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

STUART BRODY[1]* AND STANLEY A. MARTINS[2]

Department of Biology, University of California-San Diego, La Jolla, California 92093[1] and Wyeth Laboratories, West Chester, Pennsylvania 19380[2]

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Employing a fatty acid-requiring strain (*bd csp cel*) of *Neurospora crassa*, the 21.5-h period of the circadian spore-forming rhythm was manipulated by fatty acid supplementation. The addition to the medium of an unsaturated fatty acid (oleic, linoleic, or linolenic acid) lengthened the period to 26, 40, or 33 h, respectively. The period-lengthening effect of linoleic acid was proportional to its concentration up to 1.3×10^{-4} M, and also was reversed by the addition to the medium of a saturated fatty acid, palmitic acid. None of these period-lengthening effects was observed in the prototrophic strain (*bd csp cel*$^+$).

A mutant strain of *Neurospora crassa*, *bd*, displays a conidiation (spore-forming) rhythm that persists under conditions of constant temperature and darkness (17). During growth on agar medium, a mycelium alternates between phases of conidiating and non-conidiating growth. This conidiation rhythm possesses the requisite properties (16) of a circadian rhythm: (i) the rhythm has a period of approximately 1 day; (ii) it is temperature compensated; and (iii) it can be phase-shifted by light. 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 (18), it appears that this *bd* mutation simply allows the expression of an underlying rhythm. Previous studies employing this strain have shown that there are oscillations in the nucleic acid content (13), in the level of AMP (4), and in CO_2 production (21). Differences have also been found in the level of pyridine nucleotides (2) and certain enzymes (12) between the conidiating regions and the non-conidiating regions. In addition, the effects of cyclic AMP phosphodiesterase inhibitors have been reported (6) on this strain as well as the effects of mutations on the periodicity of the rhythm (7-9). This paper reports on the effects produced by exogenous unsaturated fatty acids on the periodicity of the conidiation rhythm, and is part of a larger investigation of the relationship between fatty acid metabolism and circadian rhythms. Studies on the fatty acid composition as a function of time, the effects of supplementation with other fatty acids, and the effects of temperature will be reported at a later time.

MATERIALS AND METHODS

The *bd*, *cel*, and *csp-1* strains were obtained from the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif. The *csp-1* mutation (conidial separation) interferes with the normal conidiation process (19) so that conidia are not easily detached from each other or from the aerial hyphae that produce them. The introduction of this mutation allows manipulation of the petri dishes without conidia becoming detached from the culture and reinoculating the plate. The *cel* strain has a partial requirement for saturated fatty acids (11), and has been shown to have a defect in fatty acid synthetase (5). The double mutant strain (*bd cel*) was constructed according to normal crossing procedures (3) by S. Catherine Hubbard, and then the *bd csp cel* strain was similarly constructed. All strains have been deposited and are available from the Stock Center.

Individual fatty acids of 99% purity were purchased from Sigma Chemical Co. The quality and quantity of these compounds were analyzed employing acid methanolysis (1) and found to be accurate. Solutions of these compounds when stored in ethanol and under nitrogen at −20°C showed no significant deterioration during the course of the experiments. In this paper, palmitic acid is designated as 16:0, stearic acid as 18:0, oleic acid as 18:1, linoleic acid as 18:2, and linolenic acid as 18:3.

The cultures were grown as described previously (2) on an agar medium containing inositol, maltose, and arginine. Cultures were inoculated as described previously (4), exposed to approximately 100 foot-candles (ca. 1,076 lx) of illumination for 16 to 20 h, and then placed in the dark at 21 ± 0.5°C. Large (150-mm) petri dishes containing 50 ml of medium were employed. Inoculation of the plates was at the edge, not the center, so that many days of growth could be analyzed. The plates were incubated right side up. For supplementation of the medium, individual fatty acids were separately dissolved in 95% ethanol to a concentration of 2% fatty acid. Small volumes were then added to the autoclaved medium just before pouring. The small volume of 95% ethanol added to the medium had no noticeable effect on the growth rate or periodicity of the strains.

