioactive fatty acids and following their fate during subsequent growth in unlabeled medium.

Sterol content of ciliary membranes is regulated

Sterols were the only neutral lipids present in isolated cilia; only when deciliation was accompanied by cell lysis

(release of catalase) were sterol esters or triglycerides detected in the ciliary fraction. We found that the phospholipid-to-sterol ratio in ciliary membranes was nearly invariant (4–5:1) with growth stage in bacterized medium, and was the same in axenic cells (5:1) during exponential and early stationary phase. Only in late stationary phase and shortly after inoculation of a new culture did this ratio rise. Nozawa et al. (19) made a similar observation with *Tetrahymena;* starvation induced substantial changes in the ratio of phospholipid to sterol in cell body membranes (microsomes, mitochondria) but in ciliary membranes this ratio was much less affected by starvation.

The cilium is a highly specialized organelle, and it is reasonable to suppose that its membrane is similarly specialized. Dunlap (20) and Ogura and Takahashi (21) have shown that the ciliary membrane is the exclusive locus of the Ca^{2+} channels of the excitable membrane of *Paramecium,* and Andrews and Nelson (7) and Rhoads and Kaneshiro (8) have noted that the ciliary membrane is remarkably enriched in several phospholipids, including sphingolipids. The regulation of ciliary membrane lipid composition may be essential to the maintenance of proper ciliary function and thus to survival.

Function of sterols in ciliary membranes

Sterols have been shown to affect biological and model membranes in at least two ways: their presence alters *1*) the structure of the lipid bilayer and (perhaps indirectly) *2*) the function of membrane proteins (4, 5, 22–29). The structural effects of sterols are complex, but generally involve interactions with the fatty acyl side chains of phospholipids and consequent changes in the molecular packing and motion of these phospholipids, effects that are reflected in membrane "fluidity" (5, 22, 23, 30–34). Many intrinsic membrane proteins show altered enzymatic activity when the fluidity of the bilayer is altered. It is therefore not surprising that the kind and amount of sterol present in a membrane affects the activity of enzymes in the membrane (e.g., 24–26).

Comparative studies of phospholipid–sterol interactions in model systems and in biological membranes have provided evidence of at least three structural requirements of the sterol for "normal" (cholesterol-like) function: a planar steroid nucleus, a 3-β-hydroxyl substituent, and an intact side chain at position 17 (5, 35–37). All of the sterols found in *Paramecium* membranes (stigmasterol, sitosterol, cholesterol, and their 7-dehydro derivatives) share these structural features. In monolayers at the air–water interface, 7-dehydrocholesterol has nearly the same cross-sectional area as cholesterol, and both sterols cause condensation of the phospholipids in a mixed monolayer (36). When the cholesterol of erythrocytes is partially replaced by 7-dehydrocholesterol, there are slight, but measurable, decreases in osmotic fragility and in permeability to glycerol (35). It is therefore possible that changes in the ratio of sterol to 7-dehydrosterol such as those described in Table 4 cause subtle alterations in the physical state or function of *Paramecium* membranes.

The fact that *Paramecium*'s growth requirement can be met by certain phytosterols (stigmasterol, sitosterol) but not by other very similar sterols (e.g., cholesterol) is remarkable; it suggests that the role of sterols in the *Paramecium* membrane is not solely to provide a sterol nucleus to stabilize the lipid bilayer. It is probable that specific interactions between the sterol side chain and other lipids or proteins are required. We have confirmed that cholesterol *is* taken up by *Paramecium,* and undergoes oxidation to the 5,7-diene just as stigmasterol does (15). However, cholesterol is *not* esterified by *Paramecium* (15, this paper) suggesting that sterol esterification may be essential for cell growth. It is also possible that cholesterol is a suitable substitute for stigmasterol in membranes, but that it represses the synthesis of some other product essential to growth. We found that cells in which cholesterol had displaced at least half of the stigmasterol survived, swam normally, and even divided once before growth stopped. Forte et al. (3) have recently reported that the sterol content of the growth medium markedly influences the phospholipid composition and electrophysiological properties of ciliary membranes in *Paramecium.*

Ciliary membrane excitation is a sensitive test of sterol function

The specific function of ciliary membranes in *Paramecium* can be assessed very sensitively in two ways: *1*) by observing the regulation of swimming behavior (e.g., Ba^{2+}-induced ciliary reversals or thermal avoidance) or *2*) by measuring the electrical properties of the resting and excited membrane with a microelectrode (1, 2). The thermal avoidance assay is of particular interest in the context of this paper; Hennessey and Nelson (16) and Poff and Skokut (38) have suggested that membrane lipids may function as primary thermoreceptors that relay perceived temperature changes to associated ion channel proteins. To test this hypothesis we need to seek correlations between ciliary lipid composition and thermal avoidance behavior. We have shown here that both thermal avoidance behavior and ciliary sterol composition vary with growth medium composition and growth stage, but we have not established a close correlation between composition and behavior. We have found a correlation between the ratio of 7-dehydrostigmasterol to stigmasterol in cilia and thermal avoidance

behavior during the adaptation to changed growth temperature (40). We have also found that *ba*A, a mutant with altered phospholipid composition and an increased requirement for exogenous sterol, is defective in thermotaxis.[1] We therefore believe that the ciliary membrane of *Paramecium* presents a unique opportunity to explore the function of membrane sterols in excitable cells.

Note Added in Proof: K. E. Bloch has recently published a review of the relationship of sterol structure to membrane function (1983. *CRC Crit. Rev. Biochem.* **14:** 47–92).

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