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Effects of Medium Composition and Carbon Dioxide on Circadian Conidiation in *Neurospora*[1,2]

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ABSTRACT

Efforts to significantly perturb the timing mechanism, and thus the period, of the rhythm responsible for circadian conidiation in *bd*, a strain of *Neurospora crassa*, by altering the medium composition have been unsuccessful. Various salt solutions, sugars, and amino acids do, however, have pronounced effects on growth and conidiation, and thus on the expression and persistence of rhythmicity.

Aeration stimulates conidiation in growth-tube cultures, thereby allowing experiments which demonstrate that nearly all strains and species of *Neurospora* are capable of circadian conidiation. These results extend to *Neurospora* the generalization that physiological and developmental regulation in all eukaryotic organisms has a circadian component. Aeration also increases the persistence of circadian conidiation suggesting that the damping of rhythmicity previously observed on certain media represents the cessation of conidiation, rather than the stopping of a timing mechanism.

Aeration is apparently effective in that it maintains CO_2 levels in growth-tube cultures below a critical concentration. Carbon dioxide was shown to inhibit conidiation in both wild-type and *bd* strains, with the latter being about 200 times more resistant than the former.

The mechanisms responsible for circadian rhythmicity in eukaryotic organisms remain a mystery and source of debate. The study of circadian rhythms in *Neurospora crassa* has attracted several investigators including ourselves because of the progress made in studying the biochemistry and genetics of this species. The information available offers promise for testing the hypothesis that such rhythms are a manifestation of biochemical-genetic regulation. We are utilizing the *bd* strain of *Neurospora crassa* in these efforts because it possesses a conidiation rhythm in growth-tube cultures that is clearly defined, nondamping, and circadian by the usual criteria (16, 17). Genetic studies on *bd* (18) indicate that it is a single-gene mutant and that on some media it is phenotypically identical with the double-mutant strain, timex, from which it was derived.

One way to investigate fungal metabolism and perhaps perturb the postulated timing mechanism is to vary the growth medium. In general, perturbing the metabolism of an organism has little effect on its circadian rhythmicity (9), but changes in rhythm expression and period have been demonstrated in certain strains of *Neurospora* and other fungi by supplementing the growth medium with appropriate sugars and amino acids (3, 5, 10).

The work reported here was initiated to determine if varying the growth medium would provide useful information about the mechanisms responsible for the *bd* rhythmicity. The experiments provide a better understanding of the band-gene role in *Neurospora* rhythmicity, and suggest that aeration is an important variable in the expression of *Neurospora* rhythmicity. The effects of aeration provide new insight into the occurrence of rhythmicity in *Neurospora* and the role of carbon dioxide in regulating conidiation of *Neurospora* cultures.

MATERIALS AND METHODS

Strains. A single-mutant strain, band (*bd*), which exhibits the timex phenotype on our standard glucose-arginine medium (16) was used throughout. This strain, MLS 41-4 (*bd, a*), was isolated after three backcrosses of the original *bd* (E7a) into the St. Lawrence wild-type, 74-OR 23-1A. The sources of the other wild-type and exotic strains used have been reported, as have the methods of stock maintenance and preservation (18).

Media. The media contained Vogel's salts (22), 1.5% Difco Bacto-agar (unless otherwise stated), and the supplements listed. All percentage designations for media are weight per volume. The media were prepared, autoclaved, and dispensed (8 ml/tube) into 55-cm Pyrex growth-tubes stoppered with cotton. Heat-labile amino acids were filter sterilized and added to warm medium (45–50 C) before dispensing.

Growth Conditions. Growth-tube cultures were inoculated, incubated 18 to 20 hr at room temperature in the light, and then placed in continuous darkness at 25 C. Scoring of rhythmicity and period calculations have been described previously (16, 18).

