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Rhythms of Enzyme Activity Associated with Circadian Conidiation in *Neurospora crassa*

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The mycelial growth front of the band strain of *Neurospora* grown on a solid surface exhibits a circadian rhythm of conidiation. Enzyme assays on extracts from that mycelium have shown that the activities of 6 of 13 enzymes (nicotinamide adenine dinucleotide nucleosidase, isocitrate lyase, citrate synthase, glyceraldehydephosphate dehydrogenase, phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase) and soluble-protein content oscillate with the visible morphological change. The rhythmic enzymes associated with the Krebs and glyoxylate cycles are more active during conidiogenesis, whereas the activities of the rhythmic enzymes of glycolysis and the hexose monophosphate shunt are reduced during that phase. The absence of enzyme oscillations in wild-type and fluffy strains which do not form conidia under the conditions employed suggests that the enzyme fluctuations are associated with conidiogenesis itself. Oscillations of enzyme activity as a function of time are restricted to the growth front. A permanent record of rhythmicity associated with conidial and nonconidial regions does, however, exist in the mycelial mat behind the growth front. The activities of three enzymes (nicotinamide adenine dinucleotide nucleosidase, glucose-6-phosphate dehydrogenase, and phosphogluconate dehydrogenase) are not directly influenced by CO_2 concentration, but are correlated with the prescence or absence of conidiation which is controlled by CO_2 concentration. In contrast, citrate synthase and malate dehydrogenase activities are correlated with changes in CO_2 concentration.

The specific activities of some enzymes extracted from cells exhibiting circadian rhythmicity oscillate with the phase of the cells. It has been difficult, however, to determine whether the observed enzyme fluctuations are part of the basic clock mechanism or manifestations of it. Examples of enzymes with rhythmic changes of activity would include the first enzyme in the Calvin cycle, ribulose diphosphate carboxylase, which may vary with the circadian rhythm of photosynthesis in *Gonyaulax* (4, 42). In *Lemna*, variations in activity of the hexose monophosphate pathway appear to be responsible for circadian rhythms of CO_2 production (23). Luciferase (15) and alanine dehydrogenase (41) provide additional examples of enzymes that fluctuate in phase with circadian oscillations at the physiological level in plants. In mammals, diurnal fluctuations of succinic dehydrogenase activity have been observed in the liver (14) and adrenal gland (13). More recently, there have been indications that changes in β-hydroxy-β-methylglutaryl-coenzyme A activity may control the diurnal rhythm of the cholesterol-synthesizing pathway in rats (36).

Although the relationship between rhythmic enzyme activity and circadian control is unclear, biochemical models have been postulated which include enzymes as major components of such control mechanisms. For example, Pavlidis and Kauzmann (28) suggested that the clock may be a system involving only two enzymes subject to feedback control. Experimental evidence indicates that high frequency oscillations (periods of a few minutes) of pyridine nucleotide concentrations in *Saccharomyces* are regulated by enzymes of the glycolytic pathway and a feedback effector molecule, adenosine diphosphate (29). However, in contrast to circadian rhythms, these particular biochemical oscillations are temperature sensitive, and there is no evidence directly relating them to circadian rhythms.

In *Neurospora*, circadian rhythms of CO_2 evolution and conidiation have been described and characterized (31, 32, 50). The experiments

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described in this paper present evidence that both the soluble protein content and some but not all enzyme activities oscillate with the conidiation rhythm in the band strain of *Neurospora*. Various mutant strains of *Neurospora* were also examined to determine whether the observed enzyme rhythms might reflect processes that oscillate in the absence of conidiation or biochemical steps necessary only for conidiation. In addition, the effects of physiological age and CO_2 concentration on the circadian rhythmicity and enzyme activity were investigated. (This work was taken in part from a thesis submitted by M.L. H. to the University of Illinois in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Strains. The following strains of *Neurospora crassa* were used: wild type (74-OR8-1a); band (*bd*, MLS 41-4); fluffy (*fl*, C-1835); skin (*sk*, B106); *cot-2a* (MLS 48-1a); amycelial (*amyc*, K422); and slime (*fz*; *sg*; *os-1*). The band and *cot-2a* strains, and strain maintenance and preservation, have been previously described (34; C. Eggerding, J. A. Randall, and M. L. Sargent, manuscript in preparation). The wild-type and slime strains were provided by D. D. Perkins and H. D. Braymer, respectively, whereas all other strains were obtained from the Fungal Genetic Stock Center (FGSC), Humboldt College, Arcata, Calif.

Culture conditions. Our maltose medium contained 0.5% (wt/vol) maltose, 1.5% agar (Difco), and Vogel salts (48), whereas the medium used for isocitrate lyase induction contained 1.0% acetate and 0.1% sucrose instead of maltose. The acetate medium used for derepression of repressible alkaline phosphatase has been previously described (16). The phosphate concentration had to be reduced to 50 μM to get derepression of that enzyme on the petri-plate cultures. The agar surface of petri-plate (15 cm diameter, 35 ml of medium) and baking-dish (19 by 30 cm, 125 ml of medium; reference 22) cultures was overlaid with a single layer of dialysis tubing (4.65 inches [ca. 11.8 cm] in flat width, Union Carbide) which had been sterilized by soaking in water for 24 h and then in 70% ethanol for at least 6 h. The baking-dish cultures were covered by pieces of plate glass resting on strips of cotton taped to the sides of the dishes.

