Fatty acids of mitochondrial membranes from *Tetrahymena pyriformis*

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Abstract We have examined the fatty acid composition of the mitochondrial membranes in three strains of *Tetrahymena pyriformis*. All three had similar components and exhibited large amounts of unsaturated fatty acids. The cytoplasmic mutant, CA-10, which has a slower growth rate and unusual membrane morphology, had a slightly higher amount of *iso*-acids but was otherwise similar to the other strains in fatty acid composition. Arachidonic acid, previously undetected in extracts of *Tetrahymena*, was identified as a minor component of the mitochondrial membrane.

Supplementary key words Cytoplasmic mutant • *iso*-fatty acids • lipid:protein ratios • variation among strains

It is known that the fatty acid composition of the mitochondrial membrane is vital in maintaining the phosphorylative ability of that organelle (1, 2). Presumably the lipids act to promote the proper permeability of the membrane to small molecules and ions and serve to stabilize membrane structure. In most organisms, the fatty acid composition of the mitochondrial membranes is not known. In the course of our work on mitochondrial function in the ciliate protozoan, *Tetrahymena pyriformis*, we have examined the fatty acid composition of the mitochondrial membranes of three strains of *T. pyriformis*. These are: an amicronucleate strain, ST; a strain which exhibits conjugation, DN-5; and a mutant derived from DN-5 by treatment with nitrosoguanidine, CA-10, CA-10 is resistant to the drug, chloramphenicol, and exhibits cytoplasmic inheritance of this trait. Although the growth rate (3) and structural characteristics[2] of this organism are unusual, the fatty acid composition of the mitochondrial membranes is quite similar to other strains and thus does not account for the mutant characteristics.

MATERIALS AND METHODS

Tetrahymena pyriformis, strain D1968-5, syngen 1 (DN-5) and a chloramphenicol resistant mutant derived from this strain, CA-10, were obtained from Dr. E. Orias (Dept. of Biological Sciences, University of California at Santa Barbara). *T. pyriformis*, amicronucleate strain ST, was a gift of Dr. Y. Suyama (Dept. of Biology, University of Pennsylvania). Stock cultures were maintained axenically at room temperature in 2% proteose–peptone (Difco Laboratories, Detroit, Mich.), 0.1% yeast extract (Difco), plus 0.03% sequestrene iron chelate (Geigy Agricultural Chemicals, N.Y.). Stock cultures of strains DN-5 and ST (3 ml—approximately 2.5×10^5 cells/ml) were used to inoculate 2.8-l Fernbach flasks containing one liter of the above medium. Culture growth took place with reciprocal shaking at 65 cycles/min at 28.5°C. Because the chloramphenicol-resistant mutant grows more slowly than the parent strain, a larger inoculum was used. In this case, a stock culture of CA-10 (10 ml—approximately 1.0×10^5 cells/ml) was used to inoculate a 2-l roller bottle containing 200 ml of medium. The bottles were placed on a Rollacell apparatus (New Brunswick Scientific Co., N.J.) at a high setting of 80 and at 29°C. When the cells reached early logarithmic phase (approximately 2.0×10^4 cells/ml), they were transferred to Fernbach flasks containing 800 ml of medium and incubated under the same conditions as the parent strain. Culture growth was monitored by direct counting in a Fuchs-Rosenthal hemocytometer. Cells were harvested when they reached the mid-logarithmic phase (approximately 1.2×10^5 cells/ml).