Aeration. Aeration was accomplished by allowing a compressed mixture of oxygen (21%) and nitrogen (79%) to flow through the growth-tube cultures at 25 cc/min·culture. All percentage designations for gas mixtures are volume per volume. Other oxygen-nitrogen ratios and oxygen-nitrogen-carbon dioxide mixtures were produced by mixing gases from individual tanks in suitable proportions. Various mixtures and rates of flow were controlled and monitored by purge meters (Model 1555 from Brooks Instrument Division, Hatfield, Pa.). Oxygen-nitrogen ratios were verified with an oxygen analyzer (Type OH. 150, Servomex Controls Ltd., Crowborough, England), while CO_2 concentrations were verified with a Beckman

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Model 15a infrared analyzer. One-hole rubber stoppers with 5 cm lengths of glass tubing replaced the cotton stoppers of the growth-tubes. Clear vinyl tubing was used throughout. Humidity in the cultures was maintained at 81% by passing the gases through a series of flasks containing saturated solutions of $(NH_4)_2SO_4$ (12). Sterility was guaranteed when necessary by inserting a Metrical 0.20 μ filter (Gelman Instrument Co.) in the line proximal to the growth-tubes.

RESULTS

Effect of Medium Components on Circadian Conidiation. The medium in use at the time of the discovery of timex was a complex one containing sucrose, yeast extract, and casamino acids (16). Efforts to develop a more defined medium showed that only the casamino acids were critical for the timex phenotype. Additional experiments demonstrated that a mixture of 0.3% glucose and 0.5% arginine could satisfactorily replace the casamino acids. The glucose-arginine combination was used as a carbon source because glucose alone did not permit the persistent expression of a conidiation rhythm in growth-tube cultures, and because arginine alone was not adequate to allow a normal rate of growth, although a persistent conidiation rhythm was observed. Levels of glucose higher than 0.3% prevented persistent rhythmicity regardless of the level of arginine used, while levels of arginine higher than 0.5% decreased the growth rate. The ratio adopted allowed the maximum rate of growth with the persistent production of dense conidial bands. Vogel's salts and Difco Bacto-agar were routinely used, but other experiments have shown that the salt solutions of Fries (14) or Westergaard and Mitchell (24), or the more highly purified Noble agar do not alter the expression of timex rhythmicity significantly.

The effects of various sugars on the *bd* phenotype is shown in Table I. Clearly some sugars allow faster growth than others (lactose the slowest, glucose the fastest), and reasonably rapid but sparse growth is observed in the absence of an added carbon source.

Presumably the fungus is using the polysaccharides in the agar, or compounds present in trace amounts in the agar or Vogel's salts for growth in the apparent absence of a carbon source. In general, the slower the growth, the greater the persistence (number of bands) of the rhythm. With all sugars the *bd* strain forms few to several conidial bands of varying density, but only acetate and galactose elicit a persistent rhythm of conidiation. It is also evident that the period of the rhythm can be slightly modified by the carbon source. Repeated experiments have shown the differences in period to be real although the differences are often not so large as those in Table I.

In Table II are the results of experiments to determine the ability of various amino acids to increase the persistence of the *bd* rhythm when grown in the presence of 0.3% glucose. Whereas none of the other amino acids is as good as arginine in eliciting persistent and pronounced rhythmicity, there is a group (A) of amino acids that does permit persistent rhythmicity although band density is measurably decreased. A second group of amino acids (B) also increases persistency, but band density is very weak after 4 to 6 days. The group C amino acids have no detectable effect on growth or rhythmicity, while the group D amino acids actually decrease persistency and band density compared to glucose alone. The amino acid cysteine is unique in that it is toxic to *Neurospora*, as has been previously described (1). As in the experiments with various sugars, the period of *bd* grown on glucose-alanine is always shorter than when grown on glucose-tryptophan, but the magnitude of the difference is often less.

Table I. *Effects of Carbon Sources on bd Rhythmicity*

Growth-tube cultures were grown on Vogel's salts, 1.5% agar and 0.3% carbohydrate. >12 means that 12 bands were produced before the growth front reached the end of the growth tube, thereby terminating the experiment. Density of conidial bands ranges from no bands (—) to dense, well defined bands (++++). Circadian is defined as 20 to 24 h.

