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Circadian Rhythms in *Neurospora crassa*: Effects of Saturated Fatty Acids

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To assess their effects on the conidiation rhythm in *Neurospora*, 14 saturated fatty acids from 6 to 24 carbons long were used to supplement the *bd csp* and *bd csp cel* strains. Both strains express a circadian spore-forming rhythm when grown on solid media; the *cel* mutation confers a partial fatty acid requirement. Fatty acid supplements from 8 to 13 carbons long lengthened the free-running period of *bd csp cel* compared with the control value of 21 h; the maximal effect (33 h) was obtained with nonanoic acid (9:0) at a concentration of 5×10^{-4} M. In contrast, the period of *bd csp* remained unchanged under all experimental conditions. The short-chain fatty acids (<14 carbons) reduced the rate of advance of the growth front in both strains, compared with unsupplemented controls. However, this inhibition did not appear to be responsible for the lengthened periods in *bd csp cel*. Nor was direct incorporation of the short-chain (period-lengthening) fatty acids into mycelial total lipids responsible, since such incorporation was not observed. In fact, extensive metabolic conversion of these supplements by both strains was indicated by the disappearance of short-chain fatty acids from the agar media coupled with their absence in mycelial lipids, and by the liberation of $^{14}CO_2$ from cultures supplemented with [1-^{14}C]lauric acid (12:0).

The *cel* mutant of *Neurospora crassa* is deficient in the synthesis of 16:0 due to a defective fatty acid synthetase complex (8). (Fatty acids will be designated by the standard x:y abbreviation where x = the number of carbons and y = the number of double bonds.) In liquid medium, Henry and Keith (9) found that 10^{-3} M concentrations of 12:0, 14:0, 16:0, 17:0, and 18:0 all restored some growth to a 2-day culture of *cel* as measured by mycelial dry weight. The best supplement, 16:0, restored growth to essentially that of wild type. 12:0 was the least effective as a growth promoter, which may be explained by the inhibitory effects of short-chain fatty acids. Thus, wild-type cultures were almost completely inhibited by 10^{-3} M 6:0, 8:0, or 10:0, and the *cel* strain was inhibited by the short-chain supplements even when 16:0 was present. In this study we examine the effects of 10 even-carbon fatty acids from 6:0 to 24:0 plus 4 odd-carbon fatty acids, when supplied in solid agar medium, on auxotrophic (supplement-requiring, *cel*) and prototrophic (supplement-independent, *cel*$^+$) strains. In contrast to the situation in liquid media, where little growth is observed in the absence of supplemental fatty acid (9), *cel* is able to sustain sparse growth on unsupplemented agar media at a substantial rate, as measured by the advance of the growth front.

Incorporation of the band (*bd*) mutation (20) permits the concomitant observation of the circadian rhythm of the culture. *bd* expresses its circadian "clock" by alternating phases of conidiation ("band") with non-conidiation ("interband"). As is characteristic of circadian rhythms (19) this oscillation in spore formation displays, under constant environmental conditions, a period which is not exactly 24 h long and is relatively insensitive to a wide range of growth temperatures, and a phase which is sensitive to illumination (19, 21). In view of recent speculation on the role of membranes in circadian rhythmicity (14, 23) we supposed that alterations in the *Neurospora* fatty acid profile could lead to changes in the *Neurospora* clock, and that such alterations might be obtained by supplementing a *cel* mutant with fatty acids of varying chain length.

MATERIALS AND METHODS

Strains. The construction of the mutants *bd csp* and *bd csp cel* has been described previously (5). The technically useful marker *csp-1* (conidial separation) was included to keep the spores attached to the mycelial mat (22). Bench cultures were maintained at room temperature in 6-ml slants containing Vogel "50-X" minimal medium (24), 0.5% maltose, 0.01% arginine (20), and 2% agar (Gibco); 2 drops of 10% Tween 40 (Sigma) in ethanol was added to slants of *bd csp cel*.

Chemicals. Fatty acids were purchased from Sigma; Tween 60 was from Atlas, and 1-^{14}C-labeled 12:0 was from Calatomic. Fatty acids were dispensed to the supplemented cultures as 0.05 (22:0 and 24:0) or 0.1 M ethanol solutions.

