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Role of Lipids in the *Neurospora crassa* Membrane: IV. Biochemical and Electrophysiological Changes Caused by Growth on Phytanic Acid

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Summary. *Neurospora crassa* strain *cel*, which is deficient in fatty acid synthesis, was grown with phytanic acid supplementation. The temperature dependence of membrane potential is increased by growth on phytanic acid. A temperature change of 40 °C produces a change of 184 mV in phytanic acid-grown cells as compared to a 50 mV change for *cel* grown on palmitic acid or wild-type. Membrane resistance (measured as DC input resistance) of phytanic acid-grown cells did not differ from *cel* grown on palmitic acid or wild-type. Lipid analysis of *cel* grown on phytanic acid revealed ~7 mole percent phytanic acid incorporation into phospholipids, no change in phospholipid base composition, a reduction of ergosterol content from 80 to 30 percent, and the induction of β sitosterol, a sterol not usually present in *Neurospora*. β sitosterol accounted for ~60 percent of the sterol present. Incorporation of 7 mole percent phytanic acid into phospholipids lowers the phase transition temperature by ~5 °C, and decreases the heat content of the phase transition (ΔH) slightly. Results are discussed in relation to Refsum's disease, a human neurological disorder associated with high plasma levels of phytanic acid. It is proposed that high intracellular phytanic acid concentration induces novel sterol synthesis and that the incorporation of the novel sterol into the membrane is responsible for the increased temperature sensitivity of membrane potential. The excitable membrane deficits observed in patients with Refsum's disease may also be explained by such a mechanism.

Key words membrane potential · fatty acids · sterols · β sitosterol · Refsum's disease · phospholipids

Introduction

Plasma membranes have the awesome responsibility of maintaining cell integrity. Were it not for the plasma membrane and its abilities to exclude admission of some molecules, selectively admit others, and permit equilibration of still other molecules across the cell membrane, life as we know it could not exist. Since the plasma membrane is composed of both lipid and protein, the biochemical research approach has been to study these molecules. The inherent weakness of this approach is the lack of relationship between extraction chemistry and *in vivo* assembled membrane function. We believe our research overcomes this weakness by applying biochemical, physical chemistry and electrophysiological techniques to an organism unique in its abilities to permit us to alter its membrane lipid composition while monitoring its membrane function *in vivo* (Friedman, 1977*a*, *b*; Friedman & Glick, 1980). Since lipids comprise approximately fifty per cent of the cell membrane, and have been implicated as determinants of both the physical structure and the physiology of the cell membrane (Razin, 1975; Blok, van Deenen, de Geir, Op den Kamp & Verkleij, 1977; Jain & White, 1977; Claret, Garay & Giraud, 1978), we have chosen to focus on the role of membrane lipids in controlling membrane physiology.

By using *Neurospora crassa* it is possible to alter the individual lipid components of the cell membrane through the use of metabolic mutants while assessing the effect of such alterations on *in vivo* membrane physiology. Intracellular microelectrodes are used to monitor membrane transport.

Numerous reports (Wang, 1970; Haslam, Spithill & Linnane, 1973; Farias, Bloj, Morero, Sineriz & Trucco, 1975; Robertson & Thompson, 1977; Tsao & Lands, 1980) including two of our own (Friedman, 1977*a*, *b*), have concerned themselves with the function of membrane phospholipid molecules and in particular their alkyl chain moieties. Such studies attempt to demonstrate that the alkyl chains determine: (1) the physical state of the membrane, and (2) the transport properties of the cell membrane.

One method of verifying these possible functions would be to incorporate a branched-chain fatty acid into membrane phospholipids. Insertion of such a molecule would disrupt the membrane bilayer by increasing the spacing between the polar head groups of the phospholipid molecules and thereby: (1) affect the physical state of the membrane, and (2) alter the protein-lipid interactions necessary for membrane transport.

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To pursue the use of a branched-chain fatty acid as a probe of membrane structure and function, we grew the *cel* mutant of *Neurospora crassa* on media supplemented with phytanic acid. The *cel* mutant is unable to synthesize fatty acids, and, in consequence, requires an exogenous supply of fatty acid for growth (Henry & Keith, 1971; Elovson, 1975). By growing *cel* on phytanic acid-supplemented media, we hoped to load the cell membrane with phytanic acid. Phytanic acid (3, 7, 11, 15 tetramethylhexadecanoic acid) has a cross-sectional area that is fifty percent greater than that of straight-chain fatty acids (O'Brien, 1967) and is therefore a choice candidate for membrane disruption.