The media in petri-plate cultures were inoculated with a conidial suspension (20 μliters) to induce uniform germination. Baking-dish cultures were inoculated with conidial suspensions using a procedure previously described (22). Inoculation was done on top of the dialysis tubing, and the resulting growth occurred there. After inoculation, cultures were synchronized with laboratory light (20 h for band, 12 h for fluffy and wild type) and incubated in the dark at 22 C (petri plates) or 25 C (baking dishes). Cultures were kept in either a light-tight darkroom (baking-dish cultures) or growth chambers (petri-plate cultures) draped with a black cloth to seal out light. The only illumination utilized came from ruby-red safelights (GE BBX, 40 W) which do not affect *Neurospora* rhythmicity (32). For routine harvesting, approximately 5 mm of the growth front (mycelia and conidia) were scraped off the dialysis tubing and immediately lyophilized.

For CO_2 concentration experiments, petri-plate cultures with a 1.5% sucrose medium (including Vogel salts and 1.5% agar) were inoculated with 2×10^5 conidia (0.1 to 0.2 ml of inoculum) spread evenly over the dialysis tubing. The cultures were incubated in the dark (25 C) in a sealed fish tank (32 by 61 by 41 cm) through which various mixtures of CO_2 and compressed air flowed. Rates of flow were controlled and monitored by purge meters (model 1555, Brooks Instrument Division, Hatfield, Pa.). After 48 h of growth, the entire mycelial mat of these cultures was scraped off the dialysis tubing and lyophilized.

Growth conditions and harvesting procedures for the wild-type, band, and *cot* strains for determination of nicotinamide adenine dinucleotide nucleosidase (NADase) activity were the same as those used in the CO_2 experiments. The aconidial strains, fluffy, skin, amycelial, and slime, were inoculated with vegetative hyphae. The fluffy strain was harvested after aerial hyphae were formed (2 days), whereas the skin, amycelial, and slime strains were harvested after 6 days of growth and lyophilized. Shake cultures (Vogel salts and 1.5% sucrose) of the fluffy strain were grown for 3 days (25 C) in Florence flasks and harvested by vacuum filtration. Cultures of the fluffy strain with synchronized aerial hyphae were produced using a method described by Stine and Clark (40). The mycelial mat and aerial hyphae were pressed dry after 7 h of growth and lyophilized.

Cell extraction. Cell extracts for all enzyme assays were prepared by grinding the mycelial pads in a Ten Broeck tissue grinder using the buffer of the enzyme assay (0.5 mg/ml). Cell debris was removed by centrifugation at 20,000 $\times$ *g* for 20 min (4 C).

Enzyme assays. Except where specified otherwise, all enzyme assays were carried out at 25 C and had a final volume of 1.0 ml, containing 0.1 to 0.2 ml of mycelial extract and 100 μmol of phosphate buffer (pH 7.4). All organic acids utilized were sodium salts except where specified otherwise. All absorbency measurements were made with a model 240 Gilford recording spectrophotometer.

To measure NADase (EC 3.2.2.5) cell extracts were incubated at 37 C for 3 min with 1 μmol of NAD (17). The reaction was stopped with 3 ml of 1 M NaCN, and the uncleaved NAD was measured at 340 nm. Phosphogluconate dehydrogenase (PGDH) (EC 1.1.1.44) and glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) activities were assayed by following the increase in absorbency at 340 nm caused by the formation of reduced nicotinamide adenine dinucleotide phosphate (7). The assay mixtures contained 1.0 μmol of nicotinamide adenine dinucleotide phosphate and 3 μmol of either 6-phospho-D-gluconate or glucose-6-phosphate. Glyceraldehyde-phosphate dehydrogenase activity (GAPDH) (EC 1.2.1.12) was also measured at 340 nm in a system containing 300 μmol of tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.5), 1 μmol of NAD, 60 μmol of Na_2AsO_4, 60

μmol of NaF, 1.66 μmol of glyceraldehyde-3-phosphate (barium salt), and 10 μmol of cysteine (18). Glutamate dehydrogenase (GDH) (EC 1.4.1.4) and malate dehydrogenase (MDH) (EC 1.1.1.37) activities were measured by following the decrease in absorbency at 340 nm caused by the oxidation of reduced NAD (NADH) or NADPH (24, 46). The GDH assay mixture contained 100 μmol of Tris-hydrochloride (pH 7.8), 125 μmol of NH_4Cl, 100 μmol of NADH (NADPH), and 15 μmol of α-ketoglutarate. The cell extract and buffer were incubated together for 20 min (25 C) to achieve full activation of the enzyme. The MDH assay system contained 1.0 μmol of oxaloacetic acid and 0.1 μmol of NADH.

The citrate synthase (EC 4.1.3.7) assay solution contained 0.05 μmol of acetyl-coenzyme A, 2.0 μmol of oxaloacetic acid, 4.0 μmol of $MgCl_2$, and 0.5 μmol of 5,5′-dithiobis (2-nitrobenzoic acid) (DTN) (1). The increase in absorbency caused by DTN reacting with the enzymatically released coenzyme A was followed at 412 nm. Malate synthase (EC 4.1.3.2) was assayed using a similar solution with glyoxylate as substrate instead of oxaloacetic acid (9). Since DTN inhibits this reaction, it was used to terminate the reaction. The isocitrate lyase (EC 4.1.3.1) assay solution contained 100 μmol of phosphate buffer (pH 7.0), 5 μmol of $MgSO_4$, 20 μmol of phenylhydrazine, 10 μmol of cysteine, and 6.6 μmol of isocitrate (G. H. Dixon and H. L. Kornberg, Biochem. J. **72**:3P, 1959). The increase in absorbency caused by the formation of a complex between enzymatically released glyoxylate and phenylhydrazine was followed at 324 nm.

The constitutive alkaline phosphatase (EC 3.1.3.1) and repressible alkaline phosphatase (no EC number) assay solutions contained 300 μmol of Tris-hydrochloride (pH 9.0) and 37.5 μmol of β-glycerophosphate. The repressible alkaline phosphatase solution also contained 7.5 μmol of ethylenediaminetetraacetic acid which inhibits the constitutive alkaline phosphatase (26). The acid phosphatase (EC 3.1.3.2) assay mixture contained 150 μmol of acetate buffer (pH 5.6) and 37.5 μmol of β-glycerophosphate (19). All three phosphatase assays (final volume 1.5 ml, 37 C) were terminated after 20 min with 0.5 ml of 3 M trichloroacetic acid. The enzymatically released phosphate was measured by a method previously described (30).