The commercial surfactant Tween 60 is a mixture of polyethoxylated sorbitol anhydrides that have been monoesterified with 18:0. To prepare Tweens esterified exclusively with other fatty acids, prior removal of the 18:0 group was required. Methanolysis of the ester linkage of Tween 60 in refluxing CH_3OH with 1% H_2SO_4 was followed by concentration and partition between ligroin and water to remove methyl stearate. Extraction of the aqueous phase with CH_2Cl_2 led to successively lighter-colored fractions. The initial amber fractions, after drying over $MgSO_4$ and flash evaporation, gave a syrupy product (Tween 0) contaminated with a few percent of aliphatic protons as determined by nuclear magnetic resonance (Varian EM 390). Subsequent light-yellow and clear fractions were free of fatty acid as determined by nuclear magnetic resonance; from 200 g of Tween 60 were obtained 90 g of fatty-acid-free Tween 0. The molecular weight of the Tween 0 was estimated to be about 740 by comparing the nuclear magnetic resonance areas of OCH_2 protons to (i) 18:0 protons in Tween 60, (ii) hydroxy protons in Tween 0, and (iii) methoxy protons in the carbomethoxy derivative of Tween 0 prepared with methyl chloroformate and Et_3N in CH_2Cl_2.

Tween-17:0 and Tween-20:0 were prepared in a Dean-Stark apparatus by esterifying Tween 0 with 17:0 or 20:0 in refluxing benzene-H_2SO_4. The product mixtures were swirled with solid $NaHCO_3$ and partitioned between ligroin and water. After several water extractions, the aqueous layers were extracted repeatedly with CH_2Cl_2, which was then dried over $MgSO_4$ and flash evaporated to give a 62% yield of Tween-17:0 and a 30% yield of Tween-20:0. Ratios of fatty ester to ethoxylated sorbitan were determined by nuclear magnetic resonance so that ethanol solutions of appropriate fatty acyl group concentration could be prepared for subsequent supplementation.

Culture conditions. Petri dishes (15 cm) containing about 45 ml of bench culture medium plus fatty acid supplements were inoculated at the edge of the agar with small balls of conidia. Dishes to be harvested were overlaid before inoculation with sheets of dialysis tubing (4) which had been perforated over their entire area with holes about 1.5 mm apart. Perforation was performed with an unthreaded sewing machine. The use of dialysis tubing assured the absence of agar in harvested samples and facilitated collection of the sample; perforation facilitated the use of the fatty acid supplements by the cultures. Inoculated plates were synchronized by standing in light overnight (5), followed by transfer into a dark 22°C incubator. Experiments performed at other incubation temperatures will be reported at a later time. Daily observations of the growth front were made in the presence of a red safe-light. A few long-term cultures were grown in 23- to 32-cm-long "race" tubes (18) containing media with 3% agar, as described in the 5-day-effect section of Results.

Calculations. The calculation of period length has been previously described (5). Briefly, superposition of banding phase positions on growth rate plots allowed the determination of the period between each band. These periods were collected from among the several dishes in each experimental group, and averaged to obtain the mean period and standard error. Individual values lying more than 2 standard deviations from the mean period (typically 10% or fewer of the values) were discarded, and a new mean was determined.

Analysis of fatty acids. Agar samples equal to 1% of the dish area were removed and dried overnight under a stream of air before the addition of 1.5 ml of extraction solvent (6) (methanol-benzene-H_2SO_4 [20:10:1] with 0.006% butylated hydroxytoluene). Samples of *Neurospora* were removed by scraping 1% of the dialysis tubing overlay area, typically by using a banding area 1 day behind the growing front, from a culture 3 to 5 days old. This material was immediately placed in the extraction solvent. Methyl esterification (of free fatty acids) and transesterification (of *Neurospora* total lipid fatty acyl groups) was accomplished by heating the samples in dichromate-cleaned, Teflon-liner-capped vials at 65°C for 3 to 5 h. After cooling, the samples were partitioned between 1.5 ml of saturated NaCl and 1 ml of distilled hexanes. The organic layers were dried over $MgSO_4$ and the aqueous layers were re-extracted with 0.5 ml of hexanes, which were then dried and added to the first extracts. Samples containing fatty esters shorter than 16 carbons were analyzed without concentration to avoid loss of the volatile methyl esters; control experiments showed that adequate recoveries were obtained with these procedures. Samples with longer fatty esters were concentrated under a stream of air and dissolved in about 0.3 ml of CS_2 for analysis. A known quantity of the methyl esters of either 17:0 or 20:0 in pyridine was added to each vial as an internal standard. Samples were analyzed on a Varian Aerograph 1200 gas chromatograph with flame ionization detector, using a Supelco 3% SP-2300 column (6 feet by $\frac{1}{8}$ inch [ca. 1.8 m by 0.3 cm]) at 190°C for samples without short-chain fatty esters and with temperature programming from 120 to 190°C for samples with short-chain esters. Peak areas were estimated by height times the width at half-height; relative moles of sample fatty esters were calculated from the standards by assuming the proportionality of detector response to the number of non-carboxyl carbons (1).