Materials and Methods

Growth of cel (Chain Elongation Mutant)

Neurospora crassa cel (Fungal Genetics Stock Center strain number 165) was grown on Vogel's minimal medium (Vogel, 1956) supplemented with 2% sucrose and phytanic acid (0.24 mg/ml). Both liquid, shake and solidified agar plate cultures were used in these experiments. Liquid shake cultures were grown in 1 liter flasks at 31 °C on a rotary shaker operating at 160 rpm. Liquid cultures were used for the isolation and characterization of membrane lipids as well as for differential scanning calorimetry experiments. Petri plate cultures were used for electrophysiological experiments. Agar medium differed from liquid medium only in the presence of 1.5% agar. Agar plate cultures were grown by the technique initially described by Slayman (1965).

Phytanic acid was supplied to cells as the Tween detergent. Tween-phytanic acid was synthesized by a transesterification procedure similar to that described by Brody and Allen (1972). The fatty acid composition of the Tween-phytanic acid detergent was determined by gas chromatography and found to be: 55.4% phytanic acid, 23.3% capric acid (C 10:0) 11.4% stearic acid (C 18:0), 9.4% palmitic acid (C 16:0), and 0.5% myristic acid (C 14:0). The synthesized detergent was placed in absolute ethanol 20 mg/ml (wt/vol), aliquots of which were added to the culture medium prior to sterilization.

Electrophysiology

The techniques used in our electrophysiological monitoring of membrane properties have been described elsewhere (Friedman, 1977*b*). Briefly, *cel* was grown on sterile, scratched cellophane discs, excised from culture with scalpel and forceps, and placed on the undersurface of a metal-reinforced coverglass (5 × 15 mm). The coverglass was then positioned so that the *Neurospora*-containing cellophane was supported with the *Neurospora* facing downward into a solution-containing chamber.

Care was taken to insure the uniformity and physiological condition of hyphae selected for impalement. Impaled hyphae were taken from approximately 1 cm behind the growing edge of the plate culture. Impaled segments did not contain branches. Both voltage-recording and current-passing electrodes were placed in the same hyphal segment. Hyphae exhibiting an initial membrane potential of less than 100 mV were rejected.

Electrophysiological measurements were made while *Neurospora* hyphae were bathed in an aqueous solution containing (in mM): 20 NaCl, 10 KCl, 1 $CaCl_2$ and 85 sucrose, buffered with 10 mM Tris and HCl to pH 8. Bath temperature was controlled by a fluid-cooled microscope stage as described elsewhere (Friedman, 1977*b*). Bath temperatures were monitored and recorded as described elsewhere (Friedman, 1977*b*).

Membrane potentials were recorded from hyphae using hooked, glass capillary microelectrodes filled with 2.8 M KCl and having tip diameters of less than 1 micron. In a few experiments we were successful in introducing a second, current-passing microelectrode at a distance of 100 μ from the first. Such electrodes were used to pass depolarizing current pulses which generated "*IR*" drops detected by the voltage-sensing electrode. The amount of current passed, and the magnitude of the *IR* drop induced, were used to calculate the DC input resistance of the hypha. The equipment and procedures used for these experiments are detailed elsewhere (Friedman, 1977*b*).

Separation of Phospholipid and Neutral Lipid Fractions

Lipids were extracted from freeze-dried, washed cells using the chloroform-methanol extraction and "Folch" wash procedures detailed by Christie (1973, p. 39). Lipid extracts were evaporated to dryness under nitrogen. Traces of remaining solvent were removed by freeze-drying samples overnight. The dried, purified lipid samples were dissolved in chloroform and stored under nitrogen at −10 °C.

Neutral and phospholipid fractions were separated on a silicic acid column as previously described (Friedman, 1977*a*). Neutral lipids had to be eluted with a minimum of ten column volumes of chloroform, followed by the elution of total phospholipids in ten column volumes of absolute methanol.

Phospholipid Analysis

Phospholipids were analyzed using the methodology of Friedman and Glick (1980). Total lipids were extracted and dissolved in chloroform (10 mg/ml). A 30 λ aliquot was spotted on thin-layer chromatography plates and developed using a chloroform/methanol/ammonium hydroxide solvent system (65:35:5). Individual phospholipids were identified by spraying the plates with a 55% sulfuric acid, 0.6% potassium dichromate solution and heating the plates on a hot plate until color developed. Positive identification of unknowns was made by co-chromatography with known phospholipid standards.

Identified phopholipids were quantified by phosphorus determinations. The methodology employed was that of Christie (1973, p. 189), modified to obviate the possibility of silica gel interfering with an accurate, quantitative phosphorus determination (Shen & Dyroff, 1962; Doizaki & Zieve, 1963). The procedure is described by Friedman and Glick (1980).

Fatty Acid Analysis

The fatty acid composition of the phospholipids was determined using the previously reported procedures (Friedman, 1977*a*). Phospholipid fatty acids were converted to their methyl esters via a transesterification procedure. Fatty acids were identified by comparison of the retention times of their methyl esters with standards chromatographed under identical conditions. The weight percentage of each fatty acid present was determined by peak integration.

