Biochimica et Biophysica Acta 898 (1987) 23–36
Elsevier

BBA 73411

Circadian rhythms in *Neurospora crassa*: membrane composition of a mutant defective in temperature compensation

Gary G. Coté * and Stuart Brody

Department of Biology, C-016, University of California, San Diego, La Jolla, CA 92093 (U.S.A.)

(Received 16 July 1986)
(Revised manuscript received 5 November 1986)

Key words: Circadian rhythm; Clock mutant; Fatty acid composition; Membrane phospholipid; Temperature compensation; (*N. crassa*)

The *cel* mutant of *Neurospora*, partially blocked in fatty acid synthesis and lacking temperature compensation of its circadian rhythm below 22°C, had a phospholipid fatty acid composition in liquid shaker culture distinctly different from that of a *cel*$^+$ control strain. During growth, *cel*$^+$ exhibited a reproducible increase in its linoleic acid level from about 32 to a plateau at 63 mol%, and a corresponding decrease in its linolenic acid level from about 40 to a plateau at 10 mol%. The level of palmitic acid was constant at 19 mol%. In the *cel* strain, the linoleic acid level was constant at 54 mol% while the palmitic acid level increased from about 12 to about 23 mol%. Supplementation with palmitic or linoleic acids altered the patterns of fatty acid composition of *cel*, but did not affect the pattern of *cel*$^+$. Altered fatty acid composition cosegregated with the *cel* marker. The mitochondrial phospholipids of *cel* in liquid culture also had abnormal fatty acid composition, as did the whole mycelial phospholipids on solid medium. These results are consistent with the involvement of membrane homeostasis in the temperature compensation of circadian rhythms.

Introduction

Many, if not all, eukaryotic organisms exhibit circadian rhythms, that is, diurnal physiological or biochemical oscillations which are apparently endogenous since they persist under constant conditions. An important property of circadian rhythms is that the period of the rhythm is nearly the same at different temperatures, a property known as 'temperature compensation'. This term was chosen to emphasize that rhythmicity is not independent of temperature since temperature shifts and pulses can change the phase of the rhythm, but that the effect of temperature on the period of the rhythmic system was somehow compensated for biochemically [1].

Although unusual, temperature compensation is not unique to circadian rhythmicity. For example, many organisms, particularly those of intertidal habitat show temperature compensation of their metabolism (for review see Ref. 2).

Several theories have been proposed to explain temperature compensation of circadian rhythms. Hastings and Sweeney [1] have speculated that the rate of the rhythm has normal temperature dependence but that this is compensated for by the action of an inhibitor of the rhythm, the production of which has similar temperature dependence. Ehret and Trucco [3] have suggested that circadian rhythmicity might be temperature insensitive if the rate-limiting step were aqueous diffusion. Pavlidis

* Present address: Department of Molecular and Cell Biology, U-125, University of Connecticut, Storrs, CT 06268, U.S.A. Correspondence: S. Brody, Department of Biology, C-016, University of California, San Diego, La Jolla, CA 92093, U.S.A.

and Kauzmann [4] have proposed a temperature-dependent reduction in the levels of the enzymes involved in rhythmicity to compensate for their increased activity at higher temperature.

A number of workers have proposed that cellular membranes are involved in circadian rhythmicity [5–8], and Njus, Sulzman and Hastings [5] have further proposed that the rhythm-generating system may be protected from temperature changes by alterations in membrane lipid composition in response to temperature changes. Such temperature-dependent changes in membrane lipid composition have been documented in bacteria, protists, fungi, plants, and animals (for review see Ref. 9), and have been studied in *Neurospora* by Martin et al. [10–12], and by Vokt and Brody [13].

The *cel* (chain elongation) mutant of *Neurospora crassa* [14] has a defect in fatty acid synthesis resulting from levels of bound 4′-phosphopantetheine cofactor on the fatty acid synthetase molecule which are less than 2% of the wild-type levels [15]. To obtain maximal growth of the mutant, supplemental saturated fatty acids must be added to the medium [14]. This mutant has lost temperature compensation below 22°C [16], and certain supplemental fatty acids, when added to the growth medium, alter the temperature at which temperature compensation is lost [16]. Since loss of temperature compensation is associated with a defect in fatty acid synthesis it seemed worthwhile to test the possibility that the *cel* strain is unable to maintain optimal membrane fatty acid composition at various temperatures, resulting in temperature-dependent changes in membrane properties. If the rhythm-generating system is, in fact, wholly or partly membrane-localized this would explain the loss of temperature compensation in this mutant.

As a preliminary test of this possibility the fatty acid composition of the membrane phospholipids of the *cel* mutant was compared to that of the control (cel^+) strain. The effects of supplemental fatty acids on membrane composition in these two strains were also examined. We here report that the membrane fatty acid composition of the *cel* strain is indeed different from that of the cel^+ strain, and that supplemental fatty acids strongly affect the composition of the *cel* strain while having no effect on that of the cel^+ strain.

Methods

Strains

All strains used in this study contained the *bd* (band) mutation [17] which makes conidiation (formation of asexual spores) relatively insensitive to carbon dioxide and so allows a clear rhythm of conidiation to be visible on closed petri plates. The underlying circadian oscillator is apparently not affected by this mutation [18]. Strains grown on petri plates also contained the *csp-1* (conidial separation) mutation [19] which reduces contamination through self-inoculation during manipulation of the plates. The *bd, csp-1,* and *cel* strains were obtained from the Fungal Genetics Stock Center (Department of Microbiology, University of Kansas Medical School, Kansas City, KS). Standard crossing techniques [20] were used to create the *bd cel, bd csp-1,* and *bd csp-1 cel* strains.

Stock cultures on Vogel's minimal medium [21] plus glucose and agar were kept at 4°C. Medium for stock cultures of *cel* strains also contained 0.04% (v/v) Tween-40, a source of 16 : 0. From these stock cultures, fresh cultures on the same medium were prepared and allowed to grow for seven to 14 days at room temperature before being used for inoculation in an experiment.

Chemicals

The agar was Difco Bacto-Agar (Difco Laboratories, Detroit, MI). HPLC grade solvents were obtained from Fisher Scientific, Fair Lawn, NJ. Tris base, butylated hydroxytoluene, 16 : 0, 18 : 2, and phenylmethylsulfonyl fluoride, were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were commercial reagent grade.

Measurement of growth rate and period on plates

The medium used was as described [22]. Supplemental fatty acids, when used, were added to autoclaved medium as 1% (w/v) ethanolic solutions to a final concentration of $4 \cdot 10^{-3}$% (w/v) fatty acid (156 μM 16 : 0 or 143 μM 18 : 2).

Linear growth rate on plates and the period of the conidiation rhythm were measured as described [23]. Calculations were performed on an Apple IIe computer with an Apple Graphics Tablet

using a program developed by Robin Ralls and one of us (G.G.C.).

Growth in liquid culture

The medium was Vogel's minimal medium [21] plus 2% glucose. Supplemental fatty acids, when used, were added as described above. The final fatty acid concentration was $4 \cdot 10^{-3}$% (w/v) (156 μM) 16:0, but only $1 \cdot 10^{-3}$% (36 μM) 18:2 as the *cel* strain would not grow in liquid culture at the higher 18:2 concentration. Cultures of the *cel* strain often did not grow even at the low concentration of 18:2. On the possibility that germination might be inhibited by 18:2 some cultures were also grown for fatty acid analysis in which the 18:2 was added 24 h after inoculation.