The period length was determined in the following manner. The edge of a growing mycelium was marked in ink on the back of the plates every day under a red safelight. At the termination of the experiment, the plates were removed from the dark, and the distances of the mycelial edges from the point of inoculation were plotted as a function of time. After a lag, the growth rate, calculated in millimeters per hour, was linear with respect to time (see Fig. 1). After removal from the dark, the positions of the conidiating regions, expressed in millimeters from the inoculation point, were also measured on each plate. These positions were then marked on the growth rate plot from that particular plate, and the hours corresponding to these distances were thereby obtained. The hours for the beginning and ending of each conidiating region of a given plate were then used to calculate a center for that region. The interval between consecutive centers was taken to be a period. The statistical analysis was performed on the values for the periods collected from many different plates and often from experiments on different days. In most cases, the periods that were used were those that occurred after 40 to 45 h of growth. For cultures exhibiting long periods (Fig. 1), data were collected after 80 to 90 h.

RESULTS

Effects of fatty acids. No effects of fatty acids in the media on the periodicity of the *bd csp* strain were observed. Cultures of this strain supplemented with fatty acids 16:0, 18:0, 18:1, 18:2, or 18:3, either individually or as mixtures, had periods of 22 ± 0.5 h. However, the unsaturated fatty acids did slow the growth rate of the *bd csp* strain from 1.3 to 1.1 mm/h.

In contrast, the periodicity of the *bd csp cel* strain was affected greatly by unsaturated fatty acid supplements (Table 1). The unsupplemented *bd csp cel* strain grew about 45% slower than the *bd csp* strain, and the mycelia had a sparse appearance. Nevertheless, conidiation did occur, and the periodicity of the weak conidiation rhythm was 21.5 h (Table 1). When 16:0 was present in the medium at 1.3×10^{-4} M, both increased conidiation and growth rate were observed, although the period length remained the same (21.6 h). Twofold-higher amounts of 16:0 did not increase the growth rate further. The presence of 18:1 at 1.3×10^{-4} M in the medium increased the period by ~20% to 26 h (Table 1). Similarly, 18:2 at 1.3×10^{-4} M lengthened the period by ~90% to 40.5 h, and 18:3 at 1.3×10^{-4} M lengthened the period by ~50% to 33 h. Mixtures of 18:2 and 18:3, as indicated in Table 1, and in other ratios, did not lengthen the periods of these cultures past ~40 h. Mixtures of all of the unsaturated fatty acids in roughly the proportions found in the cellular lipids (1) also did not lengthen the periods past 40 h.

Cultures exhibiting the 26-, 33-, and 40-h rhythms were similar to the normal 22-h-period *bd csp* cultures in that abundant conidia were observed during the conidiation phase. This is in contrast to certain cultures of *Neurospora* which can exhibit a noncircadian, hyphal branching rhythm (10). Additional contrast between these types of rhythms was evidenced by the fact that a light/dark entrainment regime of 12 h of light/12 h of dark can entrain the 40-h-period cultures to 24 h (Fig. 1). This is of interest in itself since it indicates that the entraining light/dark signals can override or negate the metabolic factors that give rise to 40-h periods.

Proportionality studies. Figure 2 indicates that the period of the *bd csp cel* cultures was

TABLE 1. *Growth rate and periodicity of the conidiation rhythm in cultures of the bd csp cel strain of Neurospora crassa as a function of fatty acid supplement*

Fatty acid supplement	Fatty acid concn[a] ($\times 10^{-4}$ M)	Growth rate (mm/h)	Period[b]		
			Avg value (h)	SE	*n*
None		0.7	21.5	0.4	59
16:0	1.3	1.1	21.6	0.3	121
18:0	1.3	1.0	21.7	0.3	65
18:1	1.3	0.9	26.0	0.9	63
18:2	1.3	0.6	40.5	0.8	61
18:3	1.3	0.8	33.0	1.1	51
18:2 and 16:0	1.3 2.6	1.0	21.7	0.8	59
18:2 and 18:3	1.3 1.3	0.5	40.0	0.9	60

[a] Added as free fatty acids, as given in the text.
[b] Average value was determined as given in the text. SE, Standard error. *n*, Number of determinations of period.

