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Saturated Fatty Acid Requirer of *Neurospora crassa*

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Dietary saturated fatty acids containing 12- to 18-carbon atoms satisfy growth requirements of *Neurospora crassa* mutant *cel* (previously named *ol;* Perkins et al., reference 11); unsaturated fatty acids are synthesized by direct desaturation when an appropriate saturate is available. Odd-chain saturates, 15 carbons and 17 carbons long, satisfy the requirement, and elaidic acid (18:1 Δ^9 *trans*) results in slow growth. Oleic acid and other *cis*-unsaturated fatty acids do not satisfy growth requirements; however, oleic acid plus elaidic acid result in growth at a faster rate than elaidate alone. The use of a spin-label fatty acid reveals that hyphae produced by *cel* during a slow basal level of growth have lipids that reflect a relatively rigid state of viscosity compared to wild type. *cel* Supplemented with fatty acids and wild type supplemented in the same way have lipids of the same viscosities as reflected by electron spin resonance.

The requirement and importance of double bonds in the fatty acid content of all organisms more complex than viruses are well known. In dealing with fatty acid compositions in general the emphasis has always been placed on the growth promotion and properties of unsaturated components and on the physical properties conferred on membrane lipids by double bonds. The present report demonstrates an equally stringent requirement for saturated fatty acids in *Neurospora crassa*.

A number of organisms including a mutant of *Neurospora* (9), have been known to require an unsaturated fatty acid. Specific unsaturated fatty acid requirements have been established for an auxotroph of *Escherichia coli* (14, 15), anaerobic yeast (1, 10, K. Bloch, Fed. Proc. **21:** 921-927), auxotrophs of yeast (6, 12, 18), mammals (4), and *Mycoplasma* (3).

The experiments presented here indicate that a saturated fatty acid is a requirement for growth in a mutant of *Neurospora* (*cel*) which had previously been described as an oleic acid requirer, *ol* (11). Furthermore, this mutant demonstrates the possibility of isolating mutants with different steps in fatty acid biosynthesis altered.

MATERIALS AND METHODS

Chemicals. The purified fatty acids, palmitoleate (16:1 Δ^9 *cis*), oleate (18:1 Δ^9 *cis*), linoleate (18:2 $\Delta^{9,12}$ *cis cis*), linolenate (18:3 $\Delta^{9,12,15}$ *cis, cis, cis*), elaidate (18:1 Δ^9 *trans*), petrosolenate (18:1 Δ^6 *cis*), were the products of Sigma Chemical Company, St. Louis, Mo. *cis*-Vaccinate (18:1 Δ^{11} *cis*) was obtained from Applied Science Laboratories. *trans*-Vaccinate (18:1 Δ^{11} *trans*) was supplied by K & K Laboratories, Inc. The purity of the above compounds was verified by analytical gas chromatography and found to be >99%, except for oleate which was >97%. Laurate (12:0), myristate (14:0), palmitate (16:0), stearate (18:0), pentadecanoate (15:0), heptadecanoate (17:0), and nonadecanoate (19:0) were obtained from the Hormel Institute, Austin, Minn. The purity of the compounds obtained from the Hormel Institute was accepted as claimed (>99%) and no further analysis was performed. Sodium malonate, butyrate (4:0), caproate (6:0), caprylate (8:0), and caprate (10:0) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. These compounds were all better than 95% pure, with fatty acids of a chain length 2 carbons above and 2 carbons below nominal constituting more than 4% of the remainder.

The nonionic detergent, Tergitol NP-40 was obtained from Union Carbide, Institute, W. V.

12-Nitroxide-stearate (12NS) was synthesized as previously described (15). 12NS (50 μg/ml of medium) was added to the medium of developing *Neurospora* 24 hr prior to harvest. All use of 12NS was carried out on cultures which had 24-hr exposure to 12NS. Hyphae were harvested, excess water squeezed out, and 10 to 30 mg of spin-label hyphae were placed directly in the electron spin resonance (ESR) spectrometer for analysis.

A Varian X band electron paramagnetic resonance spectrometer (Varian, Palo Alto, Calif.) equipped with the Varian variable temperature accessory was used for all measurements. The temperature accessory was calibrated with an iron constantan thermocouple to an estimated accuracy of $\pm$1.5 C.

Organism. The fatty acid requiring strain of *N. crassa* was obtained from the Fungal Genetics Stock

Center; stock no. FGSC 165 and 819. The genetics and isolation of this mutant have previously been described (11). Wild-type *N. crassa* no. 74-OR8-1A of the Oak Ridge Neurospora Culture Collection was kindly supplied by Patricia St. Lawrence.

Growth was measured by inoculating 125-ml Erlenmeyer flasks containing 20 ml of liquid minimal medium (16) with 10^5 conidia. The flasks were incubated in the dark at 34 C without agitation for varying lengths of time. The hyphae were filtered from the medium, dried overnight at 65 C, and weighed. The weight obtained in this manner was used as the measure of growth. Fatty acids were solubilized in the growth medium with Tergitol at concentrations of 1 to 3%. Stock cultures of *cel* were maintained on agar which contained minimal medium and 1% Tween 80.

