Mutations Resulting in an Unsaturated Fatty Acid Requirement in *Neurospora*. Evidence for Δ^9-Desaturase Defects†

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ABSTRACT: Unsaturated fatty acids were supplied to the *ufa* (unsaturated fatty acid) mutants of *Neurospora* as Tergitol complexes. Marked changes in the fatty acid composition of mutant lipids were achieved. The 18:2$\Delta^{9,12}$-*cis,cis* or 18:3$\Delta^{9,12,15}$-*cis,cis,cis* supplements comprised up to 75% of the lipid fatty acids. Characteristically, lipids of these enriched cells lacked monounsaturated fatty acids. In contrast, supplementation with 18:1Δ^{11}-*cis* leads to an enrichment of 50–70% with little or no polyunsaturated fatty acids present in the mutant lipids. Endogenous fatty acid synthesis, as monitored by [^{14}C]acetate incorporation into fatty acids, was comparable in the *ufa* mutants and wild type. From 60 to 70% fatty acid synthesized in wild type was recovered as unsaturated fatty acids. In contrast, synthesis of only saturated fatty acids was detected in the majority of the mutants. This and the accumulation of 18:0 suggested that the *ufa* mutants lack the Δ^9-desaturase. Fatty acid analyses of *ufa* mutants fed 18:1Δ^9-*cis* indicated these strains could desaturate the supplement to 18:2 and 18:3 and, as a result, did not accumulate 18:1. Growth of the mutants in [^{14}C]-18:1Δ^9-*cis* substantiated this supposition and further suggested the Δ^{12}- and Δ^{15}-desaturases are unaffected by the *ufa* mutations.

Many efforts have been made to elucidate the mechanisms, substrates, and regulation of aerobic fatty acid desaturation (Fulco, 1974). The components of the Δ^9-(stearyl coenzyme A) desaturase have been separated in detergent solubilized preparations of microsomes and shown to involve cytochrome b_5 reductase, cytochrome b_5, and the cyanide-sensitive desaturase (Holloway, 1971; Shimakata et al., 1972; Strittmatter et al., 1974). In most eukaryotic organisms which synthesize polyunsaturated fatty acids, the Δ^9-desaturase is the dominant activity. Mutants deficient in the Δ^9-desaturase therefore would greatly facilitate the study of the oleyl desaturase and subsequent steps in the formation of polyunsaturated fatty acids in these systems. In addition, the influence of fatty acid composition on fatty acid desaturation and other membrane functions can be revealed by supplementation techniques with unsaturated fatty acid (*ufa*) mutants. Enrichment of cellular lipids with exogenous fatty acids has been achieved with such mutants in *E. coli* (Silbert and Vagelos, 1967) and yeast (Keith et al., 1973), organisms which contain a single desaturase, and dependence of a number of membrane-associated functions in *E. coli* on membrane fluidity has been shown (Silbert et al., 1974).

Bearing these possibilities in mind, we isolated *ufa* mutants of *Neurospora* (Scott, 1977). This lower eukaryote offers several advantages. It is easily manipulated and its genetic system is well characterized. *Neurospora* can synthesize polyunsaturated fatty acids when grown on media containing inorganic salts, glucose, and biotin (Figure 1) by the action of at least two different desaturases (Baker and Lynen, 1971). Our previous studies indicated that the *ufa* mutants are grouped in two complementation loci, *ufa-1* and *ufa-2*, on the same linkage group (Scott, 1977). Fatty acids of different chain length (C_{16}–C_{20}), degree of unsaturation, and steric configuration support the growth of mutants at both loci. We therefore examined the fatty acid composition, synthesis, and desaturation in these strains under different supplementation conditions. The evidence presented here indicates that the majority of the *ufa* mutants contain a defective Δ^9-desaturase.

Materials and Methods

Fatty acids were the products of Analabs, Inc., North Haven, Conn., or Sigma Chemical Co., St. Louis, Mo. Tergitol NP-40 and Tween 80 were purchased from Sigma. Radioactive compounds, [1-^{14}C]acetate (59.5 Ci/mol), [1-^{14}C]-18:0 (56 Ci/mol), and [1-^{14}C]18:1 (59.7 Ci/mol), were obtained from Amersham/Searle Corp., Arlington Heights, Ill. Plates coated with silica gel were products of Supelco, Inc., Bellfonte, Pa. These were activated for 1 h at 100 °C prior to use, as were plates coated with silica gel containing 15% $AgNO_3$ (Analabs). Acid-washed silica gel (Supelcosil-ATF 120, 100–200 mesh), purchased from Supelco, was activated at 100 °C overnight. Organic solvents were of the highest purity available.

