Circadian Rhythms in *Neurospora crassa*: Oscillations in Fatty Acids†

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ABSTRACT: In the band strain of *Neurospora crassa*, a circadian rhythm of spore formation is expressed at the growing front of the mycelia by sequential periods of conidiating and nonconidiating growth. A region, ca. 7 mm wide, of the growth front of the mycelium of the band (*bd csp-1*) strain was sampled at different times over a 40-h interval, and the amount and composition of the fatty acids in the total lipids and in the phospholipid subfractions were determined. In the growing front, the mole percentages of linoleic acid (18:2) and linolenic acid (18:3) oscillated out of phase with each other in the total lipids and in the phospholipids. The oscillations in 18:2 and 18:3 content nearly compensated for each other; i.e., when the 18:2 content in the total lipid from *bd csp-1* increased from 34 to 38 mol %, the 18:3 content decreased from 41 to 36 mol %. No oscillations could be detected in the mole percentages of the other fatty acids or in the total lipid content. The oscillations in the content of 18:2 and 18:3 were shown to have two properties of a circadian rhythm: their periods were about 20-h long and they were phase set by an initial exposure to light. While firm conclusions as to whether or not these oscillations have a role in the mechanism of the circadian clock cannot be drawn from the available data, several criteria and experimental approaches to this problem are discussed. The possibility that the oscillations were merely part of the developmental conidiation rhythm was eliminated by demonstrating similar oscillations in the 18:2 and 18:3 content in the total lipid and in the phospholipid subfractions of samples isolated from a strain (*bd*$^+$ *csp-1*) that does not express the spore-forming rhythm under the growth conditions used.

Circadian rhythms are apparently ubiquitous among eukaryotic organisms (Bünning, 1973). The mechanism whereby these oscillations are generated at the biochemical level has remained elusive. A standard approach has been to add exogenous compounds to the culture medium in order to determine whether or not they alter either the period or phase of any observable circadian oscillation (Bünning, 1973). Since models for the mechanism of circadian oscillations have been proposed that focus on membrane phenomena (Njus et al., 1974; Sweeney, 1976), it seemed reasonable to determine the effects of exogenous fatty acids upon circadian rhythms.

Neurospora crassa was chosen for this series of studies in part because genetically useful markers can be introduced by standard crossing procedures (Davis & de Serres, 1970). When the band strain (*bd csp-1*) of *Neurospora* (Sargent & Briggs, 1967) grows from a point source across the surface of an agar medium under conditions of constant darkness and temperature, its growth front alternates between phases of conidiating growth (bands)[1] and nonconidiating growth (interbands). This developmental rhythm satisfies the three requirements for being a circadian rhythm: (i) the rhythm has a period close to, but not exactly, 24 h; (ii) its period is relatively insensitive to a wide range of growth temperatures; and (iii) it can be phase-shifted by light. Apparently the *bd* mutation allows the expression of an underlying rhythm since it has been shown that under certain conditions many of the wild-type strains of *Neurospora* will also exhibit an identical circadian rhythm of conidiation (Sargent & Kaltenborn, 1972). Introduction of an additional mutation, *cel*, into the *bd* strain yielded a double mutant that has a partial requirement for saturated fatty acids (Henry & Keith, 1971). The *cel* strain has been shown to be deficient in the synthesis of palmitic acid (16:0) due to a defective fatty acid synthetase complex (Elovson, 1975). When the *bd csp-1 cel* strain was supplemented with exogenous unsaturated fatty acids (Brody & Martins, 1979) or short-chain fatty acids (Mattern & Brody, 1979; Mattern et al., 1982), the period increased from about 21 h to up to 40 h, depending upon the supplement. These observations prompted our initial investigations of the fatty acid compositions of various strains of *Neurospora* that ultimately led to the discovery of the low amplitude oscillations in the two major fatty acid components of *Neurospora* lipids. This paper reports and compares rhythmic changes in the mole percentages of the linoleic (18:2) and linolenic (18:3) acids in both the total lipids and the phospholipid fractions of the growing front samples of *bd csp-1* and *bd*$^+$ *csp-1* cultures of *N. crassa* as a function of the number of hours of growth in constant darkness.

Materials and Methods

Strains. The *bd* and *csp-1* strains were obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, CA. The double mutant strain (*bd csp-1*) was constructed according to standard crossing procedures (Davis & de Serres, 1970) by Linda Forman. Introduction of the *csp-1* mutation (conidial separation) allowed the Petri dish cultures to be manipulated without the conidia becoming detached from the culture and reinoculating the plate (Selitrennikoff et al., 1974). The *bd csp-1* strain has been deposited and is available from the Stock Center.

Culture Conditions. The cultures were grown as described previously (Brody & Harris, 1973) on an agar medium containing inositol, maltose, and arginine. Large (150 mm) Petri plates containing 35 mL of medium were overlaid with circular sheets of sterile dialysis tubing to facilitate the harvesting of the samples. Plates were inoculated approximately 1 cm from

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[1] Abbreviations: band, conidiating region; interband, nonconidiating region; FAME, fatty acid methyl ester; RDW, residual dry weight; BHT, 2,6-di-*tert*-butyl-*p*-cresol. Fatty acids are designated by the standard *x*:*y* abbreviation where *x* = the number of carbons and *y* = the number of double bonds.

