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Circadian Rhythm in Amino Acid Uptake by *Synechococcus* RF-1[1]

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ABSTRACT

In the prokaryote *Synechococcus* RF-1, circadian changes in the uptake of L-leucine and 2-amino isobutyric acid were observed. Uptake rates in the light period were higher than in the dark period for cultures entrained by 12/12 hour light/dark cycles. The periodic changes in L-leucine uptake persisted for at least 72 hours into continuous light (L/L). The rhythm had a free-running period of about 24 hours in L/L at 29°C. A single dark treatment of 12 hours could initiate rhythmic leucine uptake in an L/L culture. The phase of rhythm could be shifted by a pulse of low temperature (0°C). The free-running periodicity was "temperature-compensated" from 21 to 37°C. A 24 hour depletion of extracellular Ca^{2+} before the free-running L/L condition reduced the variation in uptake rate but had little effect on the periodicity of the rhythm. The periodicity was also not affected by the introduction of 25 mM $NaNO_3$. The uptake rates for 20 natural amino acids were studied at 12 hour intervals in cultures exposed to 12/12 hour light/dark cycles. For eight of these amino acids (L-Val, L-Leu, L-Ile, L-Pro, L-Phe, L-Trp, L-Met, and L-Tyr), the light/dark uptake rate ratios had values greater than 3 and the rhythm persisted in L/L.

Circadian rhythms have been documented for organisms throughout the eukaryotic kingdoms (3). Circadian rhythmicity had not been detected in any prokaryote until recently, when endogenous circadian timing in nitrogen-fixation (5, 8, 12) and cell division (12, 16) were reported in some strains of unicellular cyanobacteria belonging to the genus *Synechococcus*.

In eukaryotic organisms, endogenous circadian changes in the uptake rates of histidine and lysine have been observed in the yeast *Saccharomyces cerevisiae* (4).

In this report, we investigated the uptake rates of natural amino acids in the prokaryotic *Synechococcus* RF-1 in an attempt to observe endogenous circadian variations. For comparison, we also investigated uptake rates of L-leucine in *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803.

MATERIALS AND METHODS

Synechococcus RF-1 (PCC 8801) was cultured as described previously (8). The culture was illuminated with white light at about 35 μE m^{-2} s^{-1} from fluorescent lamps (Toshiba FL20D/18, Taiwan Flouresent Lamp Co., Taiwan) under 12/12 h L/D[2] cycles or L/L. *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 were kindly provided by Dr. Louis Sherman at Purdue University. The cells were cultured under the same conditions as *Synechococcus* RF-1 except that the culture medium included 18 mM $NaNO_3$.

L-[^{14}C]Tryptophan was obtained from DuPont-New England Nuclear; other ^{14}C-amino acids were from Amersham, UK. Other chemicals were from Sigma.

Uptake of Amino Acids

Cells were cultured without shaking in BG-11 medium (15) free of nitrate and EDTA but containing 10 mM EPPS (*N*-[2-hydroxyethyl]piperazine-*N'*-3-propanesulfonic acid) buffer, pH 8.0. To start the uptake experiment, 180 μL cell suspension was added to 20 μL of 17 μM ^{14}C-amino acids and incubated for 30 min. One hundred microliters of labeled cell suspension was then filtered by suction through a prewetted 1.2 μm Millipore filter. The filter was collected after being rinsed once with 5 mL cold medium and its radioactivity was measured with a Beckman LS9800 liquid scintillation counter. To correct for background counts, 180 μL of cell suspension was heated to 90°C for 20 min, cooled in an ice bath, and mixed with 20 μL of ^{14}C-amino acid solution, whereafter the suspension was immediately filtered and the cells processed as above for the live cells.

RESULTS AND DISCUSSION

Circadian Rhythm in Leucine and AIB Uptake

When cells of *Synechococcus* RF-1 were cultured under 12/12 h L/D cycles, the uptake rates of leucine (Fig. 1A) and non-metabolizable AIB (Fig. 2) fluctuated periodically and were several times higher during the light period than during the dark period. After the cultures were subsequently exposed to L/L, the periodic variations in leucine and AIB uptake persisted for at least 72 h (Fig. 1B) and 60 h (Fig. 2A), respectively, without a noticeable change in periodicity. The average rhythm period under free-running conditions was about 24 h. The amino acid uptake rhythm was about 12 h out of phase with the nitrogen-fixation (5, 8) circadian rhythm of *Synechococcus* RF-1.

The uptake rate of leucine became steady after the culture was adapted to L/L for a long time. A single dark treatment

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[2] Abbreviations: L/D, light-dark; L/L, continuous light; AIB, 2-amino isobutyric acid.