Conidial suspensions were prepared by adding 5 to 7 ml of sterile water to freshly grown slants of *Neurospora,* vortexing, and filtering through sterile glass wool. The concentration of conidia in the suspensions was determined with a hemacytometer. The suspensions were diluted with growth medium and 1-ml aliquots of the dilution were used to inoculate 125-ml Erlenmeyer flasks containing 50 ml of growth medium. Enough conidia were inoculated to give 10^5 conidia/ml. The cultures were allowed to grow in a water bath shaker at 25°C in constant light.

To determine growth as a function of time, cultures were harvested at various times after inoculation by filtration on a Buchner funnel. The harvested mycelia were dried overnight at 60°C and weighed.

Cultures grown for the measurement of fatty acid composition were harvested on a Buchner funnel, pressed between sheets of filter paper to remove excess moisture, diced with a razor blade, and placed in vials containing methanol plus 100 μg/ml butylated hydroxytoluene. Except in the case of cultures with little mass, the harvest from each culture was divided between two vials and the phospholipid fatty acid composition of each fraction was determined separately.

Preparation of mitochondria

Neurospora was grown in liquid culture and harvested as described above. The mycelia were rinsed in the Buchner funnel with ice-cold isolation buffer [24] (0.25 M sucrose, 1 mM disodium EDTA, 10 mM Tris-acetate (pH 7.2), containing 0.5 mM phenylmethylsulfonyl fluoride, a protease inhibitor), pressed to remove excess moisture, and placed immediately on ice. A crude organellar preparation was made as described [25], with a few changes: the buffer described above was used; the first supernate was filtered through six layers of cheese-cloth instead of a glass-fiber filter; and the final pellet was resuspended in 4 ml buffer with several gentle strokes of a homogenizer.

Mitochondria were isolated from the crude preparation by gradient centrifugation. A two-step gradient of 4 ml each of 1.6 M sucrose and 0.8 M sucrose in the buffer described above was prepared in a 15-ml Corex tube, and the crude preparation was layered on top. The gradient was centrifuged at $23\,500 \times g$ for 70 min. The mitochondria, which banded at the interface between the two lower gradient layers, were removed with a Pasteur pipet and transfered to a clean Corex tube. Enough fractionation buffer was added to make 5 ml and a 0.5 ml sample was removed for protein determination by the method of Lowry and co-workers [26]. The washed mitochondria were collected by centrifugation at $20\,000 \times g$ for 20 min. The final pellet was suspended in methanol containing 100 μg/ml butylated hydroxytoluene.

Determination of fatty acid composition of plate cultures

Cultures were grown on solid medium as above with dialysis tubing overlays as described [22]. The dialysis tubing had been pierced over its entire surface with small holes a few millimeters apart. All experiments performed with solid medium used strains carrying the *csp-1* mutation to minimize contamination.

Several plates of each strain, called timer plates, were inoculated exactly 24 h in advance of the rest of the plates. All plates were placed under fluorescent light for exactly 24 h after inoculation to synchronize rhythmicity, and then transferred to a 22°C constant darkness incubator.

As *Neurospora* grew across the plates the circadian rhythm was manifested as a series of bands of conidia-forming areas separated by interband regions of vegetative growth. When a suitable band had appeared on the timer plates the

time of the end of this band was calculated by the same principles used for the calculation of periods [23]. The remaining plates were marked roughly one quarter circadian cycle before this time, i.e., 10 h before the calculated time in the case of *bd csp-1 cel* on 18 : 2 supplement, or 5.25 h before the calculated time otherwise. At the calculated time of band end the plates were again marked and all the mycelia which had grown since the previous mark were collected with a teflon scraper, and placed in a vial containing 5 ml of methanol plus 100 μg/ml butylated hydroxytoluene. Mycelia from several plates were combined for each sample. Several plates were marked but not harvested to demonstrate whether or not the plates had been harvested at the end of the band as intended.

Preparation of phospholipid fatty acids as methyl esters

To reduce contamination in the biochemical procedures described below all glassware was washed with dichromate cleaning solution. Vials used were sealed with teflon-lined caps. In addition, chloroform, methanol, and hexanes were either commercial HPLC grade or were redistilled from commercial analytical reagent grade. Contamination was monitored by running several blank vials through all the procedures concurrently, and the amounts of fatty acids found in the blanks were subtracted from the amounts in each sample. Contaminants were usually less than 10 nmol 16 : 0 and 18 : 2, and much less of the other fatty acids. This constituted about 0.5% of the 16 : 0 and about 0.2% of the 18 : 2 in most samples and about 3% of the 16 : 0 and about 2% of the 18 : 2 in the smallest samples.

To reduce oxidation of double bonds in unsaturated fatty acids, butylated hydroxytoluene, an anti-oxidant, was present in the solvents at all steps in the lipid extraction and methylation procedures. In addition, samples were sealed under nitrogen and stored in the freezer between all manipulations.

Lipids were extracted from mycelia as follows. The mycelia were suspended in 5 ml methanol containing 100 μg/ml butylated hydroxytoluene, and extracted in this solvent at −20°C at least overnight. The solvent was filtered through glass wool, concentrated under a stream of nitrogen to about one milliliter, and mixed with 2 ml chloroform. The mycelia were re-extracted with 4 ml chloroform/methanol (2 : 1, v/v) containing 33.3 μg/ml butylated hydroxytoluene, and then twice with 2 ml of the same solvent, each time for at least one hour at −20°C. These extracts were filtered and combined with the first extract. The combined extracts were twice Folch washed as described [27], dried with a small amount of anhydrous sodium sulfate, and transferred to a clean vial.

The residual material remaining in the vials after extraction was dried at 60°C overnight and weighed to determine residual dry mass.

Tests in which the residues of extraction were subjected directly to acid-catalyzed transesterification demonstrated that even if large samples of mycelia (up to 150 mg residual dry mass) were used, about 97% of the fatty acids were extracted. This number represents a minimum yield of the extraction since some of the residual fatty acids might be bound to macromolecules and not extractable in chloroform/methanol.

Lipids were extracted from mitochondria by the same procedure as from mycelia with minor modifications. Before the methanol extract was removed the suspension was centrifuged at 1000 × *g* for 15 min to collect the fragments of the mitochondrial pellet at the bottom of the tube. Similarly, the first chloroform/methanol extract was also centrifuged, at 7700 × *g* for 15 min. Finally, the third extraction with chloroform/methanol was omitted.

Phospholipids were purified from the total lipid extract by silicic acid chromatography. Columns (5 cm × 5 mm) were prepared by pipetting a methanol slurry of Bio-Sil A (100–200 mesh, Bio-Rad Laboratories, Richmond, CA) into a Pasteur pipet containing a plug of glass wool, and a layer of sand. Each column contained approximately 0.5 g of Bio-Sil. The columns were washed with 10 ml methanol, followed by 10 ml chloroform. The lipid extracts were dissolved in 0.5 ml chloroform and loaded on the columns, the vials were washed with about a half milliliter of chloroform and the washes were also loaded onto the columns.

