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Circadian Rhythms of Nucleic Acid Metabolism in *Neurospora crassa*

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Wild-type, band, and fluffy strains of *Neurospora crassa* exhibit circadian rhythms of ribonucleic acid and deoxyribonucleic acid content in the growth-front hyphae of cultures grown on a solid medium. There is also a rhythm of ^{3}H-uridine incorporation into the nucleic acids of the band strain. Maximum incorporation precedes the peaks of nucleic acid content which occur during conidiation. As cultures age, ribonucleic acid content decreases rapidly and deoxyribonucleic acid content decreases gradually in standing, shake, and bubble cultures. A reduction of ribonuclease activity with age is also noted in standing and shake cultures. The nucleic acid content, nuclease activity, and changes associated with age vary with the culture conditions.

Circadian rhythms of nucleic acid content and biosynthesis have been described in many organisms. Early attempts to detect a rhythm of nucleotide incorporation into *Gonyaulax* (11) were unsuccessful, but later studies, e.g., in *Acetabularia* (23), did find such rhythms. Mouse liver (10), rat liver (9, 16), and other mammalian tissues (5, 13) have also been found to demonstrate rhythms in various phases of nucleic acid metabolism. More specifically, Steinhart (21) has reported a diurnal variation of template activity in mouse-liver chromatin, and temporally unique messenger ribonucleic acid (mRNA) species have been described in *Tetrahymena* (2). At least one theory (8) proposes that oscillations in nucleic acid transcription are basic to the mechanism of biological timekeeping.

In this paper we present evidence for circadian rhythms of nucleic acid content and biosynthesis in *Neurospora crassa*. Other data show the effect of physiological age on nucleic acid content and ribonuclease activity in cultures grown under various conditions. These preliminary studies are necessary for the eventual biochemical analysis of the single-gene mutants affecting the expression (19) and period length (J. F. Feldman and M. N. Hoyle, Biophys. Soc. Abstr., 1973, p. 148a) of *Neurospora* rhythms, and represent part of a larger effort (17, 18) to elucidate the basic mechanism of the biological clock in *Neurospora*.

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MATERIALS AND METHODS

Strains. The following strains of *N. crassa* were used: wild-type (74-OR8-1a); fluffy (*fl*, P605a); and band (*bd*, MLS 41-4). Origins, maintenance, and preservation of wild type and band have been previously described (19). The fluffy strain was kindly provided by D. D. Perkins.

Media. Minimal sucrose medium contained Vogel's salts (24) and 1.5% (wt/vol) sucrose. Our maltose medium contained Vogel's salts and 0.5% maltose. Media were solidified when necessary with 1.5% Difco agar.

Growth conditions. For shake cultures, Florence flasks (500 ml) half filled with minimal sucrose medium were inoculated with approximately 10^6 conidia per ml and placed in the dark on a reciprocal shaker at 100 strokes per min. Mycelia were harvested by filtration at 24-h intervals and washed with cold (4 C) distilled water. The same flasks, medium (400 ml), and inoculum were used for bubble cultures, which were vigorously aerated with compressed air and harvested at 12-h intervals. Standing cultures were grown in 250-ml Erlenmeyer flasks containing minimal sucrose medium (100 ml). The flasks were inoculated, exposed to laboratory light for 24 h, and then moved to constant darkness. Conidia, when needed in large numbers, were collected in a Buchner funnel from cultures grown in 2.5-liter low-form flasks (250 ml of solid, minimal sucrose medium). The flasks were inoculated, grown upright for 48 h, and then inverted and aerated with compressed, humidified (18) air for six days. All cultures were grown at 25 C. The conidial and mycelial samples were lyophilized immediately after harvesting, and were ground in a Wiley mill by using a 60-mesh screen.