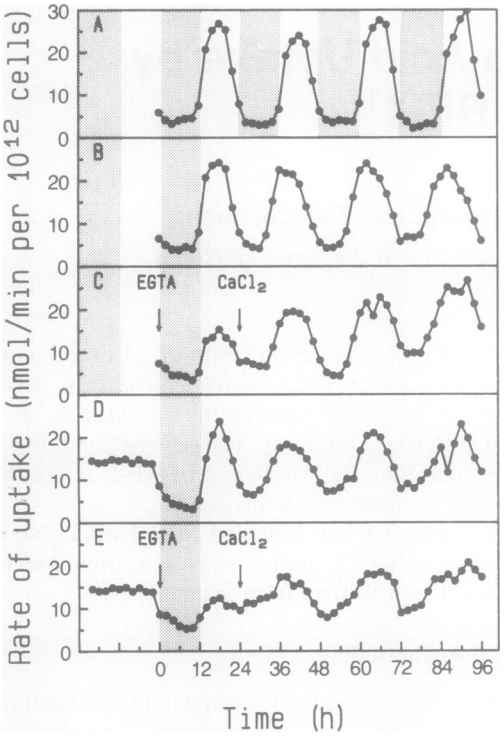

Figure 1. L-Leucine uptake circadian rhythm. In A–C, cultures of *Synechococcus* RF-1 were entrained in 12/12 h L/D cycles for 1 week before the uptake experiment was started at time 0. Shaded areas represent dark periods. In D and E, cultures were incubated in the dark for 24 h and then in L/L for longer than 8 weeks before they were exposed to a single 12-h dark period from time 0 to initiate the rhythm; whereafter they were kept in L/L. Arrows indicate when 1.5 mM EGTA or 1.5 mM $CaCl_2$ was added.

of 12 h was sufficient to turn on the expression of the circadian clock (Fig. 1D) and cause the uptake rate of leucine to fluctuate rhythmically, even though it appeared less effective than multiple 12/12 h L/D treatments (Fig. 1B).

Effects of Extracellular Ca^{2+} on Circadian Rhythm

When the extracellular Ca^{2+} concentration of a *Synechococcus* RF-1 culture that was entrained in L/D cycles was lowered for 24 h by treating the culture with 1.5 mM EGTA at the start of a dark period, the amplitude of the diurnal variations in leucine uptake was reduced to about 50% of that of the controls during the first 36 h after treatment. The EGTA treatment, however, did not affect the free-running periodicity of the process (Fig. 1C). A similar depletion of extracellular Ca^{2+} caused the rhythm for AIB uptake to disappear for 24 h, but the normal rhythm reappeared after Ca^{2+} was added back to the culture (Fig. 2B). Why the depletion of Ca^{2+} was more effective in affecting the diurnal variations in AIB uptake is not clear.

When the extracellular Ca^{2+} of the culture was depleted during the single L/D cycle that was used for the induction of the circadian leucine uptake rhythm (Fig. 1E), the variations in uptake rate were greatly reduced during the first 36 h, but the normal rhythm manifested itself later.

In *Synechococcus* RF-1, extracellular Ca^{2+} appears to be required for the expression of the rhythmic variation in amino acid uptake, which is controlled by the endogenous circadian clock, without affecting the time-keeping ability of the clock. The lowered extracellular Ca^{2+} concentration probably reduced the uptake rate in the light period of L/D cycles by lowering the photosynthetic rate (data not shown). Ca^{2+} depletion also causes photosynthetic oxygen evolution in *Euglena gracilis* (11) to be uncoupled from the circadian clock.

In the eukaryotic *Euglena,* a prolonged phase shift in the circadian rhythm of cell division can be brought about by a single increase or decrease in the extracellular Ca^{2+} concentration (18). Although a single decrease in the extracellular Ca^{2+} concentration can cause a temporal phase shift in the rhythm of nitrogen fixation (8), a prolonged phase shift has not been observed in *Synechococcus* RF-1.