Sterol Analysis

For sterol analysis the neutral lipid fractions, eluted from silicic acid columns in chloroform (as described above), were taken to dryness under nitrogen, and redissolved in chloroform in an approximate concentration of 10 mg/ml. The sterols present in this fraction were separated on a Perkin Elmer 3920B gas chromatograph equipped with a 6 foot, $^1/_4$ inch glass column containing 3% SP-2250 on 100/120 Supelcoport (Supelco, Inc., Bellefonte,

Pa.) and a flame ionization detector. The column was run at an oven temperature of 260 °C, and with an injection port and interface temperature of 300 °C. Nitrogen was used as the carrier gas at a flow rate of 20 ml/min. Approximately 1 λ (10 µg) of sample was injected into the chromatograph for each run. Sterols were identified by comparison of their retention times to standards chromatographed under identical conditions. The weight percentage of each sterol present was determined by peak integration.

Differential Scanning Calorimetry

The transition temperature and heat content (ΔH) of phospholipids extracted from *cel* were determined using the differential scanning calorimetry mode of a Dupont 990 Thermal Analyzer. Microgram quantities of lipid dissolved in chloroform were deposited in DSC pans, and evaporated to dryness under nitrogen. Open sample pans were freeze-dried overnight to remove all traces of solvent. Water in excess ($\sim 1\lambda$ water/mg lipid) was added to each pan before sealing. All samples were sealed in Dupont "volatile sample" pans and run against an empty, sealed pan as standard.

Results

Effect of Growth with Phytanic Acid Supplementation on Membrane Physiology

Our studies of the effect of phytanic acid on the membrane properties of *cel* were, in part, encouraged by the report of Brody and Allen (1972) indicating abnormal cell morphology and increased osmotic fragility of the *cel* mutant when it is grown with phytanic acid supplementation. Both these observations suggest a plasma membrane defect. As might be anticipated, impalement of an osmotically fragile cell proved diffucult. With persistence, however, we were able to penetrate a sufficient number of cells to explore the relationship between membrane potential and temperature in the approximate temperature range of 0 to 40 °C. The data obtained from these experiments are plotted in Fig. 1, and suggest an increase in resting membrane potential with increased temperature. The linearity of the relationship between membrane potential and temperature, was assessed by "best fit" linear regression. The correlation coefficient of the data to the "best fit" line is +0.825. In terms of membrane physiology, when *cel* hyphae, grown on phytanic acid, are subjected to a temperature change from 0 to 40 °C, their intracellular membrane potential rises from −102 to −286 mV. Thus, a change in bath temperature of 40 °C produces a change of 184 mV in resting membrane potential. This change in resting membrane potential is far in excess of the change in resting membrane potential observed for wild-type *Neurospora* and *cel* grown on palmitic acid. The latter two strains exhibit an approximate 50-mV change in resting membrane potential when subjected to a 40 °C temperature change (Friedman, 1977*b*). To graphically demonstrate the difference between the data of *cel* grown on phytanic acid and the other data, we subjected the wild-type and Tween 40 data to the same linear regression-best fit data analysis used for *cel* grown on phytanic acid. The "best fit" linear regression lines generated for wild-type and *cel* Tw40 are shown in Fig. 2. The two lines have similar slopes and consequently similar magnitudes of membrane potential. The correlation coefficients of the data to the lines for wild-type and Tween 40 are +0.72 and +0.86, respectively. The linear regression line for *cel* grown on phytanic acid, in contrast, has a much steeper slope, indicating a stronger temperature dependence. The data and statistical analysis demonstrate that the increased temperature dependence of membrane potential seen for *cel* grown on phytanic acid is a consequence of growth on phytanic acid.

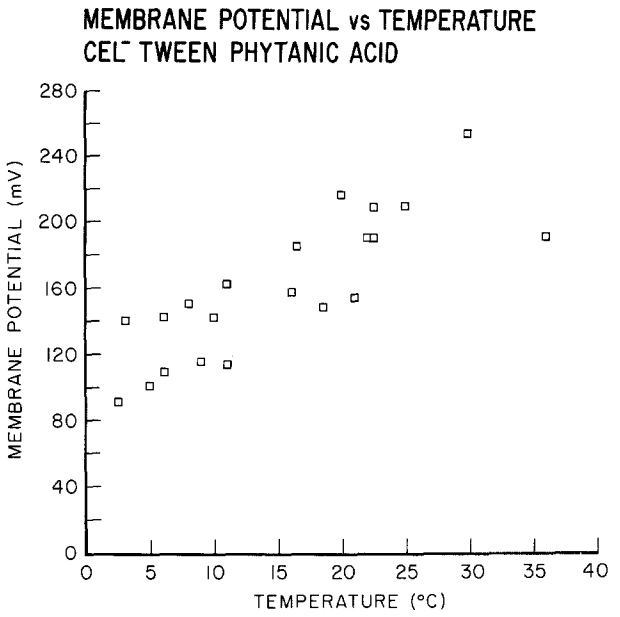

Fig. 1. Relationship between resting membrane potential and temperature for the *cel* mutant grown with phytanic-acid supplementation. Membrane potential values plotted are the steady-state potentials observed after 5 min of equilibration at a constant temperature

