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Rhythms in Glutamine Synthetase Activity, Energy Charge, and Glutamine in Sunflower Roots[1]

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ABSTRACT

Roots of sunflower plants (*Helianthus annuus* L. var. Mammoth Russian) subjected to L12:D12, L18:D6, and L12:D12 followed by continuous light all display rhythms of about 12 hours for glutamine synthetase (GS) activity (transferase reaction) with one peak in the 'light phase' and one in the 'dark phase.' Root energy charge (EC = ATP+½ADP/ATP+ADP+AMP) is directly correlated with GS, but the GS rhythm is better explained as the result of a rhythmic adenine nucleotide ratio (ATP/ADP+AMP) that regulates enzyme activity through allosteric modification. When L12:D12 plants are subjected to free-running conditions in continuous darkness, only diurnal rhythms for GS and EC, with peaks in the dark phase, remain. The 12-hour root rhythms for GS and EC appear to be composed of two alternating rhythms, one a diurnal, light-dependent, incompletely circadian light phase rhythm and the other a light-independent, circadian dark phase rhythm.

Only glutamine, of the root amino acids, displays cyclical changes in concentration, maintaining under all conditions a 12-hour rhythm that is consistently synchronized with, but nearly always inversely correlated with, GS and EC rhythms.

The phenomenon of endogenous biological oscillation in plants has been found to occur at all levels of organization: organ, cells, organelle, and enzyme (7). Almost universally, the light:dark regimen has been identified as the entraining factor and numerous examples of light-entrainable rhythms have been reported for physiological or biochemical processes in higher plants (14). Most rhythms appear to be circadian with periodicities under free-running conditions of approximately 24 h. Also reported, however, are rhythms with a period clearly less than 24 h and these have been termed 'ultradian' (10). In view of the correlation demonstrated in this laboratory (34) between the activity of sunflower root GS[3] and energy charge as defined by Atkinson (2) ([ATP]+½[ADP]/[ATP]+[ADP]+[AMP]), it has been of particular interest to note reports concerned with *Chenopodium* seedlings of an ultradian rhythm with a period of 12 to 15 h for ATP (8) and of a circadian rhythm with a period of 21 to 24 h for energy charge (31). These results have led the Wagner group to suggest that the rhythmic activity of a number of enzymes is a direct result of rhythmic changes in the energy state of the cell (32). The *Chenopodium* studies, together with a report of an ultradian rhythm for GS activity in the leaves of soybean (9), have led to the present investigation in which an effort has been made to determine whether energy charge and GS vary in a cyclical manner in sunflower roots, a portion of the plant not expected to be directly affected by light functioning as a 'Zeitgeber.' In view of the fact that previous studies have indicated a role for amino acids in the regulation of higher plant GS (22, 27), we have also examined variations in the concentrations of these substances in sunflower roots with respect to rhythmicity.

MATERIALS AND METHODS

Plant Culture. Sunflower seeds (*Helianthus annus* L. var. Mammoth Russian) were germinated in vermiculite in a controlled temperature chamber (26–27°C) under artificial light with a light:dark regimen specific to a particular experiment (L12:D12, L18:D6). Eight-d-old seedlings were transferred to a previously described (35) continuous flow apparatus that provided enclosure of the root system in blackened tubes. Complete culture solution containing nitrogen as 10 mM NO_3^- was allowed to pass over the roots for the remainder of the growth period. On day 28, the plants, having previously been subjected to a specific light:dark regimen (entrainment period), were maintained for up to 48 h in either continuous light or continuous darkness (free-running period). During either the entrainment or free-running periods, root tissue, harvested every 3 h from a randomly selected plant, was subjected to analysis for GS activity and for levels of the adenine nucleotides and amino acids.

Methods of Analysis. Acetone powders prepared by the method of Loomis (18) were utilized both in the determination of GS activity and for content of AMP, ADP, and ATP. The glutamyl transferase activity of GS was determined as previously described (34) by a modification of the procedure of Hubbard and Stadtman (15). Protein in the enzyme preparation was estimated by the method of Lowry *et al.* (20). Ion-exchange chromatography was utilized in the analysis of adenosine mono-, di-, and triphosphates with a modification (34) of the method of Keys (16). Amino acids extracted from fresh root tissue by the method of Bieleski and Turner (4) were separated by two-dimensional TLC on cellulose plates and determined quantitatively by the ninhydrin reaction.