Cultures. The *ufa* mutants, isolated in this laboratory as described previously (Scott, 1977), were maintained on agar slants of minimal medium (Vogel, 1964) supplemented with 0.1% Tween 80 and 50 mg/L inositol. The wild-type strain, RL3-8A, was stored on slants of minimal agar medium. Cultures were grown at 34 °C on a reciprocal shaker set at 102 strokes/min. Fatty acid supplements were suspended in the nonionic detergent, Tergitol NP-40 (Henry and Keith, 1971), and added to 50 mL of minimal medium containing 1% glucose and 50 mg/L inositol in 125-mL Erlenmeyer flasks. After sterilization, the cultures were inoculated with small bits of mycelia from agar cultures. The final concentrations of detergent and fatty acid supplement were 1% and 1 mM, respectively.

Mutants (Scott, 1977) representing both complementation groups, *ufa-1* and *ufa-2*, which lead to an unsaturated fatty acid requirement for growth were analyzed in this study. The *ufa-1* locus is represented by a single mutant. Of the 23 known *ufa-2* mutants, most have similar growth rates and growth responses to different unsaturated fatty acid supplements. Analyses of the fatty acid compositions of five of these strains chosen at random revealed no significant compositional differences after enrichment with different unsaturated fatty acids. One strain, TR232, was employed as a representative

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TABLE I: Fatty Acid Composition of Wild-Type *Neurospora* Lipid.

Medium or supplement[a]	Fatty acid:mole %						
	16:0	16:1	18:0	18:1	18:2	18:3	% unsat./% sat.
Minimal	17.0	2.9	2.7	26.1	48.0	3.3	4.1
Minimal-Tergitol	19.3	2.4	3.1	24.9	48.2	2.1	3.5
16:1Δ^9-*cis*	17.5	19.7	8.3	26.1	27.2	1.2	2.9
18:1Δ^9-*cis*	15.8	1.5	1.9	44.4	35.0	1.4	4.7
18:2$\Delta^{9,12}$-*cis,cis*	15.9	1.0	2.2	9.6	68.1	3.5	4.5
18:3$\Delta^{9,12,15}$-*cis,cis,cis*	15.3	3.3	2.1	17.4	27.1	34.8	4.8

[a] The wild-type strain *RL3-8A* was grown in minimal medium containing 1% glucose in the presence and absence of 1% Tergitol. Fatty acids were suspended in Tergitol medium at a final concentration of 1 mM. Fatty acid compositions of total lipids were determined as described under Materials and Methods.

mutant for further studies and is designated as *ufa-2* throughout this study. TR221 is a slow growing *ufa-2* mutant which does not respond to a number of unsaturated fatty acids utilized by the majority of the *ufa-2* mutants. Results for TR221 are presented separately where appropriate.

Lipid Extraction. *Neurospora* cultures were rapidly harvested on Büchner funnels with the aid of gentle suction. Mycelia were washed with distilled water and weighted. After removal of a portion of the mycelia for dry weight analysis, lipids were extracted by the procedure of Folch et al. (1957) at 0 °C. Mycelial pads, suspended in chloroform–methanol (2:1 v/v), were broken into fragments at 0 °C by grinding in a mortar and further dispersed in a Ten-Broeck homogenizer until a fine suspension resulted. A two-phase system was formed by the addition of 0.2 volume of 0.58% NaCl to the filtered extract. The lipid phase was washed as described by Folch et al. (1957) and dried with 1 g of Na_2SO_4–$NaHCO_3$ (4:1 w/w). BHT (2,6-di-*tert*-butyl-*p*-cresol) was added to a final concentration of 50 μg/mL. After reduction of the volume under vacuum, the lipid extract was stored at −20 °C under nitrogen in tubes sealed with Teflon-lined screw caps.

Separation of Lipid Classes. *Neurospora* lipids were separated into phospholipids, free fatty acids, and neutral lipids by thin-layer chromotography as described by Keith et al. (1968). Areas of the chromatogram corresponding to the phospholipids and neutral lipids were scraped directly into scintillation vials, and the radioactivity of each was determined in Aquasol by scintillation counting after the addition of 1 mL of water. Silica gel containing free fatty acids was scraped into small columns containing glass wool plugs and eluted with diethyl ether containing 2% formic acid (Keith et al., 1968). After evaporation of the solvent, radioactivity was determined.

For fatty acid analysis, neutral lipids and phospholipids were separated on 0.5 × 4.0 cm columns of acid-washed silica gel. Neutral lipids were eluted with 3 mL of chloroform and the phospholipids with 3 mL of methanol. Both solvents contained 50 μg/mL of BHT.

Argentation Chromatography. Fatty acid methyl esters of varying degrees of unsaturation were separated by thin-layer chromatography on silica gel plates impregnated with 15% $AgNO_3$. After development of the chromatogram in benzene, lanes containing standard mixtures of fatty acid methyl esters (24 μg of each ester) were sprayed with a solution of 0.2% dichlorofluorescene in methanol and visualized under ultraviolet light. Areas of the sample lanes corresponding to the fatty acid standards were scraped into small columns and eluted with 5 to 7 mL of chloroform. Radioactivity was determined as described above.