Whole washed hyphae were ground in chloroform-methanol (2:1 v/v). This extract was filtered through anhydrous Na_2SO_4 and then washed, employing the Folch procedure (2). Extracts obtained in this manner were either used directly for thin-layer chromatography or were saponified with 2N KOH in methanol. Fatty acids were prepared for gas-liquid chromatography by methylating (dry methanolic hydrochloride) the saponified lipid extracts.

Chromatography. A Varian Aerograph, model 600D, analytical and a Varian Preparative, model 700, gas chromatographs were employed. The preparative columns (0.63 cm by 4.57 m) were aluminum, packed with 45/60 mesh Chromasorb W (acid washed), coated with 15% diethylene glycol succinate (DEGS). Analytical columns (0.32 cm by 2.29 m) stainless steel, packed with 60/80 mesh Chromasorb W (acid washed), were coated with 18% DEGS. Preparative collections were carried out in standard Varian autoprep collection tubes. These tubes were then rinsed into scintillation counting tubes with standard scintillation counting fluid (Toluene-2,5-diphenyloxazole).

Thin-layer chromatography was used to separate the lipids into free fatty acids, neutral lipids, and phospholipids as previously described (7).

Radioactive fatty acids. The following radioactive tracers were used: stearate-*1*-^{14}C, specific activity 9.19 mCi/mmole from Nuclear Chicago; palmitate-*9,10*-^3H from New England Nuclear, activity 5.0 mCi/4.3 mg; and ^3H-acetate prepared with water from acetic anhydride from Amersham C. Searle, specific activity of the anhydride 100 mCi/mmole; 18:1-*1*-^{14}C, specific activity 50 mCi/mmole; and acetic acid-*1*-^{14}C, activity 0.25 mCi/1.8 mg were obtained from New England Nuclear Corp. The fatty acids were analyzed using preparative gas-liquid chromatography followed by liquid scintillation in toluene with 4 g of 2,5-diphenyloxazole per liter. All were found to be >99% except stearate-*1*-^{14}C which was better than 98% pure.

The synthesis and purification of 12NS is discussed in detail elsewhere (15). 12NS-*9,10*-^3H was synthesized by treating methyl ricinoleate (2 g) with 6 Ci of $^3H_2$ in the presence of Pd on charcoal (15%) in methanol for 24 hr. The resulting 12-hydroxy-stearate-*9,10*-^3H was treated in the same way as previously described in the synthesis of 12NS. The 12-hydroxy-stearate was oxidized to the corresponding ketone with CrO_3 in glacial acetic acid. The keto derivative served as the carbon skeleton for the formation of the dimethyloxazolidine ring on carbon 12. This product was oxidized to 12NS-*9,10*-^3H with *meta*-chloroperbenzoic acid in ether. Final purification was carried out with hexane-diethyl ether (7:3) on preparative thin-layer plates of Silica Gel-G to a homogeneous band which had 5.92×10^{23} spins/mole. Five-tenths curie was purified and diluted in ethanol to a concentration of 1 μCi/ml. The remaining unpurified material was stored at −20 C.

Acetate-^3H (100 μCi) or ^{14}C-fatty acid (2 μCi) or both were used for each 20 ml of liquid culture medium. ^3H-palmitate (5 μCi) was used for each 20 ml of liquid culture. Additional amounts of nonlabeled acetate and fatty acid were added to bring total concentrations to 10^{-4} M. Tergitol (0.5%) was added to solubilize the tracer during the labeling period. Cultures for tracer analysis were prepared by inoculating 20 ml of liquid minimal medium in 125-ml Erlenmeyer flasks with 10^5 conidia. Mutant cultures and some of the wild-type controls were supplemented with 10^{-3} M 16:0 solubilized with 3% Tergitol. Growth took place in the dark without agitation for 36 hr. In some instances the hyphae were transferred after 24 hr growth to medium containing no fatty acid supplement and allowed to grow for an additional 12 hr. At the end of 36 hr, radioactive tracers were added for a ½-hr period during which the cultures were agitated in a water bath at 34 C. After the labeling period the flasks were plunged into a water bath at 85 C. The fatty acids were subsequently extracted and analyzed by the methods described above.

RESULTS

Solubilization of fatty acids. A variety of detergents were tested for toxicity to *Neurospora* and their efficiency in solubilizing fatty acids. The Tween detergents were unsuitable as solubilizing agents because of inhomogeneity in fatty acid composition. One per cent Tween 20, Tween 40, Tween 60, or Tween 80 in minimal medium supported growth of *cel* without further supplementation; however, Tween 20 was detectably inhibitory to wild-type *Neurospora*. As an example of the inhibitory effect of many of the detergents tested, Triton X in concentrations as low as 0.1% was completely inhibitory to growth of wild-type *Neurospora*. After testing many such detergents (anionic, cationic, and nonionic), it was found that Tergitol NP-40 solubilized fatty acids at detergent concentrations which allowed wild-type growth at a reduced rate. Tergitol NP-40 at concentrations of 1 to 3% reduced growth of wild type by 50%. This detergent does not serve as a substitute for a saturated or unsaturated fatty acid and appears not to be metabolized by the organism in any way.