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Table I: Incubation Times for *bd csp-1* Cultures[a]

expt	light (h)	dark (h)	total (h)
Constant Light Set: Variable Total Time			
B1[b]	12.7	34.6–70.8	47.3–83.5
B5	12.5	35.3–71.0	47.8–83.5
Variable Light Set: Similar Total Time			
B2a	18.8	51.5–58.0	70.3–76.8
B2b	10.5	60.5–66.5	71.0–77.0
B2c	5.8	67.5–72.5	73.3–78.3
B3a	17.5	53.5–59.8	71.0–77.3
B3b	10.7	61.6–67.6	72.3–78.3
B3c	6.0	66.3–73.0	72.3–79.0
Variable Light Set: Variable Total Time			
B4a	9.8	38.0–45.5	47.8–55.3
B4b	14.0	50.0–57.8	64.0–71.8

[a] Cultures for each experiment were exposed to light for the indicated time and then incubated in constant darkness for variable lengths of time within the indicated range. [b] The B stands for band strain.

the edge with inocula prepared by rolling mycelia and conidia from fresh slants into tiny balls (ca. 1-mm diameter) on the surface of a Petri dish (Brody & Martins, 1979; Brody & Harris, 1973). The band strain (*bd csp-1*) slants were 3 days old; the nonconidiating strain (*bd*$^+$ *csp-1*) slants were 2 days old. Cultures were synchronized by stacking them right side up, 30 cm in front of a vertical fluorescent lamp. They were then placed in a dark incubator for 18–73 h at 22 °C. Data for the band strain (*bd csp-1*) were obtained from five experiments: B1–B5, where B stands for the band strain. In experiments B1 and B5, the length of the initial exposure to light was kept constant at 12.5–12.7 h (Table I), and samples were harvested as a function of the total incubation time. In experiments B2 and B3, the length of the initial exposure to light was varied, while the total incubation time was kept nearly constant (Table I). In experiment B4, both the times in the light and dark were varied in order to obtain additional data at certain phases of the conidiation rhythm. By varying the time of initial illumination, it was possible to determine whether the phase of the observed fatty acid oscillations was a function of the total growth time or, as was observed for the circadian conidiation and AMP rhythms (Delmer & Brody, 1975), was a function of the number of hours the cultures grew in the dark. Since the phases of the fatty acid oscillations and conidiation rhythms were shown to be a function of the time the cultures grew in constant darkness, data from all five experiments could be plotted on a single graph. Varying the initial light exposure was convenient in terms of facilitating harvest times and optimizing growth. In the case of the nonconidiating strain (*bd*$^+$ *csp-1*), which grows more rapidly and much thinner than the band strain, samples could only be harvested between 41 and 54 h. Initial illumination exposures of 11, 18, and 23 h had to be used in each of the three *bd*$^+$ *csp-1* experiments, in order to obtain data from a full circadian cycle. These experiments are labeled N1, N2, and N3, where N stands for the nonconidiating strain. All observations and the harvesting of the mycelia were performed under a red safelight. The times at which the band strain formed conidiating regions and the period lengths of the conidiation rhythm were determined as described previously (Brody & Martins, 1979).

Harvesting. Samples were harvested by using a sharpened metal spatula to scrape the most recent 6–7 mm of mycelial growth from the growing front of each culture. Since the growth rate of *bd csp-1* averaged 1.3 mm/h, this sample width included only mycelia (and aerial hyphae) formed during the most recent one-fourth of a circadian cycle. Five or six plates generally yielded about 2 mg of residual dry weight from older *bd csp-1* cultures that were in the end of the band or early interband phases, since aerial hyphae were present. Twenty or more plates were often needed to obtain the same yield from younger cultures, cultures in the interband or early band phases, and *bd*$^+$ *csp-1* cultures, since the growing fronts of these cultures were not very dense.

Extraction of Lipids. All solvents were analytical reagent grade. The mycelial fragments that were scraped from a given sample set of plates were immediately submerged in 5 mL of ice-cold methanol and stored under nitrogen at −20 °C overnight. The methanol extract, which contained 90% of the lipids, was removed with a Pasteur pipet and concentrated. Two further extractions of the residue with chloroform–methanol (1:1 v/v) (4 mL; then 2 mL), and washing of the combined extracts with 0.88% KCl, were carried out as described earlier (Hubbard & Brody, 1975). The lipid-containing chloroform layer was made homogeneous by mixing it vigorously with a small amount of methanol and divided into aliquots for total lipid analysis and phospholipid purification. The aliquots were then evaporated, redissolved in methanol–toluene (1:1 v/v) or chloroform, respectively, and stored under nitrogen at −20 °C. The residual mycelial fragments from each sample were dried overnight at 135 °C, and the residual dry weight was determined on a microbalance.

Isolation of Phospholipids. Aliquots (1 mL) of total lipids (60–350 μg) in chloroform were loaded onto silicic acid columns (1.2 × 7.0 cm, Bio-Sil A, 100–200 mesh, Bio-Rad Laboratories) that had been prewashed with methanol and equilibrated with chloroform. After the neutral lipids were eluted from the columns with 50 mL of chloroform, the phospholipids were eluted with 135 mL of methanol. The methanol eluates from each column were evaporated to dryness with a rotary evaporator and transferred to test tubes by twice rinsing the flasks with 3 mL of methanol–toluene (1:1 v/v). The samples were again evaporated and resuspended in 1 mL of methanol–toluene.

Transesterification. Alkaline methanolysis, neutralization, and column filtration were performed as described earlier (Hubbard & Brody, 1975) except that the weaker ion-exchange resin, Bio-Rex 70 (H^+ form, Bio-Rad Laboratories) was used to neutralize the potassium hydroxide. The emulsion containing the fatty acid methyl esters was extracted 3 times with 3 mL of petroleum ether (30–60 °C). The petroleum ether extracts were evaporated, redissolved in a small amount of petroleum ether, flushed with nitrogen, and stored at −20 °C.

Gas–Liquid Chromatography. Quantitative analysis of the fatty acid methyl esters was accomplished on a Varian Aerograph 1200 gas chromatograph equipped with a flame ionization detector, using a Supelco 3% SP-2300 column [6 ft × $^1/_8$ in. (ca. 1.8 m × 0.3 cm)]. Conditions were as follows: injector temperature, 230 °C; column temperature, 190 or 195 °C; detector temperature, 235 °C; nitrogen flow rate, 25 mL/min. Prior to analysis, a known quantity of the methyl ester of 17:0 (Sigma) in pyridine was added to each sample vial as an internal standard. After the solvent was evaporated, the contents of each sample vial were redissolved in carbon disulfide. Approximately 2 μL containing 0.5–2.0 μg of FAMEs was injected into the chromatograph for each run. Fatty acid methyl esters were identified by comparison with the retention times of reference compounds chromatographed under identical conditions.

