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Effects of Respiratory Inhibitors on Respiration, ATP Contents, and the Circadian Conidiation Rhythm of *Neurospora crassa*[1]

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ABSTRACT

Effects of respiratory inhibitors on the circadian clock, respiratory activity, and ATP content were examined in *Neurospora crassa*. All inhibitors, potassium cyanide, sodium azide, antimycin A, and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), shifted the phase of the conidiation rhythm. All the phase response curves were similar and resembled that for cycloheximide, but were different from the phase response curve for light. Phase shifting by azide and CCCP was proportional to the lowering of respiratory activity and ATP content, but such a correlation was not observed for cyanide and antimycin A. In particular, cyanide at a concentration of 0.5 millimolar completely depleted ATP of the cultures but did not significantly shift their phase. Their results suggest that large shifts caused by these inhibitors are not due to a decrease in energy from respiratory activity.

Circadian rhythms are observed in many organisms from lower eukaryotic plants to higher animals. However, the molecular mechanism of the clock which regulates these rhythms is still not known. Bünning (2) proposed that the clock is composed of two different phases: a physiological night phase which requires energy and a physiological day phase in which energy is not required. In fact, deprivation of oxygen for short times (1, 18) or treatment with respiratory inhibitors (3, 6, 9–11) resulted in phase shifting at specific phases in several organisms. These results suggest that certain phases of the clock, in particular the subjective night, may be energy dependent. Koyama and Feldman treated *Neurospora* with several respiratory inhibitors to examine the energy dependence of clock function (cited in Feldman and Dunlap [7]). They obtained phase-shifting data but did not measure inhibition of respiration or ATP levels, nor have such measurements been made in other organisms. The correlations such measurements might provide are needed before it can be concluded that energy depletion caused by these inhibitors results in phase shifting of the clock. In this report, both phase shifting by respiratory inhibitors and levels of the respiratory activity and ATP were measured.

MATERIALS AND METHODS

Culture Conditions and Analysis of the Conidiation Rhythm. The bd (band) strain of *Neurospora crassa* was used. Procedures for maintaining stock cultures and for liquid culture were the same as reported previously (12). Conidia (13×10^5) suspended in distilled H_2O were added to 25 ml of liquid medium containing Fries' salts (8), 0.3% glucose, and 0.5% arginine. Conidial concentration were determined by *A* at 480 nm. After culturing for 36 h in continuous light at 26°C, discs were cut from the mycelial mats with a cork borer 11 mm in diameter. Six discs from each mat were transferred to a 125-ml Erlenmeyer flask with 25 ml of liquid medium containing Fries' salts, 0.03% glucose and 0.05% arginine and cultured in continuous darkness with reciprocal shaking (100 cycles per min) at 26°C. The medium was adjusted to pH 7.0 before autoclaving by addition of NaOH. After treatment with chemicals, discs were transferred to race tubes with 8 ml of solid medium containing Fries' salts, 0.15% glucose, 0.25% arginine, and 1.5% agar and cultured in continuous darkness at 26°C. All manipulations after transfer to darkness were done under red acrylic plate filters (Acrylite, Mitsubishi, Japan). The phase of the conidiation rhythm was defined as the time when the first conidial band occurs in the race tubes. This time was calculated using the method described by Dharmananda and Feldman (4). The phase in the liquid culture was calculated from the first band in the race tubes on the assumption that the clock runs in liquid medium with the same period length as on agar medium and that it is not affected by transfer from liquid to solid medium (12, 15). All data are the average of 6 race tubes.

Determination of O_2 Consumption. Oxygen uptake rates were determined polarographically using a Clark-type O_2 electrode connected to a model 53 YSI oxygen monitor. After treatment with inhibitors for 3 h, six discs were immersed in 5 ml of the culture medium in which they had been cultured. The rate of oxygen consumption was determined in red safe light.