Conditions adequate for growth. The saturated fatty acids laurate (12:0), myristate (14:0), pentadecanoate (15:0), palmitate (16:0), heptadecanoate (17:0), and stearate (18:0) all restored growth in the mutant with varying degrees of efficiency (Fig. 1). Palmitate was especially efficient

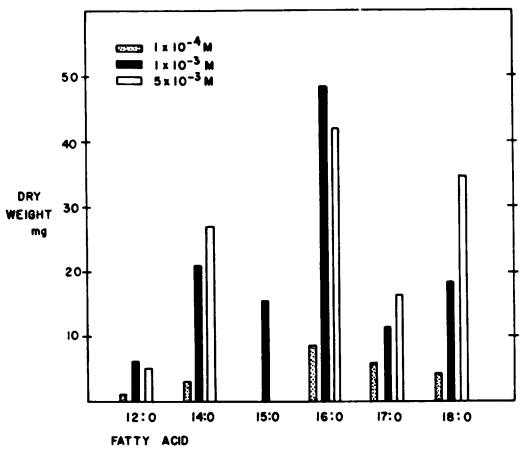

FIG. 1. *Growth of cel as a function of fatty acid supplementation. Growth period was 48 hr.*

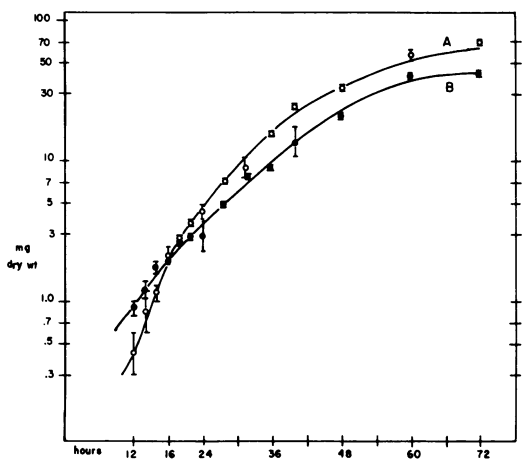

FIG. 2. *Growth of wild type and cel.* O, *wild type grown on minimal medium plus 3% Tergitol;* ●, *cel grown on* 10^{-3} M *16:0 plus 3% Tergitol. Vertical bar shows range of observations in duplicate experiments.*

in restoring growth, as 10^{-3} M of 16:0 almost restored with wild-type growth rate (Fig. 2). Even though 12:0, 14:0, and 15:0 were somewhat inhibitory to wild type, they still supported limited growth of *cel* (Fig. 1 and 3). The two straight-chained saturates, nonadecanoate (19:0) and eicosanoate (20:0), failed to support growth of *cel* on minimal medium at concentrations of 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, and 5×10^{-3} M measured at 48 hr, 72 hr, 6 days, and 12 days. After 24 days there was some growth in the culture medium containing 19:0 at 10^{-3} M (Table 1). At the same time a control culture of the mutant in minimal medium with Tergitol alone gave no measurable growth during this period. The *trans*-unsaturated fatty acids, elaidate (18:1 Δ^9 *trans*) and *trans*-vaccinate (18:1 Δ^{11} *trans*) at a concentration of 10^{-3} M with 3% Tergitol and minimal medium, supported no growth of *cel* after 48 hr, but at 6 and 12 days measurable amounts of growth had occurred (Table 1).

cel was tested for growth employing a variety of combinations of fatty acids. Each fatty acid in all the mixtures was maintained at 10^{-3} M and was solubilized with 3% Tergitol. Growth of *cel* on 18:1 Δ^9 *trans* or 18:1 Δ^{11} *trans* was enhanced by additional supplementation with oleate (18:1 Δ^9 *cis*) or palmitoleate (16:1 Δ^9 *cis*) but not by additional supplementation of linoleate (18:2 $\Delta^{9,12}$ *cis, cis*), linolenate (18:3 $\Delta^{9,12,15}$ *cis, cis, cis*), petrosolenate (18:1 Δ^6 *cis*), or vaccinate (18:1 Δ^{11} *cis*). 18:1 Δ^9 cis and 16:1 Δ^9 *cis* in combination with 19:0 and 20:0 produced growth which was measurable after 12 days (Table 1).

Conditions inadequate for growth. *cel* failed to grow in minimal medium supplemented with malonic acid in concentrations ranging from 10^{-4} to 10^{-2} M. These concentrations had no inhibitory effect on the growth of wild type. *cel* gave no detectable growth in minimal medium supplemented with any of the following unsaturated

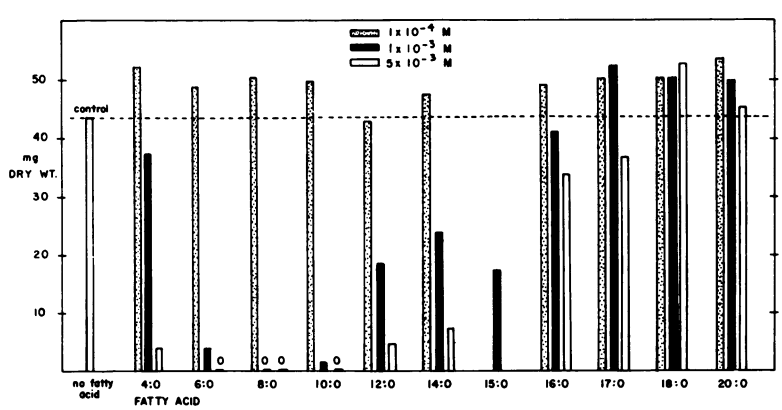

FIG. 3. *Growth of wild type in response to saturated fatty acid chain lengths. Growth period was 48 hr.*

