Circadian Rhythms of Chemotaxis to Ammonium and of Methylammonium Uptake in Chlamydomonas

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ABSTRACT

Chlamydomonas reinhardtii expresses a well-documented circadian rhythm of phototaxis, which peaks in the subjective daytime. We find that vegetative cells also express circadian rhythms of chemotaxis to ammonium and ammonium uptake (as gauged by uptake of [14C]methylammonium). The chemotaxis rhythm peaks in the subjective night. Methylammonium uptake is light dependent, and its rhythm peaks at subjective dawn. Unlike vegetative cells, gametes are not attracted to ammonium. We believe this to be the first report of a circadian rhythm of chemotaxis.

The unicellular alga Chlamydomonas reinhardtii displays several circadian rhythms, including phototaxis (1) and “stickiness” to glass (24). Of these, the phototaxis rhythm has been investigated most extensively: Bruce and Bruce (2) isolated clock mutants, drugs have been assayed for their ability to phase shift the rhythm (4), and Kondo et al. (11) measured action spectra for light-induced phase resetting.

Phototaxis of Chlamydomonas cells is high during the day phase of an LD cycle and is low at night. This rhythm persists in DD or LL, i.e. phototaxis remains high in subjective daytime and low in the subjective night (1). The rhythm is a true change in phototactic response per se and is not a daily change of motility (12, 19). Thus, the cells are motile at night, even though they do not display phototactic behavior. Is this nighttime motility useful to the cells? We wondered whether there might be some other adaptive motile behavior that the cells exhibit at night. An obvious candidate would be chemotaxis to nutrients. Hirschberg and Rodgers (7) reported chemoresponses of Chlamydomonas cells to several amino acids, fatty acids, and inorganic salts, but in a later study, Sjoblad and Frederikse (23) were only able to confirm potent chemotaxis to ammonium. Therefore, we tested whether Chlamydomonas cells are attracted to ammonium at night.

We found that Chlamydomonas not only displayed chemotaxis to ammonium at night but that the chemotaxis was rhythmic. On an LD cycle, chemotaxis was much higher at night than during the day. This rhythm persisted after the cells were transferred to various LL regimens. This indicates that ammonium chemotaxis is controlled by a circadian clock.

Ammonium uptake into the cells, as assayed by light-dependent [14C]methylammonium uptake, also displays circadian rhythmicity. Surprisingly, the peak of chemotaxis to ammonium does not correspond with the peak uptake of methylammonium: the peak of methylammonium uptake is approximately 6 h after the peak chemotaxis. Therefore, although ammonium chemotaxis is maximal in the middle of the subjective night, maximal uptake of the nutrient into the cells does not occur until subjective dawn.

MATERIALS AND METHODS

Cell Strain and Growth Conditions

All cells used in the experiments described in this paper belong to the CC-125 strain of Chlamydomonas reinhardtii maintained at the Chlamydomonas Genetics Center at Duke University and were provided by Dr. Elizabeth Harris. Cells were grown in 125-mL Erlenmeyer flasks with approximately 75 mL of HSM (5) containing 3.1 mM ammonium chloride (hereafter referred to as 0.3 HSM; the medium is about pH 7.0). Before entrainment, 5 mL of cells was transferred to 50-mL Erlenmeyer flasks containing 20 mL of fresh 0.3 HSM and incubated with gentle shaking under cool-white fluorescent light of about 25 μmol m–2 s–1 (Li-Cor quantum meter) at 22 to 23°C (all light used in this study was cool-white fluorescent light).

Cells were entrained to LD cycles of 12 h of light alternating with 12 h of darkness (LD 12:12) before use in an experiment. Cell cultures were grown under these conditions for 7 to 12 d to a cell density of about 2.0 × 105 cells/mL. Cell density was measured (a) by the culture’s A600 and/or (b) by counting cells with a Reichert-Jung Bright Line hemacytometer.

