Plant Physiol. (1981) 67, 404–407
0032-0889/81/67/0404/04/$00.50/0

Assay and Characteristics of Circadian Rhythmicity in Liquid Cultures of *Neurospora crassa*[1]

Received for publication March 3, 1980 and in revised form August 21, 1980

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ABSTRACT

Previous work on circadian rhythms of *Neurospora crassa* has been done almost exclusively with cultures expressing rhythmic conidiation and growing on solid agar medium. Such conditions severely restrict the kinds of biochemical experiments that can be carried out. We have now developed systems which allow indirect assay of circadian rhythmicity in liquid culture. *Neurospora* was grown in glucose and acetate liquid media under conditions which result in a range of growth rates and morphologies. Liquid media were inoculated with conidia and the cultures were grown in constant light for 33 or 48 hours, by which time floating mycelial pads had formed. Experimental pieces of mycelium then were cut and placed in fresh new liquid medium. As controls, other pieces of mycelium were cut and put directly on solid agar medium in race tubes. All cultures were transferred to constant darkness at this time. This light-to-dark transition set the phase of the circadian clock of both the liquid and solid cultures. At various times after the light-to-dark transition, the mycelial pieces in the liquid were transferred in the dark to solid medium in race tubes, where they grew normally and conidiated rhythmically. Comparison of the phase of the rhythm in these race tubes to the controls demonstrated that, under appropriate conditions, the circadian clock of the liquid cultures functions normally for at least two cycles in constant conditions. Using these culture systems, a significantly greater variety of biochemical studies of circadian rhythmicity in *Neurospora* is now possible.

Neurospora crassa has become an increasingly useful organism for the study of the cellular and molecular basis of circadian rhythms. There is much knowledge of its biochemistry and genetics, the *bd*[3] strain has a well-characterized circadian rhythm of conidiation or vegetative spore formation (21), and genetic analysis of its circadian clock has proved rewarding (see ref. 6 for a review). However, an important technical feature which *Neurospora* has lacked is a system in which biochemical manipulations and analyses can be carried out easily inasmuch as the conidiation rhythm is assayed on solid agar medium.

There are at least two kinds of biochemical experiments which have been done on a number of organisms expressing circadian rhythms. In the first type, various chemical agents were added to determine their effects on period length or phase of the rhythm, whereas, in the second type, biochemical parameters were measured through the circadian cycle in a search for oscillations which might be correlated with clock function. Although both types of studies have been carried out with *Neurospora*, there have been a number of problems with cultures growing on solid medium. For example, a variety of chemicals have been added to the solid medium to test their effects on period length (2, 5, 8, 9, 14, 19, 22). Two problems arise with these experiments: (*a*) often the agent inhibits conidiation itself and thereby prevents assay of its effects on the rhythm (One example is cycloheximide [19], which has been used in studying many other circadian systems.); (*b*) pulsing the agent to test for effects on phase might overcome the latter problem, but it is difficult to administer controlled pulses to agar-grown cultures.

The second type of study, in which various biochemical parameters were assayed through the cycle, has also been done using *Neurospora*. Such measurements have included the levels of cofactors (1, 3), enzyme activities (11), and nucleic acid metabolism (13). These studies also utilized rhythmically conidiating cultures on solid medium, which present several difficulties for this type of experiment as well: (*a*) although it has recently been established that areas behind the actively banding growing front are rhythmic, these areas are not in phase with each other or with the growing front (4) (Therefore, only small amounts of material from one region of a culture can be used for biochemical analysis.); (*b*) In most cases, only material from the growing front has been used, but the fact that such material is rhythmically differentiating, *i.e.* conidiating, has complicated the interpretation of any observed biochemical oscillations.

Additionally, any oscillations in biosynthetic rates which are part of the clock mechanism might be difficult to detect in rapidly growing cultures with a high "base line" rate of biosynthesis. We therefore wanted to develop culture systems in which the circadian clock functions and in which both differentiation and growth are minimized.

