Phase Shift of the Circadian Rhythm of *Lemna* Caused by Pulses of a Leucine Analog, Trifluoroleucine

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ABSTRACT

Pulses of a fluorinated analog of leucine, 5',5',5'-trifluoroleucine, reset the phase of the circadian rhythm of K+ uptake in *Lemna gibba* G3 under continuous light conditions. The trifluoroleucine pulse caused the largest delay phase-shifts during the early subjective phase but it caused only small phase advances. The action of trifluoroleucine was investigated and the following results were obtained. (a) The uptake of trifluoroleucine was essentially the same at all circadian phases, even though phase shifting was dramatically different at different phases. At effective phases, the magnitude of phase shifting was well correlated with the amount of trifluoroleucine taken up by the duckweed. (b) The trifluoro-

leucine pulse lowered the endogenous content of valine and leucine but these decreases did not correlate with phase shifting. (c) Protein synthesis was not affected by trifluorooleucine pulses which caused large phase shifts. (d) Pulses of 4-azaleucine, a different structural analog of leucine, also caused phase shifting. However, neither the direction nor the effective times of phase shifting were similar to those of trifluoroleucine. Taken together, these results negate the proposition that trifluoro-

leucine and azaleucine caused phase shift by disturbing amino acid metabolism and/or inhibiting protein synthesis, but they suggest instead that these analogs are incorporated into some protein(s) which are necessary for normal clock operation.

Physiological activities of many eukaryotic organisms fluctuate in a diurnal fashion. This rhythmicity, known as a circadian rhythm, may be ecologically advantageous for the organism to adapt to an environment which is changing with a 24 h periodicity (3). These circadian rhythms are controlled by an endogenous oscillator, or 'biological clock.' Little is known about the molecular mechanism of this underlying oscillator (11).

In many organisms, inhibitors of cytoplasmic protein synthesis on 80S ribosomes, such as cycloheximide (16, 18, 24, 28) or anisomycin (20, 23), have been reported to shift the phase of the circadian rhythm. As the phase of the circadian rhythm reflects the operation of the master clock, phase shifting by protein synthesis inhibitors suggests that, at some level, protein synthesis is an important step of the circadian oscillation. Several clock models which include protein synthesis as a step of the oscillating feed-back loop have also been proposed (2, 22). Proteins which might be involved in such a putative loop have been reported in *Aplysia* (29) and *Acetabularia* (8).

Cycloheximide also shifts the phase of the K+ uptake rhythm in the duckweed, *Lemna gibba* G3 (T Kondo, unpublished data). In this study, I report effects of pulse administration of amino acid analogs on the phase of the K+ uptake rhythm of *Lemna*. Many amino acid analogs are known to inhibit protein synthesis or to be incorporated into proteins and make them unstable or in active (7). If some proteins are important for the unimpeded operation of the clock, analogs might be expected to affect the function or synthesis of those clock proteins and thereby phase shift the rhythm. Indeed, Hastings et al. (9) also found that amino acid analogs, such as canavanine, disturbed and phase shifted the circadian bioluminescence rhythm of *Gonyaulax*.

The circadian rhythm in K+ uptake of *Lemna* (15) is suitable for examination of the action of the amino acid analogs on the circadian clock. As *Lemna* is an aquatic floating plant, we can administer chemicals quantitatively and remove them from the medium without disturbing the organism. Moreover, *Lemna* grows on simple amino acid-free medium and shows active uptake of many amino acids if they were administered to the medium (5, 12). An exogenously administered amino acid analog might therefore enter the cells and perturb the metabolism of *Lemna*.

This report demonstrates that pulses of two leucine analogs (FLE and ALE) can phase-shift the circadian K+ uptake rhythm of *Lemna*. Furthermore, the lack of effect of FLE on the endogenous levels of amino acids and protein synthesis suggests that these analogs are acting by being incorporated into proteins whose function is crucial to the clock mechanism.

MATERIALS AND METHODS

Duckweed Culture. Aseptically grown cultures of the long-day duckweed *Lemna gibba* G3 were started from a single four-frond colony in 100 ml of M medium (10) supplemented with 1% sucrose in 200-ml Erlenmeyer flasks. To prevent floral induction, the cultures were exposed to a short-day conditions (9 h light + 15 h darkness) for about 3 weeks at 26°C. The plants were illuminated with 6.5 W/m² light from white fluorescent tubes (Hitachi, Sunline FL 15 SW) during the light period. Light intensity rate was measured with a YSI 65 A radiometer.