Chemotaxis Assay by Hemacytometer Counts

One hour before the time of chemotactic assay, samples were withdrawn from the culture flasks with a sterile pipette and transferred to 15-mL polypropylene centrifuge tubes. Samples were centrifuged for 3 min at approximately 2000g...
in a clinical centrifuge, and the resulting supernatant was aspirated. The cells were then resuspended in nitrogen-free HSM (HSM without any nitrogen source). Cells were centrifuged and resuspended once more to ensure that the cells were effectively deprived of ammonium ions in the medium, and the samples were then returned to the prior illumination conditions (e.g., darkness or light).

After 1 h of nitrogen deprivation, the cells were removed from the light boxes and dispersed in dim red light into wells of a Lucite chemotaxis chamber of the type designed to assay bacterial chemotaxis (18). Each chamber has two wells, each of which holds a total of 550 μL of a cell suspension, and a lane connecting the two wells allows immersion into the cell sample of a 5-μL glass capillary tube (Drummond Scientific Co.) filled with test or control medium. Unless specified otherwise, the test medium is 1.0 HSM (HSM medium containing 9.3 mM ammonium chloride), and the control medium is nitrogen-free HSM. To prevent interference by phototaxis reactions, the chemotaxis chambers were placed into darkness for the 30-min assay period.

After 30 min, the chambers were removed from darkness, the capillary tubes were gingerly removed from the chambers with forceps, and excess medium was carefully blotted from the exterior of the capillary tube with a Kimwipe tissue. The tubes were carefully expelled into 25 μL of a mild iodine solution to immobilize the cells.

After the suspension was mixed, it was transferred to the hemacytometer, and the cells in 9 × 10^4 mL were counted. This is the cell number plotted on the ordinates of the figures that illustrate chemotaxis. In all experiments, accumulation of cells into capillary tubes containing test medium (e.g., 1.0 HSM) was compared with accumulation into capillary tubes containing control medium (nitrogen-free HSM). Usually, triplicate assays of test and control medium were performed on each sample, although occasionally duplicates or quadruplicates were measured.

We found three features of the culture conditions to be critically important for potent expression of chemotaxis to ammonium. First, ammonium chemotaxis is dependent upon extracellular calcium, as reported by Sjoblads and Frederikse (23). We repeated their experiments, finding that calcium levels in the medium (both inside and outside the capillary tubes) must be >100 μM for maximal chemotaxis to ammonium. Second, we found that the ambient temperature is crucial for optimal expression of the chemotactic rhythm. At temperatures >25°C, chemotaxis is depressed, and the daily rhythm is not expressed (see later). Finally, we found that the cells must come from medium containing significant ammonium levels before the resuspension in nitrogen-free HSM.

To check this, we routinely tested the ammonium content of the original medium with EM Quant ammonium test strips (EM Science, Gibbstown, NJ) to be sure that the ammonium level was >10 mg/L. As shown in “Results,” cells in cultures that have become depleted of ammonium for more than 10 to 16 h no longer show chemotaxis to ammonium. Depletion of ammonium from the medium can occur if the cells grow to a density >4 × 10^5/mL in 0.3 HSM.

Chemotaxis Assay Using Radiolabeled Cells

In preliminary experiments, we quantified chemotactic accumulation using cells labeled with ^35S in the chemical form of sulfate. We suspended cells in sulfate-free medium and then incubated them overnight with [^35S]sulfate in doses of 2 to 4 μCi/mL of cell suspension. Chemotaxis was assayed with capillary tubes as described above; the assay capillaries were emptied into scintillation fluid (10 mL) and counted on a scintillation counter. This method gave the first indications of rhythm chemotaxis, at which point we switched to the more direct method of chemotaxis assay described above.

Assay of Methylammonium Uptake

Chlamydomonas cells were resuspended in nitrogen-free HSM by the same procedure described above for chemotactic assay. After 1 h of incubation, [^14C]methylammonium was added to the cultures (0.5 μCi/mL; [^14C]methylammonium obtained from New England Nuclear). At each time point thereafter, 0.5 mL of cell suspension was filtered through a GF/C filter under vacuum and washed with 10 mL of ice-cold 1.0 HSM plus 10 mM nonradioactive methylammonium. The radioactivity in the samples was assessed by liquid scintillation counting. When the uptake was assayed in the light, the samples were illuminated during the incubation with [^14C] methylammonium with cool-white fluorescent light (25 μmol m^-2 s^-1).