Gas–Liquid Chromatography. Fatty acid methyl esters were prepared from extracted lipids by adding 2 mL of hydrochloric acid–methanol and 1 mL of benzene to samples in screw-capped tubes which were flushed with nitrogen, sealed, and heated overnight at 80 °C. A 6% solution of hydrochloric acid in methanol was freshly prepared by the addition of 1 volume of acetyl chloride to 9 volumes of methanol. After allowing the reaction mixture to cool, 1 mL of water and 2 mL of hexane were added. The hexane layer was removed and the water layer was washed with an additional 2 mL of hexane. The combined hexane fractions were dried for 1 h with 1 g of Na_2SO_4–$NaHCO_3$ (4:1 w/w), filtered by gravity, and reduced in volume under nitrogen. The samples were dissolved in CS_2 and analyzed by gas–liquid chromatography. Separations were accomplished isothermally at 220 °C on 20 ft × $\frac{1}{8}$ in. stainless steel columns containing 15% OV-275 on 100–200 Chromosorb P AW/DMCS (Supelco). The carrier gas (N_2) flow through the column was set at 10 mL/min as recommended (Supelco, Bulletin 752) for maximal resolution. To achieve optimal sensitivity of the detectors, the instrument was fitted with a make up gas fitting (Brownlee and Silverstein, 1968) through which additional nitrogen was added to the column effluent at 20 mL/min. The flow rates of air and hydrogen to the detector were 300 and 10 mL/min, respectively. The injector temperature was set at 250 °C, and the detector oven was maintained at 225 °C. Fatty acid methyl esters were identified by comparison of retention times with those of authentic standards (16A, Nu Chek Prep, Elysian, Minn.).

Results

Fatty Acid Compositions under Different Supplementation Conditions. Our previous studies (Scott, 1977) indicated that various unsaturated fatty acids support the growth of the *Neurospora ufa* mutants when suspended at a final concentration of 1 to 2 mM in minimal medium containing 1% of the nonionic detergent, Tergitol NP-40. In control experiments, this level of detergent was found to allow the growth of wild-type *Neurospora* as noted by Henry and Keith (1971), but at a reduced rate. The fatty acid analyses in Table I (compare lines 1 and 2) demonstrate that inclusion of detergent in the growth medium has no effect on the fatty acid composition of wild type.

As shown in Table I, *Neurospora* lipids contain a high degree of unsaturation: 18:1 (oleate) and 18:2 (linoleate) comprise over 70% of the total fatty acid. These fatty acids and the other naturally occurring unsaturated fatty acids, 16:1 (palmitoleate) and 18:3 (linolenate), of *Neurospora* were supplied to wild-type cultures as Tergitol complexes, and the fatty acid compositions of total lipids from the supplemented cultures determined (Table I). These analyses provide controls for the *ufa* mutants similarly supplemented, and also indicate the

TABLE II: Fatty Acid Composition of Neurospora *ufa* Mutants.

Strain	Supplement[a]	Fatty acid:mole % 16:0	16:1	18:0	18:1	18:2	18:3	% unsat./% sat.
ufa-1	16:1Δ^9-*cis*	15.9	31.6	28.3	21.3	2.9		1.3
ufa-2		17.1	37.8	23.0	20.8	1.3		1.5
TR221		9.4	54.3	16.3	19.0	1.1		2.9
ufa-1	18:1Δ^9-*cis*	20.3	Trace	30.2	14.5	32.0	3.0	1.0
ufa-2		18.6	Trace	30.4	18.3	29.4	3.3	1.0
TR221		13.0		6.2	68.0	12.8	Trace	4.2
ufa-1	18:2$\Delta^{9,12}$-*cis,cis*	17.1		43.5	0.9	29.5	9.0	0.7
ufa-2		12.8		11.3		70.3	5.6	3.2
TR221		14.8		16.7		64.7	3.7	2.2
ufa-1	18:3$\Delta^{9,12,15}$-*cis,cis,cis*	18.1	Trace	32.2		3.1	46.6	1.0
ufa-2		13.9		12.1		Trace	74.0	2.9
TR221		13.8		10.4		Trace	75.8	3.1

[a] The *ufa* mutants were grown in minimal medium containing 1% glucose and 1% Tergitol. Fatty acids were added to minimal-Tergitol medium at a final concentration of 1 mM. Fatty acid compositions of total lipids were determined as described under Materials and Methods.

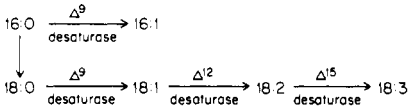

FIGURE 1: Hypothetical scheme for the synthesis of unsaturated fatty acids in *Neurospora crassa*.

degree of enrichment possible with exogenously supplied fatty acid in lipids of the normal organism. The greatest change in fatty acid composition of wild type was obtained on supplementation with the minor fatty acids of wild type, namely 16:1 and 18:3. The levels of 16:1 and 18:3 were increased 8- and 13-fold, respectively, when supplied exogenously. Both 18:1 (1.7-fold) and 18:2 (1.4-fold) were accumulated to a lesser extent. Enrichment with unsaturated fatty acids, however, had little effect on the degree of unsaturation of the wild-type lipids. It appeared that adjustments in the level of 18:2 were primarily responsible for maintaining this constant degree of unsaturation. Accumulation of 16:1 or 18:1 led to a corresponding decrease in 18:2 content. Similarly, incorporation of 18:3 reduced the 18:2 level, and to a lesser extent, the level of 18:1. It should also be noted that enrichment with palmitoleate leads to a 3-fold increase in the percentage of 18:0, whereas the C_{18} unsaturated fatty acids produced no changes in the saturated fatty acid content.