It was determined for all enzymes that the assays were linear with respect to time, dependent on the substrate supplied, and proportional to the amount of enzyme extract added to the reaction mixture. Units of activity for the following enzymes are: NADase, micromoles of NAD cleaved per min; isocitrate lyase, nanomoles of glyoxylate liberated per min; all three phosphatase enzymes, micromoles of phosphate cleaved per h. Units of activity for all other enzymes are defined as the increase or decrease in absorption per min. Specific activity is defined as units per mg dry weight of mycelium.

All experiments were done at least twice, and assays were done in duplicate or triplicate. Repeated assays of a given enzyme from the same powder sample routinely yielded values within 0 to 4% of the mean. A data point in Fig. 2 represents an individual assay, whereas in Fig. 3 to 10 it represents the average of two to three assays.

Soluble protein of cell extracts was determined by the method of Lowry et al. (21), using bovine serum albumin as a standard. All chemicals used in the assays came from Sigma Chemical Co.

Gel electrophoresis. The procedure described by Ornstein and Davis (6, 27) was utilized to make polyacrylamide gels. Approximately 100 μg of protein from cell extracts was mixed with the sample gel and subjected to electrophoresis for 1 h at 2.5 mA. Proteins were fixed and stained with 0.05% Coomassie blue (Colab Laboratories, Inc.) in 10% trichloroacetic acid. Densitometer tracings of the gels were made at 553 nm using a Gilford spectrophotometer with a linear transport accessory.

RESULTS

Nature of circadian growth. The band strain inoculated in the center of a petri-plate culture produced concentric rings of conidial bands (Fig. 1). A sparse first band of conidia (Fig. 1A) began to form after approximately 8 h of growth in the dark, and a more dense second band (Fig. 1C) was initiated after 29 h. The area between the 1st band and the 2nd band was designated as the 1st interband region (Fig. 1B). To obtain longer growth, the petri plates were inoculated near the edge of the dialysis tubing, and from these cultures a 2nd interband region

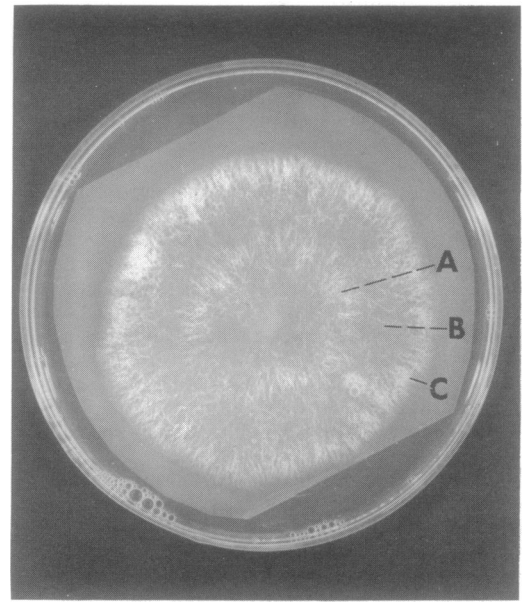

FIG. 1. *Band strain inoculated on dialysis tubing in the center of a petri plate (15 by 2 cm) and grown (maltose medium) for 20 h in the light followed by 36 h in the dark (22 C). (A) 1st band region; (B) 1st interband region; (C) 2nd band region.*

and 3rd band was harvested. At 22 C the growth rate of the band strain in these cultures was 34.4 ± 1.4 mm/day, and the period was approximately 20.5 h. Under these growth conditions, the wild-type strain had a growth rate of 54.4 ± 4.1 mm/day and exhibited little or no conidiation.

Under constant conditions (continuous darkness after 20 h of light, 25 C) the band strain produced eight distinct bands of conidia in the larger baking-dish cultures (22). In such cultures, the wild type also formed some conidia, and sparse banding patterns were observed. Reduced conidiation in closed growth containers (petri-plate cultures are presumably less well aerated) has been shown to be a result of CO_2 accumulation (33). The aconidial strain, fluffy, also exhibited a weak banding pattern in these baking dishes, with tufts of aerial hyphae and areas of increased mycelial branching being formed where conidial regions would have been produced by the band strain.

During the conidiation cycle, the growth front of the band strain transitioned from a strictly vegetative state through the stages involved with producing conidia, and then back to the vegetative stage. These morphological oscillations (periodic differentiation) possess characteristics consistent with the criteria for a circadian rhythm (31, 32).

Circadian oscillations of enzyme activity. The data in Fig. 2 and 3 show changes in enzyme activity as a function of time in the growth front (the first 5 mm) from cultures grown on the maltose medium in petri-plate cultures. The activities of NADase, citrate synthase, and isocitrate lyase were high during conidiogenesis and decreased during the vegetative growth phase. These enzymes showed their largest change in activity between the 1st interband region and the 2nd band, with 415, 98, and 74% increases, respectively. Isocitrate lyase activity could not be detected in cultures grown on the maltose medium, so the data for the enzyme represent activity in cell extracts from mycelia grown on 1.0% acetate and 0.1% sucrose. No visible differences in morphology or banding pattern were observed when the acetate-sucrose medium was utilized instead of the maltose medium. With acetate alone the cultures grew poorly, and thus 0.1% sucrose was used to enhance mycelial production.

The activities of GAPDH, G6PDH, and PGDH were low during conidiogenesis (especially the later stages) and increased during the vegetative growth phase. The largest change in activity occurred between the 2nd band and 2nd interband, with increases of 117, 400, and 195%, respectively.