For each light regimen utilized, GS, adenine nucleotide, and amino acid analyses were performed on tissues obtained from at least two separate experiments. Only minor variations occurred between experiments of any one light regimen and the results reported are of one trial. For each experiment, mean values are shown for enzyme analyses performed in triplicate and for adenine nucleotide and amino acid analyses performed in duplicate.

RESULTS

Glutamine Synthetase. Initial experiments were performed with plants entrained to a L12:D12 regimen with lights on at 6 AM and off at 6 PM, EST. Analysis for GS during days 27 and 28 revealed a rhythm during entrainment that was characterized by two peaks

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[3] Abbreviation: GS, glutamine synthetase (EC 6.3.1.2).

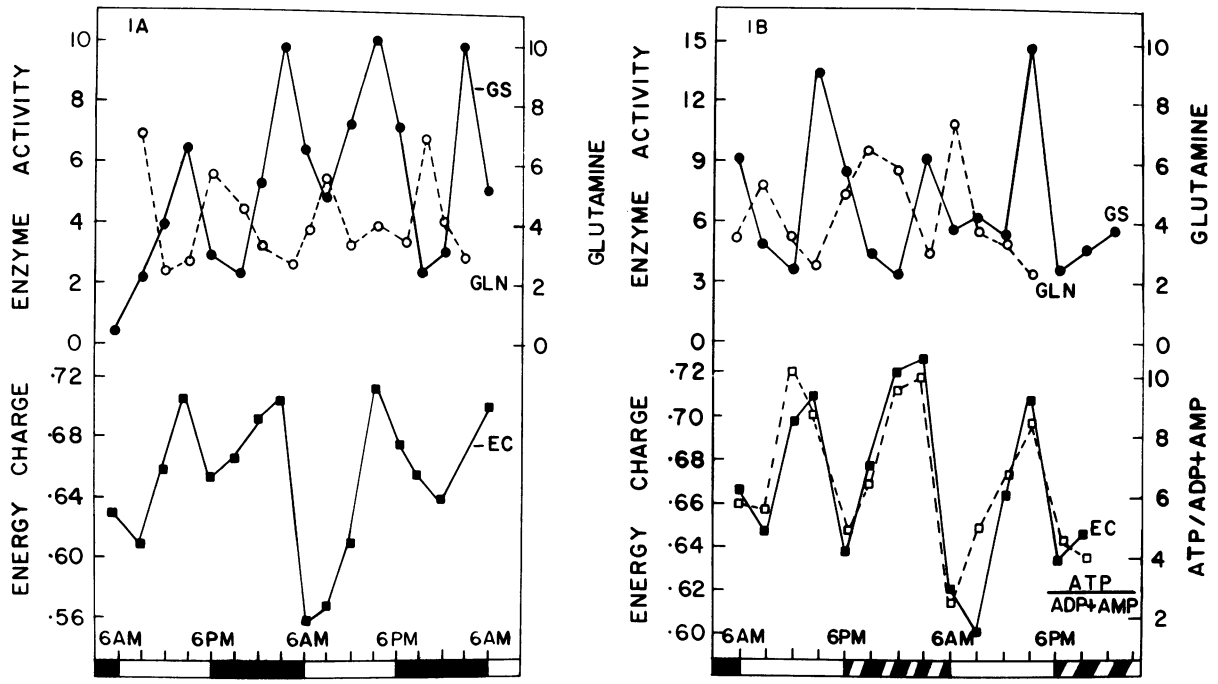

FIG. 1. Rhythms in sunflower root tissue harvested after plant growth under the following conditions: lights on 6 AM-off 6 PM; A, L12:D12; B, continuous light after L12:D12. GS, GS activity (transferase reaction; μmol/mg protein·h); GLN, glutamine concentration (μmol/g fresh weight); EC, energy charge; ATP/ADP+AMP, adenine nucleotide ratio.

of activity approximately 12-h apart in a 24-h period (Fig. 1A). In another experiment, root tissue was harvested during continuous light after a L12:D12 entrainment. Figure 1b demonstrates that the GS rhythm of approximately 12-h established during entrainment was maintained during the free-running in continuous light. In all experiments, whether plants were entrained or allowed to free-run, one peak of root GS activity was attained 6 to 9 h into the 'light phase' while the second peak appeared 6 to 9 h into the 'dark phase.'