Neutral lipids were eluted with 10 ml chloroform and discarded; phospholipids were then eluted with 15 ml methanol into a tube containing

0.75 mg butylated hydroxytoluene in 0.5 ml chloroform (to replace the original butylated hydroxytoluene which eluted with the neutral lipids). Tests with standard phospholipids showed that all the major phospholipids of *Neurospora* had cleared the column within the first 11 ml of methanol. The eluate was concentrated under a stream of nitrogen while being warmed in a tub of warm tap water, and then transferred to a fresh vial. The phospholipids were concentrated to dryness under a stream of nitrogen, dissolved in 3 ml of acid methylation solvent [28], and stored overnight at −20°C.

Methyl esters of the phospholipid fatty acids were prepared as described [28] except that incubation at 65°C was for 5 to 7 h rather than 3 to 5 h, and that butylated hydroxytoluene was not added to the methylation solvent since it was already present in the phospholipid preparation.

Gas chromatography

Mixtures of fatty acid methyl esters were analyzed on a Varian Aerograph series 1400 gas chromatograph with a 1/8 inch × 6 feet column of 10% SP-2330 on either 100/120 Supelcoport or 100/120 Chromosorb (Supelco Inc., Bellafonte, PA). Samples were dissolved in 0.3 to 1 ml of carbon disulfide and 0.5 to 1.5 μl were injected into the chromatograph. Duplicate injections of each sample were routinely analyzed. Injector and detector temperatures were 265°C and the column temperature was 210°C or 220°C.

During preparation of the fatty acid methyl esters, a known quantity (50 to 1000 nmol, depending on the amount of starting material) of methyl 17 : 0 was added as an internal standard as described [28]. Quantitation of the observed peaks was performed by a Hewlett-Packard model 3390A recording integrator, which compared the area of each peak to the area of the standard peak. Further calculations on the data were performed on an Apple IIe computer using the Visicorp Flashcalc™ version 1.0 spreadsheet program (© 1984, Nereid Systems Inc.). This program subtracted the fatty acids found in the blanks, calculated the mole fraction of each fatty acid as a per cent of the total, averaged the two injections, and combined the data from the two fractions of cultures which were divided and analyzed separately.

Statistical methods

Standard regression techniques were used to fit lines to the data of fatty acid levels as functions of residual dry mass. Extra sum of squares analyses were performed to determine whether the regression patterns of a given fatty acid in two different strains were significantly different. In this technique the data from both strains are combined and regression calculations are also performed on the combined data. The sum of the squares of the deviations of the data about the two separate regression patterns is subtracted from the sum of the squares of the deviations about the combined regression pattern. This difference is compared to the total variation within the combined data to determine if there is significantly less deviation from the regression patterns in the two-regression model, compared to the deviation in the combined-regression model. We thank Dr. John Rice of the Department of Mathematics of the University of California, San Diego, for explaining this technique to us.

Results

Growth of strains on solid medium

Table I shows linear growth rates and periods of the *bd csp* and *bd csp cel* strains grown on solid medium at 22°C and at 25°C with and without fatty acid supplements. Fatty acids were added as ethanolic solutions and control plates without fatty acid supplement contained ethanol at the same concentration. The period of *bd csp* varied little with supplement or temperature, while that of *bd csp cel* was markedly lengthened with 143 μM 18 : 2 at 22°C, but not with 36 μM 18 : 2 at 25°C. The first set of conditions, 143 μM 18 : 2 at 22°C, was that used for assaying fatty acid composition on solid medium, while the second set, 36 μM 18 : 2 at 25°C, was that used for assaying fatty acid composition in liquid culture.

Growth of strains in liquid culture

In liquid shaker culture with no fatty acid supplement, (Fig. 1A), the *cel* strain grew much more slowly than the control strain and showed much greater flask to flask variability. In addition the *cel* strain appeared to undergo a lag period of about 60 h before significant growth occurred.

TABLE I

GROWTH RATES AND PERIODS OF *bd csp-1* AND *bd csp-1 cel*

The *bd csp-1* and *bd csp-1 cel* strains were grown on solid medium in constant darkness at the temperature shown. Note that 18:2-supplemented media used at the different temperatures had different 18:2 concentrations. The fatty acid supplements were added as ethanolic solutions and unsupplemented control medium contained ethanol at the same concentration. Linear growth rates and periods were determined as described.

Strain	Fatty acid supplement	Growth rate (mm/h)	Period (h)	S.E. [a]	N [b]
At 22°C					
bd csp-1	None	1.4	21.2	0.3	51
	156 μM 16:0	1.5	20.8	0.5	28
	143 μM 18:2	1.0	20.2 [d]	0.2	125
bd csp-1 cel	None	0.9	22.1	0.4	47
	156 μM 16:0	1.1	20.6	1.0	20
	143 μM 18:2	0.6	38.9 [c,d]	0.7	68
At 25°C					
bd csp-1	None	2.0	22.0	0.6	9
	156 μM 16:0	1.9	22.4	0.4	6
	36 μM 18:2	2.0	19.9 [d]	0.6	9
bd csp-1 cel	None	1.5	21.1	0.4	18
	156 μM 16:0	1.6	20.1 [c]	0.5	16
	36 μM 18:2	1.8	20.9	0.8	12

[a] Standard error of the mean period.
[b] Number of periods used to calculate average.
[c] Significantly different ($P < 0.05$) from the value for *bd csp-1* on the same medium at the same temperature (t-test).
[d] Significantly different ($P < 0.05$) from the value for the same strain at the same temperature but without fatty acid supplement.

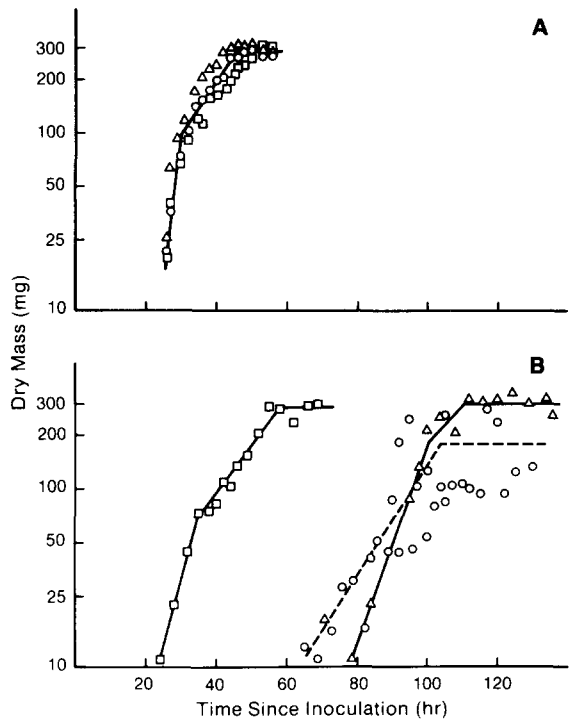

Fig. 1. Growth of *cel* and *cel*$^+$ strains in liquid shaker culture. Cultures of *bd* and *bd cel* were grown in liquid shaker culture with 2% glucose at 25°C in constant light. Supplemental 16:0, when used, was added to a final concentration of $4 \cdot 10^{-3}$% (w/v), while supplemental 18:2, when used, was added to a final concentration of $1 \cdot 10^{-3}$% (w/v). Cultures were harvested at various times, the mycelia were dried overnight at 60°C and weighed, and the logarithm of mass was plotted against time. Representative curves are shown for each strain on each supplement. The *bd cel* strain with no supplemental fatty acid was particularly variable. The lines drawn through the data were fitted by eye. (A) Growth of the *bd* strain. (B) Growth of the *bd cel* strain. Symbols: ○, unsupplemented; □, with 16:0 supplement; △, with 18:2 supplement.

