THE EFFECTS OF BRANCHED CHAIN FATTY ACID INCORPORATION INTO *NEUROSPORA CRASSA* MEMBRANES

Stuart Brody and Barry Allen

Biology Department
University of California, San Diego
La Jolla, California 92037

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Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), an unusual branched chain fatty acid thought to disrupt the hydrophobic regions of membranes, can be incorporated into the lipids of growing *Neurospora* cultures. The phytanic acid must be supplied in a water soluble form, esterified to a Tween detergent (Tween-Phytanic). This fatty acid and its oxidation product, pristanic acid, were found in both the phospholipid and neutral lipid fractions of *Neurospora*. In phospholipids of the wild-type strain, phytanic acid was present to the extent of 4 to 5 moles percent of the fatty acids and pristanic acid, about 41 moles percent. The neutral lipids contained 42 and 4 moles percent of phytanic and pristanic acids respectively. By employing a fatty acid-requiring mutant strain (*cel*$^-$), the phytanic acid level was raised to a maximum of 16 moles percent in the phospholipids and to 63 moles percent in the neutral lipids. Under this condition, the level of pristanic acid was reduced to about 6 moles percent in phospholipids and 1 mole percent in the neutral lipids. The phytanic acid levels could not be further elevated by increased supplementation with phytanic acid or by a change in the growth temperature. In strains with a high phytanic acid content, the complete fatty acid distribution of the phospholipids and neutral lipids was determined. In the neutral lipids, phytanic acid appeared to replace the 18 carbon fatty acids, particularly linoleic acid.

The presence of phytanic acid in the phospholipids was confirmed by mass spectrometry, and by the isolation of a phospholipid fraction containing this fatty acid via silicic acid column chromatography. Most of the phytanic acid in phospholipids appeared to be in phosphatidylethanolamine, and 2 lines of evidence suggest that it was esterified to both positions of this molecule. In the fatty acid-requiring mutant strain (*cel*$^-$), the replacement by phytanic acid of 10 to 15% of the fatty acids in the phospholipid produced an aberrant morphological change in the growth pattern of *Neurospora* and caused this organism to be osmotically more fragile than the wild-type strain. The lack of noticeable effect of the high levels of pristanic acid in the phospholipids suggests that it is not just the presence of the methyl groups in a branched chain fatty acid which leads to the altered membrane function in this organism.

1. INTRODUCTION

Previous studies in this laboratory have shown that certain mutants of the fungus, *Neurospora crassa*, have a severe deficiency in a particular fatty acid

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in their membranes (1). We have been attempting to alter the fatty acid composition of these mutants by supplementing them with the missing fatty acid. The most successful tactic for this purpose has been to synthesize special detergents as a source of the fatty acids, rather than adding a mixture of detergent and free fatty acid. Employing this procedure, we have been able to raise significantly the level of almost any one of the 5 fatty acids normally found in *Neurospora*, including the highly unsaturated linolenic acid, in the membranes of these mutants. Therefore, we have explored this technique as a means of introducing unusual fatty acids into cellular membranes.

One unusual branched chain fatty acid, phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is thought to have serious consequences when found in any appreciable quantities in membranes. A genetic disorder in humans (Refsum's disease) which leads to the accumulation of this fatty acid in parts of the nervous system is characterized by demyelination and death (2). A thorough review of this disorder was published in 1967 (3). It has been proposed that the presence of phytanic acid disrupts the hydrophobic regions of the membranes (4), but little work has been done on characterizing such membranes. We have performed this initial study to point out how *Neurospora* and *Neurospora* mutants can be employed as a model system for studying the effects of phytanic acid on membranes. This paper describes the methods employed for the incorporation of phytanic acid and its oxidation product in mammals, pristanic acid (2,6,10,14-tetramethylpentadecanoic acid), into membranes of growing cells in significant quantities and some of the effects produced by their incorporation.

2. METHODS

(a) *Strains and growth conditions*

A wild-type strain (RL3-8A) and a fatty acid requiring strain, *cel*$^-$ (chain elongation) were employed in this study. The *cel*$^-$ strain (FGSC 165) was obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif. All cultures were liquid shake cultures. Fifty ml of Vogel's minimal medium containing 2% glucose in 125 ml flasks were employed as described previously (1).