Cultures for the circadian-rhythm experiments were grown in Pyrex baking dishes (19 by 30 cm) containing 125 ml of maltose-agar medium. The

surface of the agar was overlaid with a single layer of dialysis tubing (4.65 in flat width, Union Carbide) which had been sterilized by soaking in water for 24 h and then in 70% ethanol for not less than six h. The plates were inoculated by using narrow (2 mm) strips of Whatman 3MM chromatography paper impregnated with a conidial suspension. Mycelial suspensions for similar inoculations of fluffy were prepared by harvesting 72-h shake cultures, and coarsely homogenizing the mycelium in sterile water with a Sorvall Omni-Mixer (setting 6, four 30-s pulses). Cultures were synchronized with light (24 h for band, 8 h for fluffy and wild type) after inoculation, and then moved to constant darkness at 25 C. The growing fronts (approximately 5 mm for band, 8 mm for fluffy and wild type) were harvested with a straight-edged spatula at six-h intervals, and were lyophilized. These mycelial samples were homogenized in cold distilled water (5 mg/ml) in a Ten Broeck homogenizer immediately before assay.

Nucleic acid extraction. The extraction procedure used is a modification of that designed by Schmidt and Thannhauser (see Munro and Fleck, 15, p. 159). The procedure was started with either samples of ground, lyophilized mycelium, or conidia suspended (25 mg/5 ml) in cold (2 C) water, or with samples homogenized in water as described above. Low molecular weight molecules were extracted with perchloric acid, and the RNA was digested with KOH. After acidification and centrifugation, RNA nucleotides were determined by light absorption (260 nm). The pellet was extracted twice with 2.5 ml of 0.5 N $HClO_4$ at 80 C for 10 min, and the extracted deoxyribonucleic acid (DNA) was assayed by using Burton's diphenylamine test (6). RNA from Torula yeast (Sigma Type II-S) and calf thymus DNA (Sigma Type I) were used as standards.

In the liquid-culture experiments, assays were done in triplicate on three samples of lyophilized powder from a given time point. A data point from Fig. 1 to 4 thus represents the average of three assays on a given powder sample. There were many instances when the averages from different powder samples were identical, thus, one data point represents six to nine assays. Standard errors for these determinations (n = 9) are given in the individual figure legends. Slight differences in the efficiency of extraction from different powder samples accounted for the major source of variability. Repeated assays for RNA or DNA from the same powder sample routinely yielded values within 1 to 2% of the mean. Low yields of mycelium from the baking-dish cultures occasionally prevented equivalent repetition for the determinations given in Fig. 6 to 8. In these experiments the data points again represent the average of the assays for a given powder sample.

Ribonuclease activity was determined as described elsewhere (M. L. Sargent, Biochim. Biophys. Acta, 1973, in press).

Pulse labeling. Incorporation of ^{3}H-uridine into DNA and RNA was used to measure nucleic acid synthesis in the mycelial growth front. Baking-dish cultures were inoculated as described above and pulsed at 6-h intervals. Strips of dialysis membrane

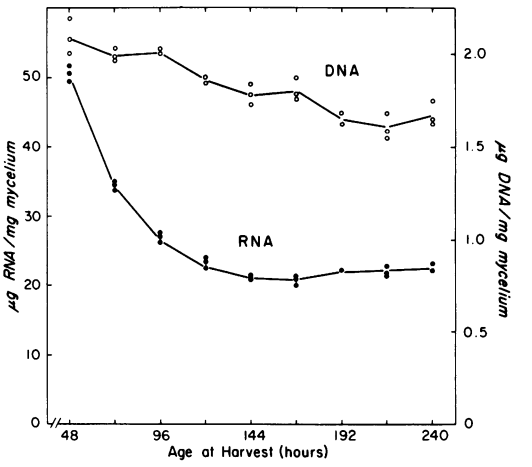

FIG. 1. *Nucleic acid levels in standing cultures of wild-type N. crassa grown in minimal sucrose medium. The standard errors ranged from 0.017 to 0.036 and 0.17 to 0.32 μg of mycelium per mg for the DNA and RNA, respectively.*