To demonstrate that the rhythm for root GS was the result of photoperiod entrainment rather than cyclical factors outside the immediate environment of the growth chamber, a set of plants was grown with a photoperiod shifted 6 h from the original regimen, lights on at 12 noon (N) and off at 12 midnight (M), EST. Another set of plants was subjected to the same entrainment regimen and then placed in continuous light. GS activity continued to demonstrate an ultradian rhythm characterized by two peaks in a 24-h period during both the entrainment and continuous light phases. In both situations, peak GS activity in the roots of the '12 N on-12 M off' plants exhibited a shift of approximately 6 h compared to that displayed by '6 AM on-6 PM off' plants (Fig. 2).

The association of a 12-h cycle of activity with a L12:D12 entrainment regimen suggested that rhythm frequency might be determined by a specific photoperiod. To examine this possibility, plants were grown with a photoperiod of either L6:D18 or L18:D6. Six of light in a 24-h period failed to yield adequate growth of either shoot or root and no enzyme data could be obtained. Plants grown on the L18:D6 regimen displayed the same GS ultradian rhythm characteristic of L12:D12 plants, *i.e.* with peak activities 12 h apart (Fig. 3).

Among the expected characteristics of an endogenously regulated biological rhythm is its maintenance during a free-running period (36). This characteristic of the GS rhythm described here has been partially verified by the observation that 12-h rhythms established during L12:D12 entrainment periods were indeed maintained into subsequent continuous light periods (Fig. 1B). However, when plants entrained to a L12:D12 photoperiod were subsequently maintained in continuous darkness rather than continuous light, the 12-h rhythm characteristic of the entrainment period was clearly altered. Root GS activity of plants that had been maintained in darkness after the end of a regular dark phase did not peak in activity in the time span that would have been the next light phase under the L12:D12 entrainment (Fig. 4A). Instead, only the peak produced during what would have been the next regular dark phase remained. When GS activity was determined in the roots of entrained plants that had been maintained in darkness for 30 h beyond the end of a regular dark phase, again light phase peaks failed to appear and the only peaks were those occurring in what would have been regular dark phases (Fig. 4B). These peaks were separated by a period of approximately 24 h, were lower in amplitude than those developed early in continuous darkness (Fig. 4A), and were shifted forward 3 to 6 h from what would have been their normal position in a light:dark regimen. Thus, unlike the 12-h ultradian rhythm developed during entrainment and maintained in continuous light, the rhythm during a free-running period of darkness is of circadian periodicity.

Energy Charge. The high degree of correlation between GS activity and the energy level of sunflower root tissue noted in a previous study (34) was also found in the present investigation. For both entrainment and continuous light free-running periods, L12:D12 plants subjected to a 6 AM on-6 PM off regimen displayed a rhythm of approximately 12 h for energy charge that was directly correlated in time with the rhythmic activity of GS (Fig. 1, A and B). A direct correlation between GS and energy charge was also evident in L12:D12 plants grown with a photoperiod phase shift of 6 h (Fig. 2, A and B). This was also true for plants grown under the L18:D6 regimen and for plants placed in continuous darkness (Fig. 4).

To test the possibility that adenine nucleotides might exert a regulatory influence upon GS by a mechanism other than or in addition to its effect on the energy level of the cell (19, 23), we examined the individual components of energy charge. Data from roots harvested during the continuous light phase of L12:D12 plants (6 AM on-6 PM off) show both variation in total adenine nucleotide concentration and changes in ATP, ADP, and AMP