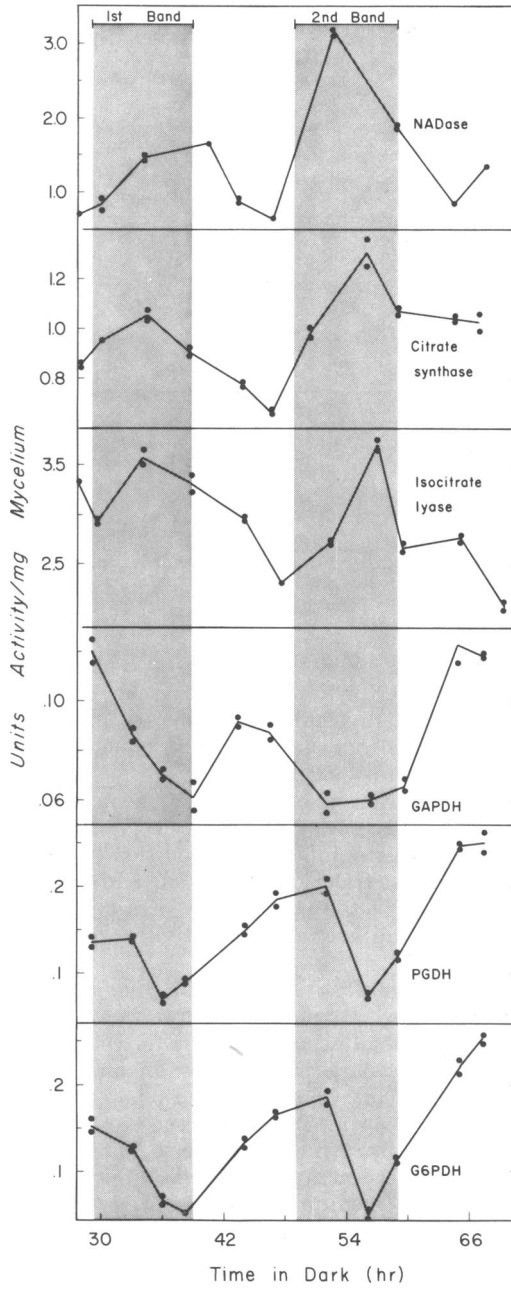

FIG. 2. *Enzyme activities in the growth front of the band strain in petri-plate cultures. Bars at top indicate periods of conidiation. Each point represents one assay.*

After 4 to 6 days of growth (band strain) in baking-dish cultures (Fig. 3), both NADase and

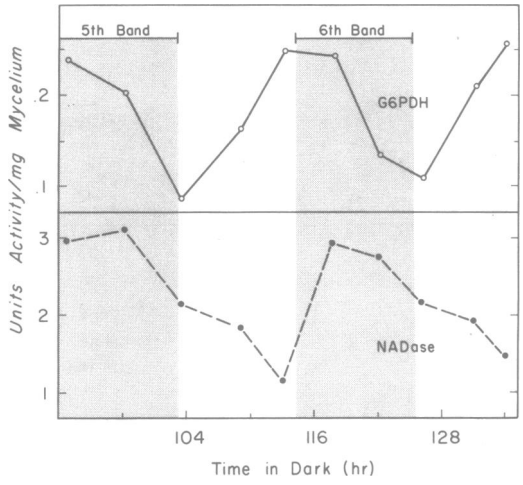

FIG. 3. *Enzyme activities in the growth front of the band strain after 4 to 6 days of growth in the dark (baking-dish cultures). Bars at top indicate periods of conidiation. Each point is the average of two determinations.*

G6PDH still exhibited pronounced oscillations of activity (20.5-h periods), thereby demonstrating that rhythmicity persists in older cultures and is not a short-term phenomenon associated with inoculation.

In comparison, none of the enzyme activities in Fig. 4 appeared to be influenced significantly by circadian rhythmicity. In addition to these five enzymes, GDH (NAD-linked) and malate synthase exhibited no evidence of a circadian oscillation and had very low activities (maltose medium).

Effect of maltose on alkaline phosphatase derepression. To obtain derepression of the repressible alkaline phosphatase for the previous experiment, a medium low in phosphate was utilized (16). Changes in phosphate concentration (0 to 44 mM) affected the growth rate and amount of mycelia produced, but had no apparent effect on conidiation rhythmicity. On the maltose medium, however, even without phosphate, derepression of the enzyme did not occur. To confirm the suspected repression of this enzyme by maltose, plates with different media (maltose or sucrose-acetate) were inoculated with vegetative mycelia which already had high levels of repressible alkaline phosphatase (1.98 units/mg of mycelium). After 58 h, the growing front from plates with maltose in the medium had no detectable enzyme activity, whereas growing fronts from plates with sucrose-acetate medium had high levels of activity (2.05 units/mg of mycelium) comparable with the original.

Permanent record of circadian enzyme oscillations. To determine whether or not the oscillations in enzyme activity occurred throughout the fungal culture, various parts of the mycelial mat were harvested after 98 h of growth (78 h in the dark). Conidial regions possessed higher NADase activities and lower G6PDH activities than the interband regions (Fig. 5). When the same band (2nd band) and interband (1st interband) were harvested over a period of 66 h (one petri-plate culture harvested every 6 h from a group of synchronized cultures), the band region exhibited higher NADase but lower G6PDH activities throughout that time period (Fig. 6). As a culture aged, the NADase activity decreased and the G6PDH activity increased in both the band and interband regions. Therefore, the amount of enzyme to be found in a particular location of a culture is determined both by the phase of the circadian rhythm when that region was the growing front and the age of that region. The enzyme activity may slowly increase or decrease as the culture ages, but there is no evidence for pronounced