Two previous reports (17, 24) had suggested that *Neurospora* could exhibit circadian rhythmicity in liquid culture. We now report the development and characterization of liquid culture systems which permit a large degree of control over growth rate, differentiation, and morphology and in which circadian rhythmicity is reproducibly demonstrated.

MATERIALS AND METHODS

STRAINS

The *bd* and *pan-2* (allele 153M66) strains were obtained from the Fungal Genetics Stock Center, Arcata, CA. *Pan-2* is a (pan)-requiring auxotroph. The easily monitored circadian rhythm of conidiation of *bd* has been described (21). The *bd,pan-2* double mutant had been previously isolated in our laboratory (7) and was chosen so that growth rate and morphology could be manipulated by the amount of pan present in the medium.

[1] This work was supported by Grant GM-22144 from the National Institute of General Medical Sciences and by a grant from the Faculty Research Committee, University of California, Santa Cruz.

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[3] Abbreviations: *bd*,band; pan, pantothenate; PRC, phase-response curve; L-D transition, light-to-dark transition.

MEDIA

Stocks were maintained in constant light on slants of Horowitz (12) complete medium. Liquid media were glucose medium [0.3% glucose-0.5% arginine-Fries' (10) salts], glucose-plus-pan medium (same as glucose medium, but with 0.001% pan added), acetate medium [1.2% sodium acetate-0.05% casamino acids (Difco)-Vogel's (23) salts], and acetate-plus-pan medium (same as acetate medium, but with 0.001% pan added). Solid media in race tubes contained 1.5 or 2.0% agar, and, in a given experiment, the carbon source, amino acids, and salts were the same throughout. Solid media for the *bd,pan-2* strain always contained 0.001% pan.

CULTURE METHODS

General Outline. Conidial inocula were from 7-day-old slants. Conidial concentrations were estimated using a Klett-Summerson colorimeter with a blue filter. Manipulations after the L-D transition were carried out under red safelight (General Electric BCJ, 60 w). All experiments were at 25 C. Growth curves were obtained by harvesting groups of six "discs" (described below), which were dried at 60 C before weighing.

Details of the culture method are given below, but the procedure may be outlined as follows. Petri dishes containing liquid media supplemented with pan were inoculated with *bd,pan-2* conidia. After standing in the light for 33 or 48 h, floating mycelial pads formed. Discs of mycelium were cut from the pad using a cork borer and washed in the same liquid medium but without pan. Sets of six experimental discs then were placed in Petri dishes containing the same pan-free medium, whereas control discs were put directly into race tubes containing solid pan-supplemented medium. All cultures were immediately placed in constant darkness. This L-D transition sets the clock to a unique phase point from which "freerun" begins. After various amounts of time in the dark, experimental discs were removed from liquid culture and put on pan-supplemented race tubes where, like the controls, the discs grew and rhythmically formed conidial bands. At the end of the experiment, the phases of the bands on the experimental race tubes were compared to controls.

Glucose Medium. In experiments involving the *bd,pan-2* strain and glucose media, 25-ml aliquots of glucose-plus-pan liquid medium were dispensed into 150-mm disposable Petri dishes. Conidia were suspended in distilled H_2O and filtered through glass wool. The Petri dishes were inoculated to a density of 5×10^4 conidia/ml and grown in constant light for 33 h. Floating mycelial mats formed; at this time, small numbers of conidia were occasionally observed near the edges of the mats. Using a brass cork borer (11 mm diameter), six discs were cut from the center area of each mat where the morphology appeared uniform and mycelial. The discs were transferred to glucose liquid medium without pan as follows. First, they were thoroughly washed twice in 25 ml medium/20 discs to remove external pan. Six discs were each put in a race tube at this time to serve as controls. Experimental groups of six discs then were transferred to 125-ml Erlenmeyer flasks containing 25 ml of the respective medium, again without pan. The cultures were immediately placed in constant darkness; on solid medium this L-D transition sets the circadian clock to a unique phase point and allows the rhythm to freerun thereafter (21). In these conditions, the *bd,pan-2* strains has a freerunning period of about 21.5 h.