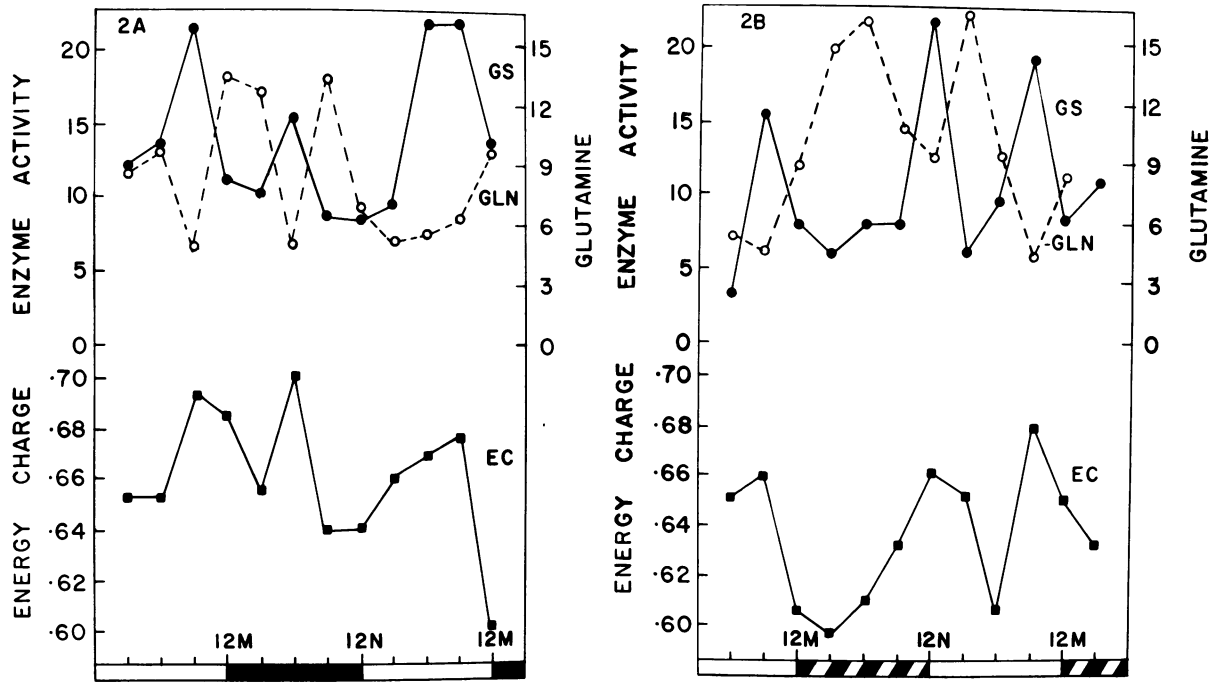

FIG. 2. Rhythms in sunflower root tissue harvested after plant growth under the following conditions: lights on 12 N-off 12 M; A, L12:D12; B, continuous light after L12:D12. GS, GS activity (transferase reaction; μmol/mg protein·h); GLN, glutamine concentration (μmol/g fresh weight); EC, energy charge.

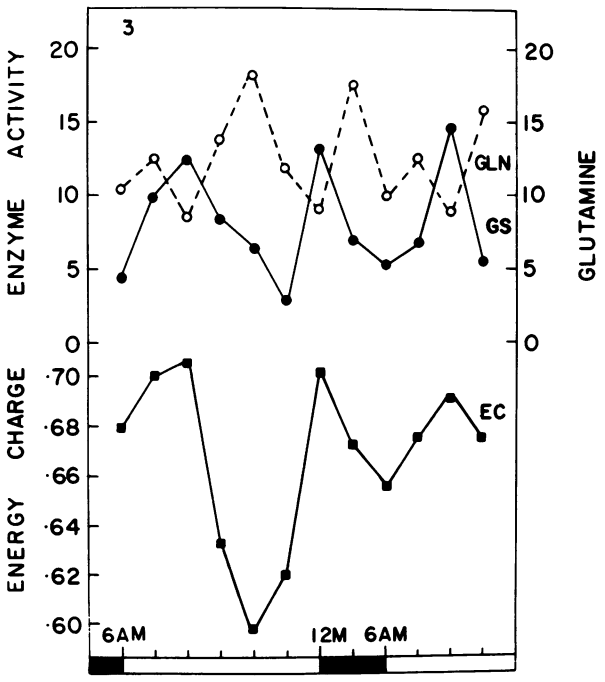

FIG. 3. Rhythms in sunflower root tissue harvested after plant growth under L18:D6 conditions. GS, GS activity (transferase reaction, μmol/mg protein·h); GLN, glutamine concentration (μmol/g fresh weight); EC, energy charge.

calculated as percentages of total adenine nucleotide (Fig. 5A). Although variation in total adenine nucleotide is evident, there is no pattern of rhythmic change. Similarly, no rhythm is discernable for AMP. ATP and ADP, however, display clear 12-h rhythms with ATP directly correlating and ADP inversely correlating with GS activity (Figs. 1B and 5A). Most striking is the very close association in time of high ATP-low ADP periods with peaks in the activity of the enzyme. A similar correlation between ATP and GS activity together with an inverse correlation between ADP and GS activity was detected in roots of entrained plants placed in darkness (Figs. 4A and 5B). These observations strongly suggest that rhythmic activity of GS may be dependent upon the fluctuations in relative concentrations of specific adenine nucleotides.