Effect of NO_3^-

Sodium nitrate (24 mM) is effective in inhibiting the expression of the nitrogen-fixing rhythm of *Synechococcus* RF-1 by

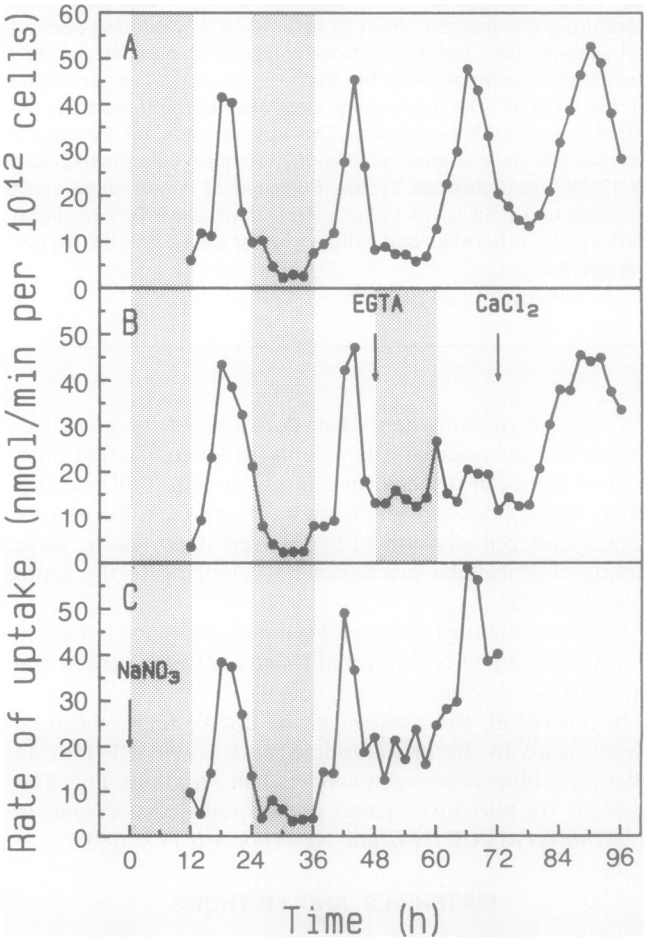

Figure 2. Effects of Ca^{2+} and $NaNO_3$ on AIB circadian uptake rhythm. Cultures of *Synechococcus* RF-1 were entrained in L/D cycles for 48 h before time 0. Shaded areas represent dark periods. A, Control experiment without EGTA or $NaNO_3$. B, Effects of Ca^{2+}. Arrows indicate when 1.5 mM EGTA or 1.5 mM $CaCl_2$ was added. C, Effects of $NaNO_3$. The arrow indicates when 25 mM $NaNO_3$ was added.

repressing nitrogenase synthesis at the transcription level (7). After 25 mM $NaNO_3$ was added to a *Synechococcus* RF-1 culture entrained in 12/12 h L/D cycles, the periodic fluctuation in the AIB uptake was not affected when the culture was subsequently exposed to L/L (Fig. 2C). The expression of the AIB uptake rhythm, therefore, appears to be independent of the expression of the endogenous nitrogen-fixation rhythm.

Phase-Shifting by Low Temperature Pulse

The 6-h low temperature pulse (0°C) shifted the phase of the leucine-uptake rhythm in *Synechococcus* RF-1 (Fig. 3). The phase-response curve shows both delay and advance phase shifts, which is similar to the phase-response curve of another unicellular organism, *Gonyaulax polyedra,* to 3-h low temperature pulse (13).

Effect of Temperature on Rhythm

When cultures of *Synechococcus* RF-1 that were entrained to 12/12 h L/D cycles at 29°C were transferred to different temperatures in L/L, a noticeable phase advancement in the rate of leucine uptake was observed in cultures maintained at 33 and 37°C (Fig. 4). In contrast, phase delay was evident in the 21°C culture. Although the rate of leucine uptake increased linearly and Q_{10} value (ratio of the rate of uptake after the temperature has been increased by 10°C, to the original rate) reached 1.46 initially, there was no observable change in