(b) *Chemicals*

Tween 40 and Tween 60 were obtained from Pierce Biochemicals. They contain predominantly palmitic and stearic acids, respectively, plus other fatty acids. The amount of Tween 40 added to 50 ml cultures was 0.2 ml of a 10% solution (w/v) in alcohol. The phytanic acid (Aldrich Chemicals) was generously provided by Dr. D. Hutton. It contained significant amounts of pristanic acid. The detergent Tween-phytanic (Tween P) was synthesized by transesterification as described below. The amount of Tween P added to cultures was 0.2 ml of a 20 mg per ml solution.

(c) *Preparation of Tween-phytanic (Tween P)*

One half g of Tween 60 and 1 g of phytanic acid were added to a flask containing 5 ml of t-butyl alcohol and 0.5 ml of 12 N HCl. This mixture was refluxed under nitrogen for 4 hr. The solution was cooled, and as much material as possible was removed by vacuum distillation at 125° to 130°. The residue was adjusted to a phytanic acid concentration of 20 mg per ml with absolute alcohol. The fatty acid composition as determined by gas-liquid chromatography (GLC) was 76% phytanic acid, 4% pristanic acid, and 20% stearic acid.

(d) *Fatty acid analysis*

Cells from cultures were harvested, washed with water, and lyophilized. The lyophilized powder was soaked overnight in methanol and the residue extracted twice with 2:1 chloroform-methanol. The separation of neutral lipids from phospholipids was performed on a silicic acid column as described previously (1). Dried samples of fatty acids were converted to methyl esters by the following transesterification procedure. An individual sample was incubated with 1 ml of benzene, 0.2 ml of 96% w/w H_2SO_4, and 2 ml of methanol in a teflon-capped tube at 65° for 5 hr. The mixture was extracted with 5 ml of petroleum ether (30° to 60°), and the ether layer washed 3 times with 1 ml portions of water. The water washes were extracted with 1 ml of petroleum ether and the ether layers combined. The petroleum ether solution was dried by shaking with 0.5 g of activated alumina and then decanted. The alumina was washed with 0.5 ml of 3% ethyl ether in petroleum ether and the ether solutions were combined. The fatty acid composition of the ether solution was then determined by GLC.

The determination of the phytanic and pristanic acid content listed in Table 1 was performed employing a stainless steel column, 1/8 inch x 10 feet, at 170°. This column contained 8% HiEFF-2BP and 1.5% SE-30 on Gas Chromosorb G, 80 to 100 mesh (Applied Science Laboratories). The determination of the fatty acid distribution listed in Table 2 was performed employing a glass column 1/8 inch x 6 feet, containing 3% EGS-SX on Chromosorb P, 100 to 125 mesh (Applied Science Laboratories). This column was operated at 190°, and its elution profile is given in Fig. 1. The mass spectrometer analysis was performed on the eluate from a glass column, 1/4 inch x 6 feet containing 3% SE-30. The calibration of the flame ionization detector of a Varian 1200 was as described previously (4). The phytanic and pristanic acids were assumed to give a similar detector response for a given amount of material as stearic acid.

TABLE 1

Pristanic and phytanic acid content of Neurospora *under different conditions*

Strain	Growth conditions	Total µmoles fatty acid per g dry weight		Moles percent of the total fatty acids in each lipid fraction			
				Pristanic acid		Phytanic acid	
		Phospholipids	Neutral lipids	Phospholipid	Neutral lipids	Phospholipids	Neutral lipids
Wild type	24°, Tween P	40,44	210,238	40,41	3,4	5,5	40,44
Wild type	24°, Tween P and Tween 40	39,42	188,199	9,20	3,3	6,4	30,40
Cel⁻	24°, Tween P	37,38, 36,31	112,109, 98,104	2,9,6,1	0,1,2,0	13,4,8, 16	60,63, 61,63
Cel⁻	24°, Tween P and Tween 40	35,39, 29	115,124, 108	4,6,8	1,0,4	13,9,6	56,54, 51

The procedures employed for the separation of phospholipids from neutral lipids and for the determination of the branched chain fatty acid content are given in Methods.