Amino Acids. Twelve amino acids were identified (alanine, γ-amino-butyric acid, asparagine, aspartic acid, glutamic acid, glutamine, glycine, leucine, lysine, serine, tyrosine, valine) in extracts of sunflower root tissue subjected to TLC. Of these, alanine, aspartic acid, glutamic acid, and serine gave sporadic evidence of rhythmic variation. Only glutamine demonstrated a consistent cycle of concentration change with rhythms of approximately 12 h in plants exposed to photoperiods of either L12:D12 or L18:D6 (Figs. 1A, 2A, and 3). Similar glutamine rhythms of approximately 12 h were found in the roots of plants exposed after entrainment to a free-running period of either continuous light or continuous darkness (Figs. 1B, 2B, 4A, and 4B). The root glutamine rhythm had a lower amplitude after an extended period of continuous darkness (Fig. 4B). Unlike the direct correlation with GS activity displayed by energy charge, a generally consistent inverse relationship is seen to exist between glutamine concentration and activity of GS. In the roots of plants subjected to a regimen of either L12:D12, L18:D6, or to continuous light after L12:D12 entrainment, low glutamine concentration was correlated with high GS activity while high glutamine was associated with low GS activity (Figs. 1–3). Only in the roots of entrained plants subjected to continuous darkness could exceptions to the inverse relationship be noted (Fig. 4).

DISCUSSION

Reports of enzymic rhythms in roots are few in number. Nitrate reductase displays a circadian rhythm in roots of wheat seedlings germinated and grown in continuous light (30) and Duke and Koukkari (10) have observed both a circadian rhythm for glutamate dehydrogenase in pea roots and 1- to 6-h oscillations in the activity of this enzyme in pea root mitochondria. Although a 12-

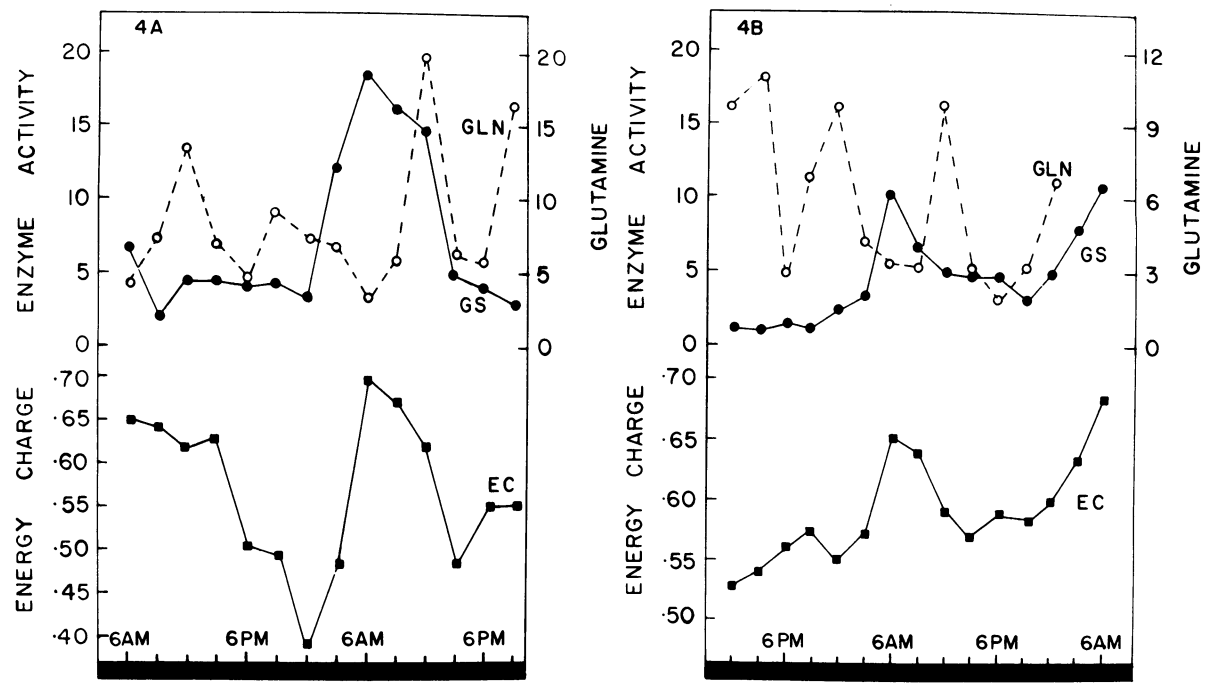

FIG. 4. Rhythms in sunflower root tissue harvested after plant growth under the following conditions: A, continuous darkness, analyses beginning after last dark phase of L12:D12; B, continuous darkness, analyses beginning 30 h after last dark phase of L12:D12. GS, GS activity (transferase reaction, μmol/mg protein·h); GLN, glutamine concentration (μmol/g fresh weight); EC, energy charge.

