Modification of the Period of a Noncircadian Rhythm in Nectria cinnabarina

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ABSTRACT

The sporulation rhythm in Nectria cinnabarina Tode ex Fr. is expressed by either concentric rings or spirals. The period is inversely proportional to the temperature and does not approach 24 hours; the rhythm is therefore noncircadian. The period of the sporulation rhythm is lengthened by culturing the fungus on a dialysis membrane over the agar surface, on media solidified with an increased amount of agar, or on media containing deuterium oxide. These observations suggest that the transfer of materials by diffusion between the colony and the substrate is a significant factor in regulating the period of the rhythm.

The most commonly observed of the periodic processes in fungi is the formation of concentric hyphal zonations or rings of sporulation as the colony develops. Although many instances of zonation are attributable to recurring environmental influences, such as alternating light and darkness, some are the expressions of endogenous rhythms. The endogenous rhythms of fungi may be circadian (11), noncircadian (1, 5, 12), or non-circadian but with many features of circadian rhythms (1, 8). Although circadian rhythms have been the subject of intense study in the recent past, the fundamental mechanism of circadian oscillation has not been elucidated. One reason that the timing mechanism of circadian rhythms resists analysis is that the approximately 24-hr period is difficult or often impossible to alter (2, 14, 15). Possibly an examination of a less complex and presumably more primitive oscillator system may provide a more direct insight into the basic mechanism of rhythmic processes.

The period of the sporulation rhythm in Nectria cinnabarina Tode ex Fr. does not approach 24 hr, the phase is apparently not shifted by light, and its period is dependent upon the temperature (1). The potential for altering the period of the rhythm in N. cinnabarina provides a system for studying this rhythm at a physiological level.

MATERIALS AND METHODS

One strain of N. cinnabarina was used in all experiments. All cultures were single spore cultures. Cultures were initiated by germinating conidia on 2% (w/v) agar for 18 to 24 hr and then transferring a single germinated spore on a small block of agar to the center of a culture plate.

The basic medium consisted of 48 g/liter Bacto-potato dextrose broth and 20 g/liter Bacto-agar (Difco). This medium was modified in some experiments by varying either the potato dextrose broth or the agar content or by the replacement of potato dextrose broth with 48 g/liter malt extract (Difco). In other experiments deuterium oxide was substituted for a portion of the water in the basic medium. In one series of experiments a 4-cm disc, cut from the wall of dialysis tubing, was autoclaved and placed on the surface of the basic medium. The fungus was then seeded and grown on this dialysis membrane.

All cultures were incubated in 5-cm plastic Petri dishes with continuous illumination provided by Grolux fluorescent lamps at 4.8 × 10⁶ erg cm⁻² sec⁻¹ intensity. The cultures were maintained in constant temperature chambers at 20 C unless otherwise specified. A minimum of 15 replications of each treatment was used.

The first clearly defined sporulation band was marked by inserting a fine sterile Pyrex needle in the band. The cultures were then incubated until 8 to 10 additional bands had developed. Marking the first ring with a needle and determining the period of the rhythm from beyond that point permitted avoidance of variations in the onset of the rhythm as well as ambiguities near the center of the colony. Insertion of a needle was not practical in those experiments involving dialysis membranes. In these experiments the rate of radial expansion of the colony was computed, and the distance between the sporulation rings was measured. Since the rate of growth of the colony is linear, the distance between bands is a function of time. Calculating the period by either method yielded comparable values.

RESULTS

Under the standard conditions described above, our strain of N. cinnabarina produces one sporulation band of conidia in approximately 15 hr. As has been reported previously (1), these bands, which are usually concentric rings, frequently develop as an Archimedes' spiral that produces one revolution in approximately 15 hr. Occasionally two spirals develop within a single culture, each of which produces one revolution in approximately 30 hr. On two occasions we have observed three spirals within a single culture, each forming a revolution in 45 hr. Regardless of whether the zonation is concentric rings or single, double, or triple spirals, one additional band is formed every 15 hr.

The period of the rhythm is inversely proportional to temperature. The period ranges from 8.4 to 16.5 hr between 18 and 26 C. Over the same temperatures, growth is little affected (Fig. 1). The Q₁₀ value for growth at these temperatures is 1.6, while the Q₁₀ of the period is 2.4 over the same temperature range.