TABLE 2

Fatty acid distribution[a] *in phospholipids and neutral lipids of* Neurospora

Strain	Growth conditions	Total content[b]	Palmitic	Pristanic	Phytanic	Stearic & oleic	Linoleic	Linolenic
			Phospholipids					
Wild type	24°	40	27			10	49	14
Wild type	24°, Tween 40	38	25			12	49	14
Wild type	24°, Tween 40 and Tween P	39	18	20	4	8	40	10
Cel⁻	24°, Tween 40	45,39,52	29,27,27			6,12,10	49,47,49	15,14,14
Cel⁻	34°, Tween 40	34,33	38,48			19,12	37,32	5,8
Cel⁻	34°, Tween 40 and Tween P	36	25	3[c]	7[c]	13	44	8
			Neutral Lipids					
Wild type	24°	70	23			30	34	13
Wild type	24°, Tween 40	91	26			28	33	13
Wild type	24°, Tween 40 and Tween P	188	20	3	30	10	27	10
Cel⁻	24°, Tween 40	152,123	18,26			14,15	39,35	28,24
Cel⁻	34°, Tween 40	129,154	18,17			15,19	53,47	13,17
Cel⁻	34°, Tween 40 and Tween P	94	16	4[c]	64[c]	4	8	4

[a] Expressed as moles percent. [b] Expressed as total micromoles of fatty acid per g of dry weight.

[c] Confirmed by mass spectometry.

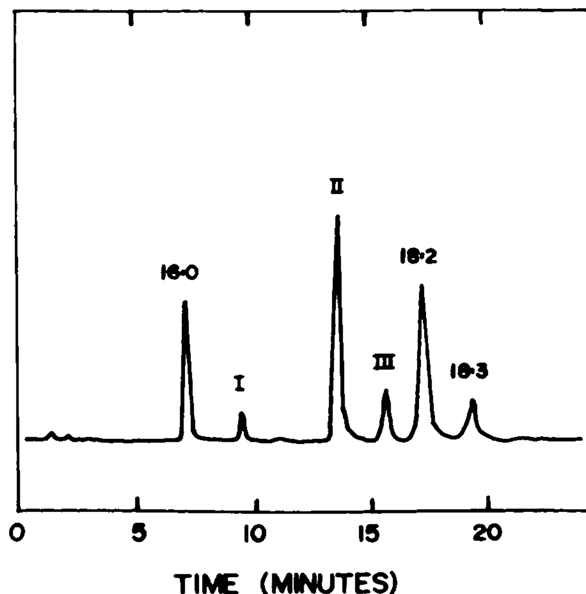

Fig. 1. Elution profile of the fatty acids from the neutral lipid fraction. Chromatographic separation was achieved by employing a column of 3% EGS-SX on Chromosorb P. Roman numeral I designates the peak location of pristanic acid, II for phytanic acid, and III for a mixture of stearic and oleic acids.

(e) *Phospholipid analysis*

Cultures of the *cel*$^-$ strain were grown in the presence of Tween P and [^{32}P]PO_4. The total lipid extract from 200 mg of lyophilized cells were applied to a silicic acid column (4), and the neutral lipids were eluted with 75 ml of chloroform. A phospholipid fraction was eluted with 100 ml of chloroform-methanol (9:1). Additional phospholipids were eluted with higher concentrations of methanol. One half of the eluate was analyzed for fatty acids, and the other half was analyzed for specific phospholipid content. Our procedure for phospholipid analysis is based on the work of Robert Lester (personal communication). Radioactive phospholipids were carefully deacylated, and solutions of the water-soluble products were neutralized, concentrated, and then chromatographed in 2 dimensions on TLC cellulose sheets (Eastman). The solvents were ethanol:NH_4HCO_3:NH_4OH (70:29:1) for the first dimension and isobutyric acid:NH_4OH:H_2O (66:1:33) for the second dimension. After localization of the individual phospholipids by autoradiography, the spots were cut out and the radioactivity determined by liquid scintillation counting.