Determination of ATP Content. ATP was extracted and assayed as previously described (14). After treatment with inhibitors for 3 h, 18 discs were put into 4 ml of ice-cold 6% $HClO_4$ and homogenized in a glass homogenizer. After standing for 2 h in an ice bath, the homogenate was centrifuged and the precipitate was mixed with 1 ml of 6% $HClO_4$ and centrifuged. All supernatants were combined and neutralized with 1 N KOH using a double-junction reference electrode after addition of glycylglycine to a final concentration of 10 mM. The neutralized solution was filtered with a Millipore filter and used for assay of ATP. Standard sample of authentic ATP was carried through all steps after the grinding. ATP was determined using a CHEM-GLOW photometer (Aminco). The reaction mixture was composed of 0.25 ml of medium containing 16 mM glycylglycine, 20 mM $MgCl_2$, 4 mM KH_2PO_4, and 50 μl of firefly extract prepared from desiccated fire-fly lanterns (Sigma) by the method of McElroy (17). Each sample (0.1 ml) was rapidly injected from the injection port and the amount of light emitted in the first 5 s after injection was used for determination of ATP. Results shown in the figures are averages ± standard deviation from four different experiments.

RESULTS

Phase Response Curves for Respiratory Inhibitors. Mycelial discs were treated with one of several respiratory inhibitors for 3

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h from 24 to 48 h after the beginning of continuous darkness. Just after treatment, each disc was transferred to a race tube and cultured in continous darkness. The phase of the rhythm was compared with that of the control series which was transferred from control medium without addition of chemicals. Discs treated with CCCP[2] or antimycin A, which require dissolving in ethanol, were compared to control discs which were treated with the same concentration of ethanol (final 0.4%); this concentration of ethanol does not affect progress of the clock function (13). Treatment with all respiratory inhibitors, cyanide, azide, CCCP, and antimycin A, resulted in very similar phase response curves with maximum phase delays at CT 3 and maximum phase advances at CT 6 (Fig. 1). (CT 12 is defined as the time of transfer from light to dark). After CT 6, the magnitude of phase advances decreased until there was no phase shift at CT 12. After CT 20 there were gradually increasing phase delays. Maximum phase shifting was an approximate 10-h phase advance at CT 6.

The shape of the phase response curves is very similar to that of cycloheximide, shown in the same figure. Light perturbation also causes phase shifting with maximum phase delay at CT 16 and maximum phase advance at CT 22. The phase response curve for light, then, is completely different from those for the

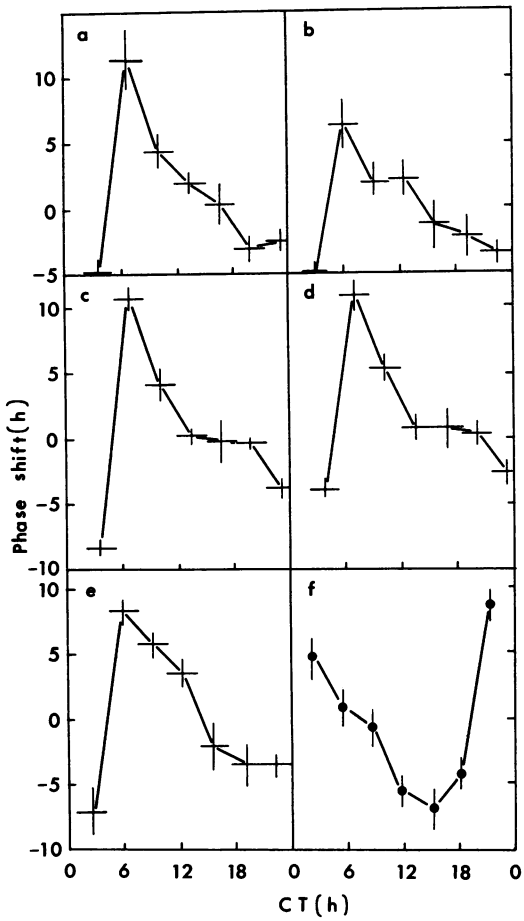

FIG. 1. Phase response curves for respiratory inhibitors, cycloheximide, and light in *Neurospora crassa*. Mycelial discs were treated with 30 μM CCCP (A), 2 mM potassium cyanide (B), 4 mM NaN_3, (C), 1 μg/ml cycloheximide (D), and 0.7 μg/ml antimycin A (E) for 3 h and transferred individually to race tubes, or they were irradiated with white light (1000 lux) (F) for 5 min at different circadian times, cultured for 3 h, and then transferred to race tubes. Error bars are ± SD.