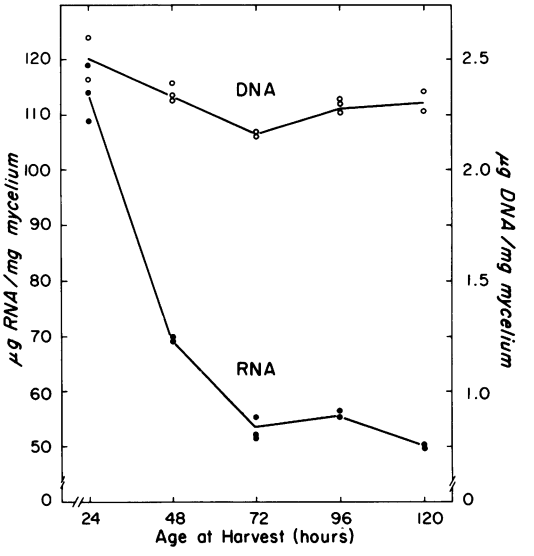

FIG. 2. *Nucleic acid levels in shake cultures of wild-type N. crassa grown in minimal sucrose medium. The standard errors ranged from 0.010 to 0.028 and 0.11 to 1.47 μg of mycelium per mg for the DNA and RNA, respectively.*

containing the growth front were cut away and transferred to a 15-cm petri dish containing liquid, maltose medium with 1 μCi of [5,6-^{3}H]uridine per ml (New England Nuclear Corp.; 40 Ci/mmol). After a 40-min pulse, the mycelia were collected and lyophilized. Nucleic acids were extracted and counted in Bray's liquid scintillation cocktail (3) with a Packard Tri-Carb (model 3375) liquid scintillation spectrometer.

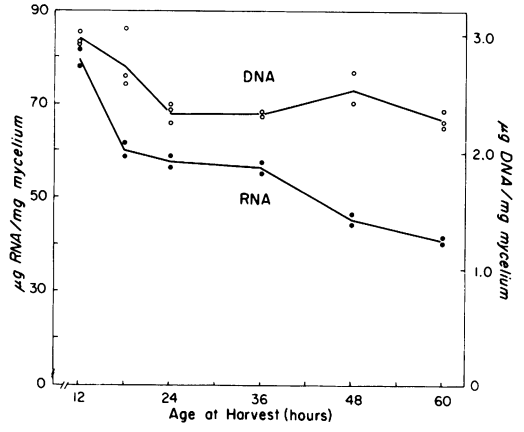

FIG. 3. *Nucleic acid levels in bubble cultures of wild-type N. crassa grown in minimal sucrose medium. The standard errors ranged from 0.020 to 0.086 and 0.31 to 0.62 μg of mycelium per mg for the DNA and RNA, respectively.*

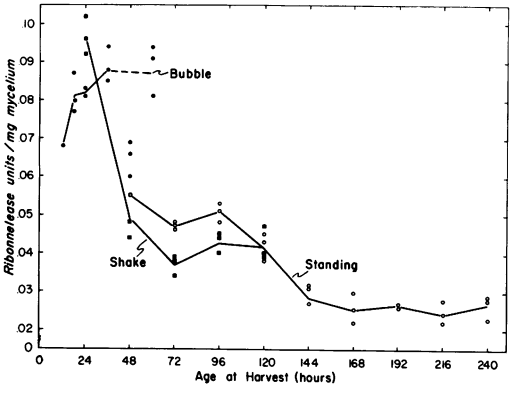

FIG. 4. *Ribonuclease activity in standing* (○), *shake* (■), *and bubble* (●) *cultures of wild-type N. crassa grown in minimal sucrose medium. The standard errors ranged from 0.0009 to 0.0019, 0.0013 to 0.0026, and 0.0006 to 0.0026 units of mycelium per mg for the standing, shake, and bubble cultures, respectively.*

RESULTS

Effects of physiological age. Nucleic acid content and ribonuclease activity were determined in conidia and the mycelia from standing, shake, and bubble cultures (Fig. 1 to 4). All cultures show a slow decrease of DNA and a rapid decrease of RNA content with time. The decreases in nucleic acid content are of similar magnitude in all types of cultures, with the aerated cultures (shake and bubble) exhibiting higher levels of both DNA and RNA than standing cultures. Conidia were found to contain more RNA (57 to 59 μg/mg) and DNA (4.1 to 6.2 μg/mg) than mature mycelia, but nuclease activity in the conidia was considerably lower (0.01 units/mg). Ribonuclease activity decreases rapidly with time in shake and standing cultures, but remains relatively constant in bubble cultures.