Radioactive tracer. Glass petri dish bottoms (9 cm) were prepared with agar medium containing 5×10^{-4} M 12:0 (32 nCi/ml of 1-^{14}C-labeled 12:0). They were overlaid with perforated dialysis tubing and placed inside 15-cm glass petri dishes. When the inoculated plates were placed into the dark, 8 ml of 2 N KOH solution was added to the outer petri dish as a trap for liberated CO_2 (2). Agar samples were removed before and after mycelial overgrowth. After growth reached the dish edge (5 to 7 days), *Neurospora* was harvested from the middle 25% of each dish, and the aqueous solutions were removed and diluted to 10 ml with rinsings. Agar samples were briefly heated with 0.5 ml of 30% H_2O_2 before the addition of 10 ml of scintillation cocktail (2) (20:6:1 Triton-X 100-toluene-liquifluor). Harvested *Neurospora* was esterified as described above, and the radioactivity in the resulting organic and aqueous layers and mycelial residue was

determined separately (Beckman LS-230 counter). Tris buffer (1 ml, pH 8) was added to KOH samples before the addition of the cocktail.

RESULTS

Growth and period effects. The effects of the 10 even-carbon saturated fatty acids and 4 odd-carbon acids on the prototroph *bd csp* are shown in Table 1 at two concentrations of supplement. Short-chain supplements, especially 8:0 to 13:0, were growth inhibitory compared with controls supplemented with ethanol only. The strongest inhibitor, 11:0, decreased the growth rate to 43% of the control value, at the higher concentration. Similar inhibitory profiles for the saturated fatty acids have been reported with other fungi; for example, intermediate-length fatty acids were the strongest fatty acid inhibitors of growth of *Cunninghamella* at pH 7.2 (13), caused maximal leakage of UV-absorbing materials from cultures of *Boletus* (15), and inhibited oxygen uptake in cultures of *Boletus* (15) and *Penicillium* (11) at low pH. The periods for *bd csp* were circadian in all cases. The average value was 21.2 h per period with an average standard error of 0.87.

Growth rate and period data for the auxotroph *bd csp cel* are also shown in Table 1. Supplementation with 16:0 increased the growth rate to a value near that of the unsupplemented prototroph. Longer fatty acids and 14:0 had no effect or were somewhat inhibitory. The short-chain supplements (<14 carbons) were markedly inhibitory. The strongest inhibitor, 10:0, diminished the growth rate to 49% of the control value. At a higher concentration of supplement (10^{-3} M), the dose-response trends of inhibition or stimulation were maintained (data not shown). A comparison of the inhibitory effects of these fatty acids on both the prototroph and auxotroph is shown in Fig. 1.

For *bd csp cel*, supplements from 8:0 to 13:0 lengthened the circadian period to values as high as 33 h per period (Table 1). This lengthening was not substantially increased by twofold-higher concentrations of supplement (data not shown). The ratio of the auxotrophic period to the circadian prototrophic period for each fatty acid supplement is shown in Fig. 2.

The period-lengthening effect of 12:0 could be reversed by simultaneous supplementation with 16:0 at twice the concentration of 12:0, in a manner similar to the "titration" of the 18:2 effect with 16:0 observed earlier (5). In addition, cultures supplemented with 12:0 could be entrained to 24-h periodicity with a light-dark reg-

TABLE 1. *Growth rate of bd csp and growth rate and period of bd csp cel supplemented with saturated fatty acid*[a]