TABLE 1. *Induced growth of cel by combinations of supplemented fatty acids*

Fatty acid	Additional fatty acid	Growth, 3 mg (dry weight)				
		3 days	6 days	12 days	20 days	24 days
18:1 Δ^{9trans}	None	0	2.4	18.0	...	...
	18:1 Δ^{9} *cis*	15.1	52.0	...	...	...
	16:1 Δ^{9} *cis*	0	10.5	39.5	...	...
	18:1 Δ^{6} *cis*	0	0	1.4	...	...
	18:1 Δ^{11} *cis*	0	0	0	0	0
	18:2 $\Delta^{9,12}$ *cis, cis*	0	1.5	...	...	...
	18:3 $\Delta^{9,12,15}$ *cis, cis, cis*	0	0	0	0	0
	19:0	0	0.3	22.5	...	...
	18:1 $\Delta^{11trans}$	0	6.5	29.5	...	...
18:1 $\Delta^{11trans}$	None	0	0	3.0	...	...
	18:1 Δ^{9} *cis*	0	5.0	44.4	...	...
	16:1 Δ^{9} *cis*	0	0.7	29.2	...	...
19:0	None	0	0	0	...	4.5
	18:1 Δ^{9} *cis*	0	4.8	63.7	...	...
	16:1 Δ^{9} *cis*	0	0.5	4.6	...	...
	18:1 Δ^{11} *cis*	0	0	0	0	0
	18:1 $\Delta^{11trans}$	0	4.7	46.8	...	...
	18:2 $\Delta^{9,12}$ *cis, cis*	0	0	0	0	0
	18:3 $\Delta^{9,12,15}$ *cis, cis, cis*	0	0	0	0	0
20:0	None	0	0	0	0	0
	18:1 Δ^{9} *cis*	0	...	18.0	60.3	0
	16:1 Δ^{9} *cis*	0	0	...	32.7	34.9
No fatty acid No Tergitol	None	0	0	0	1.3	3.0
No fatty acid +3% Tergitol	None	0	0	0	0	0

fatty acids: 16:1 Δ^{9} *cis*, 18:1 Δ^{9} *cis*, 18:2 $\Delta^{9,12}$ *cis, cis* or 18:3 $\Delta^{9,12,15}$ *cis, cis, cis*, at concentrations of 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and 5×10^{-3} M. The growth periods were 48 hr, 72 hr, 6 days, 12 days, and 24 days. The 18:1 Δ^{11} *cis* and 18:1 Δ^{6} *cis* were tested at 10^{-3} M only and showed no growth in the same periods up to 24 days. None of the above fatty acids were inhibitory to wild-type *Neurospora* at the concentrations used, except for 18:3 $\Delta^{9,12,15}$ *cis, cis, cis* which at 5×10^{-3} M restricted wild-type growth to approximately 50% for a 48-hr period. These fatty acids also failed to inhibit *cel* grown on medium supplemented with 10^{-3} M 16:0 in addition to any of the just mentioned non-growth promoting fatty acids.

Several other fatty acids which are potential metabolic intermediates and are potentially growth promoting to *cel* were also tested for growth stimulation. The fatty acids, butyrate (4:0), caproate (6:0), caprylate (8:0), caprate (10:0), laurate (12:0), myristate (14:0), and pentadecanoate (15:0), were all inhibitory in varying degrees to wild type (Fig. 3) and to *cel* (Fig. 4) grown in medium which was also supplemented with 10^{-3} M 16:0. This inhibition was correlated with chain length. Octanoate resulted in the maximum inhibition. Since 10^{-3} M 6:0, 8:0, or 10:0 allowed no growth of the wild type, no information about the ability of these fatty acids to support growth of the mutant was obtained.

The mutant grew at a very slow rate on minimal medium containing no fatty acid and no Tergitol. The addition of Tergitol completely inhibited this limited amount of growth. An inoculum of 10^{5} *cel* conidia in 20 ml of minimal liquid culture with no Tergitol produced 3 mg (dry weight) after 20 days as compared to 43 mg of (dry weight) for wild type after 48 hr.