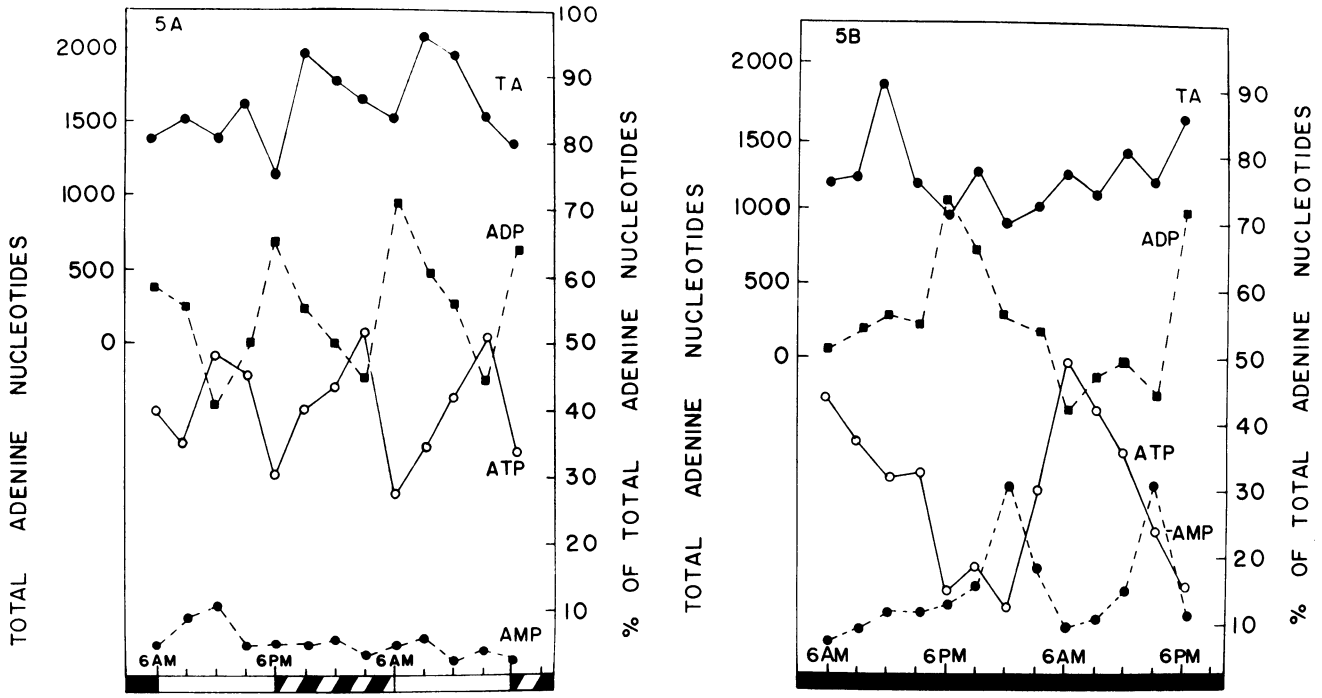

FIG. 5. Adenine nucleotides in sunflower root tissue harvested after plant growth under the following conditions: A, lights on 6 AM-off 6 PM; continuous light after L12:D12; B, continuous darkness, analyses beginning after last dark phase of L12:D12. TA, total adenine nucleotide (nmol/g acetone powder); ATP, ADP, and AMP plotted as percentages of total adenine nucleotide.

h ultradian rhythm has been reported for the GS of soybean leaves (9), the present study is the first to demonstrate rhythmic activity of this enzyme in roots. That the GS rhythm is dependent upon photoperiodically-induced processes rather than upon other fluctuating environmental factors is demonstrated by the 6-h shift in rhythm that occurred with a 6-h shift in the light:dark regimen (Fig. 2).