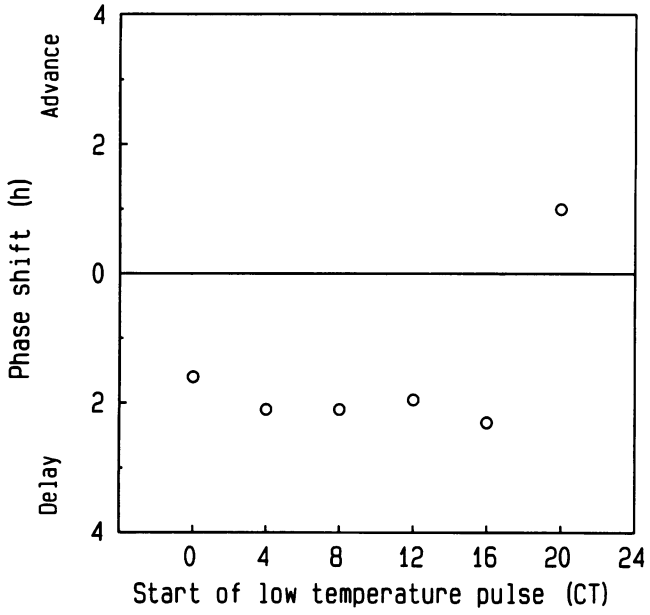

Figure 3. Phase-response curve with low temperature pulses. Cultures of *Synechococcus* RF-1 were entrained on L/D cycles for 2 weeks before the uptake experiment. After being transferred to L/L at time 0, cultures were exposed to 0°C for 6 h at 4-h intervals by resting the flasks on ice and then returning them to 29°C for the remainder of the experiment. The phase of the leucine-uptake rhythm on the third day after temperature perturbation was used to determine any phase shift with regard to leucine uptake that might have been induced by the cold treatment. CT = circadian time.

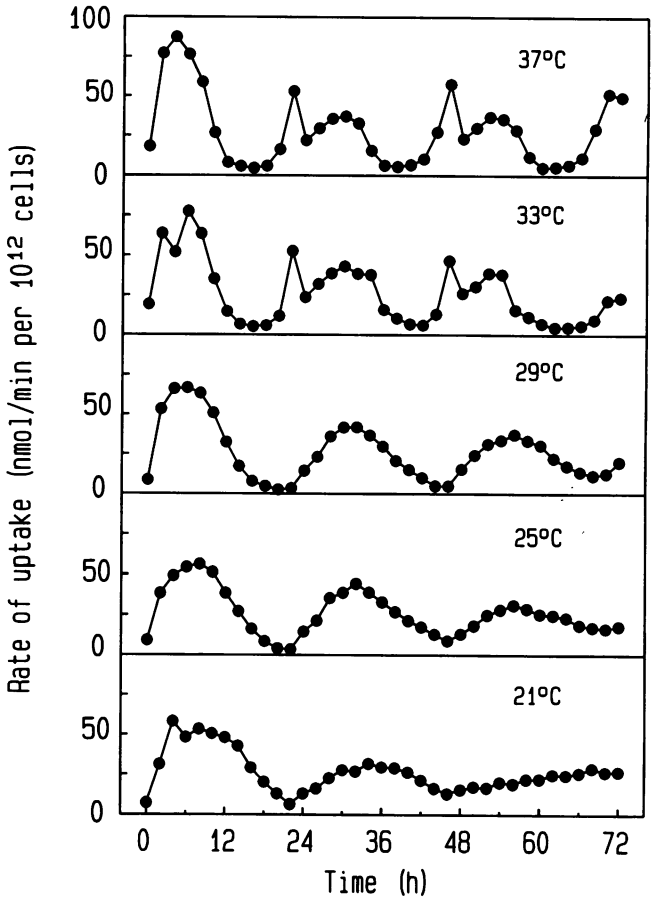

Figure 4. Effects of temperature on the leucine-uptake rhythm. Cultures of *Synechococcus* RF-1 were entrained in L/D cycles for 2 weeks at 29°C before the uptake experiment was started at time 0 in L/L at different temperatures.

the rhythm periodicity from 25 to 37°C. This "temperature compensation" property is similar to the rhythm of nitrogenase activity in the same organism (8) and the circadian rhythms of eukaryotes (2, 9, 17).

The rhythms observed in 33 and 37°C treatments show additional small peaks that maintain the main periodicity as the main peaks. Thus, it suggests that the cultures were in "transient" states of the phase-shifting process. The cultures of *Synechococcus* RF-1 do not maintain well in low temperature (<21°C); therefore, the gradual loss of leucine-uptake rhythm in 21°C culture during the 3-d experiment is likely the result of a decaying culture that failed to take up leucine actively.

Is There a Circadian Rhythm in the Uptake of All Amino Acids?

The uptake rate for 20 natural amino acids by *Synechococcus* RF-1 was measured in the middle of a light and a dark period for a culture that was adapted to growing in 12/12 h L/D cycles. For each amino acid, the ratio of the uptake rates of the two phases was calculated. For most of these 20 amino acids, the uptake rates were found to be higher during the

Table I. *Synechococcus RF-1 Amino Acid Uptake Rates*

Cells were cultured under 12/12 h L/D conditions for 1 week before being used in the uptake experiment. The L/D cycles were continued up to the end of the first 24 h of the experiment, whereafter the cells were exposed to L/L for the next 48 h. Cell samples were taken at 12-h intervals, with the first sample labeled at 6 h (ZT18) after the start of darkness. V, rate of uptake; ZT, environmental time (ZT0 corresponds to the beginning of a light period); CT, circadian time (CT0 indicates the phase point of a free-running rhythm that corresponds to the onset of light in a 12/12 h L/D reference cycle).