Fatty acid composition and metabolism. Tracer analyses with ^{3}H-acetate showed that the ability of *cel* to incorporate acetate into its fatty acids was much reduced compared to wild type (Table 2). Acetate-*1*-^{14}C was taken up from the medium by whole hyphae of *cel* and wild type at about equal rates (Table 3). The ability of the mutant to desaturate 18:0-*1*-^{14}C was relatively unimpaired (Table 4). The 18:0-*1*-^{14}C was also incorporated

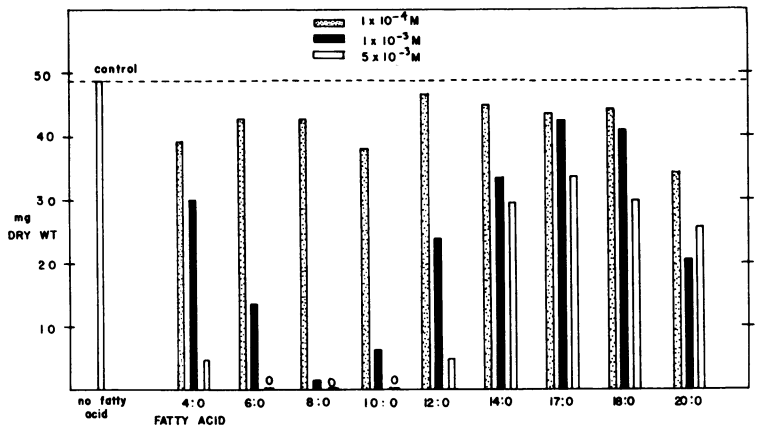

FIG. 4. *Growth of cel in response to saturated fatty acid chain length. cel was supplemented with 16:0 in all cases and the fatty acid indicated above was also included to test for possible alterations of growth. Growth was reduced with additional supplementation in most cases. Growth period was 48 hr.*

TABLE 2. *Distribution of ^3H from ^3H-acetate into fatty acids*[a]

Strain	Expt	Fatty acid fraction								
		<12	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Wild type	I	11	4	21	1613	47	406	2165	480	37
	II	6	2	12	1257	33	313	1758	392	26
cel	I	3	4	5	14	1	6	25	12	5
	II	1	3	1	8	0	3	17	9	2

[a] Data are expressed as counts per minute per milligram dry weight. Growth period was 36 hr and the ^3H-acetate was added ½ hr before harvest.

TABLE 3. *Counts from ^{14}C-acetate in solubilized whole tissues*[a]

Strain	Expt	Counts per min per mg dry weight
Wild type	I	81,496
	II	61,924
cel	I	67,343
	II	74,891

[a] Growth period was for 36 hr, and 4 μCi of ^{14}C-acetate was added 1 hr before harvest.

into phospholipids and neutral lipids by *cel* (Table 5).

The incorporation of 18:1 Δ^9 *cis-1-^{14}C* into lipid classes was similar in wild type and *cel* (Table 6). The incorporation pattern of 12NS-*9,10-^3H* was also similar in *cel* and wild type (Table 7). As might be expected, 18:0, 18:1 Δ^9 *cis*, and 12NS all show somewhat different patterns among themselves. The similarity in incorporating pattern of *cel* and wild type of all three acids indicates no gross enzyme lesions of the acylase type and no gross permeability problems with fatty acids.

cel grows slowly over a prolonged period on minimal medium without supplementation of any fatty acid. Table 8 shows the fatty acid composition of wild type, *cel*, and both *cel* and wild type with two different fatty acid supplementations. The unsupplemented *cel* has a shorter average chain length and a lower content of unsaturated fatty acids than wild type.

The supplementation of both *cel* and wild type with 18:1 Δ^9 *cis* and 18:1 Δ^9 *trans* resulted in a relatively high level of unsaturation in both strains. Under these conditions the two strains are similar and are both considerably higher in unsaturation than unsupplemented wild type. Supplementation with 17:0 results in 17:0 being the major fatty acid component in both strains. Although the numerical fatty acid composition appears different when development is on 17:0, the two strains have similar unsaturated-saturated fatty acid content. The relative proportion of saturated-unsaturated fatty acids may be more important in determining the physical properties of lipids than the relative percentage of each fatty acid species; in fact, the ESR data indicates that the two 17:0 supplemented strains have lipids with similar viscosities over a 30 to 50 C temper-

TABLE 4. *Distribution of ^{14}C from 18:0-1^{14}C into fatty acids*[a]

Strain	Expt	Fatty acid fraction								
		<12	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Wild type	I	3	1	5	6	2	706	75	18	6
	II	1	0	4	2	0	611	62	13	5
cel	I	1	4	3	4	0	897	58	15	7
	II	0	3	2	3	1	688	49	12	6

[a] Data are expressed as counts per minute per milligram dry weight. Growth period was 36 hr and the 18:0-*1*-^{14}C was added ½ hr before harvest.

TABLE 5. *Distribution of ^{14}C from 18:0-1-^{14}C into lipid classes*[a]

Strain	Expt	Lipid fraction		
		Phospho-lipids	Neutral lipids	Free fatty acids
Wild type	I	989 (77%)	211 (17%)	78 (6%)
	II	607 (75%)	174 (21%)	32 (4%)
cel	I	625 (47%)	387 (29%)	330 (24%)
	II	382 (51%)	119 (16%)	243 (33%)

[a] Data are expressed as counts per minute per milligram dry weight. Growth period was 36 hr and the 18:0-1-^{14}C was added ½ hr before harvest.