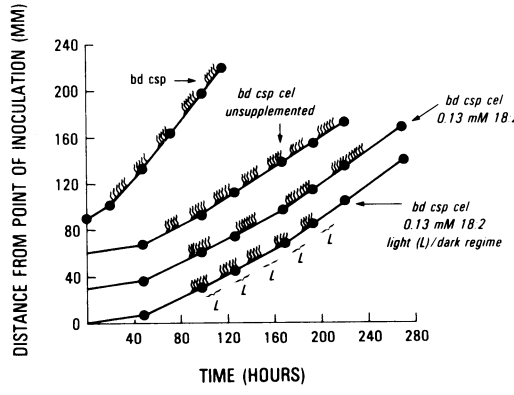

FIG. 1. *Plot of growth rate and periodicity for four Neurospora cultures. The plots of the upper three cultures have been offset 30 mm each on the graph for visual purposes only. These three cultures were in constant darkness after the initial overnight illumination. Wavy lines indicate areas of conidiation; solid circles indicate the distance from the point of inoculation to the edge of the growing mycelia at a given time.*

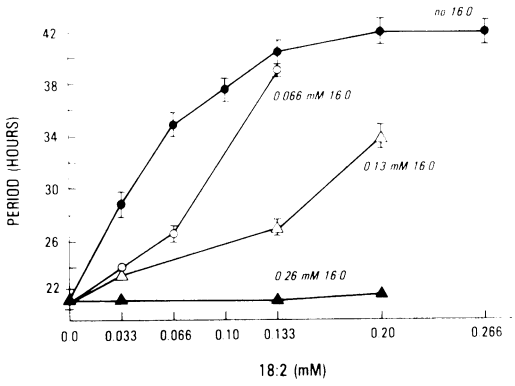

FIG. 2. *Periodicity of bd csp cel cultures as a function of 18:2 and 16:0 concentrations.*

roughly proportional to the 18:2 concentration in the medium up to the 10^{-4} M range and then reached a plateau. A replot of these data on semi-log paper indicates that the period value was linear with respect to the log of the 18:2 concentration up to 1.3×10^{-4} M. The growth rate of the colony was increasingly inhibited by the level of 18:2. At a concentration of 2.6×10^{-4} M, 18:2 cultures had a growth rate of 0.5 mm/h. The period-lengthening effects of 18:1 and 18:3 were also roughly proportional to their concentrations up to 1.3×10^{-4} M, where they reached their maximum. Concentrations of 18:1 or 18:3 above 1.3×10^{-4} M also caused progressive decreases in the growth rate of the cultures.

Reversibility studies. The period-lengthening effects of the unsaturated fatty acid, 18:2, could be reversed or "titrated" by the simultaneous addition of the saturated fatty acid, 16:0, to the medium (Fig. 2). The reversibility was proportional to the amount of 16:0 in the medium at the different 18:2 concentrations studied. Equimolar concentrations of 18:2 and 16:0 yielded cultures of *bd csp cel* whose period was 60 to 70% less than with 18:2 alone. The effect of the exogenous 18:2 on the growth rate of the cultures was also overcome by the inclusion of 16:0 in the medium (Table 1). The period-lengthening effects of 1.3×10^{-4} M 18:1 or 18:3 were also completely reversed by the addition of 2.6×10^{-4} M 16:0 (data not shown). It is not known whether reversibility of the effects of the unsaturated fatty acids by 16:0 is due to competition for uptake or to internal titration.