(f) *Osmotic sensitivity of cells*

The *cel*$^-$ strain was inoculated into 50 ml of medium containing Tween 40 with or without Tween P. The cultures were grown as shake cultures for 3 days at 23°. One half ml of the medium was carefully removed, diluted with 2.5 ml of water, and the absorbance at 260 nm was measured. Seven ml of a 30% NaCl solution was then added to half of the cultures, and the absorbance was again measured. The cultures were then incubated at 23° for an additional 24 hr, and the absorbance again measured. The cultures were then harvested, dried at 100°, and weighed.

3. RESULTS

(a) *Incorporation of branched chain fatty acids into the wild-type strain*

When the wild-type strain was grown in the presence of Tween P, most of the phytanic acid taken up by the cells was found in the neutral lipid fraction, which consists mainly of triglycerides (5). Only a small percentage of the phytanic acid was found in the phospholipids (Table 1). The same results were found if a mixture of Tweens was added, i.e., Tween P and Tween 40 (Table 1). No detectable phytanic acid was found in either the neutral lipid or phospholipid fractions of cells grown in the presence of phytanic acid solubilized in 1.0% Tergitol. Therefore, the detergent-bound form of this fatty acid appears to be necessary for uptake by this organism.

In wild-type cells, most of the pristanic acid was found in the phospholipids, and little in the neutral lipids (Table 1). The principal source of the pristanic acid found in the wild-type cells was apparently the contaminant in the original sample of phytanic acid. Pristanic acid was also present in the synthetic detergent, but at a far lower concentration than phytanic acid. Cells containing high levels of pristanic acid and low levels of phytanic acid appeared normal in their morphology and growth rate, suggesting that pristanic acid has little effect on membrane function, and that our Tween P preparations did not contain inhibitory compounds.

(b) *Incorporation of branched chain fatty acids into a fatty acid-requiring mutant*

The fatty acid-requiring mutant strain (*cel*$^-$) does not synthesize its own fatty acids under the conditions employed in these experiments (6). When the Tween P preparations were the sole source of fatty acids, this strain grew slowly and exhibited an aberrant morphology. Under these conditions the phytanic acid levels approached 16% of the fatty acids in the membranes and 63% of the fatty acids in the neutral lipids (Table 1). Higher levels were not achieved by the addition of more Tween P to the media or by temperature variations. The addition of Tween 40 along with Tween P considerably increased the growth rate of the mutant and decreased slightly the amount of phytanic acid in the cells (Table 1). This suggests that competition between the different fatty acids has an effect on the final amount of phytanic acid found in the cells. This same explanation could account for the lower level of phytanic acid found in wild-type cultures, where competition probably exists between endogenously synthesized fatty acids and the phytanic acid in the medium.

The pristanic acid levels in the mutant cells were much lower than in the wild-type organism, but the same pattern was found in the mutant, i.e., most of the pristanic acid was in the phospholipids. There appears to be some type of reciprocal relationship between the phytanic acid content and the pristanic acid content in both the phospholipid and neutral lipid fractions. Wild-type cells had high pristanic acid and low phytanic acid in their phospholipids, whereas the phospholipids of the *cel*$^-$ strain were higher in phytanic acid content and lower in pristanic acid (Table 1). In the neutral lipids, the same pattern was found, where an increase in the levels of phytanic acid (*cel*$^-$ versus wild-type) was associated with decreased levels of pristanic acid (Table 1).

(c) *Total fatty acid composition*

The total fatty acid composition of the phospholipid and neutral lipid fractions was determined for some of these samples, as indicated in Table 2. It can be seen that in the neutral lipids phytanic acid seemed to replace the 18 carbon fatty acids, primarily the unsaturated ones. On the other hand, the pristanic acid found in the wild-type phospholipids did not appear to prefer-

entially replace any one fatty acid, but simply lowered the content of each of the fatty acids by approximately 20%. In Table 2, the values for the stearic and oleic acids are grouped together, since our procedures did not separate these 2 fatty acids adequately (see Fig. 1). In normal wild-type cultures, 80% of the fatty acid that was found under this peak was oleic acid (4). We have no reason to believe this proportion has been changed in these experiments.