Comparable supplementation experiments with the *ufa* mutants lead to entirely different results (Table II). The lipids of the *ufa-1* and *ufa-2* mutants supplied with 18:1 contained the normal complement of the fatty acids synthesized by wild-type *Neurospora*, although the levels of 16:1 and 18:1 were decreased. In contrast, C_{18} polyunsaturated fatty acids were reduced to insignificant levels in the 16:1-grown cells, and the 16:1 content was elevated 1.6- to 2.8-fold over that of wild type grown in 16:1-containing medium. Supplementation of the *ufa* mutants with 16:1, therefore, results in an enrichment of the exogenous fatty acid and a reduced content of polyunsaturated fatty acids. When grown in 18:2- or 18:3-containing media, clear differences were evident between the *ufa-1* and *ufa-2* strains. The level of the supplemented fatty acid was considerably less in *ufa-1* lipids than those of *ufa-2*. In both mutant classes, unsaturated fatty acids of lesser degree of unsaturation than that of the supplement were conspicuously reduced or absent. One unusual feature of the *ufa-1* and *ufa-2* mutants was the accumulation of 18:0, which was evident under all supplementation conditions and ranged from 3.6- to 14-fold. The elevated 18:0 content was at least 11-fold in *ufa-1* and *ufa-2* fed monounsaturated fatty acids, but was reduced to one-third of this value in the *ufa-2* strains fed 18:2 or 18:3. In general, the ratio of unsaturated to saturated fatty acids in *ufa-1* and *ufa-2* was less than that of wild-type lipids. This value for *ufa-1* was unaffected by the number of double bonds in the supplemented fatty acids. Lipids from *ufa-2* enriched with 16:1 or 18:1 contained a similar degree of unsaturation as the *ufa-1* mutant, but the ratio increased 3-fold when supplemented with 18:2 or 18:3.

Compared with *ufa-1* or the majority of the *ufa-2* mutants, the degree of unsaturation of TR221 lipids remained relatively high under these supplementation conditions (Table II). Two factors appear to account for the differences between TR221 and the other *ufa-2* mutants as evidenced from enrichment with monounsaturated fatty acids. First, no accumulation of 18:0 was apparent in TR221 lipids. Second, TR221 was unable to metabolize 16:1 and 18:1 to the same extent as the other *ufa* mutants and wild type, as indicated by the greater accumulation of these supplements. However, when grown on polyunsaturated fatty acids, the fatty acid composition of TR221 closely resembled the more rapidly growing *ufa-2* mutants.

Wild type and the *ufa* mutants accumulated 16:1Δ^9-*trans* (Table III) and 16:1Δ^9-*cis* (Tables I and II) to the same extent. However, only the cis monounsaturated fatty acid appeared to affect desaturation of endogenously synthesized saturated fatty acid in wild type as evidenced by the elevated 18:0 content in 16:1Δ^9-*cis* fed cells. Significant amounts of 18:1, but not C_{18} polyunsaturated acids, were evident in the mutants enriched with both isomers of 16:1. Presumably, 18:1 was derived from 16:1 by chain elongation to give the 11,12-monounsaturated derivative. Conversion of 18:1Δ^{11}-*cis* (derived from palmitoleate) to 18:2 would not be expected since the double bond of 18:1Δ^{11} is adjacent to site of dehydrogenation by the Δ^{12}-desaturase (Figure 1). This was demonstrated directly by supplementation with 18:1Δ^{11}-*cis*. Accumulation of 18:1 occurred in wild-type and mutant lipids together with reduced levels of 18:2 and 18:3 (Table III), indicating a low efficiency of 18:1Δ^{11} desaturation.

Fatty Acid Synthesis and Desaturation. Incorporation of [^{14}C]acetate into lipid was employed as a monitor of endogenous fatty acid synthesis. Cultures of wild type and the *ufa* mutants were grown in medium containing 18:1 or 18:3 and

TABLE III: Fatty Acid Composition of *Neurospora* Grown in Medium Containing 16:1Δ^9-*trans* and 18:1Δ^{11}-*cis*.

Strain	Supplement[a]	Fatty acid:mole % 16:0	16:1	18:0	18:1	18:2	18:3	% unsat./% sat.
Wild type	16:1Δ^9-*trans*	15.8	15.2	2.7	26.9	38.7	0.7	4.4
ufa-1		14.1	38.6	24.5	20.4	2.4		1.6
Wild type	18:1Δ^{11}-*cis*	8.7	2.1	5.5	68.2	14.3	1.2	6.0
ufa-1		9.7	2.0	26.2	54.5	5.8	1.8	1.8
ufa-2		12.3	2.2	34.1	46.3	4.8	0.3	1.2

[a] See Tables I and II for culture conditions. The final concentrations of the fatty acids in the medium were 1 mM. Fatty acid compositions were determined as described under Materials and Methods.