Supplement	2.5×10^{-4} M concn					5×10^{-4} M concn				
	bd csp growth rate (mm/h)	*bd csp cel*				*bd csp* growth rate (mm/h)	*bd csp cel*			
		Growth rate (mm/h)	Period (h)	SE[b]	n[b]		Growth rate (mm/h)	Period (h)	SE[b]	n[b]
None	1.42	1.05	20.1	0.6	12					
Ethanol only	1.39	0.99	19.4	0.8	12	1.50	0.94	21.1	0.8	9
6:0	1.41	0.95	18.9	0.4	27	1.36	0.70	20.3	0.4	38
8:0	1.12	0.65	23.1	0.9	35	0.84	0.56	26.0	1.4	44
9:0	1.04	0.52	22.8	1.2	34	0.86	0.50	33.2[c]	1.2	34
10:0	0.91	0.61	28.0[c]	1.7	34	0.70	0.46	30.8[c]	1.7	31
11:0	0.87	0.68	23.2	1.2	30	0.65	0.53	30.1[c]	1.3	36
12:0	0.81	0.60	26.7[c]	1.1	35	0.70	0.50	28.0[c]	1.1	37
13:0	0.85	0.66	26.1[c]	1.2	30	0.66	0.48	24.4	1.2	28
14:0	1.41	0.77	21.2	0.4	24	1.35	1.00	20.5	0.6	24
16:0	1.53	1.17	22.4	1.1	16	1.54	1.22	22.2	0.8	10
17:0	1.52	0.95	20.1	0.7	20	1.60	1.05	19.4	0.5	18
18:0	1.53	0.88	22.4	1.3	20	1.58	0.97	21.1	0.8	19
20:0	1.53	0.91	19.8	0.6	23	1.56	0.86	21.5	1.4	16
22:0	1.57	0.90	21.3	1.2	16	1.57	0.79	20.2	0.5	19
24:0	1.56	0.95	20.2	1.1	19	1.52	1.00	22.0	0.9	20
Tween-17:0	—[d]	—	—	—	—	1.20	0.84	20.8	0.9	19
Tween-20:0	—	—	—	—	—	1.30	0.81	20.2	0.4	15

[a] The average period of *bd csp* cultures was 21.2 h (range, ±1.3 h). Period values include data both before and after 5 days of growth.

[b] SE, standard error. n, Number of determinations of the period.

[c] Significantly different from ethanol-only controls at the 99% confidence level (Student's t-test).

[d] —, Not determined.

imen of 6 h-18 h, again similar to results obtained with 18:2 (5). However, in contrast to the 18:2 experiments, more 12:0 (5×10^{-4} M) was required than 18:2 (1.3×10^{-4} M) to obtain the maximal period lengthening, and even at the maximum, 12:0 was less effective (32 h per period, Table 2) than 18:2 (40 h per period).

Five-day effect. Inspection of the *bd csp cel* cultures displaying altered periods revealed that the long periods characteristically appeared only after about 5 days of growth, even though growth rates were constant throughout the experiment. This 5-day effect is shown in Table 2, where periods occurring before and after 5 days of culture time are presented separately. The longest values approach 35 h per period when the earlier circadian periods are not included. Control data showing periods of normal length at culture times greater than 5 days are usually unattainable in petri dishes. Growth of *bd csp cel* on race tubes, which allowed longer observation times, indicated that unsupplemented and 16:0-supplemented cultures had 21-h periodicity before and after 5 days, whereas 12:0-supplemented cultures again showed long periods after 5 days. In long-period cultures of *bd csp cel* supplemented with 18:2, early 21-h periods were not observed (5). However, since no banding is displayed before about 4 days of growth, the question of early circadian periods cannot be addressed in those cultures.

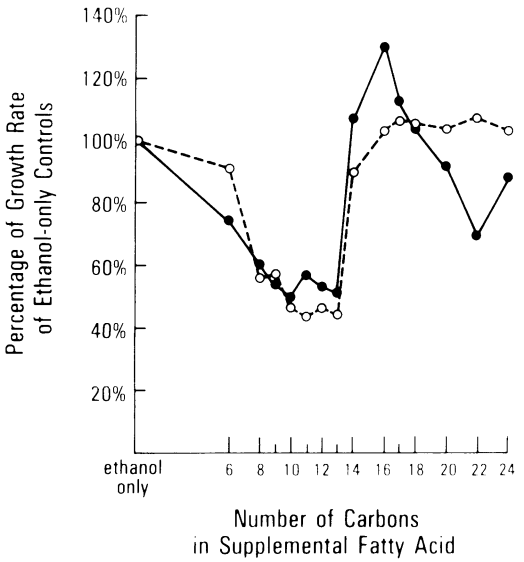

FIG. 1. *Percentage of control growth rates in cultures supplemented with* 5×10^{-4} *M saturated fatty acids. Symbols:* ○, *bd csp;* ●, *bd csp cel.*