At various times after the L-D transition, discs from the liquid culture medium were poured into sterile Petri dishes. In many cases, the discs had fused loosely during the initial limited growth, which presumably occurs before depletion of pan. Such discs were gently separated. There was no visible conidiation. Because of the absence of conidiation in glucose under these conditions, we considered it to be the medium of choice for later biochemical experiments (see "Discussion"). In preparation for such studies, which include the administration of drug pulses, we gave "sham" pulses to the discs by transferring them to 25 ml fresh glucose medium (without pan) in Petri dishes for 4 h. At the end of that time, the discs were picked up on a small spatula. Excess medium was absorbed on sterile filter paper, and the discs were put into race tubes containing glucose-plus-pan solid medium. Normal growth and conidial banding ensued, and the growing fronts of these and the control race tube cultures were marked under red safelight at 24-h intervals on subsequent days. The cultures reached the end of the 20-cm race tubes after 5 days. The times, or phases, at which the centers of the conidial bands occurred in each experimental tube were determined, and the phase of the first band was calculated by linear regression analysis (4). Phases of experimental cultures (*i.e.* those from discs which had been in liquid culture after the L-D transition) were compared to the phases of the appropriate controls (*i.e.* those transferred to race tubes at the time of the L-D transition). The first band on a control tube actually occurred 1 or 2 days before the first band on experimental tubes inasmuch as the former were inoculated earlier. Bands whose phases were compared in a given experiment occurred on the same day.

Acetate Medium. Experiments using *bd,pan-2* in acetate were the same as described for glucose with the following exceptions. Conidial concentration after the initial inoculation into liquid medium was 8×10^4 conidia/ml. Since growth in acetate was slower than in glucose, these cultures were left in constant light for 48 rather than 33 h. Acetate medium resulted in mycelial growth which was somewhat uneven and in pieces rather than a uniform pad. There was also considerable aerial growth and conidiation. When discs were cut and washed, six discs were transferred to race tubes containing acetate-plus-pan solid medium to serve as controls as previously. All cultures then were placed in constant darkness. At various times, experimental discs were transferred directly to race tubes without the 4-h "sham" pulse in fresh liquid medium. These cultures reached the end of the race tubes in 7 days. Experimental and control phases were compared as described above.

DETERMINATION OF LIGHT PRC

In order to determine whether the liquid cultures could be phase shifted by light, sets of six discs in glucose liquid medium (without pan) were exposed to a single light pulse (5 min, 50 ft-c, cool-white fluorescent) at 4-h intervals, beginning 15 h after the L-D transition. Controls were discs which received no light pulse and were transferred to race tubes at the same time as the experimentals. All discs were transferred to race tubes at the same time, 39 h after the L-D transition. For comparison, *bd,pan-2* cultures inoculated directly onto race tubes were given light pulses of the same intensity and duration at the same times after the L-D transition.

RESULTS

RHYTHMICITY IN LIQUID CULTURE

Glucose Medium. Two major aims of the study presented here were to ascertain whether rhythmicity exists in liquid cultures and to determine if such rhythmicity exhibits the same phase and period length as in solid-medium cultures. A third goal was to find culture conditions in which physiological processes of growth and differentiation unrelated to the clock mechanism are minimized so that any biochemical oscillations involved in the clock mechanism might be more easily observed. Discs of the *bd,pan-2* strain which had been grown in glucose-plus-pan liquid medium were transferred to glucose medium without pan and control discs were put directly into race tubes. All cultures were immediately placed in constant darkness. After various lengths of time, discs were transferred to fresh medium of the same composition for 4

h to simulate conditions used in a chemical-pulse experiment and then transferred to race tubes. The results of one such experiment (Fig. 1) show the difference between control and experimental phases of the bands occurring on a given day plotted as a function of h after the L-D transition when the discs were transferred. Between the first time of transfer (at 15 h) and 43 h after the L-D transition, when the last set of discs was put into race tubes, there is very little difference in phase between all the tubes. The close agreement in phase among the cultures indicates that the L-D transition sets the clock in liquid cultures to a unique phase and that the periodicity of the rhythm in the liquid cultures is very similar to that on the race tubes.