TABLE IV: Distribution of [^{14}C]Acetate in *Neurospora* Lipids.[a]

Strain	Medium or supplement	cpm in lipid[c] mg of dry weight $\times 10^5$	% of radioact.[b] Neutral lipid	Free fatty acid	Phospholipid
RL3-8A	Minimal-Tergitol	2.88	52.4	0.2	47.4
	18:1Δ^9-*cis*	0.65	69.0	0.3	30.5
	18:3$\Delta^{9,12,15}$-*cis,cis,cis*	0.73	82.4	0.2	17.3
ufa-1	18:1Δ^9-*cis*	0.70	86.5	0.3	13.2
	18:3$\Delta^{9,12,15}$-*cis,cis,cis*	0.72	83.5	0.3	16.3

[a] RL3-8A and the *ufa-1* mutant were grown in the indicated media containing 25 μCi of [^{14}C]acetate per culture. See Tables I and II for culture conditions. [b] Lipid classes were separated by TLC and the radioactivity of each was determined by liquid scintillation counting as described under Materials and Methods. [c] Radioactivity in total lipids was measured in aliquots of the lipid extracts prior to TLC analyses. Since greater than 90% of the acetate incorporated into lipid is associated with fatty acyl groups, the percentage of radioactivity in each lipid fraction is a reflection of the fatty acid content.

TABLE V: Distribution of [^{14}C]Acetate in *Neurospora* Fatty Acids.[a]

Strain	Medium or supplement	% of radioact.[b] 16:0 + 18:0	16:1 + 18:1	18:2	18:3
RL3-8A	Minimal-Tergitol	13.8	29.6	47.2	9.4
	18:1Δ^9-*cis*	41.9	16.3	37.0	4.8
	18:3$\Delta^{9,12,15}$-*cis,cis,cis*	30.1	27.2	37.1	5.6
ufa-1	18:1Δ^9-*cis*	97.0	1.0	1.6	0.4
	18:3$\Delta^{9,12,15}$-*cis,cis,cis*	95.4	1.2	1.7	1.8

[a] These analyses were performed on aliquots of the samples in Table IV. [b] Fatty acid methyl esters were separated by argentation TLC after transmethylation of total cellular lipid and radioactivity determined (Materials and Methods). Data are presented as percentages of radioactivity recovered (70%) after TLC. The specific activity of each fatty acid fraction can be estimated from the specific activity of total lipid in Table IV.

[^{14}C]acetate. TLC analysis of lipids after transmethylation indicated that greater than 90% of the acetate incorporated into lipid was associated with fatty acyl moieties. A comparison of acetate incorporation in control cultures of wild type and those containing unsaturated fatty acids further revealed that supplementation reduced endogenous fatty acid synthesis 4-fold (Table IV). A greater percentage of the radioactivity was associated with the neutral lipids of wild type enriched with 18:1 or 18:3 than in cultures grown in minimal-Tergitol medium, in keeping with the fact that the neutral lipid content increased in fatty acid-enriched cultures (see below). Levels of acetate incorporation in the lipids of the *ufa* mutants were similar to those of wild type enriched with unsaturated fatty acids as shown for *ufa-1* in Table IV, suggesting that the synthesis and acylation of fatty acids are not impaired in the *ufa* strains.

In order to measure fatty acid desaturation in the *ufa* mutants and the effect of unsaturated fatty acid enrichment on desaturation, purified fatty acid methyl esters from wild-type and mutant lipids were separated according to their degree of unsaturation by argentation TLC. The levels of radioactivity in saturated fatty acids and the olefinic analogues were determined after elution. As shown in Table V, a greater percentage of acetate incorporation was evident in the saturated fatty acids from wild-type cultures fed fatty acids than in control cultures; however, at least 50% of the radioactivity was recovered in the unsaturated fatty acids under all growth conditions. In contrast, synthesis of only saturated fatty acids could be detected in the mutants.

Incorporation and Metabolism of Exogenous Fatty Acids. Labeling patterns (Table VI) of lipids from wild-type and *ufa* cultures grown in 18:1 containing tracer amounts of [1-^{14}C]-18:0 resembled those obtained with [^{14}C]acetate. As shown in Table VII, greater than 97% of the 18:0 incorporated into mutant lipid was recovered as saturated fatty acid. Extensive metabolism of 18:0 by wild type was evident, however, in that 50% of the recovered radioactivity was associated with unsaturated fatty acids. A different picture for the *ufa* mutants

TABLE VI: Incorporation and Distribution of Fatty Acids in *Neurospora* Lipid.[a]

Strain	Supplement	Source of radioact.	cpm: mg of dry weight × 10^5	% of radioact.[b]: Neutral lipid	Free fatty acid	Phospholipid
RL3-8A	18:1Δ^9-*cis*	[1-^{14}C]-18:0	0.82	74.6	0.1	25.3
ufa-1	18:1Δ^9-*cis*	[1-^{14}C]-18:0	0.60	88.3	0.7	11.0
RL3-8A	18:1Δ^9-*cis*	[1-^{14}C]-18:1	0.31	65.1	1.1	33.9
ufa-1	18:1Δ^9-*cis*	[1-^{14}C]-18:1	0.44	67.0	0.4	32.7

[a] Culture conditions are described under Tables I and II. Each culture contained 25 μCi of [1-^{14}C]-18:0 or 25 μCi of [1-^{14}C]-18:1. [b] See Table IV for separation of lipid classes and determination of radioactivity.