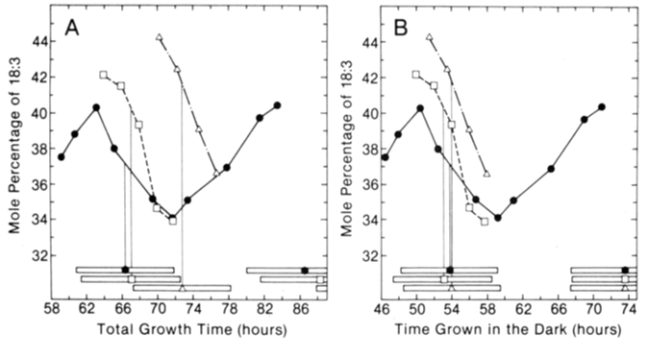

FIGURE 1: Mole percentages of 18:3 in total lipid from band strain cultures: the effect of light-induced phase shifts on the 18:3 oscillation. Samples from the growing front of cultures of the *bd csp-1* strain in *Neurospora* were harvested as a function of time and the mole percentages of 18:3 in the total lipid extracts determined. In (A), the values were plotted as a function of the total growth time. Cultures studied in experiment B1 (●) were exposed to 12.7 h of initial illumination, whereas cultures in experiments B4 (□) and B2 (△) were exposed 14.0 and 18.8 h, respectively. Bars at the bottom indicate periods of conidiation. Vertical lines have been drawn between the 18:3 curves and the midpoints of the corresponding conidiation bands to facilitate comparison. The cultures in the latter experiments were phase-delayed by approximately 1 and 6 h, respectively, in this way. In (B), the same data were plotted as a function of the time the cultures grew in constant darkness.

The peak areas were calculated by the method of Hornstein et al. (1967) since partial overlap of the 18:0 and 18:1 peaks made direct measurement of their widths at half-height difficult. The number of nanomoles per milligram of residual dry weight (RDW) of each component was calculated by dividing the peak area by the molecular weight of the FAME, the RDW of the sample, and the area per nanogram observed for the 17:0 methyl ester standard. Since little improvement in accuracy is obtained by using theoretical weight response correction factors for samples of FAMEs that contain only 17–19 carbon atoms (Ackmann, 1972), peak area was assumed to be proportional to component weight. The mole percentages were calculated by dividing the nanomoles per milligram of each FAME component by the sum of the nanomoles per milligram of all five FAMEs.

Results

Rhythmic Oscillations in 18:3 and 18:2 from the bd csp-1 Strain. In Figure 1A, the mole percentages of 18:3 in the total lipid extract of band strain (*bd csp-1*) mycelial samples are plotted as a function of time since inoculation of the cultures. The data for experiment B1 clearly show an oscillation in the mole percentage of 18:3 with a maximum of 40.3% and a minimum of 34.1%. The period of the oscillation was about 20 h, which is similar to the 21.5-h period for the circadian conidiation rhythm. The highest percentage of 18:3 occurred in samples of the growing front that were harvested when the edge of the mycelium was approximately one-fourth of the way into the conidiating band. The lowest value occurred near the beginning of the nonconidiating region.

If the rhythmic variation in the 18:3 composition is directly related to the circadian clock of *Neurospora*, then this rhythm should be phase set by light in the same manner as the conidiation rhythm (Delmer & Brody, 1975). Figure 1A shows the results of increasing the period of initial illumination from 12.7 h in experiment B1 to 14.0 h in experiment B4 and 18.8 h in experiment B2. As expected, the midpoints of the conidiation bands in experiments B1 and B4 were nearly the same, while the midpoint of the conidiation band in experiment B2 was delayed 6 h. Similarly, the extremes of the 18:3 curves

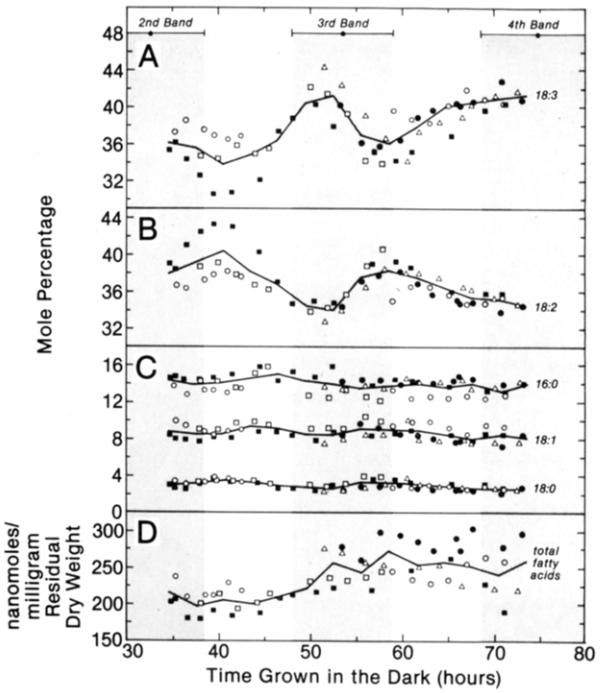

FIGURE 2: Fatty acid composition and content of total lipid from the band strain as a function of the time the cultures were grown in the dark. Samples from the growing front of cultures of the *bd csp-1* strain were harvested at various times (see Table I) and their fatty acid compositions analyzed as described in the text. Data points represent 62 separate analyses from the five experiments listed in Table I: B1 (■), B2 (△), B3 (●), B4 (□), and B5 (○). The shapes of the curves were estimated by averaging all the points within 3-h intervals. Bars at the top indicate periods of conidiation. The phase of the 6–7 mm wide mycelial samples in terms of the conidiation rhythm was taken to be the phase of the edge of the mycelial mat at the time of harvest; i.e., after 53.5 h in the dark, the growing front of the mycelial mat was at the midpoint of the conidiating region.

in experiments B1 and B4 occurred after the same total growth times, while the extremes of the 18:3 curve in experiment B2 occurred approximately 6 h later. Thus, the phase of the 18:3 oscillation was light set by the length of the initial period of illumination in the same manner as the phase of the conidiation rhythm was light set. In Figure 1B, the data are replotted as a function of the time the cultures grew in constant darkness. The midpoints of the bands agreed within ±0.5 h. The high and low points of the 18:3 curves agreed within ±1.0 h. Therefore, the data compiled for subsequent figures are plotted as a function of the total number of hours that the cultures grew in constant darkness.