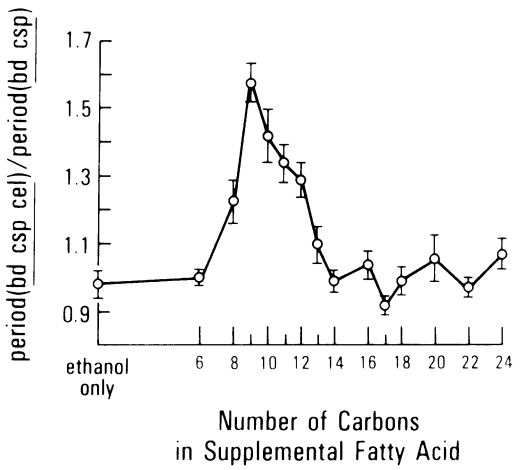

FIG. 2. *Period-lengthening effect of* 5×10^{-4} *M short-chain fatty acids on the fatty-acid auxotroph bd csp cel. Ratio of the period of the auxotroph to the period of the prototroph vs. chain length of the supplement. Error bars represent the standard error in the period of bd csp cel.*

TABLE 2. *bd csp cel supplemented with short-chain fatty acids: periods calculated before and after 5 days of growth*

Supplement	2.5×10^{-4} M				5×10^{-4} M			
	Before 5 days		After 5 days		Before 5 days		After 5 days	
	Period (h)	SE[a]	Period (h)	SE[a]	Period (h)	SE[a]	Period (h)	SE[a]
Ethanol only	20.7	0.9	—[b]	—	21.5	1.1	—[b]	—
8:0	21.8	1.4	22.8	1.0	21.2	1.2	29.1[c]	1.9
9:0	18.7	0.8	26.3[c]	1.9	26.6	2.2	35.5[c]	1.1
10:0	21.2	1.3	34.9[c]	2.0	24.8	2.3	33.1	2.0
11:0	19.6	0.6	29.1[c]	2.4	21.7	1.4	32.5[c]	1.2
12:0	22.4	0.6	34.1[c]	1.7	21.4	0.7	32.4[c]	0.9
13:0	21.5	0.5	33.5[c]	1.6	22.3	0.9	26.5	1.9

[a] SE, standard error.
[b] Not determined since culture exceeds growth area in 5 days.
[c] Significantly different from the before-5-day period at the 99% confidence level.

Utilization of supplements. The fate of supplemental fatty acids is shown in Table 3 for *bd csp* and *bd csp cel*. Extraction of fatty acids from agar before overgrowth of *Neurospora* gave the initial amounts of supplement, which might or might not be removed by the mycelia as the culture advanced. These values varied according to the particular supplement concentration in the portion of agar sampled, as well as the agar thickness. Tests for precision of these values indicated that they were no better than ±20%, and thus should be considered only estimates of the fate of the supplements. Both *bd csp* and *bd csp cel* depleted the media of short-chain supplements. The long-chain supplements (17:0, 20:0, 22:0, and 24:0) showed no substantial depletion. The status of 16:0 and 18:0 is less clear, as hyphae extending into the agar contribute endogenous 16:0 and 18:0 to these values.

Only traces of two of the unnatural fatty acid supplements were observed in the harvested *Neurospora* of either strain, namely 1 to 2% of the exogenous 17:0 and less than 1% of 20:0. In the prototroph, the incorporated 17:0 represented less than 1% of the fatty acids observed in the total lipids; in the auxotroph, about 4%. In lipids from 17:0-supplemented cultures, a fatty ester which may be tentatively identified on the basis of its retention time as 17:1 appears in quantities somewhat less than those of 17:0, implying some additional incorporation of exogenous 17:0 beyond the values given. Even so, the percentages of 17:0 incorporated into the *Neurospora* fatty acids in this study are substantially lower than the incorporation obtained in liquid cultures (9).