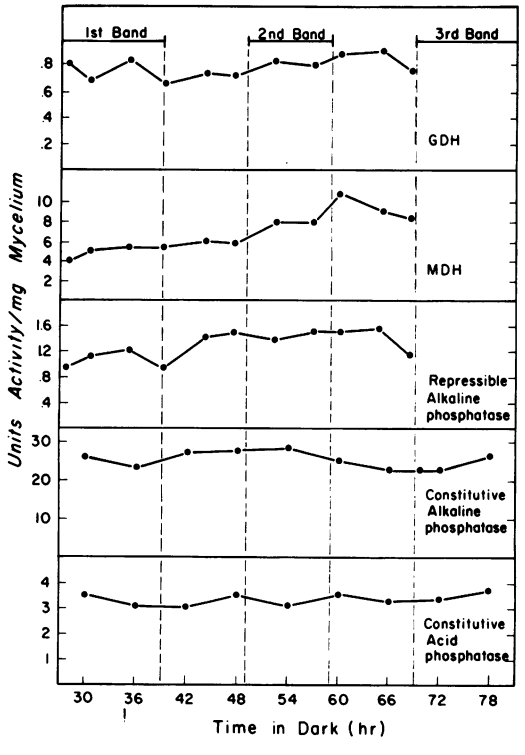

FIG. 4. *Enzyme activities in the growing front of the band strain in petri-plate cultures. Bars at top indicate periods of conidiation. Each point is the average of two or three determinations.*

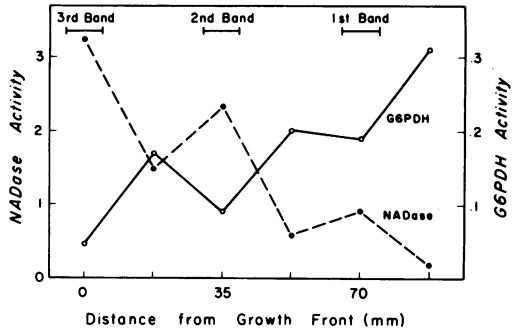

FIG. 5. *Enzyme activity (units/mg of mycelium) in different regions of a mycelial pad (band strain) harvested at one specific time (78-h petri-plate cultures). Bars at top indicate regions of conidiation. Each point is the average of two determinations.*

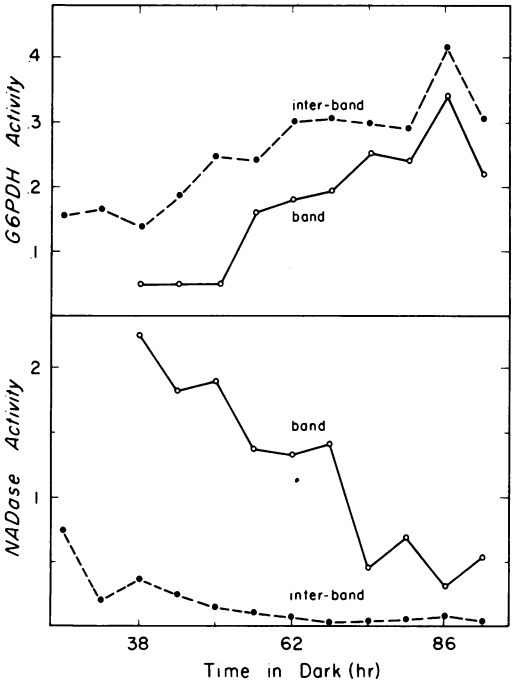

FIG. 6. *Enzyme activities (units/mg of mycelium) in a conidial band and interband region (band strain) as a function of culture age in petri-plate cultures. The 1st interband region and 2nd conidial band were harvested every 6 h from replicated cultures. Each point is the average of two determinations.*

oscillations within that region caused by circadian metabolic changes or cytoplasmic streaming.

Absence of circadian enzyme oscillations in wild type and fluffy. Enzyme activities (Fig. 7 and 8) in the growth fronts of both the wild-type and fluffy strains showed little or no evidence of circadian regulation. Also, in contrast to the band strain, no evidence of a permanent record of circadian changes in enzyme activity existed in the wild-type strain, but, as with the band strain, the NADase activity decreased and the G6PDH activity increased with culture age (Fig. 9).

NADase activity in various strains of Neurospora. Under the growth conditions utilized (petri plates, maltose medium), NADase activity was unexpectedly not detected in the fluffy strain. Since Stine (38) reported that the fluffy strain has high levels of NADase activity in all morphological stages of growth, a number of aconidial strains were tested in an attempt to resolve this inconsistency. The data in Table 1 show that the level of NADase activity in a given strain is related to its ability to produce conidia. The band strain, which produced the most conidia under these growth conditions (33) had the highest level of NADase, whereas strains that do not form conidia (skin, slime, amycelial, and *cot-2* grown at 35 C) had no detectable enzyme. Since no enzyme activity was detected in the fluffy strain from cultures

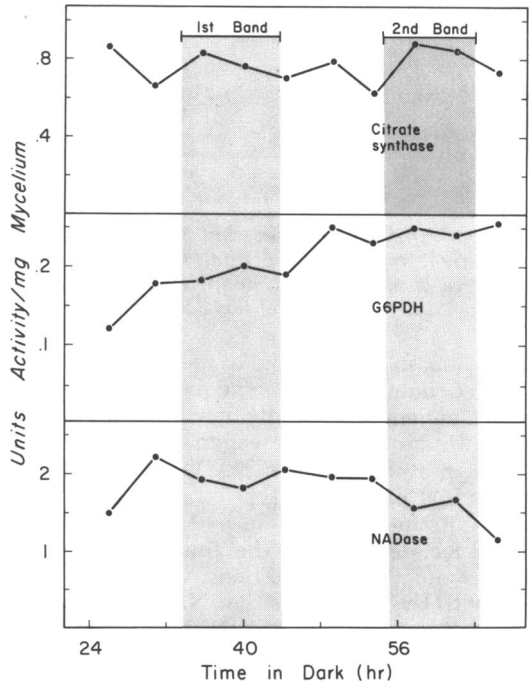

FIG. 7. *Enzyme activities in the growth front of a wild-type strain in baking-dish cultures. Bars at top indicate periods of sparse conidiation. Each point is the average of two determinations.*