Supplemental 16:0 was stimulatory to the *bd cel* strain so that growth was similar to that of the *bd* strain, while supplemental 18:2 had little effect on the growth of the *bd cel* strain. Neither fatty acid supplement had much effect on the growth rate of *bd*.

In practice, the growth of either strain varied between experiments, especially with the *bd cel* strain, because of variability of the lag period. This problem was particularly marked with supplemental 18:2, where *bd cel* strains often failed to grow at all. This variability made it difficult to harvest cultures at a predictable culture mass; therefore, fatty acid composition was examined over the entire growth period.

Fatty acid composition of the bd strain in liquid culture

The fatty acid compositions of a number of *bd*-strain cultures grown for varying periods of time were determined. The data are plotted against residual dry mass in Fig. 2. Residual dry mass is the mass of the dried residue remaining after extraction of lipids, presumably mostly cell wall material, as well as denatured proteins, nucleic acids, etc. Fig. 2 also shows regression lines fitted to this data. Levels of the saturated fatty acids, 16:0 and 18:0, showed no correlation with mass

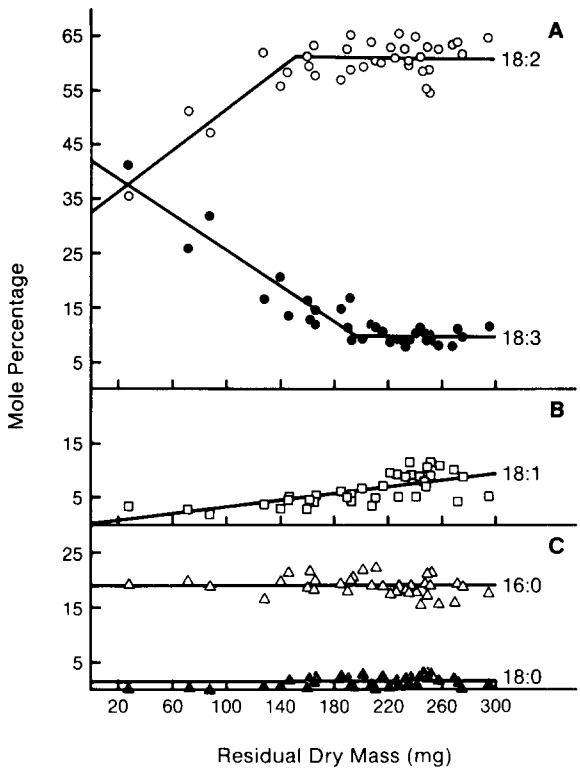

Fig. 2. Phospholipid fatty acid composition of the *bd* strain in unsupplemented liquid shaker culture. Cultures of the *bd* strain were grown in liquid shaker culture with 2% glucose at 25°C in constant light. Cultures were harvested and analyzed for phospholipid fatty acid composition as described in the text. The data were plotted against residual dry mass (see text). The lines drawn through the data are fitted regression lines. Lines are horizontal where regression analysis indicated no correlation (i.e., the 95% confidence limits for the correlation coefficient included zero). (A) Polyunsaturated fatty acids: ○, 18:2; ●, 18:3. (B) Monounsaturated fatty acid: □, 18:1. (C) Saturated fatty acids: △, 16:0; ▲, 18:0.

and thus appeared not to vary during growth. The regression line for the level of monounsaturated 18:1 showed an increase of only about eight mole per cent as mass increased from 20 to 300 mg. The correlation coefficient of this line was only 0.67, but correlation was significant at 95% confidence. The levels of the polyunsaturated fatty acids, 18:2 and 18:3, on the other hand, showed dramatic changes; the 18:2 level rose from 36 mol% at 20 mg residual dry mass to a final plateau value of 61 mol%, while the 18:3 level dropped from 39 mol% at 20 mg residual dry mass to a plateau value of 10 mol%. These changes in the levels of the unsaturated fatty acids balanced each other so that the total mole per cent of all unsaturated fatty acids showed no correlation with residual dry mass, averaging 79.5 mol% over the entire growth range. The double bond index, which is defined as moles of double bonds per 100 moles of fatty acid, decreased from 201 at 20 mg residual dry mass to a plateau value of 162, reflecting the replacement of 18:3 by 18:2.

Once the cultures had reached about 200 mg residual dry mass the changes in fatty acid composition were essentially complete. The cultures continued to grow, however, increasing in residual dry mass by as much as another 100 mg. Thus, the stabilization of fatty acid composition was not simply a result of cessation of growth.

When the *bd* strain was supplemented with 16:0 or 18:2 there was no apparent difference in the fatty acid composition at residual dry masses tested (data not shown). Observed values were within 95% confidence prediction intervals of the regression lines. Since there was no apparent effect of fatty acid supplement on the fatty acid composition of the *bd* strain, the fatty acid composition patterns of the fatty acid-supplemented *bd cel* strain were compared to the pattern for unsupplemented *bd* for which more data were available.

The fatty acid composition of the unsupplemented bd cel strain differed from that of the bd strain in liquid culture

Fig. 3 shows the growth-dependent fatty acid composition of the *bd cel* strain grown in liquid shaker culture at 25°C without fatty acid supplement. The pattern of fatty acid changes was very different from the pattern seen in the *bd* strain. The level of the saturated fatty acid, 16:0, which was a constant 19 mol% in the *bd* strain, increased from 11 mol% at 20 mg residual dry mass to 23 mol% at 300 mg in the *bd cel* strain. On the other hand, the level of 18:2, which increased during growth in the *bd* strain was a constant 54 mol% in the *bd cel* strain. The levels of the other fatty acids in *bd cel* changed in a manner similar to that in *bd*, although the levels of 18:1 rose more rapidly in *bd cel*, and the levels of 18:3 decreased more slowly and did not reach a plateau.

The total per cent of all fatty acids which were

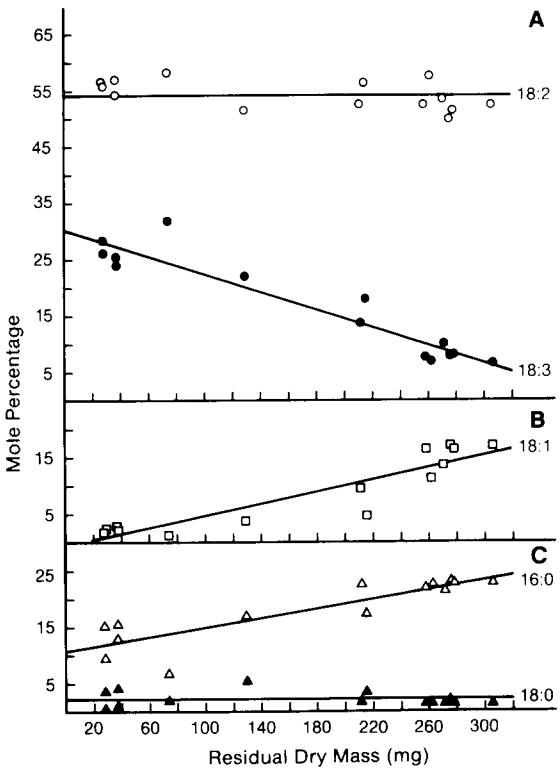

Fig. 3. Phospholipid fatty acid composition of the *bd cel* strain in unsupplemented liquid shaker culture. Cultures of *bd cel* were grown in liquid shaker culture at 25°C in constant light with 2% glucose. Cultures were harvested and analyzed for phospholipid fatty acid composition as described in the text. The data were plotted as in Fig. 2. (A) Polyunsaturated fatty acids. (B) Monounsaturated fatty acid. (C) Saturated fatty acids. Symbols as in Fig. 2.