TABLE VII: Metabolism of 18:0 and 18:1Δ^9-*cis* in *Neurospora*.[a]

Strain	Source of radioact.	% of radioact.: 16:0 + 18:0	16:1 + 18:1	18:2	18:3
RL3-8A	[1-^{14}C]-18:0	43.0	38.9	16.2	1.9
ufa-1		97.4	1.7	0.8	0.2
RL3-8A	[1-^{14}C]-18:1Δ^9-*cis*	5.0	67.3	26.5	1.2
ufa-1	[1-^{14}C]-18:1Δ^9-*cis*	2.0	50.2	42.1	5.6
ufa-2	[1-^{14}C]-18:1Δ^9-*cis*	4.7	31.8	57.0	6.6
TR221	[1-^{14}C]-18:1Δ^9-*cis*	6.5	74.3	17.8	1.4

[a] Cultures were grown in minimal–Tergitol medium containing 1 mM 18:1Δ^9-*cis* and 25 μCi of [1-^{14}C]-18:0 or 25 μCi of [1-^{14}C]-18:1. See Table V for other comments.

TABLE VIII: Fatty Acid Composition of Phospholipids and Neutral Lipids of *Neurospora* Supplemented with 18:1Δ^9-*cis*.[a]

Fatty acids in lipid fraction[b]	Strain: RL3-8A	*ufa-1*	*ufa-2*	TR221
Phospholipids				
16:0	18.7	22.3	21.3	12.2
16:1	Trace	Trace	Trace	1.2
18:0	Trace	11.2	11.2	3.0
18:1	44.4	16.4	24.8	74.4
18:2	36.9	43.9	42.7	9.2
18:3	Trace	6.2	Trace	
% unsat./% sat.	4.4	2.0	2.1	5.6
Neutral lipids				
16:0	10.1	19.6	17.4	8.1
16:1	0.6	1.5	Trace	
18:0	3.3	32.5	36.5	5.8
18:1	45.3	18.5	17.0	75.2
18:2	39.7	24.8	27.5	10.9
18:3	1.0	3.1	1.6	
% unsat./% sat.	6.5	0.9	0.9	6.2

[a] Cultures were grown in minimal–Tergitol medium containing 1 mM 18:1-*cis*. [b] Neutral lipids and phospholipids were separated on columns of acid-washed silica gel and the fatty acids of each analyzed by gas–liquid chromatography after transmethylation.

grown in media containing [1-^{14}C]-18:1 emerged than in the acetate and saturated fatty acid labeling studies. Oleate (18:1) was desaturated by the mutants to 18:2 and 18:3. Furthermore, a greater portion of the supplement was converted to polyunsaturates in the mutants than in wild type, suggesting that the exogenous 18:1 is the primary source of these fatty acids.

Fatty Acid Compositions of Neutral Lipid and Phospholipid. The fatty acid contents of the two lipid fractions from wild-type grown in minimal–Tergitol medium were comparable. In supplemented cultures, the neutral lipid fatty acid content was 2- to 3-fold greater than that of phospholipid. The amount of lipid phosphorus remained constant under all growth conditions (data not shown), indicating neutral lipid accumulation in the fatty acid enriched cultures. Similar fatty acid contents of neutral lipids (NL) and phospholipids (PL) were found for *ufa* mutants supplemented with unsaturated fatty acids. Therefore, 1 mM fatty acid allowed optimal growth of the mutant strains, but resulted in an elevated level of storage lipid.

As shown in Table VIII, the percentages of unsaturated fatty acids in wild-type fed 18:1 were comparable in NL and PL. However, NL contained more stearate (18:0) and less palmitate (16:0) than PL. This asymmetric distribution of saturated fatty acids also occurred in the lipids of the *ufa-1* and *ufa-2* mutants. The major portion of the 18:0 accumulated by the mutants was confined to NL, although a 5-fold increase in the

18:0 content of PL was evident. Unlike wild type, the unsaturated fatty acid content of the mutant lipid fractions also differed as reflected by the 18:2 levels. The percentage of 18:2 in PL of *ufa-1* and *ufa-2* was 1.7-fold greater than in NL. The higher concentration of 18:2 in PL may reflect an attempt by the mutants to compensate for the increased degree of saturation in the mutant membranes. This trend was also observed in mutants supplemented with polyunsaturated fatty acids. That the elevated 18:0 content in *ufa-1* and *ufa-2* may influence the distribution of unsaturated fatty acids between NL and PL is supported by analysis of TR221. The latter strain, which does not accumulate 18:0 (Table II), contains similar levels of individual unsaturated fatty acids (Table VIII) in both lipid fractions.