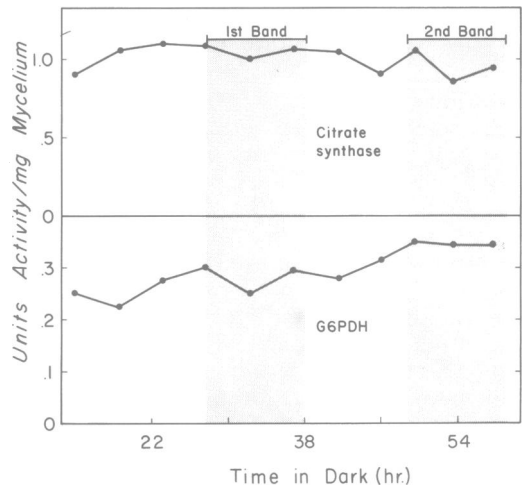

FIG. 8. *Enzyme activities in the growth front of the fluffy strain in baking-dish cultures. Bars at top indicate periods of denser growth and aerial hyphae formation. Each point is the average of two determinations.*

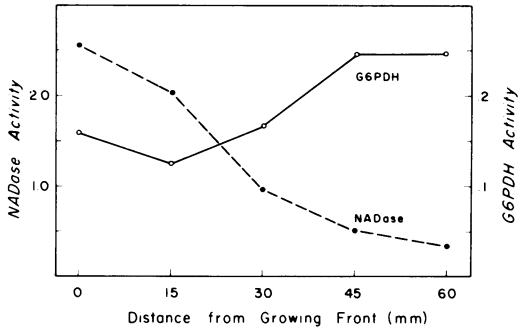

FIG. 9. *Enzyme activities (units/mg of mycelium) in different regions of the wild-type mycelial pad harvested at one specific time (petri-plate cultures). Each point is the average of two determinations.*

grown on solid medium, it was also grown in aerated liquid cultures and under conditions which enhance the production of aerial hyphae (40). Under the latter conditions, Stine (38) reported fluffy to have NADase levels many times that of the wild-type strain. However, in our experiments, only low levels of NADase could be detected in the fluffy strain when grown under these conditions, which is consistent with the hypothesis that NADase is associated with conidiogenesis (5, 39, 47, 51). The factor(s) responsible for the differences between the results reported here and those of Stine (38) have not been identified. All obvious variables, e.g., strain differences (strains of the same FGSC number were used), have been eliminated.

Soluble protein and gel electrophoresis. The amount of soluble protein that could be extracted from mycelia collected from growth fronts over a period of 2.5 days (Fig. 10) also showed evidence of a circadian oscillation, with a smaller amplitude (maximum of 33%) than any of the six cyclic enzymes. The soluble proteins were also subjected to electrophoresis in polyacrylamide gels. Even though mycelia and conidia are morphologically distinct, neither major differences nor cyclic oscillations could be detected in the gel patterns of the soluble proteins stained by Coomassie blue. Pronounced differences in the spectrum of soluble proteins were found, however, in extracts from cultures collected over a period of 11 days, indicating that significant changes can occur as cultures age.

Effect of CO_2 concentration on enzyme activity. It was previously demonstrated that conidiation in growth-tube cultures of the wild-type strain is inhibited by CO_2 concentrations of 0.13% (vol/vol) or greater, whereas the band strain is resistant to this inhibition with concentrations of CO_2 up to 20 to 25% (33). In petri-plate cultures (Table 2), conidiation of the wild-type strain was inhibited by CO_2 concentrations of 5% or greater, whereas conidiation of the band strain was not inhibited until concentrations of 30% or greater were utilized. In addition, growth in both strains could be inhibited by 30 or 50% CO_2 in such cultures.

Because CO_2 inhibited the circadian rhythm of conidiation and under these circumstances some enzyme oscillations were either damped or not observable, it was of interest to ascertain

TABLE 1. *NADase activity in various strains of Neurospora crassa*

Strain	Conidiation	FGSC no.	NADase activity (units/mg of mycelium)
74-OR8-1a	+	988	0.68
bd (MLS 41-4)	+	1859	1.64
cot (MLS 48-1a); 25 C	+	1512	1.02
cot (MLS 48-1a); 35 C	–	1512	0
sk (B106)	–	276	0
amyc (K422)	–	306	0
slime[a]	–	1118	0
fl (C-1835)	–	818	0
fl[b] (C-1835)	–	818	0.06
fl[c] (C-1835)	–	818	0.11

[a] *fz;sg;os-1* (B110;27947;B135).
[b] Grown in liquid bubble culture.
[c] Grown for 7 h in an inverted petri-plate culture to produce aerial hyphae (40).

what effect various CO_2 concentrations have on these enzyme activities. The data in Table 2 indicate that the activities of three of the enzymes associated with rhythmic conidiation (NADase, G6PDH, and PGDH) were not directly regulated by CO_2 concentration. Instead, their activities were correlated with the presence or absence of conidiation. NADase activity was high, whereas G6PDH and PGDH activities were low if conidiation occurred irrespective of the CO_2 concentration. In contrast, citrate synthase activity increased with increasing CO_2 concentration and did not appear to be completely associated with conidia formation. MDH activity, which demonstrated no evidence of a circadian component, also increased with increasing CO_2 concentration. With the highest concentration of CO_2 used (50%), G6PDH, PGDH, citrate synthase, and MDH all showed slightly decreased activities.