Figure 2 shows the fatty acid compositions and contents of the total lipid extracts of 62 samples of band strain (*bd csp-1*) mycelia as a function of the time the cultures grew in constant darkness. Data spanning nearly two periods were obtained by manipulating the total incubation time of the cultures and/or the length of the initial period of illumination as detailed in Table I. The plot of the average mole percentages of 18:3 from all five experiments vs. time (Figure 2A) oscillated in a manner similar to that described above, i.e., with a period of about 20 h, with the maximum mole percentage of 18:3 occurring about one-fourth of the way into the conidiating phase, and with the minimum near the end of the conidiating phase. While the individual experiments all showed the same relative oscillations, the median mole percentage and the values at the extrema in experiment B1 were consistently lower than the average, while those observed in experiment B5 and the first half of experiment B2 were consistently higher. The reason(s) for these differences between experiments is (are)

unknown. Please note that the partial data presented in Figure 1 have been incorporated into the composite 18:3 graph presented in Figure 2A. A slightly smaller oscillation in the 18:2 composition (Figure 2B) nearly compensated for the changes in the 18:3 composition. The maxima in the 18:2 oscillation corresponded to the minima in the 18:3 oscillation. The 18:2 oscillation also had a period of about 20 h and was phase set by initial illumination (data not shown) in the same way as the conidiation and 18:3 rhythms. The minor components, 16:0 (13.8 ± 0.9%), 18:0 (2.9 ± 0.4%), and 18:1 (8.6 ± 0.7%) showed only random fluctuations (Figure 2C). The total fatty acid content of the mycelial samples is shown in Figure 2D. The scatter in the number of nanomoles per milligram of residual dry weight may have occurred in part because of difficulties in recovering the extracted mycelial fragments, since the samples ranged from only 1.4 to 12.8 mg of RDW. The average content for the first period (207 ± 16 nmol/mg) was somewhat lower than for the second period (253 ± 28 nmol/mg), but cyclical changes in the total fatty acid content were not observed.

Rhythmic Oscillations in 18:3 and 18:2 from a Nonconidiating Strain (bd⁺ csp-1). If the oscillations in 18:3 and 18:2 are independent of the changing morphology of the *bd csp-1* cultures, they should be seen in strains that lack the *bd* mutation. This assumes that exposure to an initial period of illumination prior to transfer to constant darkness shifts all cultures to the same initial phase as it does the *bd csp-1* strain. The *bd⁺ csp-1* strain was chosen for these experiments since under the conditions used, cultures of *bd⁺ csp-1* grow as uniform, thin mats of mycelia with no conidiation. The 18:3 and 18:2 compositions of the total lipid extracts of mycelia from the nonconidiating *bd⁺ csp-1* strain (Figure 3) oscillated in a manner similar to the oscillations reported above for total lipid extracts from the banding strain. The median mole percentage of 18:3 in the nonconidiating strain, however, was 7% higher than in the band strain, while the median mole percentage of 18:2 was 6% lower. As in the band strain experiments, the period was 20–21 h and the decrease in 18:2 approximately compensated for the increase in 18:3. The minimum in the 18:3 curve occurred when the conidiation rhythm of control band strain (*bd csp-1*) cultures was at the end of the band phase. The maximum of the 18:3 curve, however, occurred when the control *bd csp-1* cultures were at the beginning of the band phase, i.e., 2–3 h earlier than the 18:3 maximum in total lipid extracts from band strain cultures. Why the oscillations in the nonconidiating strain were more symmetrical than in the band strain is unknown. It is difficult to compare the amplitudes of the fatty acid oscillations in the band strain with those in the nonconidiating strain, since in both cases the oscillations tended to gradually fade out, perhaps due to desynchronization of the cultures pooled. It is evident, however, that the amplitudes of the 18:3 and 18:2 oscillations in the nonconidiating strain (Figure 3) were a significant percentage of the amplitudes of the analogous oscillations in the band strain (Figure 2). The differences between the minimum and maximum of the first halves of the 18:3 and 18:2 curves for the nonconidiating strain were significant at the 99% confidence level, while the differences between extemes for the latter halves of these curves were significant at the 95% confidence level.

Rhythmic Fatty Acid Oscillations in the Phospholipids from bd csp-1 and bd⁺ csp-1. To determine if these oscillations in fatty acid composition also occurred in the phospholipids of *Neurospora* membranes, we subjected aliquots of the total lipid extracts of the samples investigated above (Figures 2 and 3) to silicic acid chromatography (see Materials and Methods). The mole percentages of the five major fatty acid components of the band (*bd csp-1*) and the nonconidiating (*bd⁺ csp-1*) strain phospholipids are plotted in Figures 4 and 5, respectively, as a function of the time the cultures were grown in the dark. The median mole percentages of 18:3 in the phospholipids were 5 mol % points higher than in the total lipids from the same strain, while the median mole percentages of 18:0, 18:1 and 18:2 were somewhat lower. The amplitudes of the oscillations in 18:3 and 18:2 in the total lipids and in the phospholipids from the nonconidiating strain (*bd⁺ csp-1*) were very similar (Figures 3 and 5). The amplitudes of the oscillations in 18:3 and 18:2 of phospholipids from the band strain (*bd csp-1*) (Figure 4) appear to be larger than the amplitudes of the analogous oscillations in the total lipids (Figure 2) for two reasons. First, the average curve for the phospholipids was plotted at only 2-h intervals instead of 3-h intervals. Second, the 52-h phospholipid samples from experiments B1 and B4, which had lower than average 18:3 in the analogous total lipid samples, had to be discarded due to contamination (see below). When analogous samples were compared to obtain average 18:3 and 18:2 compositions at the 52-h maximum and the differences between minima and maxima calculated, the amplitudes of the 18:3 and 18:2 oscillations in the total lipids and phospholipids from the band strain were found to be identical. One would have expected the amplitudes of the fatty acid oscillations in the phospholipids to be greater than in the total lipids if the changes in 18:3 and 18:2 content had been localized exclusively in the phospholipids. Since no difference was observed, some oscillation in these fatty acids must have also occurred in the neutral lipids.