Also included in Table 2 are the data indicating the effects of temperature on the fatty acid composition of the phospholipid and neutral lipid fractions of the fatty acid-requiring mutant strain. One can see that an increase in growth temperature from 24° to 34° led to a higher level of palmitic acid and lower levels of the highly unsaturated fatty acids (linoleic and linolenic acids) in the phospholipids. This change in fatty acid composition was not found in the neutral lipids. The finding of a shift towards a higher proportion of saturated fatty acids in the membranes at higher temperatures agrees with observations made with other organisms (7,8). One can also see that the phospholipid composition in the *cel*⁻ strain grown with Tween 40 is very similar to that of the wild-type strain (Table 2). This suggests that at a given temperature, the overall fatty acid composition of a membrane is relatively constant, independent of whether the fatty acids are supplied exogenously or endogenously.

(d) *Distribution of phytanic acid in phospholipids*

Phospholipids were fractionated on a silicic acid column employing a chloroform-methanol gradient and the column fractions analyzed as given in Methods. The first column fraction, eluted with 9:1 chloroform-methanol, contained 0.6 μmoles of phosphatidylethanolamine (PE), 0.08 μmoles of cardiolipin, all of the esterified phytanic acid (1.2 μmoles) and pristanic acid (0.20 μmoles), and approximately 3% of the total esterified palmitic acid (0.04 μmoles). These data would suggest that the bulk of the phytanic acid was associated with PE, and that both positions in this phospholipid were esterified with phytanic acid. The other column fractions, which did not contain any phytanic or pristanic acids, were pooled and their phospholipids analyzed. The amounts of the major phospholipids in this combined fraction were as follows: 0.6 μmoles of PE, 2.2 μmoles of phosphatidylcholine and 0.4 μmoles of phosphatidylinositol. Phosphatidylserine, monomethylaminoethyl-phosphatide, dimethylaminoethyl-phosphatide and phosphatidic acid were also found, but no cardiolipin was detected in this fraction. The esterified fatty acids in this fraction were palmitic acid (1.2 μmoles), stearic and oleic acid (1.5 μmoles each), linoleic acid (3.0 μmoles) and linolenic acid (1.4 μmoles). The fatty acid compositions in these 2 fractions were similar to those observed in many previous analyses, as were the proportions of the individual phospholipids. Approximately 50% of the PE and all of the cardiolipin were found in the first fraction. Control experiments were performed in which radioactive phospholipids of normal fatty acid content were fractionated and essentially the same distribution of PE and cardiolipin was found.

The silicic acid column fraction containing the esterified phytanic acid was treated with phospholipase A (Boehringer). In preliminary experiments, approximately one half of the phytanic acid was removed from phospholipid by this treatment, suggesting that the phytanic acid was esterified in both positions of the phospholipid.

(e) *Effects of phytanic acid incorporation*

Cultures of the *cel*⁻ strain which had 10% to 25% of the fatty acids in phospholipids as phytanic acid grew very slowly, and the cells had an abnormal morphology (Fig. 2) if the medium contained 8% NaCl. At lower NaCl concentrations, the effects on growth rate and morphology were not as striking. The abnormal morphology associated with phytanic acid incorporation may have been