Amino Acid	Rate of Uptake								
	First day (L/D)			Second day (L/L)			Third day (L/L)		
	V_{ZT06}	V_{ZT18}	$\frac{V_{ZT06}}{V_{ZT18}}$	V_{CT06}	V_{CT18}	$\frac{V_{CT06}}{V_{CT18}}$	V_{CT30}	V_{CT42}	$\frac{V_{CT30}}{V_{CT42}}$
	nmol/min·10¹² cells								
L-Ala	29.8	17.5	(1.70)						
L-Val	22.1	3.08	(7.17)	27.6	2.49	(11.1)	25.6	1.97	(13.0)
L-Leu	17.7	2.07	(8.55)	21.7	3.73	(5.82)	22.0	2.90	(7.59)
L-Ile	20.7	2.77	(7.46)	24.0	3.57	(6.73)	22.9	2.36	(9.72)
L-Pro	36.3	4.92	(7.38)	45.2	5.77	(7.84)	39.7	5.07	(7.83)
L-Phe	22.0	2.83	(7.78)	28.3	2.94	(9.61)	20.9	2.41	(8.68)
L-Trp	9.36	2.38	(3.93)	11.4	2.92	(3.91)	9.57	2.17	(4.41)
L-Met	13.2	2.44	(5.40)	13.2	2.91	(4.53)	12.6	2.47	(5.11)
Gly	21.6	19.1	(1.13)						
L-Ser	19.9	15.4	(1.29)						
L-Thr	47.5	32.3	(1.47)						
L-Cys	11.2	10.4	(1.08)						
L-Tyr	11.4	1.57	(7.24)	11.5	2.27	(5.06)	11.5	1.57	(7.31)
L-Asn	10.5	6.07	(1.73)						
L-Gln	29.4	13.7	(2.14)						
L-Asp	8.55	5.66	(1.51)						
L-Glu	20.5	20.7	(0.99)						
L-Lys	2.99	1.67	(1.79)						
L-Arg	0.77	0.47	(1.64)						
L-His	7.43	3.39	(2.19)						

light period than during the dark period. This was most marked for L-Val, L-Leu, L-Ile, L-Pro, L-Phe, L-Trp, L-Met, and L-Tyr (Table I). Uptake rates for these eight amino acids were further studied for the next 48 h at 12-h intervals while the culture was exposed to L/L. For all eight amino acids, the high ratios of the uptake rates persisted during the 48-h period. The results are consistent with the assumption that the uptake rates of these eight amino acids are regulated by an endogenous circadian clock.

In the cyanobacterium *Anacystis nidulans,* the uptake rates of leucine as well as AIB are found to be higher in the light than in the dark, presumably because of an increase in the proton motive force (10). Circadian changes have been observed in the membrane potential (1, 14), extracellular pH (6), and ATP content (19) of eukaryotic organisms. It is likely that similar circadian changes in the proton motive force or ATP content of *Synechococcus* RF-1 caused those amino acids, which were transported by active transport in light periods, to be taken up rhythmically.

To find out whether the rhythmic uptake of amino acids is a general property of unicellular cyanobacteria, the uptake rate of L-[^{14}C]leucine by *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 was also determined. As for *Synechococcus* RF-1, the rate of L-leucine uptake by these two organisms was much higher during the light periods than the dark periods of the L/D regimen to which the culture was adapted

Table II. *L-Leucine Uptake Rates by Synechococcus 7942 and Synechocystis 6803*

Experimental conditions were the same as those described in Table I.

Strain	Rate of Uptake								
	First day (L/D)			Second day (L/L)			Third day (L/L)		
	V_{ZT06}	V_{ZT18}	$\frac{V_{ZT06}}{V_{ZT18}}$	V_{CT06}	V_{CT18}	$\frac{V_{CT06}}{V_{CT18}}$	V_{CT30}	V_{CT42}	$\frac{V_{CT30}}{V_{CT42}}$
	nmol/min·10¹² cells								
S. 6803	79.6	15.4	5.2	67.5	57.7	1.2	62.8	53.1	1.2
S. 7942	55.6	7.8	7.1	52.8	69.8	0.8	57.4	56.3	1.0

(Table II). Consequently, the L/D uptake ratios were comparable to the ratio obtained for *Synechococcus* RF-1. However, when these cultures were transferred to L/L and studied at 12-h intervals for 48 h, the uptake rates maintained a level close to that which was obtained during the last light period of the previous L/D regimen, and the temporal variation disappeared almost completely. Unlike *Synechococcus* RF-1, a persistent rhythm in L-leucine uptake could not be observed in these two organisms when they were transferred from a L/D regimen to L/L.

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