Pulse Administration of Chemicals. Twenty colonies were transferred to fresh M-sucrose medium in Erlenmeyer flasks and exposed to four additional short days. They were then transferred to LL (6.5 W/m² at 26°C). Chemical pulses were usually given 27 to 54 h after the onset of LL, after which the duckweeds were washed three times with fresh M medium and transferred to fresh medium until the flow medium culture started. The duckweeds (ca. 0.3 g fresh weight/chamber) were transferred from Erlenmeyer flasks to the FMC chambers at 157 (i.e. 5.7 h after the onset of LL) to be monitored for their K+ uptake at 26°C. Although a growth rate was lowered, the change in the culture conditions does not influence the phase of the rhythm, for the phase in the FMC was independent from the time of the trans-

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1 Abbreviations: FLE, 5',5',5'-trifluoroleucine; ALE, 4-azaleucine; LL, continuous light; FMC, flow medium culture; CT, circadian time (in hour, 24 h in CT is equal to the period of the rhythm and CT 0 is at the beginning of the main continuous light period, which is equivalent to 'dawn'); PRC, phase response curve.

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fering. In this paper, time of the pulse was plotted as the onset of the pulse treatment. Details of the FMC were described previously (26). A 1/100 dilution of M medium without sucrose at pH 5 (abbreviated to M/100 medium) was pumped into the culture chambers at a constant rate of 5 ml/h. Used medium from the chamber was collected hourly by means of a time-controlled fraction collector. FLE was purchased from PCR Research Chemicals (Gainesville, FL) and ALE from Sigma.

**Estimation of the K⁺ Uptake Activity and Phase Shift.** The difference in K⁺ content between the used and the fresh medium was considered to be the amount of K⁺ taken up by the whole culture in the FMC chamber. The K⁺ content was estimated with an atomic absorption spectrophotometer (Perkin Elmer 603) and the K⁺ uptake per hour was automatically fed into a microcomputer. Approximately 25 measurements around a roughly estimated position of the maximum rate of K⁺ uptake (during h 140-168) were subjected to curve-fitting by multiple-regression analysis to determine the peak position (13). Phase shifts were calculated by comparing the peak positions between the treated sample and the controls. Change in the period after the pulse was always less than 0.5 h. Therefore, the error in the phase shifting due to the period change calculated as above is less than ± 2 h, for phase was compared at four cycles after the pulse.

**Amino Acid Analysis.** The duckweeds were treated with various concentrations of FLE for 6 h. After the FLE pulse was finished, the duckweeds were harvested, washed, wrapped in filter paper and centrifuged at 2000 rpm for 1 min and the fresh weight was measured. They were homogenized with 0.2 N perchloric acid in a glass homogenizer. After centrifuging to remove cell debris, the supernatant was neutralized with 5 N KOH and was frozen to precipitate potassium perchlorate. The solution was dried down and dissolved in 0.6 ml of 0.1 N HCl. The solution was analyzed with an automated amino acid analyzer (Hitachi 8011) to estimate the content of amino acids. The retention time of FLE in the analyzer column was close to that of methionine. As *Leuana* contained only very small amounts of methionine, the large peak which appeared at the methionine position is considered to be FLE.

**Protein Synthesis.** Twenty colonies of duckweeds were cultured under the same conditions as those in which the phase shifting experiments were performed. They were transferred to 10 mL M medium with 1 μCi of [³H]leucine (Amersham, 150 Ci/mmol) or [¹⁴C]glycine (Amersham, 40 mCi/mmol) in the presence or absence of FLE. After 3 h incubation at 26°C under 6.5 W/m² LL, duckweeds were rinsed with water and homogenized with 5% TCA containing unlabeled leucine and glycine. After centrifugation at 5000 rpm for 5 min, the pellet was again extracted with TCA and washed with 80% ethanol (a portion of this supernatant was retained for scintillation counting). The final pellet was dissolved in 5 N NaOH at 80°C. The dissolved protein was neutralized with HCl, precipitated with ice cold 20% TCA, and trapped on a filter paper. The radioactivity of this protein fraction was determined by liquid scintillation counting. The protein content of this fraction was determined by Sigma’s Protein Assay kit. Radioactivity of the supernatant of the first TCA extraction was also measured to estimate the uptake of labeled amino acids.