TABLE 6. *Distribution of ^{14}C from 18:1 Δ^9 cis into lipid classes*[a]

Strain	Expt	Lipid fraction		
		Phospho-lipids	Neutral lipids	Free fatty acids
Wild type	I	19,330 (39%)	23,390 (47%)	6,796 (14%)
	II	15,778 (35%)	24,095 (53%)	5,781 (12%)
cel	I	17,104 (34%)	21,638 (43%)	11,244 (23%)
	II	12,222 (30%)	16,870 (42%)	11,027 (28%)

[a] Data are expressed as counts per minute per milligram dry weight. Growth was for 36 hr and ^{14}C-18:1 Δ^9 *cis* was added 1 hr before harvest.

ature range. The supplementation of *cel* with 16:0 resulted in a very low level of unsaturation.

Spin-labeled fatty acid. In an attempt to determine if anything was detectably impaired with the way fatty acids were arranged in *cel* the spin-label fatty acids, 12NS and 12NS-*9,10-*^3H, were employed. This fatty acid is incorporated into the phospholipids and neutral lipids by *Neurospora* in both its whole mycelial lipids and mitochondrial lipids (5, 7). Parallel experiments were carried out with wild type and *cel*. Samples were prepared from cultures which developed in the presence of 12NS for 24 hr. Whole hyphae were

TABLE 7. *Distribution of ^3H from 12NS-9,10-^3H into lipid classes*[a]

Strain	Expt	Lipid fraction		
		Phospho-lipid	Neutral lipid	Free fatty acids
Wild type	I	7,842 (26%)	19,123 (64%)	2,838 (10%)
	II	6,400 (19%)	24,084 (73%)	2,418 (8%)
cel	I	7,715 (25%)	18,192 (59%)	5,023 (16%)
	II	6,052 (23%)	15,092 (58%)	4,768 (19%)

[a] Data are expressed as counts per minute per milligram dry weight. Growth was for 36 hr and 12NS-9,10-^3H was added 1 hr before harvest.

collected, squeezed free of excess water, and about 10 to 30 mg were placed in the ESR spectrometer for analysis. These experiments were carried out to determine if the physical state of 12NS in the two systems was relatively similar or different.

Spin labels are ideal for measuring molecular motion. This in turn can be related to viscosity, solvent polarity, rotational motion, translational motion, Arrhenius activation energy of some motion parameter, homo- or heterogeneity effects, surface binding, distance relations from other paramagnetic species or NMR or fluorescent probes, anisotropic effects, and perhaps other motion-related parameters. Theory has been advanced for the calculation of rotational correlation times (τ_c) from isotropic ESR spectra. τ_c is defined as the time required to go through a 90-degree arc of motion. These considerations originate from spin lattice relaxation theory which was largely developed by Bloembergen (thesis, W. A. Benjamin, Inc., N.Y.)

The equation shown below was derived from Kivelson (8) and is similar to applications used by others in recent years. This equation assumes isotropic motion. From the appearance of 12NS spectra compared to those of nearly spherical nitroxides the spectra appear to reflect isotropic motion. Seelig (13) has recently shown that varying the position of the nitroxyl group from

TABLE 8. *Fatty acid composition of cel and wild type*[a]

Strain	Supplementation	Fatty acid fraction									
		16:0	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3	% unsat./ % sat.
Wild type	None	4	21	3	...	...	8	27	32	5	(2.0)
cel	None	5	35	9	...	...	16	17	18	...	(0.8)
Wild type	18:1 Δ^9 *cis* plus 18:1 Δ^9 *trans*	6	13	4	...	...	3	61[b]	11	2	(3.5)
cel	18:1 Δ^9 *cis* plus 18:1 Δ^9 *trans*	5	10	2	...	...	4	61[b]	18	...	(4.3)
Wild type	17:0	2	15	3	38	8	8	13	13	...	(0.6)
cel	17:0	11	3	13	29	6	17	15	6	...	(0.7)
cel	16:0	traces	64	2	...	...	15	11	8	...	(0.3)

[a] Data are expressed as the molar percentage. *See* Fig. 6 legend for conditions. The <16:0 category is not included in the per cent unsaturated-saturated column.

[b] Mixture of *cis* and *trans*.

carbon 4 to carbon 12 on stearic acid largely determines the degree of anisotropy. A nitroxide on carbon 4 is highly anisotropic, and the anisotropy decreases as the distance from the carboxyl group increases until at carbon 12 no anisotropy was detectable.

$$\tau_c = KW_0 [(W_{-1}/W_0) - 1] = K (W_{-1} - W_0)$$

K is a constant which depends on the anisotropic hyperfine couplings and anisotropic g tensor values. W_0 and W_{-1} are the widths of the mid- and high-field lines in gauss; for convenience of measurement, W_{-1}/W_0 may be replaced by $(h_0/h_{-1})^{1/2}$ by assuming Lorentzean line shapes; h_0 and h_{-1} are the heights of the mid- and high-field first derivative lines. This equation is given in greater detail in reference (5). For purposes of the present report τ_c is of interest as a measure of relative velocities of molecular motion. The hyperfine coupling constant (A_N) is also of interest as a measure of local polarity to the immediate proximity of the N-O group.