In an attempt to increase the amounts of 17:0 and 20:0 incorporated into *Neurospora*, these supplements were supplied as their Tween esters. Supplemental Tween esters of phytanic acid have been shown to replace some of the natural fatty acid in shaker cultures of *Neurospora* (3). Table 3 shows that Tween-17:0 enhances the incorporation of 17:0; *bd csp* incorporates 16% of the supplement, and *bd csp cel* incorporates 8%. The incorporated 17:0 represents 4% of the prototroph's fatty acids, whereas the corresponding value for the auxotroph is 7%. The effect for Tween-20:0 is much smaller. Growth rate and period data for these Tweens at 5×10^{-4} M (Table 1) show that, in spite of the increased incorporation of 17:0, no change in period occurs.

Radiolabeling. The experiment with ^{14}C-labeled 12:0 as a supplement supports the agar-extraction data of Table 3. Portions of agar removed before overgrowth of *Neurospora* gave the initial values shown in Table 4. After overgrowth, the amount of label left in the agar probably represented 12:0 converted into hyphal constituents, since only 2% of the exogenous 12:0 was present in fatty acid assays (Table 3). The culture incorporated 5 to 16% of the label, but only 2 to 5% was in the fatty acid fraction, with

TABLE 3. *Utilization of supplemental fatty acids by Neurospora*

Supplement (5×10^{-4} M)	*bd csp*				*bd csp cel*			
	Supplement in agar[a] (nmol)		Supplement in *Neurospora*[b] (nmol)	Sum (nmol)[c]	Supplement in agar[a] (nmol)		Supplement in *Neurospora*[b] (nmol)	Sum[c] (nmol)
	Before growth	After growth			Before growth	After growth		
10:0					82	<6	0	34
11:0	105	0[d]	0	534	95	0	0	13
12:0	119	3	0	509	271	<4	0	36
13:0	158	22	0	294	206	<2	<1	47
14:0	188	56	0	481	170	14	0	17
16:0	144	144[e]	66[e]	342	197	130[e]	3[e]	13
17:0	144	175	2	346	210	144	1	22
18:0	165	147[e]	12[e]	340	148	135[e]	3[e]	31
20:0	152	178	0.4	551	165	166	1	17
22:0	110	144	0	634	146	158	0	14
24:0	166	225	0	565	134	124	0	142
Tween-17:0	99	80	16	372	116	92	8	121
Tween-20:0	161	154	2	472	163	167	1	70

[a] Representing 1% of agar volume.
[b] Harvested from 1% of dish area.
[c] Sum of all fatty acids in harvest sample.
[d] Detection limit, ca. 0.5 nmol.
[e] These fatty acids occur naturally in *Neurospora*.

TABLE 4. *Removal of radiolabeled 12:0 from agar by Neurospora*

Strain	Initial radioactivity in agar (cpm/dish)	Final radioactivity (%) in:				
		Agar	Harvested *Neurospora*			KOH solution
			Hexane fraction	Aqueous fraction	Residue	
bd csp	5.2×10^5	11	2	2	1	62
bd csp cel	1.0×10^6	16	5	4	7	50

the remaining 3 to 11% being in the aqueous wash and solid residue. The majority of the label was metabolized, released as $^{14}CO_2$, and trapped in the KOH solution surrounding the dish.

DISCUSSION

Supplementation of the *bd csp cel* strain with short-chain saturated fatty acids results in a considerable lengthening of the circadian period (Fig. 2). The growth-inhibitory properties of the fatty acids do not appear to be responsible for the long periods since (i) cultures of *bd csp cel* supplemented with 18:2 and exhibiting long periods do not have a greatly lowered growth rate (2), and (ii) cultures of *bd csp* inhibited by short-chain fatty acids demonstrate normal periodicity. These two points indicate that the inhibitory effects of the fatty acids can be separated from the period effects. Long periods are not merely the result of culture times lasting over 5 days, since (i) race tube experiments show that control cultures of *bd csp cel* have normal (circadian) periods after 5 days, and (ii) growth-inhibited *bd csp* cultures give normal periods after 5 days in petri dishes. The possibility that the long periods observed in this study are in fact the result of the observation of every second oscillation of a normal underlying circadian rhythm is unlikely for two reasons: (i) the observed period in such a case would be expected to be twice the normal period, or about 42 h per period. Instead, the values after 5 days were 30 to 35 h per period; and (ii) both the band and interband regions were expanded in the long-period cultures, as evidenced by only a small change in the percentage of culture time spent in band phase (data not shown). Thus, the short-chain fatty acids appear to be specifically and nontrivially altering the *Neurospora* clock.