Rhythm of nucleic acid content. Since preliminary experiments indicated that the amplitude of the oscillations of nucleic acid content in mycelia from standing, shake, and bubble cultures was low, baking-dish cultures were employed for the rhythm studies. As is seen in Fig. 5, such cultures have a pronounced rhythm of conidiation like that found in growth-tube cultures (17). Mycelia from baking-dish cultures were found to contain concentrations of nucleic acids similar to those from mycelia of liquid cultures in their actively growing stages. In these cultures of band growing on maltose medium, both RNA and DNA exhibit oscillations which are in phase with the circadian rhythm of conidiation (Fig. 6). Under these growth conditions, the periods of the conidia-

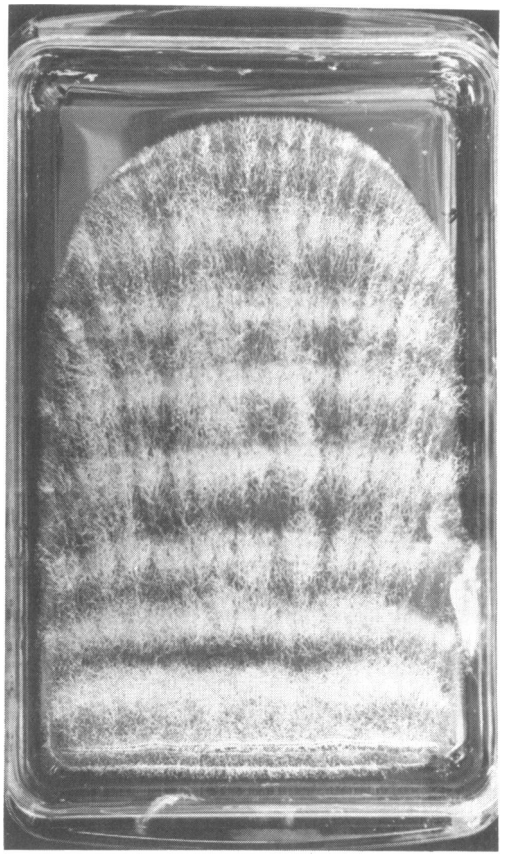

FIG. 5. *Baking-dish culture of the band strain of N. crassa grown on a glucose-arginine medium (18) to maximize contrast for illustrative purposes.*

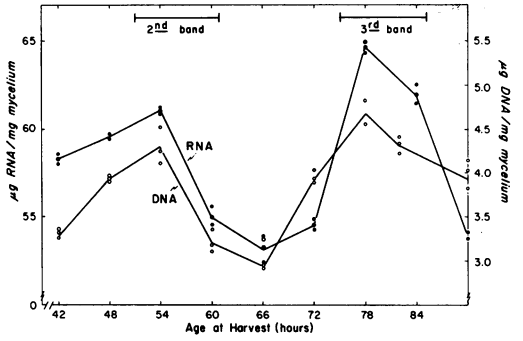

FIG. 6. *Nucleic acid content in the band strain of N. crassa grown in baking-dish cultures on maltose medium. Bars at top indicate periods of conidiation. Standard errors ranged from 0.017 to 0.14 and 0.10 to 0.27 μg of mycelium per ms for the DNA and RNA, respectively.*

tion and nucleic acid-content rhythms are about 24 h. The second cycle of both rhythms has a greater amplitude than the first.

The wild-type strain shows similar rhythms (Fig. 7) with the peaks of nucleic acid content occurring near the time of conidiation, but with the second cycle having a lower amplitude than the first. The difference in amplitude is probably related to the ability of the mycelium to produce conidia. In the band strain, maximum conidiation does not occur until the cultures are approximately 3 days old, with thick bands continuing to be produced thereafter. In the wild-type strain, only two clear bands appeared; the third band had considerably less conidia, presumably due to a buildup of CO_2 which inhibits conidiation (18).