Mitochondria were prepared for all strains by a procedure modified from (4). All the following operations were carried out at 4°C. Cells were harvested by centrifugation for 5 min at 3,000 *g* in a Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn.) and resuspended in a buffer containing 0.25 M sucrose, 0.15 M KCl, and 0.01 M Tris-HCl, pH 7.4. After washing once, the cells were resuspended in the same medium (approximately 4 ml/g wet weight of cells) and Triton X-100 was added to a final concentration of 0.01%. The cells were resedimented immediately. This Triton wash appears to weaken the cell walls so that they will break on homogenization. The cells were then suspended in a solution containing 0.35 M mannitol, 0.1 mM EDTA, and 0.02 M Tris-HCl, pH 7.4. The suspension was homogenized by hand using a Potter-Elvehjem type homogenizer. The suspension was centrifuged at 1,000 *g* to sediment debris and unbroken

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cells. The pellet was resuspended and the above procedure repeated. The supernatants were combined and centrifuged at 10,000 *g* for 15 min. The resulting pellet consisted of a tan layer of mitochondria usually covered by a lighter layer containing mostly cilia. As much of this upper layer as possible was removed with a Pasteur pipette. The mitochondria were resuspended in the above solution and recentrifuged. The resulting washed pellet was resuspended in the same buffer. The washing procedure was repeated if necessary to remove the contaminating cilia. Final concentration was 10–15 mg of mitochondrial protein per ml.

Mitochondrial membranes were prepared from washed mitochondria by sonication for 2 min with a Branson cell disruptor (Branson Instruments Co., Stamford, Conn.). After sonication, the membranes were sedimented by centrifugation at 48,000 *g* for 60 min. The pellet was resuspended in a small amount of Tris-HCl buffer, pH 7.2, and checked for the presence of mitochondrial membranes by assying for succinic dehydrogenase (5), an enzyme associated with the mitochondrial inner membrane (6). Enzyme activity was found only in the pellet; the soluble fraction of the mitochondria showed no activity. A drop of each membrane preparation was placed on a Formvar coated grid (400 mesh) and stained with 1% phosphotungstic acid in 0.1% bovine serum albumin. Grids were examined immediately in a Hitachi HU-11C electron microscope.

Lipids were extracted from mitochondrial membranes and soluble fraction with chloroform–methanol 2:1 according to the procedure of Folch, Lees, and Sloan Stanley (7).

Extracted lipids were also assayed for phosphorus. Samples were dried under a stream of nitrogen and resuspended in 2 ml of concentrated nitric acid. The suspensions were then digested for 18 hr at 85°C and ashed to a white residue. The residue was hydrolyzed with 1 N HCl in a boiling water bath for 10 min. Phosphate was determined by the procedure of Fiske and SubbaRow (8).

To determine the fatty acid composition of the mitochondrial membranes, the chloroform–methanol extract was dried under nitrogen and redissolved in a small amount of methanol–benzene 1:1. An aliquot was taken for analysis. Saponification and preparation of methyl esters was done using the methanol sodium hydroxide reagent described by Glass (9). The extent of methylation was checked by thin-layer chromatography on glass plates spread with 0.4 mm layers of silica