The rhythms displayed by the roots of sunflower plants during a L12:D12 regimen are characterized by 12-h cycles. When the light:dark regimen was modified to L18:D6, the 12-h rhythms were maintained (Fig. 3). The persistence of a 12-h rhythm over a wide-ranging light period within a 24-h photoperiod has also been observed in respiration of *Lolium multiflorum* (13). *Lolium* roots displayed diurnal fluctuations with two peaks separated by 10 to 12 h and with the frequency independent of light periods of 8 to 16 h. The similarity of rhythmic behavior between *Lolium*

root respiration and the root GS activity observed in the present study is suggestive of a physiological link between the two processes.

When considered on the basis of the 12-h GS rhythm that is maintained in the roots of plants placed in continuous light subsequent to a L12:D12 entrainment period (Fig. 1B), the GS rhythm seems to be ultradian (*i.e.* a rhythm of less than 24 h that is produced during both the entrainment and free-running phase), in nature. However, the 24-h frequency with which peak activities appear in the roots of entrained plants that have been placed in continuous darkness suggests a circadian rhythm. These data indicate that the 12-h ultradian GS rhythm engendered during normal fluctuating periods of light and darkness might be more accurately described as one composed of two independent diurnal rhythms that alternate with each other in such a manner as to yield peaks in GS activity that are approximately 12 h apart. One diurnal rhythm produces peaks of GS activity during the light phase of light:dark-entrained plants which are sustained in continuous light. When entrained plants were placed in darkness, the light phase GS rhythm was not detected (Fig. 4). This demonstrates both the incompletely circadian nature of the light phase rhythm and the dependency of this diurnal rhythm upon light. A second diurnal rhythm generates peaks of GS during the dark phase of the entrainment period but, unlike the light phase rhythm, these are maintained whether plants are placed in continuous light or continuous darkness. Thus, the dark phase rhythm, once established by entrainment, is light independent. The maintenance of the dark phase rhythm during free-running periods in either light or darkness (Figs. 1B and 4), the shift in peak position when entrained plants are subjected to continuous darkness (Fig. 4B), and the decline in peak amplitude under free-running conditions in continuous darkness (Fig. 4B) all support the view that the dark phase rhythm is completely circadian (36).

Experiments now in progress in this laboratory (17) indicate strongly that the GS rhythms in sunflower roots are not endogenous to the root system itself but are wholly dependent upon the presence of a shoot system that has been exposed to normal fluctuations of light and darkness. These observations, when considered together with the precautions taken in the present study aimed at preventing exposure of the root system to fluctuations of light and darkness, lead to the conclusion that the root GS rhythms reported here are ultimately dependent upon processes in the leaf such as photosynthesis and/or phytochrome activation of a Zeitgeber (7). The association of root rhythms with these processes could be mediated by shoot-to-root translocation of carbohydrate, substances known to affect GS activity (28). However, to date no firm evidence for rhythmic changes in the translocation of carbohydrate or in the concentration of respirable materials in roots has been presented.

Our knowledge of the role of adenine nucleotides in the control of GS activity has been enhanced by reports on the regulation of GS by energy charge (22), competitive inhibition (26), and allosteric effectors (37). Energy charge has been viewed as an index of readily available energy and has been defined as a ratio of the anhydride phosphate bonds in the adenine nucleotide system compared to total adenine nucleotide concentration (2). GS, as an enzyme utilizing the energy of the anhydride phosphate bond in a synthetic reaction, might thus be expected to be influenced by the energy charge of the cell. All data of the present study confirm the findings of previous studies with sunflower roots (34) indicating a direct correlation between energy charge and GS activity (Figs. 1–4). The question might be raised, however, as to whether the rhythmic variation in GS activity observed here is related to the demonstrated rhythmic changes in root cell energy charge or whether a more valid view would be one that linked the GS activities to the allosteric effects exerted upon the enzyme by the adenine nucleotides while still within the intact cell. It should be noted that, in the determination of transferase activity of the acetone powders prepared in the present study, a constant and relatively high concentration of ADP (4 mM) in the reaction mixture provided the enzyme material of each sample with essentially the same energy charge milieu, one that would completely negate the energy charge derived from proportions of ATP, ADP, and AMP in the tissue sample. Furthermore, the transferase reaction, rather than having a sensitivity to energy charge, is one that utilizes the catalytic properties of added ADP (21). It seems that the rhythmic variations observed for GS activity in the root tissue samples cannot have been caused by the ratio of anhydride phosphate bonds to total adenine nucleotide (energy charge) of the tissue but are more likely due to conformational changes exerted upon GS, while within the root cells, by allosterically effective adenine nucleotides.