Figure 2 shows the growth curve of *bd,pan-2* discs in glucose medium, with and without pan, in constant darkness. The mass attained by cultures deprived of pan is decreased by almost 80%; even so, the growth rate varies through the experiment from an initial mass doubling time of about 12 h to no detectable growth. Under these conditions, no visible conidia formed. Deprivation of pan is crucial to inhibition of conidiation; in other experiments using pan-supplemented liquid medium for incubation in constant darkness, there was considerable conidiation by 12 h after the L-D transition (J. Perlman, unpublished results).

Acetate Medium. Because conidial bands are narrower and therefore phase determination is possibly more precise in race tubes containing acetate instead of glucose as a carbon source, acetate might be the medium of choice for some kinds of studies. The procedure outlined above was therefore repeated with *bd,pan-2* and acetate medium. Acetate medium resulted in visible aerial growth and conidiation by the time the discs were cut at 48 h. As previously, they were washed in liquid acetate medium without pan and placed in the same medium for culture. Discs put on race tubes at this time served as controls and all cultures were placed in constant darkness. Sets of six discs were transferred from liquid culture to acetate-plus-pan race tubes at various times after the L-D transition. Figure 3A shows the phases of all bands on experimental tubes, whereas the difference in phase between experimental and control race tubes is plotted in Figure 3B. These data again show very small variability among the disc-inoculated experimental tubes and very little difference between experimental and control phases. This demonstrates that cultures in liquid-acetate medium retain the control phase under freerunning conditions for at least 50 h, and possibly longer. (We hesitate to draw conclusions from the single time point at 63 h.)

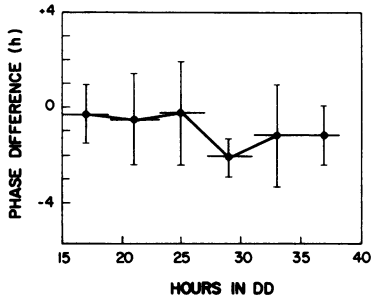

FIG. 1. Circadian rhythmicity of *bd,pan-2* in glucose liquid medium. The graph shows phase differences between control tubes, in which discs were transferred from liquid medium to race tubes at the time of the L-D transition, and experimental tubes, in which discs were maintained in liquid culture in constant dark (DD) for various times after the L-D transition, given a "sham" pulse (see text) at the time indicated by the horizontal bars, and then transferred to race tubes. Each point is the average of six replicate tubes; error bars are ±SD.

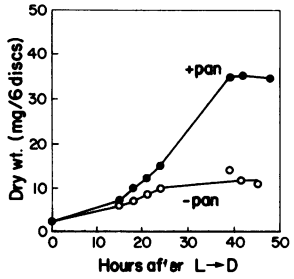

FIG. 2. Growth curves of the *bd,pan-2* strain in glucose liquid medium with and without 0.001% pan.

PRC FOR LIGHT

One of the distinguishing characteristics of a circadian clock is its phase resetting, or phase shifting, in response to pulses of visible light. The magnitude and direction (advance or delay) of phase shifts is a function of the phase in the cycle when the light pulse is given, and the phase shifts through the cycle are described by a PRC. Since this light PRC is believed to be a fundamental property of the clock (18), similarity between the light PRC for liquid cultures and that obtained for agar cultures would provide further strong evidence for the normal functioning of the clock in liquid cultures. Figure 4 shows the light PRCs for *bd,pan-2* in both liquid culture and on agar medium in race tubes. It is clear that the PRCs of the cultures are similar to each other. They are also essentially identical to PRCs of the prototrophic *bd* strain (15, 20).