**RESULTS**

**K⁺ Uptake Rhythm after FLE Pulses.** Figure 1 shows K⁺ uptake rhythms of duckweed after 6 h pulses of 10 μM FLE. Independent of the time that the pulse was applied, the mean level of the K⁺ uptake recovered to the control level within 50 h after the pulse. By contrast, both the phase and the amplitude of the rhythm were greatly modified by FLE pulses starting between CT 3 and 12 (curves 2-5). The pulse starting at CT 12 (curve 5) was the most effective and the pulses starting from CT 15 or later (curves 6-9) had little effect. The FLE pulse did not affect the period of the rhythm (usually 25.5 h) after the pulse.

**Phase Shifts Evoked by FLE Pulses of Various Concentrations.** As before, the pulse was given to static cultures before the rhythm could be monitored in FMC. We could assay the K⁺ uptake rhythm only after recovery of K⁺ uptake from transient inhibition by FLE and adaptation of the duckweed to the iono environment of the FMC. The inability to monitor the rhythm before and after the pulse makes the assignment of phase shifts as delay vs. advance phase-shifts ambiguous. Therefore, data obtained from the dose response experiments (Fig. 2) are important to identify the direction of the phase shifting. Figure 2 shows that as the FLE concentration was increased, progressively larger phase delays were induced. No discontinuity was found in any dose response curve; that is, for FLE pulses starting CT from 3 to 12, the magnitude of the phase delay increased in proportion to the concentration of FLE up to 15 μM. Phase shifts were largest for FLE pulses given at CT 12. The phase delay was 18 h by 10 μM FLE pulse at CT 12. At CT 15, slightly advanced the phase of the rhythm and FLE failed to shift the phase if given at CT 21. Pulses of FLE higher than 15 μM caused an irreversible damage to K⁺ uptake activity of the duckweed, irrespective of the phase of the FLE pulse. Figure 2 also shows that the responses to the FLE pulses given at CT 12 are blocked in the presence of 10 μM leucine. This result will be discussed later.
but it should be noted that the phase advance region was very small. The 'breakpoint' of the PRC (a discontinuity in the curve) lies between CT 12-15. Figure 3 also shows that 3 h pulses induced a similar phase shift as that caused by 6 h pulses but phase delays were smaller than those by the 6 h pulses.

To examine the possibility that FLE is retained in the cell after the pulse period, a mixture of leucine, isoleucine and valine was given to the duckweed after the pulse to confirm the FLE pulse. (Leucine at 50 μM or higher was toxic to the duckweed if given alone for 6 h.) As also shown in Figure 3, the phase shift induced by the FLE pulse was not affected by chasing the FLE pulse with 10 μM leucine.

Effects of Normal Amino Acids on the Phase Shifting by the FLE Pulse. Competition between FLE and leucine has been demonstrated in bacterial metabolism (6). In Lemna, the addition of 20 to 30 μM leucine to 10 μM FLE during the pulse nullified the phase shifting by FLE. However, competition between leucine and FLE on phase shifting was rather complex when leucine was given at concentrations lower than 10 μM. That is, the response of phase shifting to varying FLE concentrations in the presence of 10 μM leucine indicated that the phase was delayed slightly by FLE at concentrations lower than 5 μM and advanced by FLE at higher concentrations (Fig. 2). Therefore, phase shifting by 10 μM FLE in the presence of varying concentration of leucine was plotted as in Figure 4; i.e. the phase was advanced by 10 μM FLE and leucine lowered this phase-advance. The phase delay by 6 μM FLE gradually declined to 7 h delay phase-shift with increasing doses of leucine up to 4 μM; above this, there were phase advances which were decreased with increasing leucine. Unlike leucine, isoleucine diminished the phase delays by FLE by increasing its concentration and at 8 μM or higher isoleucine cancelled the FLE effects (Fig. 4). In addition, Figure 4 shows that leucine did not itself shift the rhythm. Isoleucine also did not itself shift the phase (data not shown).

Figure 5 shows the effects of other amino acids on the phase shifting by FLE. Arginine failed to prevent phase shifting by the FLE pulse, even at 50 μM (a concentration which is five times that of FLE). In contrast, phenylalanine and tryptophane reverse

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**Figure 2.** Response of the phase shift to the FLE concentration. Between h 27 (CT 3) and 51 (CT 2) of the LL, various concentrations of FLE were given to the duckweed for 6 h at the time indicated in the figure as CT (onset of the 6 h pulse). FLE pulses starting from CT 12 were also examined in the presence of 10 μM leucine (12 leu). Peak positions between h 140 and 170 were plotted against the concentration of FLE.