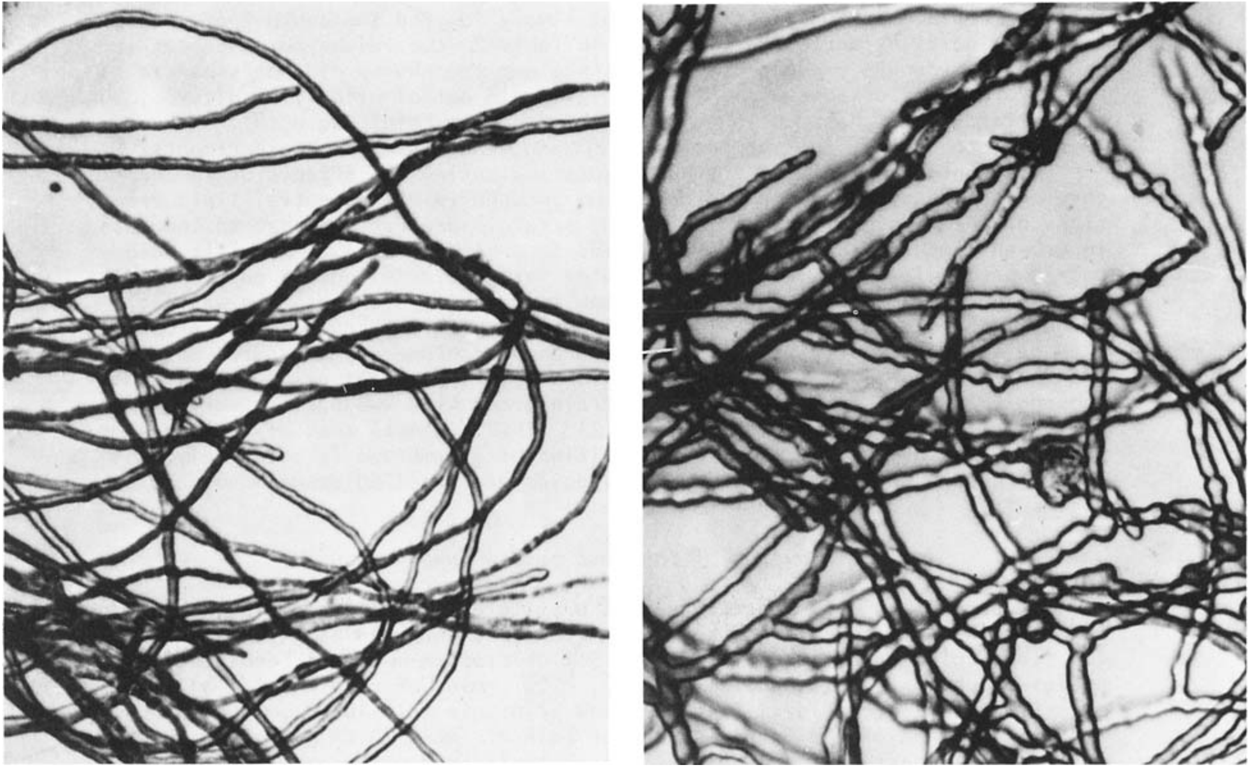

Fig. 2. The effect of phytanic acid incorporation on cellular morphology. The photomicrographs are of *cel*$^-$ shake cultures grown in the presence of Tween 40 and 8% NaCl. The culture grown on the right had, in addition, Tween P in the medium. The magnification for both pictures was 320X.

due to alterations in the secretion and assembly of the cell wall components. In fungi, the cell wall is the principal shape-determining structure and this structure appears to be quite sensitive to changes in membrane structure (9).

In a separate set of experiments the osmotic fragility of membranes containing phytanic acid was tested. Cultures of the *cel*$^-$ strain grown in the presence or absence of Tween P were stressed with an increase in the osmotic strength of the medium (see Methods). The increase in absorbance at 260 nm in the medium was taken as one indication of possible membrane alterations due to the abnormal fatty acid content. As shown in Table 3, a significant leakage of intracellular material occurred in the cells containing phytanic acid. It would appear then that under conditions of high osmotic strength, the presence of phytanic acid led to an abnormal membrane. This same effect on the membranes could account for the inability of the phytanic acid-containing organism to grow in a medium of higher osmotic strength.