Measurement of the DC input resistance of *cel* grown on phytanic acid proved difficult. We found that penetration of hyphae with a second, current-passing microelectrode (needed to make the measurement), usually caused cell lysis. However, we were occasionally successful in obtaining long-term, two-microelectrode penetrations. The DC input resistances measured at these times were comparable to our previously published values of DC input resistance for wild-type *Neurospora* (Friedman, 1977*b*). We performed a series of short-term, two-microelectrode penetrations at 5 °C temperature intervals over the 0 to 30 °C temperature region to further explore

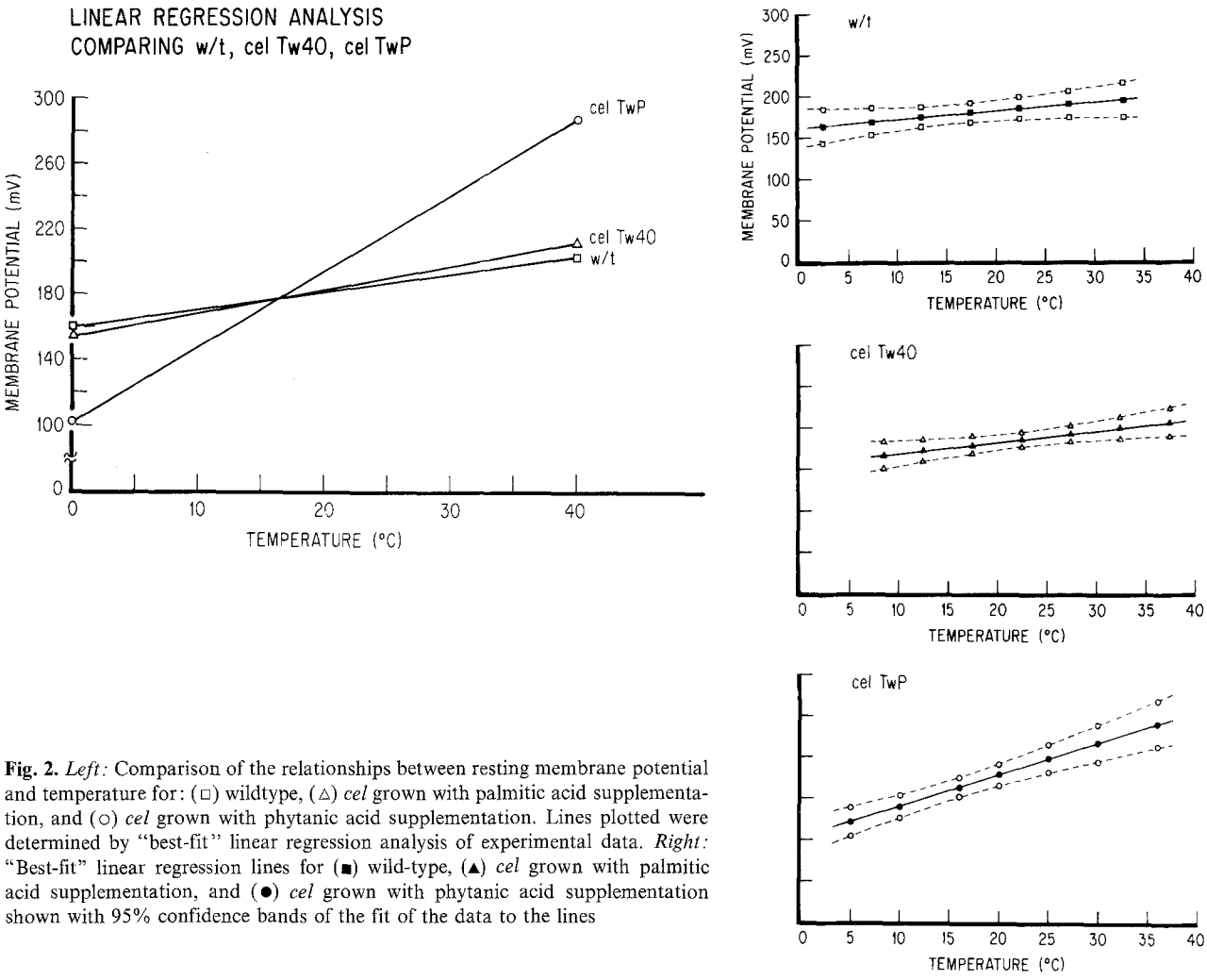

Fig. 2. *Left:* Comparison of the relationships between resting membrane potential and temperature for: (□) wildtype, (△) *cel* grown with palmitic acid supplementation, and (○) *cel* grown with phytanic acid supplementation. Lines plotted were determined by "best-fit" linear regression analysis of experimental data. *Right:* "Best-fit" linear regression lines for (■) wild-type, (▲) *cel* grown with palmitic acid supplementation, and (●) *cel* grown with phytanic acid supplementation shown with 95% confidence bands of the fit of the data to the lines

the relationship between membrane resistance and temperature. Membrane resistance values obtained were not significantly different from our previous membrane resistance data for wild-type *Neurospora.*