DISCUSSION

The mycelial growth front of *N. crassa* grown on a solid surface exhibits a rhythmic cycle of conidiation previously shown to possess characteristics consistent with the criteria for a circadian rhythm (31, 32). Enzyme activities monitored in the growth front as a function of time have clearly indicated that the specific activities of some enzymes oscillate with the visible morphological change. Although we have presented no data verifying that the enzyme rhythms are in fact "circadian" by the usual criteria, their synchrony with the conidiation rhythm which has been so verified is strong presumptive evidence that such a description is valid. These rhythms in activity represent oscillations in specific activity of the enzymes in crude extracts and thus provide no evidence regarding the level at which the circadian control is exercised. It remains to be determined whether the regulation involves transcription, translation, activation/inhibition by small effector molecules, or some other mechanism. Studies to date with other systems (e.g., reference 4) have been rather unsuccessful in determining the site of control.

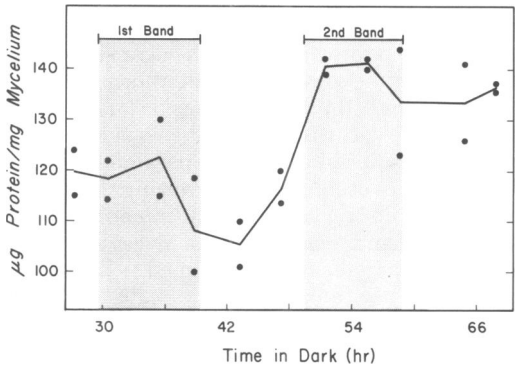

FIG. 10. *Soluble protein (extracted in 0.1 M sodium acetate, pH 5.6; 0.5 mg/ml) in the growth front of the band strain in petri-plate cultures. Bars at top indicate periods of conidiation. Each point represents the average of three determinations.*

It should also be noted that, although there is a rhythm in the amount of extractable protein, this rhythm could not be totally responsible for the enzyme oscillations that we have observed. The amplitude of the oscillation in extractable protein (about 15% above and below the median value) is smaller than the amplitude of all of the enzyme oscillations classified as circadian (40 to 200% above and below the median value). Furthermore, the extractable protein oscillations are in phase with only some of the enzyme oscillations. While the oscillations in extractable protein would, in fact, maximize the expression of NADase oscillations, at the same time they would minimize the amplitude of the oscillations in GADPH.

Another potential complication is the presence of high levels of NADase in crude extracts

TABLE 2. *Effect of CO_2 concentration on enzyme activity and conidiation in the band and wild-type strains in petri-plate cultures.*

Strain[a]	CO_2 (%)	Conidiation	Dry wt/plate (mg)	Activity (units/mg of mycelium)				
				NADase	G6PDH	PGDH	Citrate Synthase	MDH
bd	0	+	144	1.46	0.176	0.204	0.568	3.80
+	0	+	218	1.32	0.188	0.208	0.688	4.23
bd	5	+	209	1.28	0.176	0.192	0.904	6.48
+	5	−	196	0.11	0.330	0.316	1.028	7.14
bd	30	−	30	0.10	0.320	0.304	1.584	7.90
+	30	−	30	0.12	0.358	0.306	1.664	7.86
bd	50	−	14	0.13	0.232	0.212	1.252	6.51
+	50	−	28	0.08	0.237	0.218	1.180	6.34

[a] *bd*, band; +, wild-type.

that are being assayed for the three dehydrogenases (PGDH, G6PDH, and GAPDH). The NADase would be expected to hydrolyze the substrates (NAD and NADP) for these reactions. If the NADase activity in a series of extracts from different time points oscillated, then the apparent dehydrogenase activity would appear to oscillate with a reversed phase even though the actual dehydrogenase activity was constant from extract to extract. Although the rhythms of dehydrogenase activity are complicated by such a phenomenon, their existence and phase relationships are probably real for the following reasons. In the first place, the assays were done very rapidly with the increasing absorbance due to NADH or NADPH being measured within 15 to 30 s of the reaction's initiation and before the reaction became nonlinear. The addition of two- to fourfold more substrate (NAD or NADP) did not increase the measurable rates in the initial, linear reaction. Secondly, although a superficial examination of our data would indicate that high NADase activities in an extract are associated with low dehydrogenase activities, and that increasing NADase in extracts from a time series is associated with decreasing dehydrogenase activities, a closer examination of the data will show that such is not invariably true. For example, in Fig. 2 the activities of NADase, PGDH, and G6PDH are all high at 52 h, and in Fig. 3 the NADase and G6PDH activities are both decreasing during the intervals from 98 to 104 and 118 to 126 h. In addition, GAPDH is out of phase (Fig. 2) with the other two dehydrogenases, suggesting that a single factor, i.e., NADase in the crude extracts, is not responsible for the observed rhythmicity.

The six rhythmic enzymes show a sequence of activity maxima with respect to morphological change in the growth front. The NADase activity peaks early during conidiogenesis, whereas citrate synthase and isocitrate lyase exhibit highest activity during later stages of conidiogenesis. GAPDH shows highest activity during the early vegetative phase, whereas both G6PDH and PGDH peak when conidiogenesis is initiated. The phase relationships between the oscillations involving these enzymes, conidiation, light sensitivity, CO_2 production, and other biochemical parameters, are being continually studied and compared, but to date no particularly significant generalizations or predictions have emerged.

Isocitrate lyase activity exhibited rhythmicity when an acetate medium was utilized, but this rhythmicity was absent with a maltose medium. It has been demonstrated that there are at least two isocitrate lyase isozymes with different metabolic functions and that the activity of only one isozyme is enhanced by acetate in the medium (35). Since rhythmicity of isocitrate lyase is only detectable in mycelia grown on an acetate medium, it is possible that only one isozyme is regulated by the clock. A time study of the two isozymes on different media should be able to resolve whether or not they are regulated independently.