Control Experiments. Since the amplitudes of the oscillations reported above were small, it was important to consider the reliability of the methods used in obtaining these data. Three potential problems, autoxidation, recovery, and interference by contaminants, were considered. No detectable autoxidation of the purified fatty acid methyl esters of 18:2 or 18:3 was observed when samples were reanalyzed after storage for 4 months at −20 °C as petroleum ether solutions in vials flushed with nitrogen. The 18:3 composition of *bd csp-1* samples that were reanalyzed the same day deviated ±0.14 mol % unit from the mean, 5 days apart, ±0.28 mol % unit, and 4 months apart, ±0.1 mol % unit. The average composition for 18:3 in all the *bd csp-1* samples was 37.8 mol % unit. Hence, on the average, repetitive gas–liquid chromatographic analyses agreed within ±0.7%. Furthermore, since the above purified fatty acid methyl ester samples were dried 2 or more times under an air stream with no decrease in 18:2 or 18:3 content, we concluded that drying under a stream of nitrogen was not necessary. Autoxidation of 18:2 and 18:3 was not a significant problem during earlier steps of the purification procedure either since samples harvested at the same times gave nearly identical results whether or not antioxidant (0.05% BHT) was included in the solvents. In addition, samples that were prepared by using multiple precautions against autoxidation, i.e., 0 °C, little exposure to light, and nordihydroguaiaretic acid as an antioxidant, did not show increased levels of 18:2 or 18:3 compared to the samples prepared by the usual, more rapid procedure. Thus, in these experiments, use of antioxidants in the solvents, nitrogen, and other precautions gave no detectable change in the mole percentages of 18:2 and 18:3 recovered. Furthermore, in an early experiment, the total lipids in the mycelia were extracted and transesterified directly in benzene–sulfuric acid–methanol (5:1:10 v/v) at 65 °C for 5 h (Brody & Allen, 1972). While

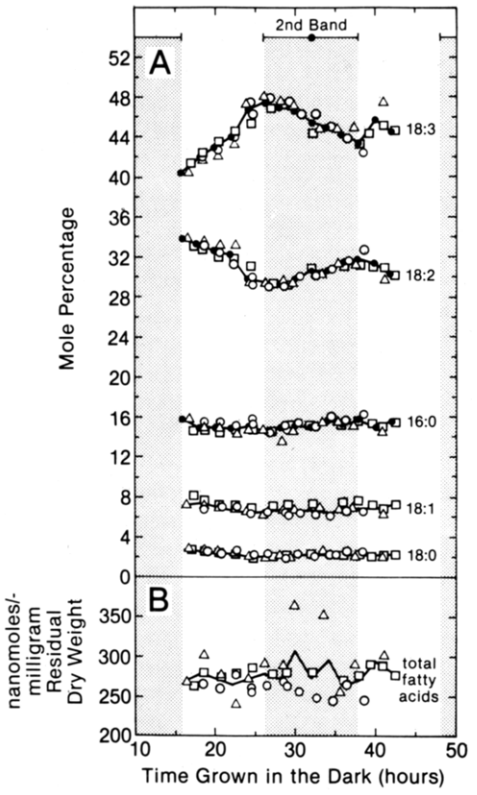

FIGURE 3: Fatty acid composition and content of total lipid from a nonconidiating strain as a function of the time the cultures were grown in the dark. Samples from the growing front of cultures of the *bd*$^+$ *csp-1* strain were harvested at various times and their fatty acid compositions analyzed as described in the text. Data points represent 28 separate analyses from three experiments: N1 (○), N2 (△), and N3 (□). The shapes of the curves were estimated by averaging all the points within ±1 h of each even-numbered hour. Bars at the top indicate times when control *bd csp-1* cultures formed conidiating bands.

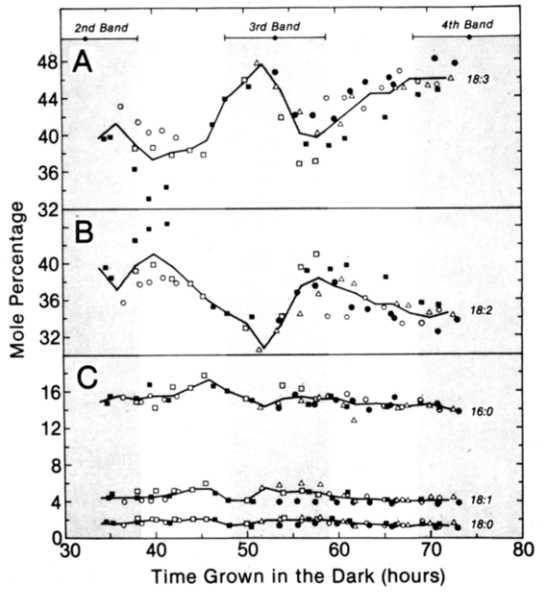

FIGURE 4: Fatty acid composition of phospholipid from the band strain (*bd csp-1*) as a function of the time the cultures were grown in the dark. Aliquots of the samples described in Figure 2 were subjected to silicic acid chromatography and the fatty acid compositions of the purified phospholipids determined. The symbols used are the same as those defined in the legend of Figure 2. The shapes of the curves were estimated by averaging all the points within 2-h intervals.

the average 18:3 content was slightly lower, perhaps due to some decomposition (Christie, 1973), the character of the 18:2 and 18:3 oscillations was the same.