Other parameters. We have also studied the effects of four other variables on the growth rate and periodicity of *Neurospora* cultures. (i) The source of the exogenous fatty acids (i.e., whether they were free fatty acids or in a Tween-detergent form [1]) appeared not to matter. The Tween-detergent (Tween 40) containing 16:0 was also capable of reversing the effects of free 18:2 in the media. The effect of a reagent we synthesized (Tween-18:2) could be reversed by the free fatty acid, 16:0. (ii) The *csp* marker had only a slight (~5%) effect on the periodicity of the cultures. *bd* and *bd cel* cultures had periods of approximately 1 h longer than the corresponding *bd csp* and *bd csp cel* cultures. The *bd cel* cultures responded essentially the same to 18:2 supplementation as did the *bd csp cel* cultures. (iii) Cultures grown on race tubes (15) gave essentially identical results to cultures grown on large petri plates, as reported here. Race tubes are long cylindrical tubes which allow longer growth, 6 days or more, depending upon the growth rate of the strain. (iv) Inoculation of the petri plates with mycelial inoculum instead of a conidial inoculum produced no discernible differences. It should be pointed out that various combinations of these parameters were tried as well, and we did not find any combination of strain differences, fatty acid source, etc. that influenced these results to any significant extent.

DISCUSSION

It is not known how the exogenous unsaturated fatty acids lengthen the period of the *bd csp cel* cultures. Two basic elements that are needed for an understanding of this mechanism are a further description of the *cel* mutation and an analysis of the metabolic fate of the fatty acids in the media. It is known that the *cel* mutation definitely leads to a partial requirement for fatty acids and a substantial loss of fatty acid synthetase activity (5). However, the analysis by Elovson (5) of this mutation could not distinguish between two possibilities: (i) the fatty acid synthetase was defective because its primary structure was altered by the *cel* mutation, making the structure unable to allow the attachment of the 4′ phosphopantetheine prosthetic group necessary for its activity, or (ii) the enzyme responsible for attaching this prosthetic group to a protein, such as the fatty acid synthetase complex, was deficient in the *cel* strain. This latter possibility would be intriguing if other proteins had this prosthetic group bound to them as well. This would mean that the *cel* mutation could cause other effects in addition to those on fatty acid synthetase. Both possibilities (i) and (ii) above are under consideration.

Analysis of the fate of the exogenous fatty acids is far from complete. It is known that some ^{14}C-labeled 18:2 is taken up by the 40-h *bd csp cel* cultures and incorporated into the lipids (P. Roeder, unpublished data). However, a great deal of additional information is required on the 18:2 effect, such as a comparison of the 40-h

cultures with the 22-h cultures with respect to the composition and content of their fatty acids, phospholipids, and steroids. In addition, it would be interesting to know the comparative levels of the free fatty acids and fatty acyl coenzyme A(s), since these compounds have in vitro effects on mitochondrial functioning (20).

The slowing of the "biological clock" in this mutant strain by unsaturated fatty acids suggests various possibilities about the clock mechanism. One obvious one is that an unsaturated fatty acid-containing component is part of the clock mechanism, and supplementation distorts the level of this component. A second possibility is that these fatty acids could be inhibiting some component of the clock that has been made sensitive by the *cel* mutation. Additional studies on the effects of short-chain saturated fatty acids and unsaturated fatty acid analogs on the *bd csp cel* strain (D. Mattern, manuscript in preparation) have been directed towards resolving these possibilities.

Although the mechanism for producing longer periodicity in these cultures is under current study, three items should be pointed out: (i) the biological clock of *Neurospora* can be manipulated easily and reproducibly to any period between 21 and 41 h; (ii) the 40-h periodicity appears to be a longer change in period than any nontransient changes observed so far in any organism (14); and (iii) the effectors for these changes are not antibiotics, inhibitors, etc., but are normal cellular components.

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