DISCUSSION

The circadian clock of *Neurospora* functions in at least two kinds of liquid medium cultures with growth rates and morphologies that differ from each other and vary during the course of the experiment. A major aim of this culture method is to permit analysis of biochemical oscillations involved in the clock mechanism. It is therefore desirable to be able to limit biochemical changes related to other developmental phenomena, such as

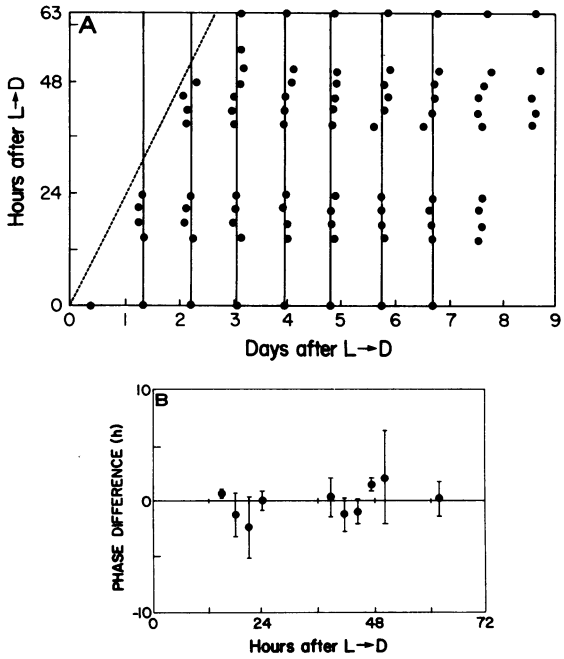

FIG. 3. Circadian rhythmicity in acetate liquid medium with *bd,pan-2*. Procedures were similar to those described for Figure 1 except that experimental cultures were not given a "sham" pulse prior to their transfer to race tubes. A, phases of all bands on race tubes inoculated with mycelial discs from liquid culture at various times after the L-D transition; B, difference of phase between control and experimental race tubes.

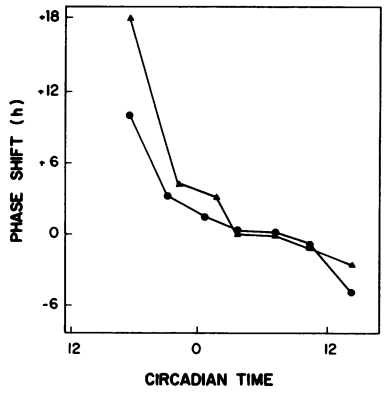

FIG. 4. PRCs to light pulses for *bd,pan-2* in glucose liquid medium without pan (●) and on solid medium supplemented with pan (▲).

growth and differentiation.

The *bd,pan-2* strain in glucose medium appears to be a good choice for a system in which growth and differentiation are very limited. If glucose is used, there is almost no conidiation, and growth is also severely limited in the absence of pan, whereas the clock continues to function. The phase of the rhythm is essentially unaffected by the transfer from glucose liquid medium without pan to glucose-plus-pan solid medium in race tubes. In addition to the experimental application they provide, these findings indicate that significant changes of growth rate in pan auxotrophs due to depletion of pan do not perturb the clock.

Bd,pan-2 cultures grown in acetate medium also show very consistent phase when transferred to race tubes (Fig. 3). However, these cultures produce significant amounts of conidia and non-uniform mycelial morphology before being transferred to minus pan medium and constant darkness. Since we are seeking to minimize variation in morphology and differentiation, this is probably not an optimal culture system for some types of studies on the biochemistry of the clock. However, because conidial bands are narrower on acetate than on glucose, phase determination is usually more accurate on acetate. As a result, this medium may be more useful for certain kinds of phase shifting experiments, such as drug or light pulses.

Neurospora grown in liquid media is amenable to many biochemical manipulations and measurements previously difficult or impossible for studies of its clock. The cultures can be assayed for various biochemical parameters or pulsed with chemical agents. For example, cycloheximide, which alters the clock in many organisms, inhibits conidiation at such low concentrations on solid medium that its effect on the *Neurospora* clock could not be determined (19). Using this liquid culture system, however, we have recently demonstrated that pulses of cycloheximide induce phase-dependent phase shifts (16). The liquid culture method for *Neurospora* should prove a useful technique in further studies of molecular mechanisms of circadian rhythmicity.

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