Four experiments were carried out in order to demonstrate that maximum recovery was achieved during the various stages of lipid purification. First, the amount of lipid remaining in the mycelial residue following each extraction step was determined by direct transesterification of the residue in benzene–sulfuric acid–methanol (5:1:10 v/v) at 65 °C for 5 h (Brody & Allen, 1972). Ninety percent of the lipid was removed by the initial methanol extraction, 9% by the first chloroform–methanol (1:1) extraction, and only 1% by the final chloroform–methanol (1:1) extraction. In an earlier study, alternative extraction methods were shown to be less efficient (Hubbard, 1974). Second, thin-layer chromatographic analysis of aliquots eluted from silicic acid columns showed that the neutral lipids were completely removed by 50 mL of chloroform, while 135 mL of methanol was required to remove all the phospholipids. The recovery of neutral plus phospholipids following column chromatography was 93 ± 7%. Third, there was no significant difference in the yield or composition when aliquots of a total lipid sample were transesterified at 0 °C by using the usual methanolic potassium hydroxide method or at 65 °C by using benzene–sulfuric acid–methanol (5:1:10 v/v) (Brody & Allen, 1972). Furthermore, use of redistilled and dried methanol (Christie, 1973) was shown to be unnecessary. Fourth, gas–liquid chromatographic analysis of a series of 17:0 standards prior to sample analysis assured the linearity of the detector response in the range used. While the recovery in nanomoles of FAMEs per milligram of RDW varied, presumably due to error in recovering the dry mycelia prior to weighing, there was no correlation between the recovery and the phase of the fatty acid oscillations.

In the experiments reported in this paper, several precautions were taken to eliminate and/or detect any contaminants. All glassware was soaked in dichromate–sulfuric acid cleaning solution and rinsed first with water and then with methanol. Vial caps were lined with foil. The purity of the solvents (analytical reagent grade) was demonstrated by running solvent blanks through the entire procedure parallel to the samples in each experiment. In spite of these precautions, 7 of the 62 phospholipid samples from *bd csp-1* and 4 of the 38 phospholipid samples from *bd*$^+$ *csp-1* from various phases had to be discarded because they exhibited a characteristic pattern of contamination, i.e., a peak that partially overlapped and hence broadened the 18:0 peak, an unusually large 16:0 peak, and two or more large, slow moving components that had to be removed by heating the GLC column to 220 °C for 30 min. In all the experiments reported above, the column was routinely heated at 220 °C for 30 min in order to detect and remove any contaminants prior to analysis of the subsequent sample. The total lipid samples showed no evidence of contamination, suggesting that the source of contamination was unique to the phospholipid purification procedure, possibly the rotary evaporation step. No further contamination was observed in experiments subsequent to those reported when this step was eliminated by purifying the phospholipids on micro silicic acid columns. We now suspect that early observations of a large amplitude oscillation in the percentage of the fatty acids that were unsaturated in *Neurospora* were produced by contamination of some of the few samples that had been analyzed up to that point. Extensive data have been accumulated and reported above showing that such an oscillation does not exist but that there is a low amplitude oscillation in the 18:2 and 18:3 content.

Discussion

The oscillations in the mole percentage of 18:3 and 18:2 in lipids isolated from growing front samples of surface cultures

of *Neurospora* were shown to have two properties of a circadian rhythm: first, the period of these fatty acid oscillations was about 20 h, similar to the period of the conidiation rhythm and, second, the maxima and minima of these fatty acid oscillations were phase set by changing the length of the initial exposure to light. Furthermore, a preliminary experiment at 30 °C (P. Roeder, unpublished results) suggested that the period at this higher temperature was the same as at 21 °C.

There are three possible roles for these fatty acid oscillations in terms of the *Neurospora* circadian rhythm. The first possibility, that the fatty acid changes were merely part of the biochemical reaction sequence involved in the conidiation process, was experimentally eliminated by demonstrating their presence in the *bd*$^+$ *csp-1* strain, which does not exhibit a conidiation rhythm. The shapes of the fatty acid oscillation curves for the band strain (*bd csp-1*) and nonconidiating strain (*bd*$^+$ *csp-1*) were similar, although in *bd*$^+$ *csp-1* the decrease in 18:3 following the maximum was more gradual than in *bd csp-1* and the amplitudes of the changes in the 18:3 and 18:2 were smaller. While some of these differences might be due to the cyclical presence of aerial hyphae and conidia in the *bd csp-1* strain, the oscillations in 18:3 and 18:2 composition were largely independent of the developmental events that allowed expression of the conidiation rhythm. The small differences between the two strains in the fatty acid oscillations might be due to their different growth rates and the ages of the cultures when sampled.

The second possibility is that these oscillations are simply another expression of the circadian clock, i.e., a "hand" similar to the visible conidiation rhythm. Since a number of enzyme and transport systems are known to be sensitive to changes in the overall fluidity of membranes and/or the fluidity of smaller domains within a membrane (Singer, 1974), it would be interesting to determine whether these fatty acid oscillations produce a measurable oscillation in the overall fluidity or some other physical property of *Neurospora* membranes. Although the oscillations in overall fatty acid composition in *Neurospora* appear to be relatively small, it might be expected that certain areas of particular membranes, such as those surrounding very hydrophobic proteins, could be sensitive to these small changes in membrane structure. For example, the F_0 portion of the mitochondrial ATPase contains a hydrophobic protein, which is soluble in chloroform–methanol, and mutations affecting this protein alter the period of the *Neurospora* rhythm (Dieckmann & Brody, 1980). It will be of interest to see if the role of fatty acids in the biological clock is related to the role of this hydrophobic protein in determining periodicity.