Carbon Source	No. of Bands	Band Density	Period	Growth Rate
			hr	*mm/day*
Lactose	6–7	+	22.2	29.5
Ribose	12–14	++	22.5	30.8
Galactose	>12	+	24.0	30.9
None	5–6	+	24.0	31.4
Acetate	>12	++	22.6	37.0
Glycerol	3–4	+	Circadian	37.2
Raffinose	7–8	+++	22.4	37.5
Trehalose	6–7	++++	22.2	40.4
Xylose	7–8	++++	22.4	41.3
Maltose	9–10	++++	22.4	43.1
Fructose	3–4	++++	Circadian	46.1
Mannose	4–5	+++	Circadian	47.0
Sucrose	3–4	++++	Circadian	47.3
Glucose	3–4	++++	Circadian	48.6

Table II. *Effect of Amino Acids on bd Rhythmicity*

Growth-tube cultures were grown on Vogel's salts, 0.3% glucose, 1.5% agar, and 0.5% amino acid. Heat-labile amino acids were filter-sterilized; media were titrated to pH 5.8 after addition of acidic or basic amino acids. *Bd* on the group B amino acids exhibited dense conidial bands for several days (left columns) followed by thin conidial bands (right columns).

Amino Acid	No. of Bands	Band Density	Period	Growth Rate
			hr	*mm/day*
Group A				
L-Arginine	>10	++++	22.2	46.7
L-Tryptophan	>10	+++	24.0	31.3
L-Histidine	>10	+++	24.3	39.2
L-Alanine	>10	++	20.0	48.5
(d,*l*)-Hydroxylysine·HCl	>12	++	22.4	26.8
Group B				
L-Aspartic acid	8–9; >4	++++; ++	21.8	41.4
L-Threonine	4–5; >5	++; +	20.9	43.7
L-Asparagine	5–6; >4	+++; +	21.2	40.7
L-Glutamine	4–5; 2–3	+++; +	21.2	37.5
L-Ornithine·HCl	4–5; >5	+++; +	21.8	41.8
L-Citrulline	4–5; >6	+++; +	23.2	35.6
Group C				
None	3–4	+++	Circadian	40.9
L-Isoleucine	3–4	+++	Circadian	43.3
L-Phenylalanine	4–5	+++	Circadian	41.9
L-Proline	3–4	+++	Circadian	42.4
L-Glutamic acid	3–4	++++	Circadian	44.6
L-Valine	3–4	+++	Circadian	38.3
Urea	6–7	+++	21.5	38.7
Group D				
L-Leucine	3–4	++	Circadian	43.3
L-Tyrosine	2–3	++	Circadian	41.6
L-Serine	4–5	++	Circadian	39.2
L-Methionine	2–3	++	Circadian	47.2
L-Glycine	2–3	+	Circadian	38.4
L-Hydroxyproline	2–3	+	Circadian	39.2
L-Cystine	2	+	Circadian	40.4
L-Lysine·HCl	1	+		31.0
Group E				
L-Cysteine·HCl				0

Table III. *Effects of Aeration on Neurospora Rhythmicity*

Growth-tube cultures were grown on Vogel's salts, 0.3% glucose, 0.5% arginine-HCl (or 1.5% sucrose), and 1.5% agar. Aeration was by compressed 79% N_2-21% O_2 using 25 cc/min·culture flow rate.

Species	Control Cultures				Aerated Cultures			
	Growth rate	Period	Band density	No. of bands	No. of bands	Band density	Period	Growth rate
	mm/day	*hr*					*hr*	*mm/day*
Glucose-arginine medium								
N. intermedia (NITa)	51.6			0	0			60.5
N. sitophila (EngSit 21a)	85.3			0	>8	+	13.5	81.7
N. tetrasperma	85.3			0	>5	+	20.9	84.5
N. crassa (STA 4)	90.5			0	>5	+++	20.9	83.0
Strains of *N. crassa*								
Abbott 12a	73.8			0	>7	++	22.7	51.8
SY4f8a	87.5			0	>6	+++	24.0	66.6
Lindegren 1A	67.9	23.5	+++	>5	>7	++	20.0	54.9
Em a	98.0	21.2	+	>4	>6	+++	21.4	73.6
Puerto Rico 18A	22.1			0	0			19.5
Liberia 4A	105.0			0	>5	++	24.5	78.3
Costa Rica A No. 205a	97.3			0	>8	++++	21.5	62.0
Minimal sucrose medium								
N. intermedia (NITa)	47.5			0	0–4	+		46.5
N. sitophila (EngSit 21a)	81.0			0	0			86.0
N. tetrasperma	68.0			0	2	+	Circadian	85.0
N. crassa (STA 4)	85.0			0	1–4	++	Circadian	87.5
Strains of *N. crassa*								
Abbott 12a	84.0			0	2	++	Circadian	80.0
SY4f8a	73.5			0	0			85.0
Lindegren 1A	71.5			0	>7	+++	Circadian	57.0
Em a	87.0			0	>4	+++	Circadian	87.0
Puerto Rico 18A	25.0			0	0			29.0
Liberia 4A	97.5			0	2	+	Circadian	89.0
Costa Rica A No. 205a	80.0			0	>5	++++	20.4	70.0