Table 9 shows τ_c values for the different lipid supplementations. For the sake of clarity in considering what these values mean, the Stokes' equation for viscosity (η) is shown where V is the volume of the spin label molecule, k is Boltzman's constant, and T is the temperature in degrees Kelvin.

$$\tau_c = (4\pi \eta r^3)/3kT = V \eta/kT \simeq \eta$$

This shows that τ_c is proportionate to viscosity, and this relationship should be valid for most considerations where spin labels are in lipid or hydrophobic domains. The hyperfine coupling constant (A_N) varied from 14.2 to 14.3 gauss at 60 C for all preparations (12NS yields an A_N = 14.2 in octadecane and = 16.1 in water at 60 C). Therefore, the A_N values show that 12NS was in nonpolar domains in all preparations. Judging from the lipid class distribution of 12NS-*9,10-*^3H this represents an average of 12NS in phospholipids, neutral lipids and free fatty acids. We assume that most of the phospholipids are in membranes and the other lipids are dispersed throughout the system, possibly including cytoplasmic aggregates.

Table 9 shows that the unsupplemented wild type has the lowest τ_c values and therefore the lowest viscosity values in the local regions surrounding 12NS. The unsupplemented *cel* has τ_c values of about three times wild type. In terms of viscosity this would mean more viscous lipid regions than wild type. Supplementation with a mixture of 18:1 Δ^9 *cis* and *trans* resulted in almost identical τ_c values for *cel* and wild type over a temperature range. Since the fatty acid composition and ratio of unsaturated-saturated values were similar a relationship between lipid viscosity and fatty acid composition is indicated. The supplementation of both strains with 17:0 resulted in τ_c values of about the same for wild type and *cel*. In this case the fatty acid composition is not very similar on a molecular species basis but does display about the same degree of unsaturation. Both are much more saturated than unsupplemented wild type. These observations establish a relationship between τ_c and fatty acid composition (or perhaps better, degree of unsaturation). This relationship is probably important to the organism in maintaining the physical properties of lipids best suited for physiological function.

DISCUSSION

cel is capable of incorporation, desaturation, and acylation of supplemented long chain fatty acids as demonstrated by its desaturation and incorporation of stearate-*1-*^{14}C into phospholipids and neutral lipids. It was originally reported (9) that oleic acid would restore growth of this mutant and it was, therefore, referred to as *ol* for

TABLE 9. *Motion of 12NS in hyphae of + and cel*[a]

Strain	Supplementation	% Unsaturated/ saturated	τ_c values at			
			30 C	40 C	50 C	30 C/50 C
Wild type	none	2.0	20[b]	16	13	1.5
cel	none	0.8	62	49	38	1.6
Wild type	17:0	0.6	57	31	17	3.4
cel	17:0	0.7	58	30	15	3.9
Wild type	18:1 Δ^9 *cis* + 18:1 Δ^9 *trans*	3.5	32	24	18	1.8
cel	18:1 Δ^9 *cis* + 18:1 Δ^9 *trans*	4.3	33	24	19	1.7

[a] *Cel* supplemented with 18:1 Δ^9 *cis* plus 18:1 Δ^9 *trans* was cultured for 72 hr. Cel unsupplemented was cultured for 24 days. All others were cultured for 48 hr, and 12NS was added 24 hr before harvest in all cases.
[b] All values are 10^{-10} sec.

oleic acid; however, we observed no growth occurred when highly purified oleic acid and other purified unsaturated fatty acids were used. These unsaturates do restore growth to double-bond requiring mutants in yeast (18), *Neurospora* (9), and *E. coli* (14). Since radioactive oleate (oleate-*1*-^{14}C) is readily incorporated into *cel* its growth requirement does not seem to be limited by permeability barriers to unsaturates. We have, therefore, renamed the mutant *cel* for fatty acid chain elongation. Tween 80, which contained approximately 14% saturated fatty acids, supported growth of *cel* as did commercial grade oleic acid containing approximately 5 to 10% saturated impurities. *cel* has the reduced ability to incorporate labeled acetate into long-chain fatty acids, and its growth is restored by a variety of saturated fatty acids. The observation that 12:0 and 14:0 are less effective in supporting growth than 16:0 is explained by some degree of growth inhibition to both *cel* (Fig. 3 and 4) and wild type and does not necessarily indicate that *cel* has difficulty elongating these fatty acids.

This mutant has provided considerable information on the specificity of the requirement for fatty acids in *Neurospora*. A saturated fatty acid is a requirement and none of the *cis*-unsaturated fatty acids tested supported detectable growth. The *trans*-unsaturated fatty acids, 18:1 Δ^9 *trans* and 18:1 Δ^{11} *trans* supported a small amount of growth (Table 1). We suggest that this was due to the similarity of the physical properties and structural arrangement of the *trans*-unsaturated fatty acids to saturated fatty acids. The observation that the *trans*-unsaturated fatty acids supported some growth even in the absence of *cis*-unsaturates suggests two possibilities. First, *trans*-unsaturates, although they substitute primarily for saturated fatty acids, may be capable of fulfilling the function of *cis*-unsaturates in biological membranes. Second, the *trans*-unsaturated fatty acids may free the small quantity of saturated fatty acids produced by substituting for them in structural positions. The saturated fatty acids could then be desaturated, thus increasing the available *cis*-unsaturates for key positions.