The third possible role for the fatty acid oscillations is as part of the actual clock mechanism. Deciding whether or not a given cellular component is part of the mechanism itself is not simple. Some criteria for analyzing the role of a given biochemical component have been stated by Engelmann & Schrempf (1980) and can be expanded somewhat as follows: (1) the level or activity of the component should oscillate in a circadian fashion; (2) changing the level of this component should produce a change in the period or phase of the component's rhythm as well as producing a change in other observable rhythms in this organism; (3) an increase or decrease in the level of this component should produce opposite effects on the period length of an observed visible rhythm; (4) changes in the phase and/or period of the visible rhythm due to entrainment or phase resetting by light, temperature, or chemicals should lead to corresponding alterations in the phase and/or period of the biochemical component as well. How rapidly the biochemical oscillation changed after a phase shift would also be important information to obtain. The criteria listed above are only meant to be a beginning for the analysis of this question and may not be sufficient criteria for deciding the role of a given cellular component. Additional criteria may become necessary once specific candidates are proposed to play a role in the clock mechanism.

Operationally, one could approach the question of the role of a component in at least three ways: (1) One could produce alterations in the level of a cellular component as a result of a mutation affecting either the synthesis, degradation, or activity of this component. These mutations could then be analyzed for clock effects. (2) One might employ mutant strains that have been isolated on the basis of their effects on clock properties and analyze such mutants for possible effects on the particular cellular component in question. Some, but possibly not all, of these mutants might show changes in the given component. It would be important to study these mutants under nonrhythmic conditions as well, in order to assess the effects of clock mutations on the level of a cellular component under conditions where the effects of different amplitudes of the oscillation due to the clock mutation would be minimized. (3) Another approach is to add the component to the organism and monitor any clock effects. Alternatively, perturbations of the level of this component might be attempted via the addition of chemical inhibitors or antibiotics.

In the *Neurospora* system, only one of these three approaches has so far been tried with respect to the fatty acid oscillations. In a fatty acid requiring strain, supplementation with either 18:2 or 18:3 leads to an increase in the period from 21 to 40 and 33 h, respectively (Brody & Martins, 1979). Studies on the oscillations in fatty acid composition in these supplemented cultures would help in deciphering the role of the 18:2/18:3 oscillations. The other approaches are being undertaken, i.e., analyses of clock mutants for fatty acid composition and the analysis of mutations affecting fatty acid metabolism for clock effects. In all of these analyses, it should be kept in mind that the evidence may not be definitive, since the change in fatty acid composition might indirectly influence the kinetics of the clock without actually being a key component in the actual mechanism. Therefore, the involvement of the 18:2/18:3 oscillations in the generation of a "pacemaker" is still an open question requiring considerably more experimental evidence.

Other biochemical oscillations have been reported in *Neurospora*, and two observations can be made about these. First, in strains containing the *bd* mutation, the rhythms in the 18:2 and 18:3 content, in the nucleic acid content (Martens & Sargent, 1974), in the AMP level (Delmer & Brody, 1975), and in CO_2 evolution (Woodward & Sargent, 1973) all exhibit either a maximum or a minimum between 19 and 22 h Subjective Circadian Time (period normalized to 24 h and the center of each conidial band designated as 2200), i.e., several hours after the onset of conidiation. It is interesting that the extremes of these oscillations correspond roughly to the time when the response to illumination switches from phase delays to phase advances (Sargent & Briggs, 1967) and do not correspond to the onset of the morphological rhythm. The second observation is that the 18:3 oscillation in *bd csp-1* and the carbon dioxide rhythm are both asymmetric with a rapid decrease during the last part of the conidial band phase followed by a slow increase. Phase response curves of *Neurospora* also show a marked asymmetry (Sargent & Briggs, 1967; Nakashima & Feldman, 1980). The possibility of a "relaxation" oscillator model in circadian rhythms as an explanation for asymmetric rhythms has been noted by many

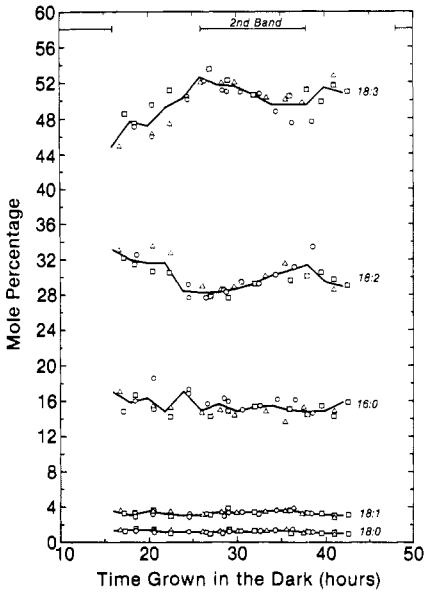

FIGURE 5: Fatty acid composition of phospholipid from a nonconidiating strain (*bd*$^+$ *csp-1*) as a function of the time the cultures were grown in the dark. Aliquots of the samples described in Figure 3 were subjected to silicic acid chromatography and the fatty acid compositions of the purified phospholipids determined. The definitions of the symbols, the bars at the top of the graph, and the method for calculating the average curves are the same as described in the legend of Figure 3.

workers and is discussed in detail elswhere (Bünning, 1973).

The primary route for synthesis of unsaturated fatty acids is by sequential desaturation of oleic acid (18:1) to linoleic acid (18:2) to linolenic acid (18:3). An oscillation in the synthesis of 18:2 and 18:3 could be produced either by cyclically activating and inactivating existing desaturases or by synthesis and degradation or dilution of new desaturases or by both mechanisms. Fatty acid desaturases have been shown to undergo relatively rapid induction or inactivation in response to sudden temperature shifts in *Bacillus megatherium* (Fujii & Fulco, 1977) and *Tetrahymena* (Nozawa & Kasai, 1978). Similar studies in *Neurospora* have not yet been reported, although the fatty acid composition does change as a function of temperature (Brody & Allen, 1972; Brody & Nyc, 1970). Since some organisms also possess the ability to form 18:3 via the elongation of 12:3 (Jacobson et al., 1973; Richards & Quackenbush, 1974), there is a possibility of the coordination of two routes for 18:3 synthesis. Regardless of the mode(s) of 18:3 synthesis, it would appear that the rapid decrease in 18:3 during the conidiating phase of the culture might occur via β-oxidation of the 18:3 and/or, in the growing tips, by dilution with phospholipids acylated with newly synthesized 18:2. This assumption is based on the fact that the fatty acid content of the membranes is constant per gram of residual dry weight and that the desaturation process is irreversible (Scott, 1977).