Methylammonium uptake seems to be a valid gauge of ammonium uptake in Chlamydomonas, because (a) ammonium competes with methylammonium (data not shown) and (b) a single-gene mutation in Chlamydomonas has been reported to alter the transport of both ammonium and methylammonium (3).

RESULTS

Daily Rhythm of Chemotaxis

Figure 1 shows that chemotaxis to ammonium is rhythmic in cells exposed to an LD cycle. Ammonium chemotaxis is maximal in the middle of the night and is quite low during the daytime. Comparison of data from many experiments indicated that the exact time of the chemotactic peak is variable but is within the subjective nighttime, usually between LDT 16 and 20. Control values, as gauged by the accumulation of cells into capillary tubes filled with nitrogen-free HSM, were low at all phases. Chlamydomonas cells are fully motile in both the control (nitrogen-free HSM) and chemoattractant (1.0 HSM) media; therefore, there is no possibility of an artifact due to “trapping” of immotile cells within the capillaries. In this experiment and in most of the other experiments described in this paper, cell densities increased during the time course of the experiment. In all cases, however, the growth rate was slow enough that 80 to 90% of the cells were motile at every assay time point. Therefore, the rhythm of chemotaxis is also not due to an artifact relating to a rhythm of cell division. Statistical analysis (two-factor ANOVA) of the data of Figure 1 indicates highly significant differences (P < 0.01) between: (a) experimental and controls, (b) time groups, and (c) interaction effect of time and treatment. The rhythmic pattern of chemotaxis to ammonium is the reverse of the pattern of rhythmic phototaxis, i.e., the phototactic rhythm peaks at phases between dawn and the middle of the day (1).
To determine whether the daily rhythm of chemotaxis was circadian, we tested whether it would persist in constant conditions. We tested several different constant regimes, i.e., in each case, the temperature was maintained at a constant level, but the pattern of illumination was changed.

The first illumination condition was simply to transfer cells from an LD cycle to DD. Figure 2 illustrates the result of an experiment in which cells in LD were transferred after 72 h to DD. The cells expressed a high-amplitude rhythm in LD. After transfer to DD, a rhythm clearly persisted for at least three cycles, even though the amplitude of the chemotaxis rhythm dampened significantly.

Figure 3 depicts the results from constant conditions experiments in which cells were transferred from LD cycles to LL. Cells were transferred from LD to constant bright LL (light intensity of bright LL = 25 μmol m⁻² s⁻¹) (Fig. 3A). In this case, the chemotaxis rhythm appeared to dampen or to become disrupted within 1 d of the onset of LL. If cells were transferred to constant dim LL (4 μmol m⁻² s⁻¹), an obvious circadian rhythm persisted for at least three cycles (Fig. 3B). In dim LL, chemotaxis continued to peak in the subjective nighttime; the peak shifted to later times after each cycle, suggesting that the free-running period is longer than 24 h, as is the phototactic rhythm in LL (11).

Finally, we tested the expression of the chemotactic rhythm under the same illumination regimen usually used to assay the phototactic rhythm: a high-frequency LD cycle of 15 min light followed by 45 min darkness (11). This LD regimen is also "constant conditions," inasmuch as the 1-h light cycle is far from the limits of entrainment for the circa-24 h biological clock. Figure 4A shows two experiments in which cells were transferred at dusk of a final LD cycle to the high-frequency LD cycle. Circadian rhythms of chemotaxis to ammonium clearly persisted under this regimen. Figure 4B also illustrates that cells were rhythmically chemotactic to methylammonium (test solution = 9 mM methylammonium), although the amplitude of this rhythm was less than that for ammonium. Because chemotaxis to ammonium displayed a rhythm that could be entrained to a 24-h LD cycle and persisted in constant conditions with a period of approximately 24 h, chemotaxis can be considered to be another circadian behavior expressed by *Chlamydomonas* cells.