**Figure 3.** PRC of the K+ uptake rhythm by FLE pulses. Between h 27 (CT 3) and 51 of the LL, 6 h (C) or 3 h (O) 10 μM FLE pulses were given to duckweed in the static culture. Some of the 6 h FLE pulses (C) were followed by mixture of leucine, isoleucine, and valine at 10 μM for 18 h. Phase shifts were calculated by comparing peaks of the experimental and the control rhythm between h 140 and 165. (The peak of the control rhythm was at hour 147 of LL.) The abscissa indicates the onset of the pulse in CT scale. CT 0 on the abscissa corresponds to h 24 of LL (subjective dawn).

**Phase Response Curve for FLE Pulses.** Figure 2 indicates the direction of the phase shifting by FLE. Based on that information, we can plot the phase shifts of the K+ uptake rhythm by FLE pulses shown in Figure 1 against the CT of the onset of the pulse (Fig. 3). Large phase delays were induced by pulses given between CT 6 and CT 12. The pulse at CT 12 induced the maximum phase delay (~18 h), while the phase was slightly advanced by pulses given at CT 15. Phase shifts were small for FLE pulses given between CT 18 and CT 24. This PRC can be classified as 'Type 0' (30) with respect to the slope of the curve,
FIG. 5. Effects of arginine (+Arg), proline (+Pro), phenylalanine (+Phe) and tryptophane (+Trp) on phase shifting by 10 μM FLE. Various doses of the amino acids were given to the duckweed for 6 h in the static culture together with 10 μM FLE from h 36 of LL (CT 12). The phase shift (ordinate) of the rhythm as measured in Figure 3 is plotted against the amino acid concentrations (abscissa).

FIG. 6. FLE and branched-chain amino acid concentrations within Lemna after FLE pulses at various concentrations. After pulses of FLE at different extracellular concentrations as plotted on the abscissa (6 h pulses given at hour 36 of LL, CT 12), the duckweeds were washed and amino acids were extracted. The tissue content of leucine (L), valine (V), isoleucine (I) and FLE (F), measured by the amino acid analyzer, were plotted against the concentration of the FLE. Data plotted are the means of duplicate or triplicate experiments. Phase shift curve identified as (0) is plotted against concentration of FLE and right side abscissa.

the FLE action in proportion to their concentrations. At 30 μM, they almost completely nullify the phase shifting by FLE. Proline at 40 μM showed a small effect in nullifying the phase shift by FLE.

Uptake of FLE and Levels of Amino Acids. At CT 12, when the FLE was most effective in phase shifting, the amount of FLE taken up by the duckweed was proportional to the concentration of FLE presented to the plant. Thus, the uptake of FLE by the duckweed was well correlated to the phase shifting by the FLE (Fig. 6). The magnitude of the phase delay increased as the amount of FLE absorbed by Lemna increased from 20 to 70 nmol/g fresh weight. Beyond 70 nmol/g fresh weight absorbed, phase shifting was saturated. Note also that there might be a contribution due to nonspecific binding of FLE to the cell surface matrix (apparent free space). Because Lemna absorbed 50 nmol/g fresh weight FLE after a 3 h incubation in 10 μM FLE (data not shown), the apparent free space can be calculated to be about 20 nmol/g fresh weight, assuming that the uptake of FLE was linear with the incubation time. The tissue level of valine declined slightly during FLE treatment. The leucine level remained essentially unchanged, while the isoleucine level increased slightly during the FLE pulse. These changes did not parallel FLE phase shifting.

The uptake of FLE during the 10 μM pulse is essentially the same at all circadian phases (Fig. 7). Uptake of FLE was slightly lower in the subjective night phase (CT 12–24) than in the day phase (CT 0–12). However, this change did not correlate with phase shifting. For example, FLE uptake was smaller at the phase (CT 12) when the FLE pulse caused the largest phase shift. Levels of free leucine and isoleucine in the control duckweed showed an apparent circadian rhythmicity, with lower values in the subjective night. Valine level was rather constant at all phases (Fig. 7). As was true at CT 12 (Fig. 6), the FLE pulse lowered the level of valine markedly and of leucine slightly. However, at no phase was a positive correlation found between the lowering in these amino acid levels by FLE and the phase shifting by the FLE pulse (Fig. 7).