Effect of Phytanic Acid Supplementation on Neutral Lipid Composition

The electrophysiological data presented above indicate a dramatic change in active transport properties of *cel* hyphae when grown on a phytanic acid-supplemented medium. Several hypotheses might explain this finding including: (1) phytanic acid incorporation into the alkyl chain moieties of membrane phospholipids, (2) phytanic acid-induced disruption of the plasma membrane bilayer, (3) phytanic acid-induced alteration of membrane phospholipid base composition, and (4) a phytanic acid-induced change in membrane sterol composition. We have investigated all these possibilities and our data indicate that phytanic acid produces a dramatic change only in membrane sterol composition.

A relationship between phytanic acid and membrane sterol composition seems plausible from the work of Fischer, Markl, Honel and Rudiger (1962) who demonstrated a relationship between phytol and sterol synthesis in plants. Phytol differs from phytanic acid only in having a Δ^2-double bond and an alcohol instead of a carboxylic acid at carbon -1. A sterol analysis of the neutral lipid fraction of our *cel* lipid extracts was therefore undertaken. As demonstrated in Fig. 3, growth of *cel* with phytanic acid supplementation produced a large sterol peak whose elution time is much slower than that of ergosterol (Peak 3 of Trace *C*). The peak appears to be β sitosterol since it has the same retention time as a β sitosterol standard run under identical column conditions. To quantitate the change in sterol composition induced by growth of *cel* on phytanic acid, peak integration of the chromatograph tracings was performed. As shown in Table 1, growth of *cel* with phytanic acid

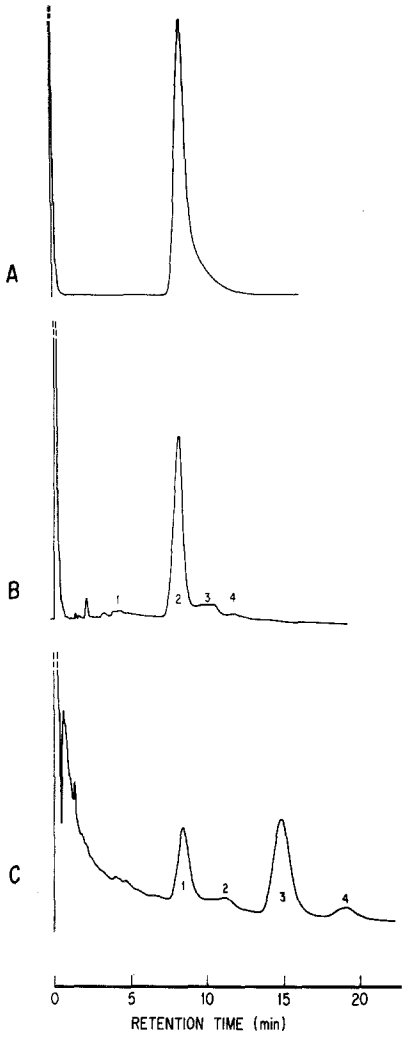

Fig. 3. Gas liquid chromatograms of sterols present in the neutral lipid fraction of whole cell extracts of *Neurospora*. Trace *A*: ergosterol standard; Trace *B*: sterols of wild-type – peak 1 unidentified, peak 2 ergosterol, peak 3 brassicosterol, peak 4 unidentified; Trace *C*: sterols of *cel* grown on phytanic acid – peak 1 ergosterol, peak 2 campesterol, peak 3 β sitosterol, peak 4 unidentified

supplementation lowers the amount of ergosterol present from ~82 to ~29 percent. It appears that the ergosterol is replaced by β sitosterol in the membrane. (The absolute values of sterols present per mg dry weight will be published elsewhere.)

To be certain of our finding, a number of control experiments were performed: (1) Since our *cel* analyses were performed on total lipid extracts, we wished assurance that the sterol profile of a whole-cell extract accurately reflects the plasma membrane's sterol profile. Using the *slime* mutant, we compared whole cell and plasma membrane sterol profiles. As can be seen in Table 1, there is good agreement between the sterol profiles of whole cell and plasma membrane extracts. Thus whole cell extracts appear to reflect the sterol profile of the plasma membrane. (2) To verify that β sitosterol is not associated with the *cel* mutation *per se*, we performed a sterol analysis of the *cel* mutant grown on Tween 80 (oleic acid, 18:1). The results of this experiment are also shown in Table 1. No β sitosterol was detected, and the distribution of other sterols is in good agreement with the data obtained for both wild-type and *slime Neurospora*. (3) To determine if β sitosterol induction was a phenomenon associated with growth on medium supplemented with Tween-phytanic acid, wild-type *Neurospora* was grown on Tween-phytanic acid-supplemented media. Wild-type, under these conditions, showed a relatively small amount of β sitosterol present, which did not affect the amount of ergosterol present.