Because of the neutral lipid accumulation in the *ufa* mutants, it is plausible that significant levels of Δ^9-desaturation may occur in phospholipids and go undetected. It should be noted that no desaturation of [^{14}C]-18:1 was detected in the isolated mutant PL.

Discussion

The *ufa* mutants of *Neurospora* are capable of fatty acid synthesis at levels comparable to that of the wild-type strain as evidenced by the incorporation of [^{14}C]acetate into long chain saturated fatty acids (Table IV). Furthermore, the uptake and acylation of 18:0 and 16:0 (data not shown) by the mutants is normal (Table VI). It would appear, therefore, that the specific growth requirement of the *ufa* strains for unsaturated fatty acids does not result from an impermeability to C_{16} or C_{18} saturated fatty acids. No appreciable desaturation of 16:0 or 18:0 was detected. In addition, all of the *ufa* mutants, with the exception of TR221, accumulate 18:0 (Table II). These results suggested that the *ufa* mutations affect the Δ^9-desaturase (Figure 1).

The *ufa* mutants readily desaturate [^{14}C]-18:1 derived from the growth medium (Table VII). Compositional analyses (Table II) of mutants fed 18:1 or 18:2 also indicate this to be the case. From these data, we have concluded that the *ufa-1* and *ufa-2* mutations do not influence the Δ^{12}- or Δ^{15}-desaturases (Figure 1). The above conclusions pertain to the *ufa-1* mutant and the majority of the *ufa-2* mutants, with strain TR221 being the exception. The *ufa-2* mutant, TR221, remains an enigma. TR221 mapped within the *ufa-2* locus as determined by complementation analysis (Scott, 1977). Reisolates of this mutant from back-crosses to wild type retain the characteristics described herein which distinguish it from the other *ufa-2* mutants, suggesting that the TR221 strain contains a single mutation or closely linked multiple mutations. TR221 did not accumulate 18:0 when enriched with monounsaturated fatty acids, except for 16:1Δ^9-*cis* and then only at levels comparable to that in wild type similarly supplemented. In addition to an inability to desaturate saturated fatty acids, TR221 also appeared to have a reduced capacity to convert 18:1 to 18:2. Both labeling studies (Table VII) and compositional analyses (Table II) supported the latter supposition. Apparently, the Δ^{12}-desaturase as well as the Δ^9-desaturase activity is affected in TR221. Enzymatic studies and additional genetic analyses are necessary to ascertain the number of mutations involved and enzymatic steps affected in this interesting mutant.

Little metabolism of unsaturated fatty acids, other than desaturation, appeared to occur in the *ufa* mutants. Lipids from strains supplemented with 18:2 were characterized by the virtual absence of 16:1 and 18:1, and 18:3 supplemented mutants by the lack of 18:2, the major fatty acid of wild type, in addition to monounsaturated fatty acids. The mutants fed 18:1Δ^{11}-*cis* contained low levels (2%) of 16:1 indicating some β oxidation of this supplement. This level of oxidation is considerably less than those reported in higher eukaryotic systems (Wisnieski et al., 1973; Williams et al., 1974; Ferguson et al., 1975).

The *ufa* mutants were selected on media containing each of the natural unsaturated fatty acids of *Neurospora* (Scott, 1977). Mutations affecting the Δ^9-desaturase and the Δ^{12}-desaturases should be recovered on selection media containing the appropriate unsaturated fatty acids provided that (i) at least two different enzymes are responsible for the desaturase sequence (Figure 1), and (ii) the organism has an absolute requirement for both monounsaturated and polyunsaturated fatty acids. However, the majority of the *ufa* mutants appear to have a defective Δ^9-desaturase, and no substantial number of Δ^{12}- or Δ^{15}-defective mutants were detected. This is to be expected from the results presented here, since the organism can survive without polyunsaturated fatty acids. As a result, Δ^{12}- and Δ^{15}-desaturase lesions would behave as silent mutations and would not be recovered in a selection scheme that depended on an unsaturated fatty acid requirement for growth.

From the data presented, *Neurospora* can tolerate a great deal of flexibility in its fatty acid composition and continue to grow. It is surprising that this is the case, for *Neurospora* like most eukaryotes contains a complex mixture of unsaturated fatty acids, the composition of which the organism undoubtedly manipulates in order to maintain a constant degree of membrane fluidity. Because of the considerable flexibility in unsaturated fatty acid composition that can be achieved by supplementation of the *ufa* mutants, these strains should be of value in studies concerned with the influence of fatty acid composition on membrane-associated functions in eukaryotic systems as well as for studies of polyunsaturated fatty acid synthesis. Currently, the levels of the Δ^9- and Δ^{12}-desaturase activities of the *ufa* mutants are under investigation. In addition, the effects of fatty acid composition on the membrane-bound adenylate cyclase are being examined.