Other investigators (9, 37, 38, 43–45, 47, 49, 51) monitored enzyme activities during conidiogenesis in an effort to determine which biochemical pathways are involved in this process. However, in these experiments, conidiation was initiated by altering growth conditions to induce spore formation. Because of the somewhat artificial means of inducing conidiogenesis, changes in enzyme activity could not be conclusively correlated with spore formation. Such factors as aging of the culture of changes in the medium might have been responsible for the observed changes in enzyme activity rather than the induction of a developmental pathway.

In our cultures, the growing front remained under constant conditions. As the front of the colony moved forward across the agar surface, it was in constant contact with a uniform, unused medium, and the mycelia used for enzyme assays were of the same physiological age. Therefore, any biochemical parameters that were shown to oscillate in the growing front were presumed to be regulated by the circadian clock mechanism and many, but not necessarily all, of these parameters were presumed to be associated directly with conidiation.

The work mentioned above does suggest, however, that certain biochemical pathways are involved in the control of conidiogenesis. As our data corroborate, at least some enzymes of the Krebs and glyoxylate cycles become more active during conidiogenesis, but only after the activities of glycolysis and the hexose monophosphate shunt are reduced. The rhythms of enzyme activity reported here correlate well with the observations that (i) inhibition of glycolysis with iodoacetate, *p*-chloromercuribenzoate, or fluoride produces conditions favorable for conidia formation (49), (ii) glucose inhibits the expression of circadian rhythmicity and conidiogenesis (33), and (iii) abnormal levels of hexose monophosphate shunt enzymes in mutant, colonial strains of *Neurospora* are responsible for the altered morphology (3, 12, 20).

In addition, Flavell and Woodward (11) showed that a glucose medium that inhibits conidiogenesis also causes repression of Krebs cycle enzymes in *Neurospora* and suggested that catabolite repression is involved. Constitutive levels of Krebs cycle enzymes are produced,

however, when Casamino Acids are added to a culture medium containing glucose (10). Furthermore, Sjogren and Romano (35) found that isocitrate lyase was repressed by glucose, but that the addition of casein hydrolysate to the medium led to a constitutive level of the enzyme being produced. The effect of amino acids on the synthesis of these enzymes presumably explains why conidial formation in race-tube cultures is enhanced by high concentrations of amino acids (33).

From available data, the enzyme rhythms appear to be restricted to the growth front. However, a permanent record of circadian rhythmicity of enzyme activity does exist in the mycelial mat behind the growing front. The amount of activity found in a particular region of the culture is determined partially by the phase of the circadian rhythm when that region was the growth front. In contrast to these results, Brody and Harris (2) reported that the differences in pyridine nucleotide content between band and interband regions of *Neurospora* are evident only in the growing tips, and are eliminated within 24 h of the growth front progressing past a given region of the mycelium.

The fact that a permanent record of enzyme rhythmicity does persist in the mycelial mat supports the concept that little cytoplasmic streaming is present in aged sections of the culture. Microscopy studies by Sternberg and Sussman (manuscript in preparation) of a wild-type colony have confirmed that rapid streaming occurs in the growth front, both forward in the direction of growth and between the main branches connected by anastomoses. They also observed some cytoplasmic streaming in older regions of the colony in anastomoses between major hyphal branches, but, as our data indicate, the streaming is apparently less vigorous in older mycelia.

None of the enzymes that oscillate in the band strain showed evidence of a circadian component in either the fluffy or wild-type strains. However, circadian oscillations of nucleic acid metabolism have been found in the wild-type and fluffy strains under these growth conditions (22). The lack of enzyme fluctuations in these strains therefore suggests that the enzyme activities are closely associated with the conidiation process itself, and are not part of the timekeeping mechanism of the clock, nor are they regulated by the clock independently of the conidiation process.

Since weak circadian morphological alterations were observed in the fluffy strain although no NADase activity could be detected, it appears that this enzyme is not essential for the expression of rhythmicity. Surprisingly, levels of both NADase and its substrate, NAD (2), are higher in the band than in the interband regions. This fact suggests that the enzyme and its substrate are incapable of interacting under certain conditions, perhaps through compartmentalization into different locations within the cytoplasm. Although the function of NADase is unclear, it is associated with conidiogenesis (Table 1) and serves, therefore, as an excellent biochemical marker for monitoring the conidiation rhythm.

Although the enzymes shown to be rhythmic in this study do not appear to be directly involved in the clock mechanism (prevention of conidiation abolishes the enzyme rhythmicity), the discovery of their rhythmic activity is potentially useful in several regards. For example, one of the enzymes could conceivably be utilized for an in-depth study of the factors that regulate enzyme synthesis or activity in a circadian manner. In choosing an enzyme for such an in-depth study, however, one would prefer an enzyme whose activity was not dependent on a morphological change. Since the enzymes in this study were chosen on the basis of a previously documented relationship to conidiation (an effort to maximize the chances of finding rhythmicity at the enzyme level), it is not too surprising that the regulation of their activity is tightly coupled to the conidiation process itself. The feasibility of such in-depth studies would be enhanced by examining enzymes involved with parameters such as nucleic acid metabolism, which appear to be rhythmic in the absence of morphological oscillations.

These findings furthermore supplement the increasing information concerning rhythmicity at the biochemical level in *Neurospora* (2, 22; D. P. Delmar and S. Brody, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 38, 1973). This knowledge will presumably be critical for (i) testing the newer biochemical models to explain circadian oscillations (25; S. Brody, unpublished data), (ii) evaluating the single-gene mutants affecting the expression (34) and period length (8) of *Neurospora* rhythms, and (iii) suggesting new models concerning this form of biological rhythmicity.

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