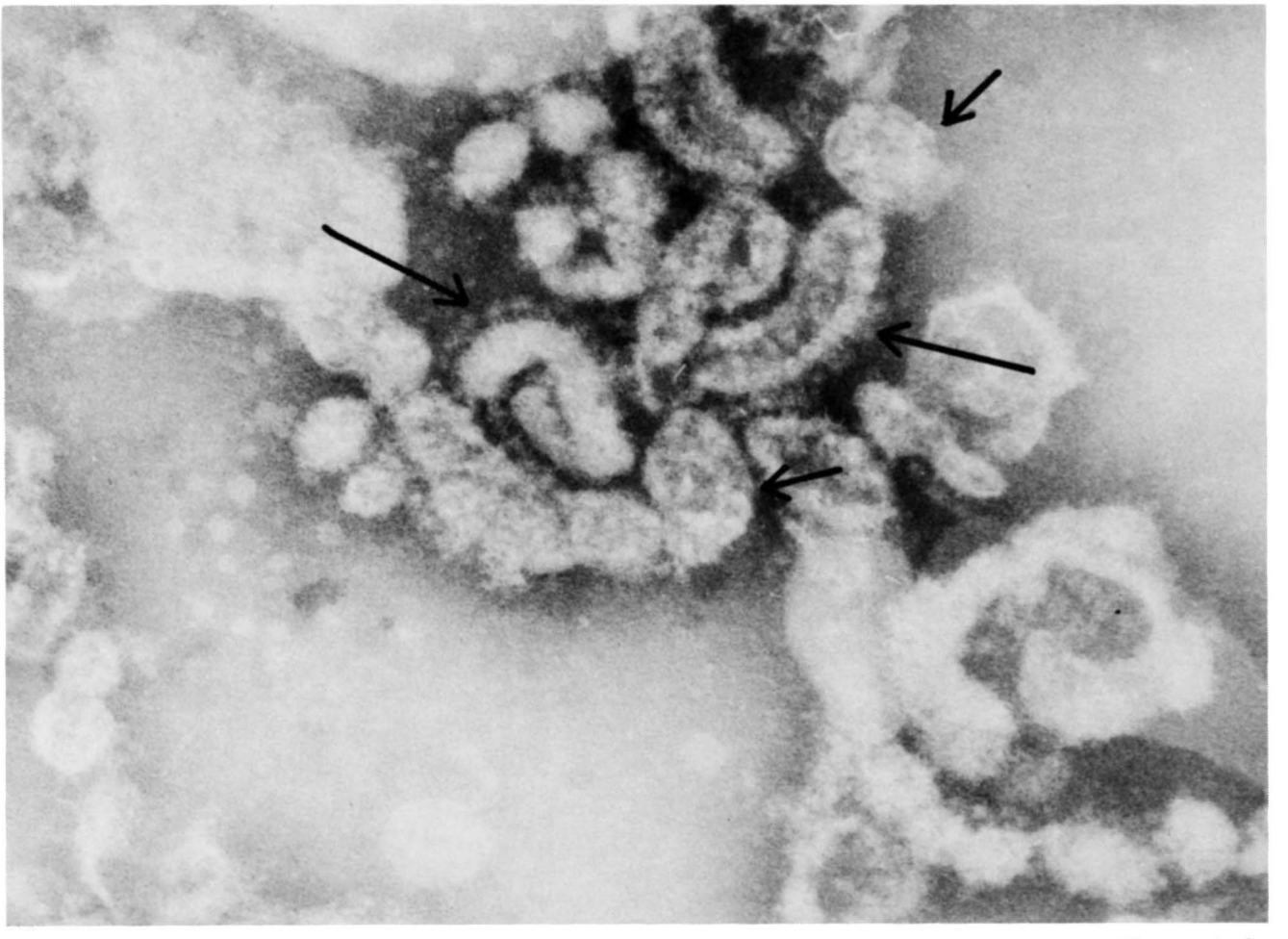

Fig. 1. Negative-stained mitochondrial fragments from *T. pyriformis* ST. Long arrows point to fragments of cristae. These retain the tubular configuration seen in micrographs of whole cells (4). Stalked particles can be seen on the periphery. Short arrows point to smooth walled vesicles which are assumed to be fragments of outer membrane. × 298,000.

TABLE 1. Lipid:protein ratios in mitochondria from *T. pyriformis*.

Strain	DN-5	CA-10	ST
		lipid:protein (mg/mg)	
Membranes	0.29	0.32	0.28
Soluble fraction	0.23	0.16	0.11
		phospholipid:protein (μmoles phosphorus/mg)	
Membranes	0.34	0.40	0.29
Soluble	0.10	0.09	0.06

gel H. The solvent system used was Skellysolve F–ethyl ether–acetic acid 85:15:1 (v/v/v). Fractions were detected by charring after spraying with sulfuric acid–sodium dichromate solution. The plates showed quantitative conversion of the triglyceride fraction to the methyl esters. Very few free fatty acids were detected in the mitochondrial membrane samples. No fatty alcohols were found. Methyl esters were analyzed by gas–liquid chromatography on a F & M gas chromatograph equipped with flame ionization detector. A 10% diethyleneglycol succinate column was used. Fatty acids were identified by comparing sample retention times with those of standard samples of fatty acid methyl esters (Hormel Institute, Austin, Minn.; and smelt rose standards, courtesy of R. L. Glass). Relative percentages of the fatty acids in each sample were determined by cutting out the peaks and weighing them. These identifications were confirmed by mass spectrometry. The analysis was performed on an LKB Type 9000 gas chromatograph–mass spectrometer. A 5% DEGS column was used. The exciting voltage was 70 eV.