unsaturated declined from 86 mol% at 20 mg residual dry mass to 75 mol% at 300 mg, but the average value was essentially the same as the constant value in *bd* (80 vs. 79.5 in *bd*). The double-bond index of *bd cel* started out at nearly the same value as in *bd* (200 at 20 mg vs. 201 in *bd*) and decreased at essentially the same rate, but, failing to reach a plateau, it dropped to much lower values than in *bd* (e.g., 141 at 300 mg).

Extra sum of squares analyses, as described in Materials and Methods, indicated that the growth-dependent patterns of the levels of 16:0, 18:1, 18:2, and 18:3, of the total unsaturated fatty acids, and of the double-bond index in *bd cel* were all significantly different from the equivalent changes in *bd* at 99.9% confidence. The patterns of 18:0 levels in the two strains, however, were not significantly different ($P > 0.05$).

Supplemental 16:0 alters the pattern of growth-dependent fatty acid composition in bd cel

The growth-dependent pattern of fatty acid composition of the *bd cel* strain supplemented with 16:0, which is shown in Fig. 4, was very different from that of the unsupplemented strain. The level of 18:2 was not constant, as it was without supplement, but increased during growth. The levels of 18:1 and 16:0, conversely, did not increase during growth as on unsupplemented medium, but were constant at 2.8 mol% and 9.5 mol%, respectively. Extra sum of squares analysis showed that levels of 16:0 and 18:2 behaved

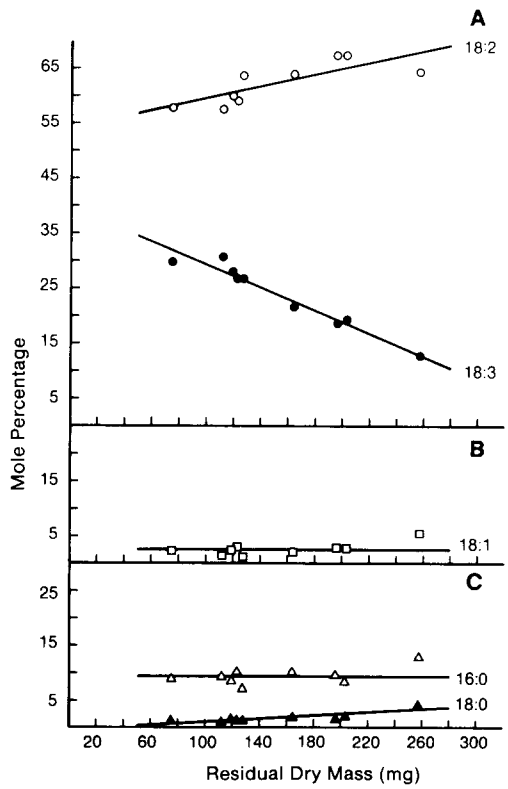

Fig. 4. Phospholipid fatty acid composition of the *bd cel* strain in 16:0-supplemented liquid shaker culture. Cultures of *bd cel* were grown in liquid shaker culture at 25°C in constant light with 2% glucose and $4 \cdot 10^{-3}$% (w/v) 16:0. Cultures were harvested and analyzed for phospholipid fatty acid composition as described in the text. The data were plotted as in Fig. 2. (A) Polyunsaturated fatty acids. (B) Monounsaturated fatty acid. (C) Saturated fatty acids. Symbols as in Fig. 2.

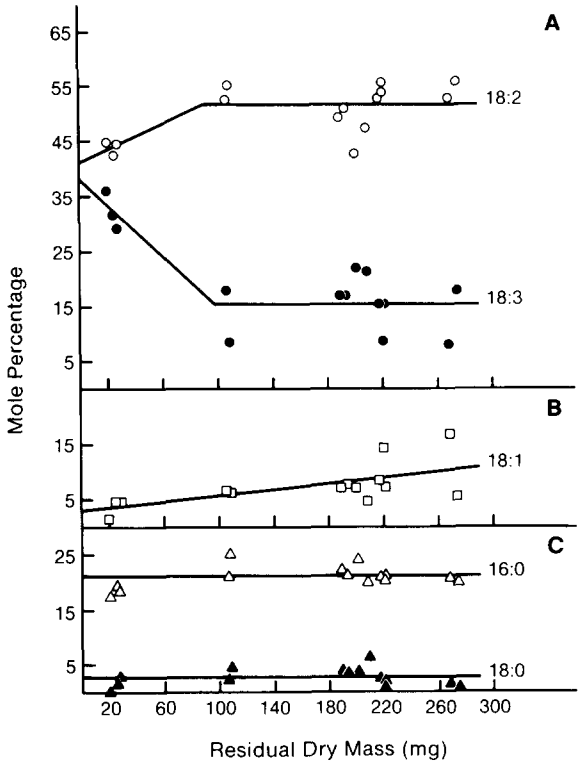

Fig. 5. Phospholipid fatty acid composition of the *bd cel* strain in 18 : 2-supplemented liquid shaker culture. Cultures of *bd cel* were grown in liquid shaker culture at 25°C in constant light with 2% glucose and $1 \cdot 10^{-3}$% (w/v) 18 : 2. Cultures were harvested and analyzed for phospholipid fatty acid composition as described in the text. The data were plotted as in Fig. 2. (A) Polyunsaturated fatty acids. (B) Monounsaturated fatty acid. (C) Saturated fatty acids. Symbols as in Fig. 2.

significantly differently in cultures with and without 16 : 0 at 99.5% confidence or better. The behaviors of 18 : 0 and 18 : 3 levels, however, were sufficiently similar with and without supplement that they were not significantly different ($P > 0.05$).

The per cent unsaturated fatty acids was constant in *bd cel* with 16 : 0 supplement, and the value, 88.6 mol%, was higher than any value attained without supplement. With 16 : 0-supplement, the double-bond index of *bd cel* was higher at 20 mg residual dry mass than it was without supplement (223 vs. 200 without supplement), and it dropped less rapidly, reaching only 170 at 300 mg residual dry mass. Extra sum of squares analysis showed that the behaviors of total per cent unsaturated fatty acids, and of the double bond index in 16 : 0-supplemented *bd cel* was significantly different from their behaviors in unsupplemented *bd cel* at 99.9% confidence.