Effect of FLE on protein synthesis. No correlation between phase shifting and the inhibition of protein synthesis by FLE was found. As shown in Figure 8A, incorporation of radioactive amino acid (leucine or glycine) into protein was not lowered by the presence of FLE up to 20 μM at CT 12 when the FLE pulse caused a large phase delay of the rhythm. This result held true at other times of the day (Fig. 8B). The uptake of radioactive amino acid into acid soluble fraction was also not affected by the presence of 10 μM FLE at any time of the day (Figs. 8A, 8B).

Phase shifting by 4-azaleucine (ALE). The phase of the K⁺ uptake rhythm was changed by pulses of ALE, a different leucine analog. Dose response curves of the phase shifting by ALE concentration (Fig. 9) indicate that the phase was delayed by ALE given at CT 3 and advanced at CT 9. Therefore, phase shifts by ALE pulses (3 and 6 h pulses) can be plotted as shown in Figure 10. The PRC for 100 μM ALE pulses was 'type 0,' but was different from that for FLE pulses. Phase shifting by ALE was bidirectional (substantial delay and advance phase shifts were induced) and the break point of the PRC occurred in the day phase (between CT 7 and 10). The presence of ALE altered...
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Fig. 8. Incorporation of amino acid into protein in the presence of FLE. A, At CT 12, 3 h FLE pulses of various concentrations (abscissa) were given to the duckweed and the uptake and incorporation into protein of $^3$H-leucine were assayed. Radioactivities of soluble fraction (uptake: U, ●) and TCA-precipitate fraction (incorporation into protein: P, ○) at the end of the pulse were plotted as the percent of control (Control = CT 12 without FLE treatment). Similar results were obtained by incubation with $[^{14}]$Cglycine instead of $[^3]$Hleucine. Data are means of duplicates or triplicates. B, At various times of the circadian cycle, 3 h FLE pulses (10 μM) were given to the duckweed and $[^3]$Hleucine uptake and incorporation were measured. Experimental procedures, presentation of data and control were essentially the same as in A.

Fig. 9. Response of the of phase shift to the ALE concentration. Experimental procedures and presentation of data were the same as in Figure 2, except that ALE was given to the duckweed instead of FLE.

The endogenous levels of branched-chain amino acids differently from FLE, ALE (50 or 100 μM) pulses for 6 h at CT 3 increased valine levels from 73 to 185 nmol/g fresh weight and isoleucine levels from 24 to 45 nmol/g fresh weight. Leucine levels were not affected by ALE treatment. However, these changes in the amino acid levels again were essentially equivalent at all circadian phases, while the magnitude of phase shifting changed considerably as a function of the circadian phase of the pulse.

Discussion

The large phase delays caused by FLE (Figs. 1, 2, and 3) indicate that FLE affects the normal operation of the duckweed clock. Because the leucine chase after the FLE pulse did not diminish the phase shifting (Fig. 3), FLE probably affects the clock during the 3 or 6 h of pulse administration.

At any given time, the amount of FLE taken up by the duckweed during the pulse is well correlated with the amount of phase shifting (Fig. 6), indicating that the phase changes are caused by FLE taken up by the duckweed. However, the rate of FLE uptake was similar at different circadian times, while the magnitude of phase shifting was quite different (Fig. 7). Thus, the change in the phase shifting with time of day cannot be ascribed to a circadian change in FLE uptake. Phase responsive-ness to FLE must therefore be due to phase-specific effects of FLE on the clock mechanism.

The dose-response curve (Fig. 2) indicates an unusual feature of the PRC with FLE, that is, the phase shifting by FLE was highly asymmetrical. In Lemna, light or temperature pulses usually cause both advance and delay phase-shifts. This is also true for the metabolic inhibitor azide (13), the protein synthesis inhibitor cycloheximide (T. Kondo, unpublished data), and temperature pulses (14). Although the precise mechanism is unknown, the action of FLE on the clock might be different from that of stimuli which cause most symmetrical PRCs.

An amino acid transporting system of Lemna is reported to have a high affinity for many nonpolar amino acids (5, 12). As FLE has a nonpolar side chain similar to that of leucine, Lemna may be taking FLE in by this nonpolar amino acid transporter. Thus it would be expected that the uptake of FLE would be competitively inhibited by adding another nonpolar amino acid such as tryptophane or phenylalanine at doses which exceed the capacity of the transporter of Lemna. Therefore, lowering the magnitude of phase shifting of FLE by the concomitant addition of tryptophane and phenylalanine (Fig. 5) could be due to decreasing the FLE uptake. In contrast, arginine failed to decrease FLE phase shifting, so a polar amino acid like arginine may not be able to compete with FLE for uptake.