In addition to the persistence of a rhythm in constant conditions with an approximately 24-h cycle, another salient feature of circadian rhythms is the phenomenon of temperature compensation. We did not rigorously test this criterion for the chemotaxis rhythm, as has been done for the phototaxis rhythm (1; also C.H. Johnson and T. Kondo, unpublished data), but we checked the expression of the chemotaxis rhythm at various temperatures. The data shown in this paper come from experiments performed at 22 to 23°C. At 18°C, a chemotaxis rhythm was expressed for at least 4 d in dim LL with a period that was indistinguishable from that at 23°C, given that our assay points were at 6-h intervals (data not shown). At temperatures >25°C, rhythmic expression was lost, with chemotaxis being low at all phases. This observation is not a problem for our interpretation that a circadian pacemaker controls chemotaxis; it merely means that tempera-

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**Figure 1.** Chemotaxis of cells in LD conditions. Chemotaxis was assayed every 6 h for 4 d by counting the number of cells accumulating in capillary tubes filled with N-free ("controls") or N-containing ("experimental") media. The number of cells, determined as described in "Materials and Methods," accumulating in tubes (ordinate) is plotted against the time in hours from the beginning of the experiment (abscissa). ○, Controls (triplicates); Δ, experimental (triplicates). Mean value points are connected by lines. Chemotaxis peaks in the middle of the 12-h period of darkness. In this experiment, cell densities increased from 1.8 to 2.7 × 10⁶ cells/mL. Two-factor ANOVA shows significant differences between experimental and controls at P < 0.01 (experiment 53).

**Figure 2.** Persistence of chemotaxis in DD. Chemotaxis was assayed as in Figure 1 for 3 d in LD and then transferred at hour 72 to DD. Chemotactic response is plotted as in Figure 1. ○, N-free HSM controls (quadruplicates); Δ, 1.0 HSM experimental values (quadruplicates). Mean value points are connected by lines. Chemotaxis in DD continues to peak in mid- to late-subjective night (CT 18-24) and is always lowest at the end of the light period (CT 12). Cell densities increased from 1.8 × 10⁶ cells/mL on the first day to a peak of 3.3 × 10⁶ cells/mL on day 4 and then decreased to 1.5 × 10⁶ cells/mL by the fourth day of DD. Two-factor ANOVA shows highly significant differences between experiments and controls at P < 0.01 (experiment 57).
Figure 3. Persistence of chemotactic rhythm in LL. A, Cells were assayed as in Figure 1 for 3 d in LD and then for 4 d in constant bright light (25 μmol m\(^{-2}\) s\(^{-1}\)). Under these conditions cell densities increased from 2.0 × 10\(^{6}\) cells/mL on the first day to a peak of 3.9 × 10\(^{6}\) cells/mL on the seventh and final day. B, Cells were assayed as in Figure 1 for 1 d of LD and then for 4 d in constant dim light of 4 μmol m\(^{-2}\) s\(^{-1}\). Under these conditions, cell densities increased from 1.7 × 10\(^{6}\) cells/mL at the start of observations to a peak of 2.2 × 10\(^{6}\) cells/mL on the fifth and final day. Ordinate, Number of cells accumulating in triplicate (B) or quadruplicate (A) capillaries; abscissa, time in hours from the beginning of observations; ○, controls; △, experimental; mean point values are connected with lines. Two-factor ANOVA indicate highly significant differences (P < 0.01) between the analysis groups as in Figure 1, except for the sixth cycle in A (times 120–138 h), which is significant at P < 0.05 (A, experiment 58; B, experiment 72).

Figure 4. Persistence of rhythmic chemotaxis to ammonium and to methylammonium in a high-frequency LD cycle. Cells were first tested as in Figure 1 for chemotaxis to ammonium (A and B) and to methylammonium (B only) under standard LD conditions. After 24 and 42 h, respectively, cells were placed in a cycle of 15 min of light (25 μmol m\(^{-2}\) s\(^{-1}\)) followed by 45 min of darkness (hatched bar). This light cycle contains no 24-h cues and has been shown to allow expression of circadian rhythms of phototaxis. It also retards the more rapid increases in cell density associated with constant bright light. A, Densities increased from 2.2 × 10\(^{6}\) cells/mL on the first day to 3.1 × 10\(^{6}\) cells/mL on the fourth day. B, Densities increased from 2.0 × 10\(^{6}\) cells/mL on the first day to 3.3 × 10\(^{6}\) cells/mL on the fourth day. Ordinate, number of cells accumulated in assay capillaries (quadruplicates in A and duplicates B); abscissa, time in hours from the start of observations; ○, controls; △, responses to 9 mM ammonium; ▲, responses to 9 mM methylammonium (experiments 62 and 68).
tempatures >25°C are out of range of circadian expression (17). Within at least 18 to 25°C, rhythms of chemotaxis are expressed with a period that is temperature compensated.