4. DISCUSSION

The amount of phytanic acid incorporated into *Neurospora* seems to be influenced by at least 3 parameters: 1) the form in which the fatty acid is supplied, i.e., detergent-bound or detergent-solubilized; 2) the competition with other fatty acids in the medium; 3) the competition with endogenously syn-

TABLE 3

The effect of phytanic acid incorporation on the leakage of intracellular material from the cel⁻ *strain of* Neurospora

Supplement	Initial absorbance at 260 nm of media	NaCl added (%)	Absorbance of medium 24 hr later	Dry weight (g)	Change in absorbance per g dry weight
Tween 40	0.960	0	1.140	0.122	1.5
Tween 40	0.820	4	0.900	0.056	1.4
Tween 40 + Tween P	1.080	0	1.260	0.118	1.5
Tween 40 + Tween P	0.900	4	1.080	0.067	2.7

The procedures employed are given in Methods. The absorbance at 260 nm of the uninoculated medium was 0.80 O.D. units per ml.

thesized fatty acids (Table 1). The observation that only detergent-bound phytanic acid was incorporated was surprising, since other fatty acids can be taken up by *Neurospora* if they are solubilized by similar concentrations of Tergitol (6). This requirement for a special form of phytanic acid may not be true for other organisms, however. Once fatty acids have been transported into the *Neurospora* cells, they can usually be found first in the neutral lipids and then in the phospholipids. This statement is based on the results of a long term pulse-chase experiment in which [^{3}H]oleic acid was supplied to the *cel⁻* strain (Brody and Allen, unpublished observation). Similar observations have been made for yeast cells (10). The observations on the distribution of phytanic acid, but not the pristanic acid, would be in agreement with the idea of the triglycerides serving as a source of fatty acids for phospholipid biosynthesis.

The relationship between pristanic acid and phytanic acid is somewhat unclear. There seems to be a general reciprocal relationship between the amount of these 2 branched chain fatty acids found in a given lipid fraction. Whether this reciprocal relationship is due to some competition between these fatty acids at some common step in their incorporation is not known. The pristanic acid found in the lipids does not appear to be due to any appreciable oxidation of phytanic acid since much of the pristanic acid found in the cells could be accounted for by the pristanic acid added to the medium. Little breakdown of either phytanic acid or pristanic acid would be expected anyway, since these cultures were grown in the presence of high levels of glucose. Perhaps the most curious aspect of their relationship, though is their differential *in vivo* effects on this organism. Phytanic acid can have a serious effect on membrane function when its concentration is 10% to 15% of the fatty acids in the phospholipids. Pristanic acid has no apparent effect, as judged by growth rate and cellular morphology, when present even in much higher amounts in the membranes. Since pristanic acid and phytanic acid have the same number of methyl groups, and these groups are in the same relative position to each other, one must consider that it is not just the presence of these methyl groups in phytanic acid which are responsible for the abnormal phenotype. Perhaps the proximity of one of the methyl groups *vis a vis* other parts of the phospholipid molecule might be the significant determinant of the abnormal phenotype. Another possibility is that phytanic acid may be capable of assuming a "twisted" configuration (11), whereas the pristanic acid may not. In addition, phytanic and pristanic acids may be esterified to different phospholipids. Phytanic

acid was found only in phosphatidylethanolamine, whereas no information is available on the localization of pristanic acid.

The phytanic acid-induced aberrations in *Neurospora* and in nervous tissue differ in certain respects. MacBrinn and O'Brien have found the phytanic acid in nervous tissue to be mainly in phosphatidyl choline (12), whereas in *Neurospora* none of it is found in phosphatidyl choline. Secondly, *Neurospora* has a very high content of unsaturated fatty acids in its membranes as compared with nervous tissue. Therefore, interactions between phytanic acid and specific types of fatty acids and/or phosphoryl bases in the phospholipids do not appear to play a common role in the effects on the 2 types of membranes.

The elucidation of the molecular basis for the effects of phytanic acid on membranes could be investigated employing the fatty acid-requiring mutant strain of this eucaryotic organism. The cells are fast-growing and easy to culture, and relatively homogenous membrane or mitochondrial preparations can be obtained. Transport can be studied, employing the well-characterized uptake systems for K^+ (13), glucose (14), or amino acids (15). In addition, certain physical-chemical studies on the protein-lipid interactions in membranes containing phytanic acid could be performed, similar to those studies already described on the membranes of this organism (16).

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