Supplemental 16 : 0 makes the growth of the *cel* strain much more similar to the growth of the control strain both in liquid culture and on plates (see Table I and Fig. 1), and extends the lower end of the range of normal temperature compensation from 22°C to 18°C in the mutant [16]. The changes in fatty acid composition seen with 16 : 0 supplement were also somewhat more similar to the changes seen in the control strain. The level of 18 : 2 increased, and the level of 16 : 0 was constant, as in unsupplemented *bd*. However, there were still differences between *bd cel* with 16 : 0 supplement and *bd*. The level of 18 : 1 which increased somewhat during growth in *bd* was constant in *bd cel* with 16 : 0 supplement. The level of 16 : 0, although constant in both strains, was about twice as high in *bd* as in *bd cel* with 16 : 0 supplement. The levels of 18 : 2 and 18 : 3 did increase and decrease, respectively, in *bd cel* with 16 : 0 supplement, as in *bd*, but neither fatty acid level reached a plateau.

The levels of 16 : 0 in unsupplemented *bd* and *bd cel* supplemented with 16 : 0 were significantly different at 99.9% confidence (*t*-test), and extra sum of squares analysis indicated that the patterns of all other fatty acid levels in *bd cel* with 16 : 0 supplement, except that of 18 : 0, were significantly different from the equivalent patterns in *bd* at 99.9% confidence. The patterns of total unsaturated fatty acids, and of double-bond index also differed significantly at the same level of confidence (*t*-test and extra sum of squares analysis, respectively).

Supplemental 18 : 2 alters the pattern of growth-dependent fatty acid composition changes in bd cel

The growth-dependent fatty acid composition pattern of the *bd cel* strain supplemented with 18 : 2 is shown in Fig. 5. As described in Materials and Methods, some of this data is from cultures grown with 18 : 2 supplement from the time of inoculation and some is from cultures to which 18 : 2 supplement was added 24 h after inoculation. The levels of 16 : 0 and 18 : 0 in the two kinds of cultures were compared by *t*-test, and the levels

of the unsaturated fatty acids were compared by extra sum of squares analysis. None showed significant variation between the two groups ($P > 0.05$) except 18 : 0 ($0.001 < P < 0.01$). Since 18 : 0 was a minor component of the fatty acid composition and since no other fatty acids were significantly different, the difference in 18 : 0 was ignored and data from both kinds of cultures were combined.

The fatty acid composition pattern of the 18 : 2-supplemented *bd cel* strain was also distinctly different from that of the same strain without supplement. The level of 18 : 2 increased, from 43 mol% at 20 mg residual dry mass to a plateau of 52 mol%, close to the control value of 54 mol% without supplement.

The level of 18 : 3 decreased faster on 18 : 2 supplement than without supplement but leveled off at 15.5 mol% while the level on unsupplemented medium continued to drop. The level of 16 : 0 in 18 : 2-supplemented *bd cel* was constant at 21 mol%, essentially the same level as was reached in unsupplemented *bd cel* at 270 mg residual dry mass. Extra sum of squares analysis showed that the patterns of 16 : 0, 18 : 2, and 18 : 3 were significantly different at 99.9% confidence.

The level of 18 : 0 was constant on both media and not significantly different (t-test, $P > 0.4$). The level of 18 : 1 increased on both media, although more rapidly on unsupplemented medium. There was no significant difference between the two strains in 18 : 1 levels, however ($0.05 < P < 0.1$).

The total proportion of unsaturated fatty acids was constant with 18 : 2 supplement, averaging 76.5 mol%, the value attained on unsupplemented medium at about 260 mg residual dry mass. The double-bond index started at nearly the same value with and without 18 : 2 supplement (approx. 200 at 20 mg residual dry mass), but dropped more rapidly with 18 : 2 supplement and reached a plateau of 158 at about 100 mg residual dry mass. Both parameters differed significantly between cultures on the supplemented and on the unsupplemented media at 99.9% confidence (extra sum of squares analysis).

As with 16 : 0 supplement, the fatty acid composition pattern of *bd cel* with 18 : 2 supplement was more similar to that of unsupplemented *bd* than to that of unsupplemented *bd cel*. However, in *bd cel* with 18 : 2 supplement the polyunsaturated fatty acids, 18 : 2 and 18 : 3, reached plateaus much earlier than in *bd*, at about 80 to 100 mg residual dry mass as against 150 to 200 mg in *bd*. The 18 : 2 plateau in *bd cel* with 18.2 supplement was about 9.5 mol% lower than in *bd* and the 18 : 3 plateau was about 5.5 mol% higher.

Extra sum of squares analysis showed that the patterns of 18 : 2 and 18 : 3 levels, and of double-bond index in *bd cel* with 18 : 2 supplement differed significantly from their patterns in *bd* ($P < 0.001$), but the pattern of the level of 18 : 1 did not ($0.05 < P < 0.1$). By t-test it was also shown that 16 : 0 and 18 : 0 levels, and the total proportion of unsaturated fatty acids were significantly different at 99% confidence or better.

In general, supplemental 18 : 2 altered the fatty acid composition pattern of *bd cel* in a manner quantitatively similar to that in which supplemental 16 : 0 altered it. However, with 18 : 2 supplement, the pattern was more similar to that of the *bd* control than it was with 16 : 0 supplement. All fatty acid levels, the proportion of unsaturated fatty acids, and the double-bond index all behaved significantly differently with 18 : 2 supplement than with 16 : 0 supplement at 99.9% confidence (t-test or extra sum of squares analysis as appropriate).

The fatty acid composition of mitochondrial phospholipids parallels that of whole mycelial phospholipids

At the same time that cultures were harvested for determination of whole mycelial phospholipid fatty acid composition, duplicate cultures were harvested for isolation of mitochondria. The phospholipid fatty acid compositions of the isolated mitochondria were also determined as described in Materials and Methods. The yield of mitochondria in the isolation procedure was variable, and, since the mycelia were destroyed during the isolation, residual dry masses could not be measured for these cultures. The fatty acid compositions of the mitochondrial phospholipids were therefore compared to the fatty acid compositions of whole mycelial phospholipids of duplicate cultures from the same experiment. In both strains the composition of the mitochondrial phospholipids was simi-

lar to the composition of the whole mycelial phospholipids (data not shown). Thus the differences between the fatty acid compositions of *bd* and *bd cel* seen in the whole mycelial phospholipids were also seen in the mitochondrial phospholipids. This similarity of mitochondrial and whole mycelial fatty acid compositions was also seen in other experiments with *frq* (frequency) [29] and *oli*^{r} (oligomycin resistant) [30] mutants (unpublished observations).

Altered fatty acid composition in liquid culture co-segregated with the growth rate and period phenotype of cel

Seven random *bd pan*$^+$ segregants of a *bd cel pan-2A* × *bd a* cross were kindly provided by Sam Ong. They were tested for the presence of the *cel* marker by assaying for linear growth rate on unsupplemented plates, for increased growth rate and thickness of growth on plates supplemented with 16 : 0, and for long period and reduced growth rate on plates supplemented with 18 : 2. By these criteria, four of the segregants were *cel* and three were *cel*$^+$. Fatty acid compositions were determined for all the segregants in unsupplemented liquid culture and in liquid culture supplemented with 16 : 0. The *cel* segregants had fatty acid compositions consistent with the regression models for the *bd cel* strain shown in Figs. 3 and 4 while the *cel*$^+$ segregants had fatty acid compositions consistent with the regression model for the *bd* strain shown in Fig. 2 (data not shown). Several progeny were also tested in liquid culture supplemented with 18 : 2; the data for the *cel* progeny were consistent with the 18 : 2-supplemented *bd cel* regression model, while the data for the *cel*$^+$ prog-

TABLE II

PHOSPHOLIPID FATTY ACID COMPOSITION OF THE *bd csp-1 cel* STRAIN GROWN ON PETRI PLATES

The *bd csp-1* and *bd csp-1 cel* strains were grown on solid medium in constant darkness at 22°C. Supplemental 18 : 2 was added to 143 μM and supplemental 16 : 0 to 156 μM. Mycelia were harvested and analyzed for phospholipid fatty acid composition as described in the text. Each *bd csp-1* sample contained mycelia from three to eight plates and each *bd csp-1 cel* sample contained mycelia from eight to 12 plates.