Circadian Rhythm of Methylammonium Uptake

Given that *Chlamydomonas* expresses a circadian rhythm of chemotaxis, we hypothesized that the cells might express a correlated rhythm of ammonium uptake. In this context, it is worth mentioning that a daily rhythm of ammonium uptake has been observed in the ryegrass, *Lolium* (6). To assess ammonium uptake in *Chlamydomonas*, we measured the cells' uptake of [14C]methylammonium. The use of methylammonium is commonly used as a gauge of ammonium uptake. In support of this interpretation, a single-gene mutation of *Chlamydomonas* has been shown to abolish the uptake of both ammonium and methylammonium, suggesting that the uptake mechanism is the same for both ions (3). Moreover, we found that extracellular ammonium inhibits methylammonium uptake (data not shown).

Figure 5 shows the uptake of methylammonium into *Chlamydomonas* cells from both midday (LDT 6) and midnight (LDT 18) phases of an LD cycle. Methylammonium uptake was strikingly dependent on the illumination conditions; light stimulated the uptake. In addition, Figure 5 clearly shows that methylammonium uptake is more robust into cells from the light phase than from the dark phase.

When we tested the possibility of a rhythm of methylammonium uptake, the data startled us. We had expected that methylammonium uptake would be highest when the cells

Figure 5. Methylammonium uptake of midday and midnight phase cells in both light and darkness. Samples were drawn from cell colonies in both the midday (LDT 6) and midnight (LDT 18) phases of an LD 12:12 cycle. Samples of each phase were then tested for [14C]methylammonium uptake in either cool-white fluorescent light (25 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) or darkness. Ordinate, cpm in units of 10^4 counts; abscissa, time elapsed from the addition of radioactive methylammonium in min; O, midday phase (LDT 6) measured in light conditions; •, midday sample measured in darkness; △, the midnight samples (LDT 18) measured in light; ▲, midnight sample measured in darkness. Cell density for this experiment was 1.3 x 10^6 cells/mL for all samples (experiment 71). The values of methylammonium uptake are not zero at the first time point (30 s); because formalin-fixed cells trap the same amount of labeled methylammonium as the living cells at the first time point, we believe this value is the result of some binding of the label to cell components.

Figure 6. Comparison of ammonium chemotaxis and methylammonium uptake in standard LD and high-frequency LD cycles. Cells were tested for ammonium chemotaxis as in Figure 1 for 24 and 48 h, respectively, and then placed into the high-frequency LD cycle described in Figures 4. At the indicated times, aliquots from the same cultures were simultaneously withdrawn and tested for [14C]methylammonium uptake. Left ordinates, mean number of cells accumulated in duplicate capillaries; right ordinates, [14C]methylammonium uptake as measured in units of 10^4 cpm/h of incubation in [14C]-containing medium; abscissa, time in hours from the start of observations. △, Mean chemotaxis values; bars, ±1 se. Control values for chemotaxis are not shown to avoid cluttering the graph. Controls never exceeded 178 and were always lower than the experimentals for the same time point. Both chemotaxis and uptake samples were deprived of ammonium for 1 h before assay, during which time the high-frequency LD cycle continued. At the end of the 1-h incubation, [14C]methylammonium was added to the uptake samples; one uptake sample was placed in the light (25 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and the other in darkness. ▲, Difference in [14C]uptake between the light and dark samples after 60 min incubation. Cell densities increased from 1.4 to 1.5 x 10^6 cells/mL in A and from 1.4 to 2.1 x 10^6 cells/mL for the experiment shown in B (experiments 70 and 71, respectively).
were most strongly attracted to ammonium. This was not so, as shown in Figures 6 and 7. Figure 6 depicts the results of two experiments of cells in LD 12:12 transferred to the high-frequency light cycle. Figure 6A shows that light-dependent methylammonium uptake was highest in the day phase of LD, when chemotaxis is lowest, and plummets at night, when chemotaxis peaks. After transfer to constant conditions (i.e. high-frequency LD cycle), a rhythm of methylammonium uptake persisted, but a difference in the phase relationship between the two rhythms still existed, i.e. the methylammonium uptake rhythm peaked about 6 h after the chemotaxis rhythm (Fig. 6). In this experiment, the chemotaxis rhythm peaked in the middle of the subjective night, whereas the methylammonium uptake rhythm peaked at about subjective dawn. In other experiments in which the peaks of the chemotaxis and uptake rhythms were more precisely mapped, the peak of the uptake rhythm lagged that of the chemotaxis rhythm by 4 to 8 h (data not shown).