Protein was determined by the biuret method (10) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Table 1 shows a comparison of the total lipid:protein and phospholipid:protein ratios of mitochondrial membranes and soluble fractions. Although both inner and outer membranes were isolated by the procedure used, quantitatively most of the membrane fraction is inner membrane due to the extensive cristae. Thus the ratios are more characteristic of inner membrane. All three strains showed typical ratios for mitochondrial membranes (11), i.e., the membrane is 70–80% protein. All our attempts to separate the two membranes by the digitonin procedure (6) were unsuccessful.

Small amounts of lipids, including phospholipids, were found in the soluble fraction or matrix. This is consistent with the work of Levy and Sauner (12) and Stoffel and Schiefer (13) who found phospholipids in the matrix of rat liver mitochondria. Acoording to these workers the phospholipids in the matrix have a different composition from those in the mitochondrial membranes. Further analysis of the phospholipid composition of *Tetrahymena* mitochondria would be necessary to determine if this variation also occurs in this organism. Alternately, the presence of phospholipids in the soluble fraction may be due to contamination with frag-

TABLE 2. Fatty acid composition of mitochondrial membranes

Chain Length	Fatty Acid	Approximate % of Total		
	strain	*DN-5*	*CA-10*	*ST*
12:0	Laurate	6.1	4.9	6.6
13:0	11-Methyl laurate	3.3	trace[a]	trace
	Tridecanoate	trace	trace	1.2
14:0	Myristate	5.6	5.1	6.6
15:0	13-Methyl tetradecanoate	trace	4.2	1.5
	Pentadecanoate	trace	trace	1.3
16:0	14-Methyl pentadecanoate	trace	trace	trace
	Palmitate	8.2	7.3	8.7
16:1	Palmitoleate	7.3	7.0	6.6
17:0	15-Methyl hexadecanoate	trace	6.0	2.9
	Margarate	trace	trace	2.6
17:1	Heptadecenoate	trace	trace	trace
18:0	Stearate	1.3	trace	3.3
18:1	Oleate	6.1	12.2	8.3
18:2	Linoleate	16.9	15.1	14.2
18:3	γ-Linolenate	39.6	32.4	29.0
19:0	Nonadecanoate	1.7	trace	1.2
19:1	Nonadecenoate	trace	trace	trace
20:0	Arachidate	trace	trace	trace
20:1	Eicosaenoate	trace	trace	trace
20:2	Eicosadienoate	trace	3.8	1.6
20:3	Eicosatrienoate	trace	trace	1.3
20:4	Arachidonate[b]	2.9	1.3	2.1

[a] Less than 1% of total.
[b] Assignment on the basis of chromatographic retention time and fragmentation pattern in mass spectrometer (15).

ments of outer mitochondrial membrane not sedimented at 48,000 *g*. Unfortunately this possibility cannot be tested by the usual technique of assaying for an enzymatic marker since no one has yet demonstrated an enzyme associated exclusively with the outer mitochondrial membrane of *Tetrahymena*. Contamination with inner membrane is unlikely because no succinic dehydrogenase activity was detected in the soluble fraction.

Electron micrographs of negative-stained membrane preparations show a large proportion of inner membrane–cristae fractions. (**Fig. 1.**) These retain the tubular configuration of the cristae seen in situ (4) even after sonication and are characterized by the presence of stalked particles on the surface as originally described by Fernandez-Moran (14) for beef heart mitochondria. Smooth-walled, rounded vesicles are assumed to be portions of outer membrane. These were, as expected, less numerous than inner membrane fractions. Negative-stained membrane preparations from all three *Tetrahymena* strains appeared similar to those seen in Fig. 1.