Incorporation of Phytanic Acid into Membrane Phospholipids

Phytanic acid is not appreciably incorporated into membrane phospholipids. We extracted phospholipids from *cel* hyphae grown on Tween-phytanic acid and methyl esters of their alkyl chain moieties. The fatty acid methyl esters were subbsequently analyzed via gas chromatography. We did not attempt to isolate plasma membrane phospholipids *per se* since phospholipids extracted from whole cells and plasma membranes of *Neurospora* have similar fatty acid profiles (Friedman & Glick, 1980). The fatty acid analyses of five phospholipid extraction experiments are shown in Table 2. Also shown in Table 2 are the results of Brody and Allan (1972) who, using different phospholipid extraction and alkyl chain methylation

Table 1. Comparison of the sterol composition of *Neurospora* strains grown with varying fatty acid supplementation[a]

Strain	Supplement	Extract	Ergosterol	Brassicosterol	Campesterol	Unidentified	β-Sitosterol
Wild-type	None	Whole cell	82.3	9.1	–	8.5	–
Slime	None	Plasma membrane	76.8	15.6	–	7.6	–
Slime	None	Whole cell	74.2	13.1	–	12.7	
cel	Tw 80	Whole cell	76.6	9.5	–	13.9	–
cel	Tw Phytanic	Whole cell	29.0	–	5.5	7.3	58.2
Wild-type	Tw Phytanic	Whole cell	91.8	–	–	–	8.2

[a] Data presented as mole %.

Table 2. Fatty acid composition (mole %) of phospholipids of *Neurospora* mutant *cel*

Fatty acid	Present result[a]	Reported result[b]	Control[c]
16:0	7.3± 3.2	25	39.3
Phytanic acid	6.6± 2.2	7	–
18:0	1.1± 0.6	13	2.3
18:1	3.2± 0.7		10.2
18:2	63.5±10.3	44	35
18:3	18.3±10.8	8	11.5

[a] Results of 5 experiments, *cel* grown on Tween-phytanic acid 0.24 mg/ml, 31 °C. Mean values ± standard deviations.
[b] Brody and Allan (1972).
[c] Control experiment, *cel* grown on Tween-palmitic acid, 34 °C

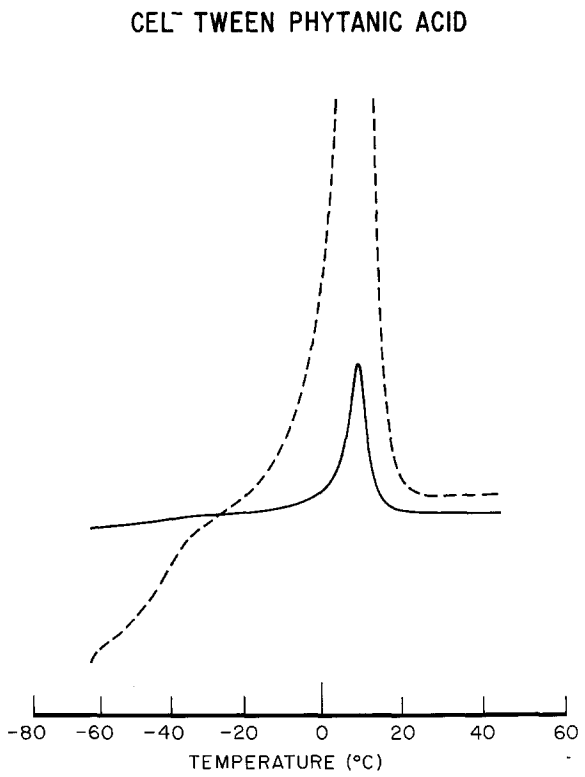

Fig. 4. Differential scanning calorimetry thermogram of phospholipids extracted from *cel* grown with phytanic acid supplementation. Upward deflections from baseline represent endothermic events. Phospholipids were scanned in excess water. Large endotherm centered around 0 °C represents the melting of ice. Smaller endotherm centered around −36 °C represents the gel-liquid crystal phase transition of the phospholipids. Sensitivity of the solid-line trace 5 mcal/sec. Sensitivity of the dashed-line trace 0.5 mcal/sec

procedures, found a similar amount of phytanic acid incorporation into *cel* phospholipids.