Fluoroleucine (19) has been employed widely as a biochemical and genetic probe (27). In microorganisms, FLE has been shown to inhibit growth in the following ways: (a) by acting as a false repressor (6) or a false feed-back inhibitor (4) for enzymes for the biosynthesis of branched-chain amino acids, (b) by interfering with the incorporation of leucine into protein (1, 25), and (c) by being incorporated into protein in place of leucine at sites usually occupied by leucine (19). Feedback regulation of biosynthesis of branched-chain amino acids is rather complex (17). Some enzymes catalyse biosynthesis pathways both for isoleucine and valine-leucine. In bacterial systems, FLE is reported to affect these enzymes and deplete the availability of branched-chain amino acids. As shown in Figures 6 and 7, both valine and
leucine levels were lowered to half of the control value by FLE. However, the phase shifting by FLE is not caused by these perturbations, because the decrease in valine or leucine levels were not positively correlated with phase shifting. FLE did not lower the isoleucine level in *Leuina*. These results negate the possibility that FLE is acting on the clock by changing the endogenous levels of amino acids in general.

In various systems, FLE was reported to act as a leucine analog. However, direct competition by leucine with FLE in phase shifting was not found. In the presence of leucine at 10 μM or lower, FLE pulse given at the maximum delay time (CT 12) advanced the phase of the clock (Fig. 2) and leucine lowered this phase advance (Fig. 4). As FLE itself advanced the phase at CT 15 or 18 (Fig. 2), the presence of leucine would postpone the FLE action on the clock rather than diminishing the phase shifting during the pulse time. These results can be explained as follows: by the simultaneous addition of 10 μl leucine to FLE pulses, uptake of FLE was not lowered (20% potentiated) and leucine level was elevated fivefold (ca. 200 nmol/g fresh weight). Therefore, the action of FLE would be blocked during the pulse time; e.g. at CT 12 (a large delaying phase), for the FLE-to-leucine ratio was lowered to one-fifth its previous value. If accumulated leucine were consumed by cellular metabolism more rapidly than FLE, FLE-to-leucine ratio would elevate after the pulse (FLE and 2–10 μM leucine) was over; e.g. at time when FLE induced phase advance (CT 15 or 18). By addition of leucine at higher than 10 μM, the ratio would elevated at the phase when FLE did not shift the rhythm.

On the other hand, because isoleucine can reverse the FLE action on the phase of the rhythm (Fig. 4), isoleucine may compete with FLE in part. However, as isoleucine did not postpone the action of FLE as leucine did, it might interfere with the action of FLE on the clock without competing for FLE.

Incorporation of radioactive amino acid into protein was not affected by FLE concentrations up to 20 μM. This is true for both [14C]glycine and [3H]leucine incorporation. Therefore, neither disturbing amino acid levels nor inhibiting protein synthesis caused the phase shifting by FLE. Unlike other amino acids, leucine is not known to have a function in living cells other than to serve as a constituent of protein (27). Thus, the most reasonable postulate for action of FLE on the clock is that FLE is incorporated into some protein(s) important for clock operation, and thereby inactivates or otherwise modifies the activity of this clock protein(s). In fact, FLE has been reported to be incorporated to protein (19). It is difficult to predict how FLE might modify the protein(s), but resultant ‘false’ protein might be unstable, inactive, or altered in its activity.

ALE, whose structure differs from that of leucine by the substitution of a skeleton carbon for nitrogen, is also known to disturb amino acid metabolism and yield ‘false’ proteins (21). If a second amino acid analog (e.g. ALE) shifts the clock through a similar disturbance of branched-chain amino acid metabolism, a PRC similar to that for FLE would be predicted. However, the PRC for ALE was quite different from that for FLE (compare Figs. 3 and 10). Possibly these amino acid analogs modify different proteins which function in the clock system in different ways or at different times. The modification of protein by the incorporation of ALE might be quite different from that of FLE, for the modification to a particular proteins by incorporation of a given analog is hard to predict. It may be possible that some protein(s) is affected by incorporated of FLE but not of ALE and another protein is affected by ALE but not by FLE; or that some proteins might be inactivated by ALE and enhanced by FLE. Regardless, these results favor the interpretation that FLE and ALE phase-shift the clock by being incorporated into proteins which have an important function in the clock mechanism.

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