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Diet-Induced Changes in Plasma Membrane Fatty Acid Composition Affect Physical Properties Detected with a Spin-Label Probe†

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ABSTRACT: The fatty acid composition of plasma membranes derived from Ehrlich ascites tumor cells was altered in vivo by changing the diet fed to the tumor bearing mice. After isolation, membranes prepared from cells grown in mice fed either a regular rodent chow (PM_{RC}) or a fat-deficient chow (PM_{FD}) were spin labeled with 12-nitroxide stearic acid (12NS). Discontinuities in Arrhenius plots indicated that the transition temperatures with the 12NS label were 31 and 19 °C for the PM_{RC} and 31.5 and 24.5 °C for the PM_{FD}. The order parameter, S, at 37 °C was higher for PM_{FD} than for PM_{RC}. The phospholipid composition, ratio of phospholipid to cholesterol, and distribution of fatty acyl chain lengths were similar in PM_{RC} and PM_{FD}. Marked differences were observed, however, in the degree of unsaturation of the two membrane preparations. PM_{FD} contained 34% saturated and 49% monoenoic fatty acids. Because of their high monoenoic acid content, the PM_{FD} contained a large band of unsaturation in the middle of the bilayer leaflet. By contrast, the fatty acid composition of the PM_{RC} was much more heterogeneous, with 36% polyenoic and only 23% monoenoic fatty acids. The overall unsaturation of the PM_{RC} was 1.4 times greater than that of the PM_{FD}, and the PM_{RC} contained large amounts of unsaturation in both the 9,10 and 12,13 positions of the fatty acyl chains. We conclude that the lower transition temperature and the smaller S values in PM_{RC} result from the increased amount and broader distribution of fatty acid unsaturation. These data indicate that diet-induced lipid modifications in a mammalian tumor cell are of sufficient magnitude to alter the physical properties of the plasma membrane.

The physical properties of membrane lipids are important in determining the biological activity of membrane related functions. For example, Eletr et al. (1974) have shown that Arrhenius plots of O_2 uptake and of spin-label motion in yeast exhibit discontinuities at the same temperature. Ca^{2+}-ATPase activity in sarcoplasmic vesicles also depends on the fluidity of the membrane lipids (Seelig and Hasselbach, 1971). In addition, poikilotherms have been shown to adjust their lipid composition as the temperature is lowered in order to prevent lipid fluidity from decreasing below a point detrimental to enzyme function (Marr and Ingraham, 1962; Lewis, 1962; Pearson and Raper, 1972). One important parameter that affects the physical properties of membrane lipids is fatty acyl chain unsaturation. Alterations in phase transition temperatures in phospholipid vesicles (Chapman, 1968) and yeast (Eletr and Keith, 1972) have been correlated with the degree of unsaturation. It has been suggested, however, that these types of changes might have less of an effect in the much more complex and heterogeneous mammalian membrane systems (Eletr and Keith, 1972).

We have observed that the fatty acid composition of Ehrlich ascites tumor cells can be altered appreciably by feeding the tumor-bearing mice diets containing different kinds of fats (Liepkalns and Spector, 1975; Brenneman et al., 1975). This is associated with large changes in the fatty acid composition of the Ehrlich cell plasma membrane (Awad and Spector, 1976). Therefore, it was of interest to determine whether these types of lipid changes in a heterogeneous mammalian system like the Ehrlich cell membrane would produce the same kinds of physical effects as in phospholipid vesicles and bacteria. Very large quantities of Ehrlich cells can be grown routinely, making the preparation of enough plasma membrane for electron spin resonance (ESR)[1] measurements feasible.

ESR of nitroxide free radical probes has been used extensively in physical studies of membranes (Raison et al., 1971; Seelig, 1970; Hubbell and McConnell, 1971; Eletr and Keith, 1972). Fatty acids labeled with a nitroxide group at various positions along the hydrocarbon chain can be added in vitro to membrane preparations (Hubbell and McConnell, 1971), and the ESR spectra of these probes can provide conformational and dynamic information about the physical state of the membrane lipids. These spectra may be interpreted in terms of relative molecular motion and the order of the environment in the region of the label, and they can be used to infer the existence of temperature-dependent phase separations. In the present work, we have modified the degree of lipid unsaturation in Ehrlich cell plasma membranes and utilized ESR spectra

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[1] Abbreviations used are: ESR, electron spin resonance; PM_{RC}, plasma membrane fraction from cells grown in mice fed regular mouse chow; PM_{FD}, plasma membrane fraction prepared from cells grown in mice fed a fat-deficient mouse chow; 12NS, 12-nitroxide stearic acid; S, order parameter; A^n, electron spin resonance nitrogen nuclear hyperfine splitting constant; Tris, tris(hydroxymethyl)aminomethane; Tricine, *N*-tris(hydroxymethyl)methylglycine; P_i, inorganic phosphate; TLC, thin-layer chromatography.