The same phase relationships are obvious after cells are transferred to constant dim light, as Figure 7 illustrates. Again, rhythms of both chemotaxis and light-dependent methylammonium uptake persisted in dim LL, with the light-dependent methylammonium uptake rhythm phase lagging the chemotactic rhythm by about 6 h. The rate of methylammonium uptake assayed in the dark was minimal at all phases in LL or in the high-frequency light cycle.

Could the methylammonium uptake rhythm be some artifact of using methylammonium and not reflective of ammonium uptake? This seems unlikely for three reasons: (a) single-gene mutations block both ammonium and methylammonium uptake (3), (b) extracellular ammonium inhibits methylammonium uptake (not shown), and (c) Chlamydomonas cells exhibit rhythmic chemotaxis to methylammonium as well as to ammonium, albeit less strongly (Fig. 4B). Consequently, we believe that methylammonium is an accurate gauge of a light-dependent ammonium uptake rhythm.

**Role of Light in Methylammonium Uptake**

Because light stimulates methylammonium uptake, we wondered whether the methylammonium uptake rhythm reflected an underlying rhythm of photosynthetic capacity, such as has been observed in other algae (13, 21). Therefore, we measured photosynthetic oxygen evolution under saturating light conditions and subsaturating/saturating bicarbonate conditions from cells tested at various circadian phases in dim LL. We could detect no rhythm of photosynthetic oxygen evolution (data not shown).

We also tested the impact of DCMU, an inhibitor of photosynthetic electron flow, on light-dependent methylammonium uptake. DCMU at a concentration that completely inhibits oxygen evolution (1 µM) did not inhibit methylammonium uptake. At a concentration of 5 µM, DCMU inhibited methylammonium uptake by about 4%, but the effect of the drug at this concentration was probably less specific for photosynthesis (DCMU data not shown).

**Gametes Do Not Respond Chemotactically to Ammonium**

Because nitrogen deprivation is the standard technique for initiating gametogenesis in Chlamydomonas, we wondered whether chemotaxis to ammonium might actually be a property of gametes instead of vegetative cells. This is clearly not true, as the results of Table 1 show. After 24 h in nitrogen-free medium in LL (standard gametogenic conditions, see ref. 5), Chlamydomonas cells differentiate into gametes, which are not attracted to ammonium. In other experiments (not shown), we found that chemotaxis decreases about 10 to 16 h after nitrogen deprivation, which is approximately the time at which mating-reactive gametes first appear (5). Perhaps this change in chemotactic behavior might be used as a gauge of gametogenic differentiation. For the purposes of this report, however, the main point is that mature gametes do not chemotax to ammonium; therefore, our results concerning chemotaxis and methylammonium uptake using cells that have been ammonium depleted for 1 h are not artifacts of some advanced stage of gametogenesis.

**DISCUSSION**

**Circadian Rhythms of Ammonium Uptake and Chemotaxis**

Our results show that Chlamydomonas cells express daily rhythms of ammonium uptake and chemotaxis which persist in constant conditions. The presence of light affects the damping of the rhythm in constant conditions; the rhythm persists
with greater amplitude in either continuous dim light (dim LL) or in a high-frequency LD cycle (15 min light/45 min dark) than in DD. This result is not surprising; circadian rhythms in many plants persist better in LL than in DD. In some cases, this enhanced persistence is undoubtedly due to the maintenance of metabolic energy by photosynthesis. In other cases, however, LL promotes a higher amplitude of plant rhythms even when the light is not used for photosynthesis (9, 10, 22).