The view that energy charge functions through the allosteric effects of the adenine nucleotides on certain enzymes has been adopted by others (19, 23) and is supported by the data of the present study. From Figure 5a, it may be observed that ATP and ADP, calculated as percentages of total adenine nucleotide in roots of L12:D12-continuous light plants, rise and fall with 12-h rhythms that are inversely related to each other and that GS activity is directly correlated with levels of ATP or inversely correlated with levels of ADP (Fig. 1B). The same correlations were noted for the roots of entrained plants placed in darkness (Figs. 4A and 5B). Furthermore, if for each harvest of L12:D12-continuous light plants an ATP/ADP+AMP ratio is calculated on the supposition that ATP is allosterically stimulatory while ADP and AMP are inhibitory, a 12-h rhythm for the adenine nucleotide ratio is obtained (Fig. 1B) that appears to be as well correlated with glutamine synthetase activity as is energy charge. The rhythmic activity displayed by root glutamine synthetase in this study, therefore, rather than finding its explanation in rhythmic changes in energy charge, is best explained in terms of the rhythmically changing allosteric effects exerted upon the enzyme by the rhythmically changing ratio of ATP/ADP+AMP in the root tissue.

The existance of a diurnal variation in the concentration of a number of amino acids in leaves has been long recognized (3, 25), but the observation in this study of an approximately 12-h cycle in the glutamine concentration of sunflower roots may be the first report of an amino acid rhythm in this portion of the plant. Also noteworthy is the regularity of this rhythm under variable conditions and its strict inverse phase relationship with the 12-h rhythm for GS (Figs. 1–3). This suggests that, in addition to the dependency of glutamine content on GS activity, other processes such as glutamine utilization or transport must have some effects. Factors which may influence the concentration of glutamine in root tissue include the release and utilization of glutamine in protein turnover, utilization of glutamine as an amide group donor (*e.g.* in the asparagine synthetase reaction), the conversion of glutamine to other amino acids by a glutamate synthase-aminotransferase system, translocation of glutamine from root to shoot, and the availability of NH_4^+ for glutamine synthesis. No evidence is yet available for rhythmic activity in amide group utilization in synthetic reactions. However, investigations with *Triticum* leaves (24) suggest that glutamate synthase displays circadian activity. Exudation studies on sunflower have revealed the existence of an endogenous diurnal rhythm for this process (12), and previously reported work from this laboratory (33) has demonstrated a rise followed by a fall in the glutamine concentration of exudate sampled periodically from 8 AM to 4 PM. Thus, the 12-h rhythm in glutamine concentration may represent a summation of processes, some of which are rhythmic in nature.

The general synchrony of the glutamine rhythm with the adenosine phosphate ratio and GS activity, albeit inverse in nature, suggests that the various rhythms are ultimately synchronized by

the same timing mechanism. Yet, it appears that the rhythm of glutamine level is regulated in some manner that differs from the regulation of adenosine phosphate and GS rhythms. This is indicated by the observation that unlike the conversion of the 12-h rhythms for energy charge and GS activity to circadian rhythms when entrained plants are subjected to continuous darkness, glutamine concentration maintains its 12-h cycle (Fig. 4, A and B).

The biochemical significance of the 12-h glutamine rhythm observed in this study is not clear, but it is of interest to note that previous investigations with yeast (11), *Chlorella* (1), and *Lemna* (27) have established an inverse relationship between the concentration of the glutamine pool and GS activity. With both soybean cell suspension cultures (5) and hepatoma tissue-culture cells (6), increasing concentrations of extracellular glutamine have been found to exert a repressive effect upon GS activity. Glutamine has been viewed as an allosteric effector of GS in *Chlorella* (1), but others have presented evidence indicative of a degradation of the active octameric form of GS to a less active tetrameric form in the presence of high levels of glutamine (29). Homeostatic considerations with regard to nitrogen assimilation in the root suggest that a rhythm for glutamine concentration could function, together with rhythmic changes in the adenine nucleotides, as a factor yielding rhythmic activity of GS. With the exception of experiments utilizing continuous darkness after entrainment (Fig. 4, A and B), the data presented here support this concept. For those instances in which low glutamine was found in low GS activity tissues, it should be noted that energy charge values were all at low levels. This could account for the low GS activity in the low glutamine environments. The overriding necessity of conserving ATP in a low energy or low ATP/ADP+AMP milieu may account for the position of priority held by the energy charge mechanism among the multiple systems of enzyme regulation acting upon GS.

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