Effects of Aeration on Circadian Conidiation. The inhibition of conidiation when cotton plugs were replaced with foam plugs, the occasional observation of thin conidial bands in the middle of growth-tube cultures, the enhanced conidiation when very large growth-tubes (diameter 7.5 cm) were used, and the reported dependence of conidiation on oxygen tension (11) all suggested that oxygen might be limiting in our cultures. This hypothesis was tested by passing air through growth-tube cultures of several strains differing in their ability to conidiate or to demonstrate rhythmicity. The results in Table III illustrate that aeration considerably increases the ability of most species and strains to conidiate in a growth-tube and thereby exhibit rhythmicity. Those two types (*N. intermedia* and *N. crassa* Puerto Rico 18A) that still did not conidiate were subjected to 60 and 100% oxygen with no apparent increase in ability to conidiate or exhibit a rhythm. The data in Table III also indicate that the glucose-arginine medium is superior to a minimal-sucrose medium in eliciting rhythmicity even when the cultures are being aerated.

Since the growth pattern of *bd* on most sugars in the absence of arginine is a rhythmic production of dense conidial bands for 4 to 6 days followed by a cessation of conidiation, it seemed likely that aeration might substitute for arginine in promoting continued rhythmic conidiation. From Table IV it is clear that aeration does increase the persistence of rhythmic conidiation on media containing no amino acids.

It may be noticed that the density of the conidial bands in the controls of this experiment is less than in similar cultures reported in Table I. Reduced conidiation is occasionally observed in all our cultures for a few weeks at a time. We have tested our stocks, chemicals, water, glassware, and environmental parameters (temperature, humidity, aeration), but have been unable to identify the responsible factor. Growth rate, period and persistence of the conidiation rhythm are not affected.

Table IV. *Effects of Aeration on Persistence of bd Rhythmicity*

Growth-tube cultures was grown on Vogel's salts, 1.5% agar, and 0.3% carbohydrate. Aeration was by compressed 79% N_2-21% O_2 using 25 cc/min·culture flow rate.

Carbon Source	No. of Bands	Band Density	Period	Growth Rate
			hr	*mm/day*
Control cultures				
None	8–9	+	20.3	23.6
Glycerol	3–4	++	Circadian	34.1
Glucose	3–4	++	Circadian	52.2
Mannose	3–4	++	Circadian	48.3
Sucrose	3–4	++	Circadian	49.9
Lactose	5–6	+	21.3	23.7
Aerated cultures				
None	>15	+	22.8	27.8
Glycerol	>14	++	20.7	21.8
Glucose	>11	+++	23.1	35.0
Mannose	>11	+++	23.1	33.5
Sucrose	>11	+++	23.1	36.5
Lactose	>11	++	22.4	22.4

Table V. *Inhibition of Conidiation in Neurospora crassa by CO_2*

Growth-tube cultures were grown on Vogel's salts, 0.3% glucose, 0.5% arginine-HCl, and 1.5% agar. Carbon dioxide was added to compressed 79% N_2-21% O_2 flowing at 25 cc/min·culture.