The non-conidiating fluffy strain was used to test the hypothesis that the observed rhythms of RNA and DNA content are a result of rhythmic production of conidia. Even in the absence of an observable conidiation rhythm (a rhythm of aerial hyphae production is observable under certain conditions though), oscillations of nucleic acid content were detected (Fig. 8). The amplitude of the nucleic acid rhythms are, however, less than those found in the conidiating strains. More extensive experiments with the CO_2-production rhythm (M. L. Sargent, unpublished data) indicate that these results with fluffy are not unique. The rhythms seem to be present in the total absence of conidiation or morphological change, but the amplitudes of the rhythms are greatly depressed. The relationships between conidiation and the amplitudes of rhythms both closely and remotely related to such morphological change need further study.

Rhythm of nucleic acid biosynthesis. Nucleic acid synthesis, as measured by incorporation of ^{3}H-uridine into DNA and RNA, is also rhythmic in the mycelial growth front of the band strain (Fig. 9). Another experiment of longer duration but less precision confirms that the one cycle seen in Fig. 9 is repeated during formation of the third conidial band. Uridine incorporation into both nucleic acids is greatest just prior to conidiation and maximum nucleic acid content as would be expected.

DISCUSSION

The experiments on nucleic acid content as a function of physiological age and culture conditions were done for two reasons. First, an evaluation of our extraction and assay procedures was needed, and second, the diversity of experimental values reported for the nucleic acid content of *N. crassa* (1, 7, 12, 14, 20, 22)

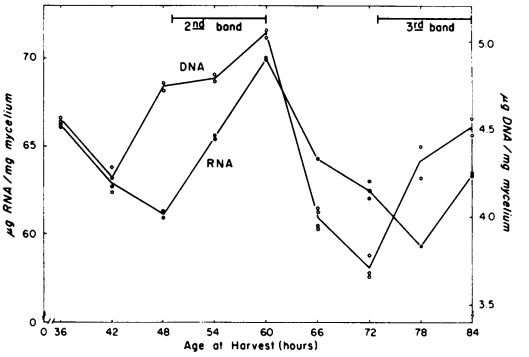

FIG. 7. *Nucleic acid content in wild-type N. crassa grown in baking-dish cultures on maltose medium. Bars at top indicate periods of conidiation. Standard errors ranged from 0.012 to 0.090 and 0.08 to 0.28 μg of mycelium per mg for DNA and RNA, respectively.*

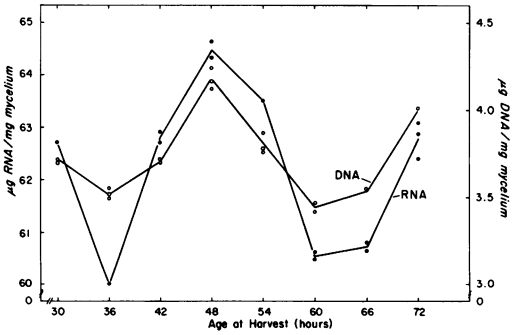

FIG. 8. *Nucleic acid content in the fluffy strain of N. crassa grown in baking-dish cultures on maltose medium. Standard errors ranged from 0.007 to 0.035 and 0.08 to 0.23 μg of mycelium per mg for the DNA and RNA, respectively.*

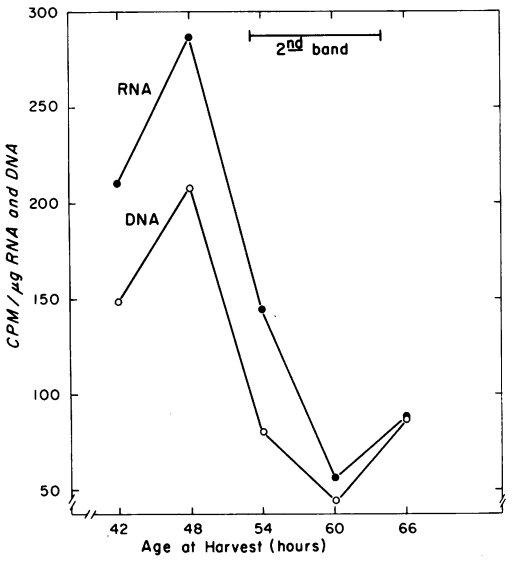

FIG. 9. *^{3}H-uridine incorporation into the nucleic acids of the growing front in the band strain of N. crassa grown in baking-dish cultures on maltose medium. Bars at top indicate periods of conidiation. Each point is the average of three determinations.*