[2] Abbreviations: CCCP, carbonylcyanide *m*-chlorophenyl hydrazone; CT, circadian time; DES, diethylstilbestrol.

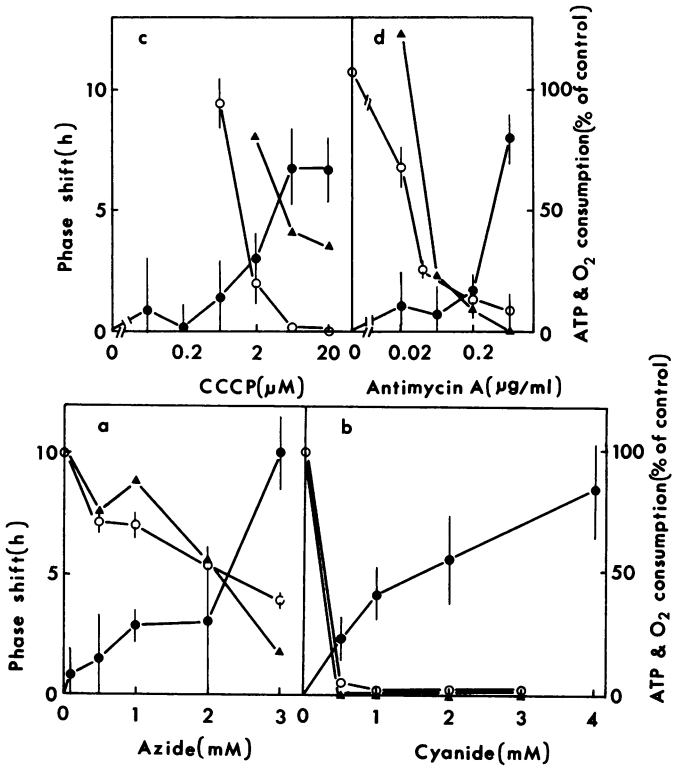

FIG. 2. Effects of respiratory inhibitors on the phase of the clock, respiratory activity, and ATP content in *Neurospora crassa*. Mycelial discs were treated with various concentrations of NaN_3 (A), potassium cyanide (B), CCCP (C), or antimycin A (D) for 3 h starting at CT 6. Six discs were transferred individually to race tubes for determination of phase shifting (●). Six discs were assayed for the rate of O_2 consumption (▲). Eighteen discs were assayed for ATP (O). Error bars are ± SD.

respiratory inhibitors and cycloheximide. The phase most sensitive to light is one that is least sensitive to all the inhibitors examined and *vice versa*.

Effects of Respiratory Inhibitors on Respiration and ATP Content. The effects of various concentrations of the respiratory inhibitors on phase, respiratory activity, and ATP content were measured at CT 6, the time of maximum phase shifting (Fig. 2). Phase shifting by azide and CCCP was proportional to the decrease of ATP and oxygen consumption. However, this correlation was not observed for cyanide and antimycin A. In particular, 0.5 mM cyanide did not affect the phase of the clock but almost completely depleted the ATP content; phase shifting occurred only at higher concentrations of cyanide. Similar results were observed in experiments using antimycin A; there was very little phase shifting when ATP levels were decreased by 90%.

After treatment with any of the respiratory inhibitors, respiratory activity was proportional to ATP levels (Fig. 2).

Recovery of ATP Content after Removal of Inhibitors. Discs were treated with 3 mM cyanide or antimycin A (0.7 μg/ml) for 3 h starting at CT 6 and transferred to Petri dishes containing the same culture medium as that of the race tubes. At various times they were harvested and assayed for ATP (Fig. 3). At these concentrations both inhibitors caused almost maximum phase shifting (Fig. 2). The ATP content of the discs increased rapidly after removal of the inhibitors and after 2 h was nearly normal in the case of cyanide, and was over 50% of control levels in the case of antimycin A.