Strain	CO_2	No. of Bands	Band Density	Period	Growth Rate
	% (v/v)			*hr*	*mm/day*
bd	40.0	0			27.3
	30.0	1	+		31.1
	25.0	1	++		53.9
	20.0	>8	+++	21.8	52.2
	10.0	>5	+++	21.3	56.0
	2.5	>7	++++	21.2	51.9
	1.0	>6	++++	20.7	59.4
STA 4 wild-type	1.00	1	+		99.8
	0.216	1	+		92.7
	0.131	1	+		99.5
	0.129	1	++		85.5
	0.119	>5	+	20.9	89.4
	0.102	>5	+	20.9	93.8
	0.08	>5	++	20.6	81.7
	0.052	>5	++	21.8	80.8
	0.032	>5	+++	20.4	82.8

Carbon Dioxide Inhibition of Conidiation. We initially supposed that the increased persistency resulting from aeration of growth-tube cultures was due to increased levels of oxygen. However, reducing the oxygen concentration to low levels (well below 1%) did not mimic the absence of aeration. Although linear growth was retarded 20 to 30% and yellow rather than pink conidia were produced, the period and persistence of the rhythm were normal and conidia were, in fact, still produced.

As an alternative, CO_2 of varying amounts was added to the compressed O_2-N_2 mixture used for aeration. From Table V it is clear that CO_2 does in fact inhibit conidiation of the two strains tested. About 0.125% CO_2 is able to inhibit conidiation of the STA 4 wild-type strain, whereas it takes 20 to 25% CO_2 to inhibit the *bd* strain. Above 25% CO_2 the *bd* strain can still exhibit circadian rhythmicity, for tufts of mycelia appear at circadian intervals until more than 40% CO_2 is used.

Other experiments in which small porcelain boats filled with KOH solutions were inserted into growth-tube cultures (STA 4 wild-type and *bd* on minimal-glucose) gave indecisive results. Conidiation was enhanced, especially in the case of *bd* on minimal-glucose, but the enhancement was never as great as with aeration. Presumably not enough KOH could be introduced into these small boats to reduce the CO_2 concentration below the critical level for each strain. More successful experiments of this type in which KOH-saturated paper strips stimulate conidiation in closed, small, test-tube cultures have been obtained in the laboratory of R. W. Siegel (personal communication).

It is also evident from these data and other experiments in which wild-type and *bd* strains were exposed to high (1–40%) and low (0.032–1.0%) CO_2 concentrations respectively, that CO_2 can stimulate growth. The optimum concentration for both strains is about 1%.

DISCUSSION

The use of various sugars and amino acids has shown that the conidiation rhythm of the *bd* strain is similar to the circadian rhythms of most other eukaryotic organisms in that it is little affected by inhibitors in general (9). These experiments further document the fundamental differences (16) between the *bd* strain and the clock strain of *Neurospora* (20) which has a rhythm effectively controlled (both expression and period) by such medium components. The rhythm of clock is considered to be non-circadian because of its insensitivity to light and sensitivity to temperature.

Berliner and Neurath (3) have shown that some sugars, *e.g.*, glucose, maltose, and fructose, can cause the clock strain to exhibit a rhythm of hyphal branching whereas sucrose causes continuous growth without evidence of rhythmic branching. In comparison, the major effect of various sugars on the *bd* rhythmicity is to alter the density and number of conidial bands, *i.e.*, the expression of rhythmicity, rather than the timing mechanism itself. The relative abilities of the various carbohydrates to stimulate conidiation in the *bd* strain are generally consistent with the results presented by Dicker *et al.* (6) for the stimulation of conidiation in the amycelial and biscuit strains of *Neurospora*. The particular effectiveness of acetate in stimulating conidiation has been previously described by Turian (21), and a general hypothesis to explain the effectiveness of various carbon sources in stimulating conidiation has been postulated by Weiss and Turian (23).