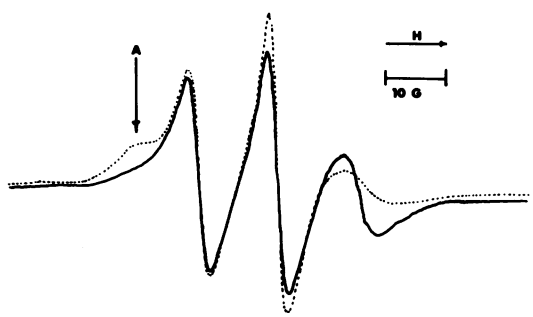

FIG. 5. *Electron spin resonance spectra of 12-nitroxide-stearate (12NS) in cel and wild type. First derivative spectra of 12NS in wild type (——) unsupplemented and cel (· · ·) unsupplemented after 24 hr of growth in the presence of 12NS (48 hr old wild type hyphae and 24-day-old cel were analyzed).*

That the *trans*-unsaturated fatty acids substituted for saturates is further indicated since the growth resulting from *trans*-unsaturated fatty acids was further stimulated by 18:1 Δ^9 *cis* and 16:1 Δ^9 *cis*. In this respect, the *trans* components were similar to 19:0 which, by itself, supported only a slow rate of growth. The addition of 18:1 Δ^9 *cis* or 16:1 Δ^9 *cis* with 19:0 considerably increased growth. When a saturated fatty acid such as 16:0 or 18:0 which can be desaturated (or 12:0 or 14:0, which can be chain elongated and then desaturated) is given to *cel*, there is no need to add an unsaturated fatty acid. Apparently, 19:0 and 20:0 cannot be efficiently desaturated since no gas chromatographic peaks corresponding to either unsaturated homologue were apparent and they were inadequate for growth by themselves. The ability of 19:0 and 20:0 to serve as saturated fatty acids in *cel* indicates that the requirement is not highly restricted to 16- or 18-carbon chain

length components. Also, an even chain length saturate is not a requirement since 15:0 and 17:0 will support a good rate of growth even though odd-chain fatty acids are not normally present in wild-type *Neurospora*. These odd-chain-length fatty acids are desaturated, and the products of desaturation are apparently acceptable as unsaturated fatty acids since no unsaturate is required to promote growth of *cel* in medium containing 15:0 or 17:0.

Information from these growth experiments is relevant to the specificity of the requirement for an unsaturated fatty acid. Only 18:1 Δ^9 *cis* and 16:1 Δ^9 *cis* supplemented *cel* growth in the presence of 19:0, 20:0, 18:1 Δ^9 *trans*, or 18:1 Δ^{11} *trans*. 18:2 $\Delta^{9,12}$ *cis*, *cis*, 18:3 $\Delta^{9,12,15}$ *cis*, *cis*, *cis*, 18:1 Δ^{11} *cis*, and 18:1 Δ^6 *cis* will not support growth in combination with a saturated fatty acid or *trans*-unsaturated fatty acid. *cel* seems to require a fatty acid with only one double-bond since 18:2 $\Delta^{9,12}$ *cis*, *cis*, and 18:3 $\Delta^{9,12,15}$ *cis*, *cis*, *cis* are unacceptable as the sole unsaturated fatty acid even though they are normally present in *Neurospora* and contain a double bond in the Δ^9 position.

18:1 Δ^9 *cis-1-*^{14}C, 18:0-*1-*^{14}C, 12NS-*9,10-*^3H, and acetate-*1-*^{14}C were all incorporated by both *cel* and wild type at about the same rate. Therefore, it is unlikely that permeability is limiting in any of the supplemented fatty acids, including 18:2 and 18:3.

The motion of a spin-label fatty acid responded to fatty acid composition. The distribution of this same spin label (12NS-*9,10-*^3H) into lipid classes was similar for both *cel* and wild type; therefore, these data should be accurate in reflecting the relative viscosities and local polarities of 12NS in *cel* and wild type. Representative spectra are shown in Fig. 5. The relative τ_c values are presented in Table 9, and the A_N for all preparations show a hydrocarbon environment. The temperature range between 30 and 50 C can also be analyzed in terms of Arrhenius activation energy (E_a). This data can be plotted as log τ_c versus $1/R°K$ where E_a is the slope. These values are proportionate to the 30 C/50 C ratio shown in Table 9 and show the temperature dependency of τ_c. It is not possible to fully interpret these ratios or activation energy values at this time, but the smallest ratio (activation energy value) and the fastest τ_c value characterize the unsupplemented wild type. It has been shown that a characteristic change in activation energy occurs at the optical melt in a variety of fatty acids and lipid systems using a variety of spin labels on Arrhenius plots (18). These τ_c values are linear for all preparations between 30 and 50 C indicating that no physical state change (phase transition) occurs in this range. Since the unsupplemented *cel* and wild type show similar 30 C/50 C ratios but have very different τ_c values, it seems probable that the state of fluidity of lipid zones is important in the maintenance of good physiological conditions.

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