needed an explanation. The results presented in Fig. 1 to 4 indicate that our procedures give values similar to those previously reported, and indicate the magnitude of error associated with these determinations. In addition, they suggest that the diversity noted is due primarily to differences between laboratories with respect to method of culturing and age at harvest. The previously reported values for single time points (12, 14, 20) or serial time points (1, 7, 22) are in agreement with our values for a comparable physiological age and method of culturing.

Our data covering longer time spans and several culture conditions confirm the suggestions of the previous authors that there is a general reduction of nucleic acid content as cultures age, presumably due to the slower growth rate and an increasing synthesis of cell wall and storage material. A parallel decrease in ribonuclease activity with age suggests that the changes detected in DNA and RNA content are not caused by changes in nucleic-acid degradation by nucleases during extraction. The data also reemphasize that important biochemical alterations can result from seemingly minor differences in culture conditions.

Since nucleic acid rhythms were not clearly discernible in the three types of liquid cultures used, the rhythm studies were done with cultures grown on dialysis tubing. The circadian rhythm of CO_2 production also has an extremely low amplitude in liquid-grown cultures (M. L. Sargent, unpublished data). Experiments in progress have not yet ascertained whether these low amplitudes result from a lack of synchronization between mycelial fragments, differentiation of a mycelial mat into metabolically active and inactive portions, suppression of the regulatory mechanisms causing oscillations, or from some other unknown reason.

The rhythms of nucleic acid content and biosynthesis (Fig. 6 to 9) reported here fit well with the observations that conidia are produced rhythmically and that the conidia have more RNA, and especially DNA, than mature mycelia. Although the amplitudes of the oscillations are small to moderate in size (10 to 20 and 3 to 8% above and below a median value for DNA and RNA, respectively), they are significant as consideration of the experimental error inherent in these determinations (Fig. 1 to 3) will verify. The phase relationships between the rhythms suggest that a period of active nucleic acid synthesis immediately precedes the rise in nucleic acid content that is associated with conidiation. Although we have presented no data verifying that the nucleic acid rhythms are in fact "circadian" by the usual criteria, their synchrony with the conidiation rhythm which has been so verified (17) is strong presumptive evidence that such a description is valid.

Since the maxima of the uridine incorporation rhythm occur just prior to the maxima of the nucleic acid content rhythms, the most straightforward interpretation of the pulse experiments would be that the respective polymerase activities vary with time. However, alternative interpretations including nucleic acid degradation, or temporal changes in uridine uptake or pool sizes are feasible and additional experiments will be necessary to decide among them.

Several other biochemical parameters have recently been reported to fluctuate with the conidiation rhythm in *N. crassa*. Pyridine nucleotide levels (4; S. Brody, Amer. Soc. Microbiol. Abstr., 1973, p. 38), energy charge (D. P. Delmar and S. Brody, Amer. Soc. Microbiol. Abstr., 1973, p. 38), and the activities of several enzymes (Hochberg and Sargent, unpublished data) have been found to exhibit circadian rhythmicity in the hyphae of the growing front. A circadian rhythm of carbon dioxide evolution has also been found to be associated with conidiation under several culture conditions (25). Although these biochemical oscillations are temporally associated with the conidiation process, their occurrence (Fig. 8; S. Brody, Amer. Soc. Microbiol. Abstr., 1973, p. 38;

Hochberg and Sargent, unpublished data) in non-conidiating cultures, e.g., fluffy, wild type, or band (conidiation chemically inhibited), indicates that they are not merely a result of the conidiation process. Analysis of possible cause and effect relationships among these various rhythms will hopefully lead to a deeper understanding of the regulatory mechanisms underlying circadian rhythmicity.

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