		Composition (mol%)						DBI [b]	*N* [c]
		16 : 0	18 : 0	18 : 1	18 : 2	18 : 3	Total UFA [a]		
cel$^+$									
No fatty	Mean	20.6	1.3	5.7	40.4	32.0 [f]	78.1	183 [f]	11
acid	S.E. [d]	1.1	0.3	0.7	0.5	1.0	1.4	2	
+16 : 0	Mean	18.1	1.1	4.8	40.0	36.0	80.8	193	8
	S.E.	0.5	0.1	0.1	0.3	0.5	0.6	1	
+18 : 2	Mean	18.6	1.4	5.1	41.7 [f]	33.2	80.0	188 [f]	5
	S.E.	0.2	0.1	0.1	0.1	0.3	0.4	1	
cel									
+16 : 0	Mean	10.4 [e]	1.4	2.7 [e]	50.6 [e]	34.9	88.2 [e]	208 [f]	5
	S.E.	0.3	0.5	0.1	0.6	0.4	0.7	1	
+18 : 2	Mean	10.5 [e]	2.6 [e]	4.4 [e,f]	57.6 [e,f]	25.0 [e,f]	86.9 [e]	194 [e,f]	4
	S.E.	0.2	0.1	0.2	0.4	0.4	0.6	1	

[a] Total mol% of unsaturated fatty acids.

[b] Double-bond index (moles double bonds per 100 moles fatty acid).

[c] Number of samples analyzed.

[d] Standard error of the mean.

[e] Significantly different ($P < 0.01$) from the value for *bd csp-1* on the same medium.

[f] Significantly different ($P < 0.01$) from the value for the same strain on 16 : 0 (t-test). 16 : 0-supplemented medium was used as the standard of comparison as opposed to unsupplemented medium since data was not available for *bd csp-1 cel* on unsupplemented medium.)

eny was consistent with the *bd* regression model (data not shown).

In addition, seven *bd csp*$^+$ *cel* progeny of a *bd csp cel*×*bd*$^+$ cross prepared earlier were retested for the presence of the *cel* marker as described above. All but one were indeed *cel*; the exception appeared to have reverted. The fatty acid compositions of these strains growing with 16 : 0 supplement were determined; all were consistent with the *bd cel* pattern in Fig. 4 except the presumed revertant which was consistent with the *bd* pattern in Fig. 2 (data not shown).

The fatty acid composition of the bd csp cel strain was altered during growth on solid medium

Table II shows the phospholipid fatty acid compositions of the *bd csp* strain on unsupplemented, 16 : 0-supplemented, and 18 : 2-supplemented plates, and of the *bd csp cel* strain on 16 : 0-supplemented and 18 : 2-supplemented plates. The composition of *bd csp cel* on unsupplemented plates could not be measured because there was insufficient material. Samples were taken of the growing front of the cultures at the time the fungus had just completed a band of conidiation.

Supplemental fatty acids did not greatly change the composition of the *bd csp* phospholipids. Supplemental 18 : 2 increased the level of 18 : 2 by less than 1.5 mol%, and supplemental 16 : 0 increased the level of 18 : 3 by 4 mol%. The double-bond index was highest with 16 : 0 supplement. The composition of *bd csp cel,* however, varied greatly with the fatty acid supplement; the level of 18 : 2 was 7 mol% higher and that of 18 : 3 was 9 mol% lower on supplemental 18 : 2 than on supplemental 16 : 0.

The composition of *bd csp cel* differed significantly from that of *bd csp* on both supplements. The level of 18 : 2 in *bd cel* was significantly higher and those of 16 : 0 and 18 : 1 significantly lower on both supplements, while the 18 : 3 level was also low on supplemental 18 : 2. The per cent of all fatty acids that were unsaturated and the double-bond index were also significantly higher in *bd cel* on both supplements.

Discussion

Since the cel-strain has lost temperature compensation and has a partial loss of the ability to synthesize fatty acids, it was decided to examine the interrelationship of the fatty acid composition with temperature-compensation in this strain. The system used for examining membrane fatty acid composition involved cultures grown in liquid medium is constant light at 25°C. Under these conditions, the control, *cel*$^+$, strain did not have a specific fatty acid composition, but, rather, the fatty acid composition varied with residual dry mass. The significance of this variation is not known. Growth-dependent changes in phospholipid fatty acid composition have been previously described in *Candida lipolytica* [31] and in *Neurospora* [13]. Under these same conditions the *cel* mutant exhibited mass-dependent variation in fatty acid composition distinctly different from that of the control strain. A number of other mutants tested under the same conditions had mass-dependent fatty acid compositions similar to that of the control strain (Coté, G., PhD Thesis, 1986, U.C. San Diego). This evidence supports the hypothesis that the *cel* defect interferes with the maintenance of normal fatty acid composition.

The reason *cel* was unable to maintain normal fatty acid composition is unknown. The only known defect in lipid metabolism in this strain is in the synthesis of 16 : 0, which is decreased but not eliminated by the mutation [15]. Elongation of 16 : 0 to 18-carbon fatty acids, desaturation of saturated fatty acids, and oxidation of fatty acids to CO_2 all occurred in this strain (Coté, G., unpublished observations). Given that fatty acid metabolism was apparently normal, save for the synthesis of 16 : 0, one might predict that the growth of *cel* would be slow but that fatty acid composition would be normal. Since normal fatty acid composition was not observed it is evident that the balanced control of fatty acid composition was altered in this strain. One possibility is that the reduced fatty acid synthesis in this strain had differentially induced other enzymes of fatty acid metabolism so that fatty acids were not produced in appropriate ratios. It would be of great interest to determine the changes which occur–or do not occur–in the fatty acid composition of *cel* during a temperature shift, and the fatty acid composition at low temperatures.

The *cel* mutant differs from the wild type not only in that temperature compensation is lost be-

low 22°C, but also in that this property is altered by exogenous supplemental fatty acids [16]. Exogenous 16:0 restores normal temperature compensation at temperatures above 18°C, while exogenous 18:2 prevents normal temperature compensation below 26°C. The liquid culture fatty acid composition pattern of *cel* was also shown to be altered by these two exogenous fatty acids.

Both the altered pattern of fatty acid composition and sensitivity of the pattern to added exogenous fatty acids co-segregated with the *cel* marker. The fact that both the fatty acid composition pattern and the phenotype of *cel* are sensitive to supplemental fatty acids supports the possibility that altered fatty acid composition is a property of the *cel* allele, and not of some allele at some other, unrelated locus linked to the *cel* locus.