Effect of Growth with Phytanic Acid Supplementation on the Physical State of Phospholipids

To determine whether the plasma membrane of *cel* grown on Tween-phytanic acid could be disrupted by ~7 mole percent phytanic acid incorporation into phospholipids, we subjected the isolated phospholipids to differential scanning calorimetry. Figure 4 presents scanning calorimetry thermograms of these phospholipids in excess water. For these experiments whole cell phospholipid extracts were used, since whole cell and plasma membrane phospholipid extracts exhibit similar calorimetric properties (Friedman & Glick, 1980). In this Figure, endothermic events appear as upward deflections from the baseline, and two such events were seen in the sample. The large endotherm, occurring around 0 °C represents the melting of ice to water. The displacement of this peak to the right is caused by the slow conduction of heat through the large volume of water present in the sample. The smaller endotherm, which occurs at −36 °C represents the gel-liquid crystal phase transition of the phospholipids. The calculated heat content of that transition (ΔH) is ~0.9 cal/g lipid. The value compares favorably to previously published values (Friedman, 1977*a*) for wild-type *Neurospora*. Wild-type phospholipids have their gel-liquid crystal phase transition occurring at −31 °C, with a heat content of 1 cal/g. Thus, calorimetric data indicate that the incorporation of ~7 mole percent phytanic acid into phospholipids does not appreciably after either the physical state nor the phospholipid interactions of the bilayer.

Effect of Growth with Phytanic Acid Supplementation on Phospholipid Base Composition

The calorimetric data presented above also indicate that growth of *cel* on phytanic acid does not alter phospholipid base composition, since model experiments (Friedman & Glick, 1980) have shown that a change in phospholipid base composition would alter the calorimetric properties of *Neurospora* plasma membrane phospholipids. However, those same experiments demonstrated that small changes in phospholipid base profile would not be detected by calorimetry. Therefore, we wished to determine if growth on phytanic acid had produced some small alteration in the phospholipid base profile of *cel*. Conceivably, an alteration of the base profile could alter binding properties of ion-transporting proteins, thereby causing the observed increase in the temperature dependence of the resting membrane potential. Table 3 presents data of two experiments in which the phospholipid base composition of *cel* grown with phytanic acid supplementation was analyzed. The data are presented as mean values ± standard deviation. For the purpose of comparing and determining if growth of *cel* on phytanic acid causes a change in phospholipid base composition, the phospholipid base profile of

Table 3. Comparison of phospholipid base composition[a]

Phospholipid base	Wild-type (Friedman & Glick, 1980)	Wild-type (Kushwaha & Kates, 1976)	Wild-type (Hubbard & Brody, 1975)	*cel* (Tw-phytanic) (0.24 mg/ml at 31 °C)
Cardiolipin	4.5±0.6	2.8	5–8	2.7±0.1
Phosphatidyl ethanolamine	35.6±1.8	25.1	20–25	24.9±0.8
Phosphatidylcholine	45 ±4.5	46.6	40–45	50 ±1.1
P-inositol/*P*-serine	11.1±3.6	4.7[b]	11–16	14.1±2.7
Phosphatidic acid	4.2±1.8	20.9	3	8.6±4.7

[a] Data presented as mole %.
[b] Serine only, no value for inositol given.

wild-type *Neurospora*, as determined by three independent investigators, is also presented. The data indicate that the mole percentages of the phospholipid bases present in *cel* grown with phytanic acid supplementation fall within the range of the values reported for wild-type. It therefore appears that phytanic acid supplementation does not alter phospholipid base composition.

Discussion

The ability of phytanic acid to alter membrane physiology has been known for some time: (1) It causes both osmotic fragility and abnormal morphology in *Neurospora* (Brody & Allan, 1972), and (2) it is found in high concentrations in persons suffering from the neurological disorder, Refsum's disease (Steinberg, 1972). Prior to the present study there has been a tacit acceptance of the hypothesis that the abnormalities associated with phytanic acid are due to the substitution of phytanic acid for straight-chain fatty acid molecules in membrane phospholipids.

The data here as well as other data in the literature indicate that this is not the case: (1) In the above-cited experiments we achieve only ~7 mole percent phytanic acid incorporation into *Neurospora* phospholipids, (2) Brody and Allan (1972), in similar experiments, achieved ~7 mole percent incorporation of phytanic acid into *Neurospora's* phospholipids, and (3) the administration of large amounts of phytanic acid or its precursors in experimental animals does not induce Refsum's disease (Steinberg, 1972).

Nevertheless, both Refsum's disease, and the abnormalities associated with growth of *cel* on phytanic acid appear to be membrane defects. For *Neurospora*, we interpret the previously observed osmotic fragility, and our present finding of increased temperature-dependence of resting membrane potential as indications of membrane involvement. In Refsum's disease, motor and sensory losses, diminished reflexes, dyscoordination, tremors, nerve deafness, and changes in ECG all indicate excitable membrane abnormalities.