Since the 18:3 and 18:2 oscillations occur in the phospholipids, the membranes of *Neurospora* are clearly involved, either directly or indirectly, in circadian rhythmicity. Preliminary data have been reported suggesting circadian changes in the fatty acid composition of the phospholipid–digalactosyl diglyceride fraction isolated from *Gonyaulax* (Adamich, 1976) and the polar lipids isolated from the laminar pulvinus of *Phaseolus* (Gardner & Galston, 1977). Daily changes in fatty acid composition and desaturase activities have also been reported in rat liver microsomes (Actis Dato et al., 1973). However, the studies with rats are complicated by external factors including the feeding cycle, which is considered to be responsible either directly or indirectly for at least some of the observed rhythms. While the specific fatty acids involved in the changes in *Neurospora*, *Gonyaulax*, and *Phaseolus* are somewhat different, it is interesting that all three organisms exhibit fatty acid oscillations that may be circadian.

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Nitrogen-15 Spin–Lattice Relaxation Times of Amino Acids in *Neurospora crassa* as a Probe of Intracellular Environment†

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ABSTRACT: The nitrogen-15 spin–lattice relaxation time, T_1, and the nuclear Overhauser enhancement (NOE) have been measured for intracellular glutamine, alanine, and arginine in intact *Neurospora crassa* mycelia to probe their various intracellular environments. The relaxations of $^{15}N_\gamma$ of glutamine, $^{15}N_\alpha$ of alanine, and $^{15}N_{\omega,\omega'}$ of arginine in *N. crassa* were found, on the basis of their NOE values, to be predominantly the result of ^{15}N–H dipolar relaxation. These relaxations are therefore related to the microviscosities of the various environments and associations of the respective molecules with other cellular components that act to increase the effective molecular sizes. For $^{15}N_\gamma$ of glutamine in the cytoplasm, the intracellular T_1 (4.1 s) was only slightly shorter than that in the culture medium (4.9 s). This indicates that the microviscosity of the cytoplasm surrounding the glutamine molecules is not much greater than 1.3 cP. By contrast, for $^{15}N_{\omega,\omega'}$ of arginine, which is sequestered in vacuoles containing polyphosphates, the intracellular T_1 (1.1 s) was only one-fourth of that in the medium (4.6 s). In model systems, the T_1 of $^{15}N_{\omega,\omega'}$ in a 1 M aqueous solution of arginine containing 0.2 M pentaphosphate was 0.95 s, whereas in an isoviscous (2.8 cP) solution without pentaphosphate, the T_1 was 1.8 s. These results suggest either that the vacuolar viscosity is substantially above 2.8 cP or that the ω,ω'-nitrogens of vacuolar arginine are associated with a polyanion, possibly polyphosphate. The implications of these results for the properties of the vacuolar interior are discussed in relation to the mechanism of amino acid compartmentation.

In many eucaryotic microorganisms, intracellular amino acids are not randomly distributed in the cell but are localized within subcellular organelles (Davis, 1975). In *Neurospora crassa*, the bulk of basic amino acids such as arginine and ornithine is compartmentalized in vacuoles (Weiss, 1973). Compartmentation plays an important role in controlling arginine metabolism; it permits the accumulation of large pools of arginine without induction of the respective catabolic enzymes or degradation by preexisting enzymes.

Several hypotheses have been proposed for the mechanism by which amino acids are sequestered in the vacuoles. One possibility is that an intracellular active-transport system moves amino acids across the vacuolar membrane and maintains the resultant concentration gradient. Alternatively, it has been suggested that basic amino acids become associated with polyanions such as polyphosphates in the vacuole (Durr et al., 1979; Urrestarazu et al., 1977). Recent studies suggest that the mechanism of compartmentation is more complex. Metabolic energy is required for the movement of arginine across the membrane, but not for retention within the vacuole (Drainas & Weiss, 1982). *N. crassa* grown in low-phosphate medium do not accumulate polyphosphate but continue to sequester arginine in the vacuoles (Cramer et al., 1980). It is possible that an active-transport system moves basic amino acids into the vacuole, but the vacuolar interior has properties which enables it to retain these amino acids. To test this hypothesis, we have investigated the physical properties of vacuolar arginine and its possible association with polyanions in living *N. crassa* mycelia.

High-resolution nuclear magnetic resonance (NMR) spectroscopy is a useful technique for studying the dynamic properties of cellular components in intact organisms. ^{15}N NMR spectroscopy has been used to probe the dynamic properties of cell wall components of bacteria [Lapidot & Irving, 1979a; see Lapidot & Irving (1979b) and references cited therein]. Recently, ^{15}N spectra of intracellular amino acids in suspensions of intact *N. crassa* mycelia have been obtained (Legerton et al., 1981) and shown to provide useful information on their in vivo metabolism (Kanamori et al., 1982). Well-resolved ^{15}N resonances were observed for both cytosolic amino acids such as glutamine and alanine and vacuolar amino acids such as arginine and lysine. These results open the way to utilizing ^{15}N NMR to probe the intracellular environments of amino acids through measurements of the spin–lattice relaxation time, T_1, and nuclear Overhauser enhancements (NOE) of their ^{15}N nuclei.

The spin–lattice relaxation time, T_1, of a ^{15}N nucleus of a molecule can be a sensitive probe of its correlation time, τ_c, which is a measure of its rotational freedom. Among the various mechanisms that contribute to the relaxation of a ^{15}N nucleus, ^{15}N–^1H dipolar interaction is ofter dominant for the protonated nitrogens of amino acids. In mobile liquids where

$$\tau_{c,\mathrm{eff}}^2(\omega_N + \omega_H)^2 \ll 1 \quad (1)$$

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