DISCUSSION

Respiratory inhibitors can phase shift the clock in several organisms. Usually, the amount of the phase shifting approxi-

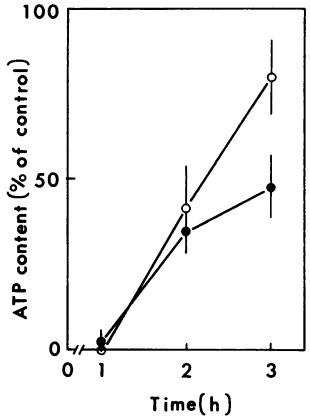

FIG. 3. Increase of ATP after removal of respiratory inhibitors. Mycelial discs were treated with 3 mM potassium cyanide (O) or 0.7 μg/ml antimycin A (●) for 3 h and transferred to solid medium not containing the inhibitors. At various times after transfer, the discs were harvested for determination of ATP content. Control ATP levels were determined by assaying mycelial discs which were harvested just before addition of the inhibitors.

mately equals the time of treatment. Bünning *et al.* (3) reported that 4-h treatments with cyanide and dinitrophenol resulted in 4-h phase shifts in *Phaseolus*. Six-h treatments with the same chemicals in *Aplysia* resulted in almost the same length of phase delays in the subjective night (6). A similar result was reported in *Phaseolus* (11). However, in *Neurospora*, such inhibitors can shift the phase by 10 h with 3-h treatments. Further, phase was drastically shifted during the subjective day (from CT 0 to CT 12), but not in the subjective night (from CT 12 to CT 24). This suggests that processes other than respiration may mediate effects of metabolic inhibitors on the *Neurospora* clock. This suggestion is supported by the fact that phase shifting by the inhibitors is not quantitatively correlated with changes in the respiratory activity and ATP content. Experiments with cyanide is a typical example of this point; depletion of ATP by 0.5 mM cyanide did not cause phase shifting (Fig. 2). Cyanide immediately lowers ATP levels after addition to the medium (16) and ATP content recovers rapidly when the inhibitor is removed (Fig. 3). If cyanide caused a 3-h phase shift with a 3-h treatment, it would be difficult to detect such a small phase change with the present culture method. It is, therefore, impossible to exclude the possibility that the clock of *Neurospora* stops immediately when the ATP content is lowered but runs normally when ATP levels recover. However, results presented here show that decreased ATP levels are not the sole explanation for large phase shifts caused by metabolic inhibitors.

It was previously concluded from studies using DES and related compounds that the plasma membrane ATPase is not a component of the clock in *Neurospora* (13). However, it seemed possible that DES indirectly inhibits plasma membrane ATPase activity by lowering ATP levels and thus shifts the phase of the clock. However, this possibility is not supported by data presented here since complete depletion of ATP by 0.5 mM cyanide does not cause phase shifting (Fig. 2).

It is difficult to speculate on the specific cell functions which are altered by the respiratory inhibitors. The phase response curves were very similar to that for DES (Fig. 1). It was previously suggested that DES shifts the phase by inhibiting some mitochondrial function(s) (13); calcium transport in mitochondria was later suggested to be important for clock function in *Neurospora* (14). The respiratory inhibitors used in experiments presented here may affect such a process and cause phase shifting. Involvement of mitochondria in the clock mechanism has been suggested for *Neurospora* (5), *Aplysia* (6), and also recently in *Lemna* (10). Further analysis of mitochondrial function is necessary to explore any possible involvement in the molecular mechanism of the circadian clock. Another possibility is that the inhibitors indirectly suppress protein synthesis by depleting energy since the phase-response curves for the respiratory inhibitors and cycloheximide are very similar. However, the disproportion between phase shifting and decrease of ATP levels (Fig. 2) does not support this possibility.

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