The data we have obtained in the present work indicate that the membrane abnormality associated with growth of *Neurospora cel* on phytanic acid is: 1) the induction of synthesis of a new sterol (β sitosterol), and 2) the incorporation of this sterol into the membrane in place of the usual membrane sterol (ergosterol). The evidence arguing for this hypothesis is compelling: (1) Growth of *Neurospora* in the presence of exogenous phytanic acid induces β sitosterol synthesis. In normal, wild-type, hyphae this synthesis is minimal. However, in *cel*, β sitosterol becomes the predominant sterol. (2) The appearance of β sitosterol is dependent upon an exogenous supply of phytanic acid. Neither wild-type nor *cel* hyphae contain any trace of β sitosterol unless exogenous phytanic acid is supplied. (3) β sitosterol appears to be incorporated into the membrane since the sterol composition of hyphae and their plasma membranes are comparable; and (4) Incorporation of phytanic acid into membrane phospholipids does not appreciably alter the physical properties of the phospholipids into which they are incorporated, as demonstrated by calorimetric experiments.

The induction of β sitosterol and its substitution for ergosterol in *Neurospora* is associated with a change in membrane physiology. The observed increase in temperature-dependence of resting membrane potential correlates with a change in sterol composition. This change in the temperature-dependence of the resting membrane potential is attributable to a change in active transport properties of the membrane. Our DC input resistance data indicate no change in passive permeability properties of the plasma membrane. We are not aware of any prior reports correlating a change in membrane sterol composition with an alteration of resting cell membrane potential.

Our findings may prove to be of clinical significance. The possibility of using *Neurospora cel* as a model for Refsum's disease was discussed by Brody and Allan (1972). They rejected the idea based upon a difference in the distribution of phytanic acid residues amongst the phospholipid bases between *Neurospora* and nervous tissue. The data presented here

suggest that sterol composition rather than phospholipid composition is the primary biochemical lesion in Refsum's disease. The *cel* mutant with its reduced ability to synthesize fatty acids, accumulates phytanic acid. We propose that the high intracellular concentration of phytanic acid affects sterol synthesis in *Neurospora*. Similarly, impaired phytanic acid catabolism, which has been demonstrated in persons affected with Refsum's, leads to high intracellular phytanic acid concentrations. Possibly by biosynthetic pathways analogous to those found in *Neurospora*, the high phytanic acid concentration effects a change in sterol composition in Refsum's disease. This mechanism would account for some of the negative findings in Refsum's disease research: (1) the reason why the animal models of Refsum's disease generated by maintaining high plasma concentrations of phytanic acid have failed is that the intracellular mechanism for the destruction of phytanic acid is still present, and (2) the reason why researchers have been unable to demonstrate incorporation of phytanic acid into the phospholipids of patients with Refsum's disease (MacBrinn & O'Brien, 1968) is that the lesion is in the sterol, rather than the phospholipid, component of the membrane.

The electrophysiological data presented here indicate that the physiological consequence of growth of *cel* with phytanic acid supplementation is an increased temperature-dependence of the resting membrane potential. Were this same consequence to occur in Refsum's-diseased patients, membrane hyperpolarization would occur at body temperature. One might expect that membrane hyperpolarization would more radically alter the performance of excitable, as opposed to inexcitable cells, thereby accounting for the predominance of neural and muscular deficits in Refsum's-diseased patients. Membrane hyperpolarization could, of course, also occur in nonexcitable cells possibly explaining the nonelectrical deficits observed in the syndrome. We are presently testing this hypothesis by: (1) determining the sterol profiles of fibroblasts derived from humans with Refsum's disease, and (2) determining if these fibroblasts are hyperpolarized in comparison to normal fibroblasts through the use of voltage-sensitive dyes to probe the membrane potential of these cells.

Our hypothesis that a change in membrane sterol composition can alter the activity of active transport mechanisms which then may inflict neurological disease is supported by existing literature: (1) Stephens and Shinitzky (1977) altered the electrical activity of neurons by increasing their cholesterol content; (2) Altered membrane cholesterol content has been shown to alter active transport pump activity in human red blood cells (Claret et al., 1978); (3) Myotonia has been induced in rats by agents which either block cholesterol synthesis (Winer et al., 1966), induce desmosterol accumulation in plasma and muscle, or alter membrane $(Na^+ - K^+)$-ATPase activity of membranes (Peter, Dromgoole, Campion, Stempel, Bowman, Andiman & Nagatomo, 1975); and (4) The hereditary myotonia of chickens appears to be associated with an elevated cholesterol content of the muscles which are affected by the disease (Stewart, Werstiuk, Vickers & Rathbone, 1977). Thus, membrane sterols may play a significant role in modulating active transport, resting membrane potential, excitable membrane physiology, neuropathies, and neuromuscular disorders.

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