The addition of 16:0 to the *cel* mutant does not completely restore the wild-type phenotype. The pattern of fatty acid composition is still quite different from that in the control strain particularly in having low 16:0 levels. Temperature compensation is still lacking below 18°C, and linear growth rate on solid medium is not completely restored to normal at 22°C or at 25°C (see Table I). It is possible that supplemental 16:0 cannot reach intracellular concentrations sufficient to restore a normal phenotype, either because, at the concentration used, it cannot be absorbed rapidly enough or because it is rapidly β-oxidized.

The phospholipid fatty acid composition of the *cel* mutant is also abnormal on solid medium is constant darkness. The fatty acid composition under these conditions was investigated to demonstrate that the altered fatty acid composition of the mutant was not a peculiarity of growth in shaking liquid culture. Rhythmicity has not been reported in log-phase liquid cultures of *Neurospora*; furthermore, constant light, as used in the experiments described here, is thought to prevent rhythmicity [18]. The data reported here for the unsupplemented *bd csp-1* strain are similar to results reported for the same strain under similar conditions by Roeder, Sargent, and Brody [32], although 18:3 levels were somewhat higher and 16:0 levels somewhat lower in their report.

The fatty acid composition observed for the *cel* strain on solid medium supplemented with 16:0 was similar to the fatty acid composition observed in liquid culture. In both systems, the *cel* strain had reduced levels of 16:0, elevated levels of 18:2, and an elevated double bond index. On the other hand, the fatty acid composition of the *cel* strain on solid medium supplemented with 18:2 was quite different from the fatty acid composition of the same strain in liquid culture. On solid medium the *cel* strain had elevated 18:2 levels and reduced 18:3 levels compared to the control strain while the opposite was seen in liquid. Furthermore, the level of 16:0 was much reduced on solid medium but only slightly reduced in liquid culture. The differences between solid and liquid culture presumably reflect differences in temperature and in supplement concentration between the two culture systems. At the temperature and supplement concentration used in liquid culture the fatty acid composition pattern was similar to that of the control strain and the period was also normal on plates (Table I). At the temperature and supplement concentration used on solid medium the fatty acid composition pattern was much different from that of the control strain and the period was very abnormal.

Feldman, Pierce, and Brown [33] have reported that a *Neurospora* strain carrying an allele at the *frq* (frequency) locus, *frq-9,* which also leads to a loss of temperature compensation, does not cause an obviously abnormal fatty acid composition. In addition, we have also noted that strains bearing other alleles at this locus, with normal or altered temperature compensation [34], also appear to have normal fatty acid composition (unpublished observations). This demonstrates that temperature compensation may also be altered by mutations which do not affect the overall membrane fatty acid composition. Perhaps such mutations alter the temperature sensitivity of the components of the rhythm-generating system themselves.

In this paper evidence has been presented suggesting that the *cel* mutant of *Neurospora* has lost the ability to maintain normal membrane composition. This suggests that some components of the circadian rhythm-generating system may be membrane-localized, and that temperature compensation may involve adjustment of membrane composition. We thus hypothesize that normal rhythmicity depends, not on any given pattern of fatty acid composition, which of course would be

different at different temperatures, but on the ability of the organism to maintain optimal membrane properties through normal adjustment of membrane composition.

Acknowledgements

This work was supported by National Institute of Health Grant GMS 21842 to S.B., and by a National Science Foundation Graduate Fellowship and a NIH Predoctoral Traineeship, USPS GM 07240, to G.G.C.

References

1 Hastings, J.W. and Sweeney, B.M. (1957) Proc. Natl. Acad. Sci. USA 43, 804–811
2 Hazel, J.R. and Prosser, C.L. (1974) Physiol. Rev. 54, 620–677
3 Ehret, C.F. and Trucco, E. (1967) J. Theor. Biol. 15, 240–262
4 Pavlidis, T. and Kauzmann, W. (1969) Arch. Biochem. Biophys. 132, 338–348
5 Njus, D., Sulzman, F.M. and Hastings, J.W. (1974) Nature 248, 116–120
6 Sweeney, B.M. (1974) Int. J. Chronobiol. 2, 25–33
7 Schweiger, H.G. and Schweiger, M. (1977) Int. Rev. Cytol. 51, 315–342
8 Burgoyne, R.D. (1978) FEBS Lett. 94, 17–19
9 Thompson, G.A., Jr. (1983) in Cellular Acclimatisation to Environmental Change (Cossins, A.R. and Sheterline, P., eds.), pp. 33–53, Cambridge University
10 Martin, C.E., Siegel, D. and Aaronson, L.R. (1981) Biochim. Biophys. Acta 665, 399–407
11 Aaronson, L.R., Johnston, A.M. and Martin, C.E. (1982) Biochim. Biophys. Acta 713, 456–462
12 Aaronson, L.R. and Martin, C.E. (1983) Biochim. Biophys. Acta 735, 252–258
13 Vokt, J.P. and Brody, S. (1985) Biochim. Biophys. Acta 835, 176–182
14 Henry, S.A. and Keith, A.D. (1971) J. Bacteriol. 106, 174–182
15 Elovson, J. (1975) J. Bacteriol. 124, 524–533
16 Mattern, D.L., Forman, L.R. and Brody, S. (1982) Proc. Natl. Acad. Sci. USA 79, 825–829
17 Sargent, M.L. and Woodward, D.O. (1969) J. Bacteriol. 97, 861–866
18 Sargent, M.L., Briggs, W.R. and Woodward, D.O. (1966) Plant Physiol. 41, 1343–1349
19 Seletrennikoff, C.R., Nelson, R.E. and Siegel, R.W. (1974) Genetics 78, 679–690
20 Davis, R.H. and De Serres, F.J. (1970) Methods Enzymol. 17A, 79–143
21 Vogel, H.J. (1956) Microb. Genet. Bull. 13, 42–43
22 Brody, S. and Harris, S. (1973) Science 180, 498–500
23 Brody, S. and Martins, S.A. (1979) J. Bacteriol. 137, 912–915
24 Sebald, W., Neupert, W. and Weiss, H. (1979) Methods Enzymol. 55, 144–148
25 Cramer, C.L., Ristow, J.L., Paulus, T.J. and Davis, R.H. (1983) Anal. Biochem. 128, 384–392
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
27 Hubbard, S.C. and Brody, S. (1975) J. Biol. Chem. 250, 7173–7181
28 Mattern, D. and Brody, S. (1979) J. Bacteriol. 139, 977–983
29 Feldman, J.F. and Hoyle, M.N. (1973) Genetics 75, 605–613
30 Sebald, W., Sebald-Althaus, M. and Wachter, E. (1977) in Genetics and Biogenesis of Mitochondria (Kaudewitz, F., Schweyen, R.J., Bandlow, W. and Wolf, K., eds.), pp. 433–440, De Gruyter, Berlin
31 Kates, M. and Baxter, R.M. (1962) Can. J. Biochem. Physiol. 40, 1213–1227
32 Roeder, P.E., Sargent, M.L. and Brody, S. (1982) Biochemistry 21, 4909–4916
33 Feldman, J.F., Pierce, S.B. and Brown, D. (1986) Plant Physiol. 80 (Suppl.), 92
34 Gardner, G.F. and Feldman, J.F. (1981) Plant Physiol. 68, 1244–1248