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The type of medium or the concentration of nutrients apparently has little effect on the period provided that suitable nutrients are supplied in sufficient quantity to support the appearance of zonations. We have been unable to develop a defined medium that will induce zonations, nor do zonations appear on potato dextrose agar that contains less than about 18 g/liter potato dextrose broth. While the sporulation bands become less distinct and more difficult to measure with decreasing levels of nutrition, only small differences in the period could be detected at nutrient concentrations between 24 and 54 g of potato dextrose broth per liter. At 20 C, periods of 16.4 and 15.0 hr were obtained on malt extract agar and potato dextrose agar, respectively.

The suggestions that rhythmic processes may be governed by diffusion phenomena or membrane behavior (6, 10, 14) prompted us to explore the effect of factors which may influence the rate of diffusion within the colony. The period of the rhythm was noticeably lengthened in colonies that developed on a layer of dialysis tubing placed over the agar (Fig. 2). At 20 C the period is 18.2 hr on dialysis membrane but only 14.5 hr on the agar surface. Similarly, the sporulation bands develop more slowly on media that is solidified with a higher agar concentration. The period ranges from 14.6 hr on media solidified with 15 g/liter agar to 21.9 hr on media containing 80 g/liter agar (Fig. 3). On the other hand, mycelial growth is little affected by increased amounts of agar.

On media containing deuterium oxide as a partial replacement for water, the period is lengthened. The results presented in Figure 4 indicate that the relationship between deuterium oxide and the period is approximately linear. The observed effect of deuterium oxide on the period appears to be separate from a less pronounced effect on hyphal growth. With increased deuterium oxide concentration the spacing between the bands is widened even though the growth rate is slightly reduced (Table I).

**DISCUSSION**

The rhythm found in *N. cinnabarina* is similar to the zonation rhythms found in some other fungi (4, 7) in that the
period is temperature-dependent and the phase is not shifted by light perturbations. Three lines of evidence seem to indicate that the physical relationship between the substrate and the colony is a significant factor in controlling the timing of the rhythm. Imposing a layer of dialysis membrane between the substrate and the fungal colony, increasing the amount of agar in the medium, and replacing a portion of the water with deuterium oxide all cause the period to be lengthened. One effect that these treatments have in common is to impede the movement of substances between the colony and the substrate. These observations with *N. cinnabarina* are probably not generally applicable to zonation rhythms in other fungi. Indeed, Chevaugeon and Van Huong (4) presented convincing evidence that control of the noncircadian hyphal growth rhythms in *Podospora anserina* and *Aspergillus immersus* is internal rather than through the substrate. Furthermore, phase shifting by light perturbations and the innateness of the period of the circadian rhythm in *Neurospora crassa* almost certainly point to an internal control mechanism (11). It should not be inferred, however, that the influence of external diffusion phenomena is unique to *N. cinnabarina*. Similar explanations have been proposed for other fungal zonation rhythms (6, 7, 10).

If diffusion is significant in controlling the timing of the rhythm, then one or two possible conditions appear likely: substances in the nutrient medium must diffuse into the colony to initiate each cycle, or the fungus produces substances which must diffuse from the colony into the medium before a new cycle can be initiated. In view of the observation that relative concentration of nutrients has little effect on the period, the latter hypothesis is favored. The period and the temperature dependence of zonations in *Alternaria tenuis* can be modified by nutritional factors (7), but such a relationship has not been found as yet in *N. cinnabarina*. Either of the explanations proposed for *N. cinnabarina* is somewhat analogous to the formation of Liesegang rings. It is known that Liesegang rings may take the form of single, double, or even multiple spirals that are remarkably similar to the sporulation spirals in *N. cinnabarina* (9).

In view of the possibility that the relationship between the fungal colony and its environment may influence the period of the rhythm, the question arises as to whether we may properly refer to this phenomenon as an endogenous rhythm. The environment in the immediate vicinity of the colony may undergo cyclic changes, and in this sense the rhythm is not endogenous. On the other hand, these changes in the environment are a direct result of fungal activity, and we therefore use the term endogenous to describe this rhythm.

These are several possible explanations for the deuterium oxide effect other than that of influencing diffusion. Among the recognized effects in deuterium oxide-water mixtures that should be considered are electrochemical changes (pH or pD), inhibition of certain enzymes (16), and active transport (17). These effects could quite conceivably be implicated in lengthening the period of the rhythm, but taken in conjunction with the effects of increased agar concentration and dialysis membranes it seems probable that at least a portion of the deuterium oxide effect is attributable to a reduction of the transfer of materials by diffusion between the colony and the substrate or perhaps within the fungal cells themselves. Of special interest in this regard are the reports that deuterium oxide lengthens the period of circadian rhythms in other organisms (2, 3, 13). This raises the possibility that diffusion processes are implicated in the timing mechanism of circadian rhythms.

**LITERATURE CITED**