The importance of amino acids to fungal rhythmicity has been discussed by several authors. Berliner and Neurath (3) have shown that the period of the clock strain can be varied from 18 to 110 hr depending on the amino acid mixture utilized, and Jerebzoff (10) has been able to control the expression of rhythmicity in several different species of fungi with amino acid mixtures. Most striking in comparison to our own experiments is the finding of Berlinear and Neurath (4) that arginine is most effective in stimulating rhythmicity in the fungus *Ascobolus*. In general, amino acids seem to stimulate conidiation, especially after conidiation would have stopped on glucose alone, *i.e.*, they are increasing the persistence of the rhythm. Our experiments have delimited a group A of amino acids that is particularly effective in promoting persistence, but the group is so varied (one neutral, one aromatic, and three basic) that no common metabolic action is apparent. However, amino acid mixtures are known to stimulate conidiation in *Neurospora* (6), and our results correspond rather well with the grouping of amino acids proposed by Subramanian *et al.* (19) to account for the induction of nitrate reductase, an enzyme synthesized under conditions normally favorable for conidiation. Our groups A and B correspond (8/10) with their groups II, III, and IV, while our groups C and D correspond (7/9) with their groups V and VI.

The insensitivity of the *bd* timing mechanism to sugars, amino acids, and various salt solutions is matched by the insensitivity of the *bd* rhythm to antibiotics. Some investigators have been able to perturb the rhythms of certain species with antibiotics, but our experiments with actinomycin D, cycloheximide, puromycin, and mitomycin have proved negative (15). These antibiotics do affect growth and conidiation of the *bd* strain but are unable to alter the period of the rhythm significantly.

The idea that variations in the growth medium are affecting primarily the conidiation process (the hands of the clock) and not the timing mechanism (the clock itself) finds additional support from the aeration experiments. They suggest that various strains differ in their sensitivity to CO_2, and that once a critical level of CO_2 is exceeded, the conidiation process is inhibited and rhythmicity is no longer evident, although linear mycelial growth is normal. The various mutant strains of *Neurospora* that exhibit rhythmicity in unaerated growth-tube cultures presumably contain genes that enhance their ability to conidiate under these conditions, and thus demonstrate their inherent rhythmicity. It is possible that some of the genes

enhance conidiation because they reduce the rate of growth and CO_2 production, thereby allowing diffusion to maintain a low CO_2 level in these cultures. This suggestion is supported by the data in Table I and by the findings of Durkee *et al.* (7), Esser (8), and Dicker *et al.* (6) that slow growing morphological strains of various fungal species tend to exhibit rhythmic growth.

These data also provide additional support for the notion first advanced by Pittendrigh *et al.* (13) that rhythmic growth or conidiation represents a coupling of those processes to a timing mechanism. The absence of overt rhythmicity presumably represents a lack of coupling between the overt process and a timing mechanism common to all strains of *Neurospora*. Sussman *et al.* (20) first gave credence to the idea by showing that several strains of *Neurospora* demonstrated rhythmic growth when incubated on a medium containing sorbose. Our results show that all but two of the *Neurospora* strains tested are capable of circadian conidiation. These two strains need to be examined for circadian rhythmicity in some other way, and the recent discovery (Sargent, unpublished) that several strains of *Neurospora* produce CO_2 in a rhythmic fashion may in fact provide the needed assay system.

The aeration experiments also suggest an answer for the question discussed previously (18) as to the reason for the damping of rhythmicity when the *bd* strain is grown on salts plus glucose. The results in Table IV show that with aeration conidiation occurs throughout the experiment rather than just the first 4 to 5 days. The most logical explanation for this effect is that aeration keeps the CO_2 concentration below a critical level throughout the experiment, whereas, without aeration, the CO_2 concentration rises to the critical level within 4 to 5 days. Accordingly, we suggest that the damping of rhythmicity on salts plus glucose is an inhibition of conidiation (uncoupling), rather than arrest of the timing mechanism.

The mechanism for the CO_2-mediated inhibition of conidiation is unknown, but the availability of *Neurospora* mutants that either are defective in one of the several CO_2-utilizing enzymes or require CO_2 for normal growth on unsupplemented media suggests that elucidation of the mechanism may be possible. Elucidation of the mechanism involved would be particularly rewarding, for although carbon dioxide is known to control morphogenesis in several types of fungi, *e.g.*, yeast-mold dimorphism in *Mucor* (2), the biochemical mechanisms involved are mostly unknown.

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