The identities and relative percentages of the fatty acids extracted from the mitochondrial membranes are shown in **Table 2.** In all cases, the principal fatty acid is 18:3 (γ-linolenate) followed by 18:2 (linoleate), 18:1 (oleate), and the C_{16} fatty acids. This differs from the distribution found in mammalian mitochondria such as those isolated from guinea pig liver, where linoleate (18:2) is the predominant fatty acid, followed by stearate (18:0) and arachidonate (20:4) (15) or rat liver mitochondria where approximately equal amounts of palmitate (16:0) and linoleate are found, followed by stearate (16). Mammalian mitochondria also contain fatty acids with chain lengths greater than 20 carbons made by elongation of α-linolenate and desaturation. The process in *Tetrahymena* does not appear to go beyond the C_{20} fatty acids. The fatty acid distribution of *T. pyriformis* mitochondria, however, more closely resembles that of mammalian organelles than that of mitochondria from aerobically grown yeast. In *Saccharomyces cerevisiae*, oleate is the predominant mitochondrial fatty acid and polyunsaturated fatty acids such as linoleate are not found in this organism (17). Although the relative amounts found in the mutant (CA-10) differ from the parent strain (DN-5), these variations probably are not sufficient to cause significant alterations in membrane properties. A comparison of the CA-10 composition with that of an unrelated strain, ST, shows that the composition is fairly typical of *Tetrahymena* membranes. The fatty acid components of mitochondrial membranes are qualitatively similar to those reported for whole cells (18). However, some additional fatty acids were identified in these extracts. These are the *iso*-fatty acids (i.e., those having a methyl branch at the penultimate carbon atom), 11-methyl laurate and 15-methyl hexadecanoate. CA-10 especially had a relatively large amount of this latter acid. The *iso*-acid derivatives give a mass spectrum similar to normal chain esters, but exhibit a small peak at m/e = M-65 which is characteristic of a penultimate methyl branch (19). *Iso*-acids can also be identified by the ratio of intensities of the M-29/M-31/M-43 peaks (20). The relative abundance of these *iso*-acids may reflect the high amino acid composition of the growth medium, particularly leucine (21). The C_{20} fatty acids previously reported (18) were separated and identified. The 20:4 acid was identified as arachidonic acid on the basis of retention time and fragmentation pattern in the mass spectrometer (22).

The proportion of unsaturated fatty acids also appears to be greater in these mitochondrial membrane extracts than that found by Jonah and Erwin (23). This may be due to the fact that these workers used another strain of *Tetrahymena* or that they analyzed cells grown to the stationary growth phase. Studies (24) have shown that the ratio of saturated to unsaturated fatty acids in *Tetrahymena* is increased by lowering the oxygen tension which occurs in older, more dense cultures. The high content of unsaturated fatty acids in the lipids of *Tetrahymena* mitochondria (63% of total in strain ST and 72% in DN-5 and CA-10) is in agreement with analyses of organelles from other organisms. It appears that proper mitochondrial function is dependent on a high level of unsaturation in the lipid component of the membrane. The enzymes hydroxybutyrate dehydrogenase (25) and oligomycin-sensitive ATPase from beef heart particles (26) require lipids containing unsaturated fatty acids for full activity. In addition, Haslam, Proudlock, and Linnane (27) have demonstrated, using a fatty acid desaturase mutant of yeast, that yeast mitochondria having an unsaturated fatty acid composition of less than 20% are unable to carry on oxidative phosphorylation, although such organelles appear normal in electron micrographs and have a full complement of cytochromes (28). Thus, the amount of unsaturated fatty acids found in the mitochondrial membranes is probably quite similar for most strains of *T. pyriformis* grown under conditions that promote a high rate of aerobic metabolism.

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