The short-chain fatty acid supplements not only yield long periods (Table 1); they are, in general, also the same supplements that are removed from the agar by *Neurospora* (Table 3). The only exception is 14:0, on the borderline between the short and normal-chain supplements, which is partially removed from the agar but does not affect the period. It would seem reasonable to suppose, therefore, that short-chain supplements are significantly incorporated as such into the organism's lipids, and that a resulting change in membrane fluidity (7) might have an effect on the kinetics of the clock (14). This scheme does not appear to be correct, however, since supplements which yield long periods were not detected in the *Neurospora* total lipids (phospholipids and neutral lipids) (Table 3). In fact, the only unnatural supplements whose appearance in the total lipids could be demonstrated were 17:0 and 20:0, two supplements which do not appreciably alter growth rate or periodicity. Substantial changes in overall membrane composition via direct incorporation of the short-chain supplements cannot, therefore, be responsible for the observed period effects. This is in contrast to earlier experiments using supplemental 18:2, where direct changes in internal 18:2 levels remain a possible mechanism for period lengthening (5).

The disappearance of the short-chain acids from the agar beneath growing mycelia, as well as the loss of the carboxy carbon as CO_2 from radiolabeled 12:0, indicate that the short-chain acids are extensively metabolized by *Neurospora*, presumably via β-oxidation. An acetate-requiring mutant of *Neurospora* has been shown (12) to be capable of obtaining acetate through the β-oxidation of the even-carbon fatty acids from 4:0 to 14:0, but not of 16:0 or 18:0. This is consistent with the supplement-utilization data in Table 3, which shows removal from the agar of fatty acids with 14 or fewer carbons. In addition, a *Penicillium* strain has been shown to produce β-oxidation products only from short- and medium-length fatty acids. (11)

Among the remaining possible fates for exogenous fatty acids are their incorporation or localization as free fatty acids, fatty acyl coenzyme A's, or acylated lipid into some lipid or enzyme compartment at levels that are too small (<1%) for detection with our present methods. Another alternative is their conversion to other fatty acids through elongation and/or desaturation. For example, one may speculate that metabolic conversion of the short-chain supplements ultimately results in the increase of the level of a naturally-occurring unsaturated fatty acid, which can itself lengthen periods when presented as a supplement (5). Although presently lacking for *Neurospora*, evidence for the conversion of some short-chain fatty acids directly to

18:3, another naturally occurring fatty acid which lengthens the *bd csp cel* period (5), exists for spinach (10) and *Penicillium* (16). In these systems, 12:0 is presumably desaturated to 12:3, and then elongated to 18:3. Such a conversion would explain the potency of 12:0 and its potential precursors 10:0 and 8:0, as well as the impotency of 14:0, in altering the *Neurospora* clock. However, the period-lengthening effects of the odd-carbon short-chain acids, especially 13:0, are more difficult to reconcile with a simple elongation-desaturation scheme.

Further investigation of this scheme will require determination of the effects of short-chain fatty acid supplements on unsaturated fatty acid levels in our system. However, because these levels undergo substantial circadian changes in *Neurospora* (17), reliable comparisons of fatty acid levels must await the detailed determination of fatty acid oscillations under various supplemented conditions.

The *bd csp* and *bd csp cel* strains, when grown at 22°C, exhibit circadian rhythms which are similar with respect to period, phase response curve to light, and light-dark entrainment (unpublished data). These studies show that *bd csp* and *bd csp cel* are similar in several other respects as well. When saturated fatty acids are supplied exogenously in solid media, the short-chain acids inhibit the growth rate of both strains to a comparable percentage of their control rates. Further, the disappearance of these supplements from the agar underneath both strains, and the release of considerable label from 12:0 tracer as CO_2, indicate that both strains are capable of incorporating the supplements, activating them to fatty acyl coenzyme A's, and transferring them to the mitochondria, where both strains are competent in the β-oxidation of the supplements. However, the two strains do differ in that short-chain fatty acid supplements slow the circadian clock in *bd csp cel* while leaving the clock in *bd csp* unaffected. It therefore appears that the *cel* mutation renders the *Neurospora* clock sensitive either to trace accumulation of exogenous short-chain fatty acids or to the normal metabolism of these supplements.

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