The rhythms of ammonium uptake and chemotaxis appear to be under the control of a circadian pacemaker(s), as has already been shown for phototaxis (1, 11). Are these various rhythms controlled by the same circadian pacemaker? We have no data that answers this question. Clearly, the phase relationships of these rhythms are different; phototaxis peaks at subjective early- to midday, chemotaxis to ammonium peaks in the subjective night, and methylammonium uptake peaks at about subjective dawn. Nevertheless, these rhythms could all be controlled by the same pacemaker: e.g. there is no apparent desynchronization among these rhythms during constant-condition free running for one to four cycles. Although evidence exists that supports the possibility of multiple oscillators in higher organisms (20), there is no conclusive evidence to date for multiple pacemakers in unicellular organisms (16). Therefore, this issue remains unresolved at the present time.

Proximal Bases for Chemotactic and Uptake Rhythms

A viable strategy for discovering the mechanism of a circadian pacemaker is to track the control pathway of an overt rhythm upstream to the clock mechanism (8). We are, therefore, interested in tracking the control pathways of the chemotactic and uptake rhythms. First, the chemotactic rhythm does not seem to be controlled by the ammonium uptake rhythm, because the phase relationships of these rhythms do not coincide. Second, we hypothesized that rhythms of calcium transport might be involved in the control network, because chemotaxis to ammonium is dependent upon extracellular calcium (ref. 23; our own data, not shown). Therefore, we measured $^{45}$Ca uptake at midday and midnight phases in ammonium-depleted cells. Ammonium failed to stimulate or inhibit $^{45}$Ca uptake in a phase-dependent manner. Therefore, we presently have no data supporting a rhythmic change in calcium transport that might control rhythmic chemotaxis or ammonium uptake.

Because methylammonium uptake is highly dependent upon light, we tested whether a rhythm of photosynthetic capacity might underlie the rhythm of ammonium uptake. We found no evidence for a rhythm of photosynthetic capacity. This is not to say, however, that ammonium uptake is not dependent on photosynthesis; it may well be that ammonium uptake depends upon ATP generated by photophosphorylation (which is not very sensitive to DCMU). But the rhythm of ammonium uptake is not controlled in a rate-limiting fashion by a rhythm of photosynthesis.

It could be that the rhythm of ammonium uptake is due to a rhythm of activity of enzymes in the ammonium assimilation pathway, e.g. glutamine synthetase and glutamate synthase (15). Interestingly, daily (not necessarily circadian) rhythms of glutamine synthetase and glutamate synthase activity have been reported during the cell division cycle of *Chlamydomonas* (14). We have not yet measured the activities of these enzymes during the circadian cycle of nondividing cultures, but such experiments may provide a clue for the basis of the ammonium uptake rhythm.

Adaptive Significance of Rhythms of Ammonium Chemotaxis and Uptake

*Chlamydomonas* cells express two taxis rhythms: phototaxis and chemotaxis. Phototaxis peaks in the daytime, which seems reasonable because it will allow the cell to find an optimal environment for photosynthesis. When the cells cannot expect to photosynthesize (nighttime), phototaxis turns off and chemotaxis to ammonium turns on. Presumably, nighttime chemotaxis allows the cells to find a nutrient-rich environment.

The phase relationship of the methylammonium rhythm, however, initially puzzled us. Why move toward ammonium maximally at midnight but then wait 6 h to maximally utilize this nutrient? We proffer that the key to this riddle lies in the fact that ammonium uptake/assimilation is a light-dependent mechanism. Because ammonium uptake/assimilation must wait for dawn in nature, the uptake/assimilation system is not fully activated until that time. Perhaps chemotaxis to

### Table I. Response of *Chlamydomonas Gametes* to Chemotactic Stimulation

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ammonium at night enables the cells to find a nutrient-rich environment to exploit whence comes the new dawn. At that time, cells can assimilate as much ammonium as possible and switch into a predominantly phototactic mode.

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LITERATURE CITED