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Glutamine Synthetase Regulation by Energy Charge in Sunflower Roots[1]

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ABSTRACT

Energy charge [(ATP) + ½ (ADP)]/[(ATP) + (ADP) + (AMP)] and glutamine synthetase activity (transferase reaction) of roots increase in a near congruent manner when decotyledonized sunflower plants (*Helianthus annuus* L. var. Mammoth Russian) are grown in nitrate for 9 days. Replacement of nitrate with ammonium for the final 2 days leads to a higher energy charge and increased enzyme activity. Similar correlations occur when nitrate plants are placed on a zero nitrogen regimen and when they are subjected to continuous darkness. A rank order correlation of 0.72 is obtained for all data. Control concepts such as adenylylation-deadenylylation and ammonium inhibition of enzyme synthesis are not supported by the data. Energy charge-enzyme activity plots support the view that glutamine synthetase of sunflower roots is subject to control by end products of glutamine metabolism. Alanine appears to exert a modulating effect on the regulation of glutamine synthetase by energy charge.

In recent years the regulation of enzyme synthesis and the control of enzyme activity in higher plants has received the attention of a number of investigators (21). Studies with bacteria have led to the suggestion (1) that enzymes utilizing ATP for biosynthetic purposes increase in activity in accordance with the energy level of the cell. Only limited efforts have as yet been made to determine the extent to which energy charge, defined by Atkinson (1) as [(ATP) + ½ (ADP)]/[(ATP) + (ADP) + (AMP)], influences the activity of biosynthetic enzymes found in higher plants (20, 23, 28). An enzyme utilizing the energy of ATP in bond formation is glutamine synthetase (EC 6.3.1.2.). Responsible as it is for the formation of glutamine from glutamic acid, ammonia, and ATP, the enzyme is also known to function *in vitro* as a glutamyl transferase (19). Evidence has previously been presented indicative of a correlation between the energy charge of soybean and sunflower roots and the glutamyl transferase activity of these tissues (28). In a further effort to investigate the relationship between energy charge and the specific activity of a biosynthetic enzyme, the present study has examined glutamine synthetase activity (transferase reaction) and adenosine phosphate content in sunflower root tissue of plants supplied with nitrate after removal of cotyledons, of plants transferred from a nitrate culture solution to an ammonium solution, of plants subjected to a zero nitrogen medium, and of plants treated to an extended period of darkness.

In microorganisms (31) and in rice roots (12) the synthetic and transferase activities of the enzyme have been demonstrated as sensitive to inhibition by various amino acids generally viewed as end products of glutamine metabolism. Accordingly, the influence of amino acids in the regulation of glutamine synthetase has been examined in this study by a determination of the concentration in the root tissues of a possible end product, alanine, together with the levels of glutamic acid and glutamine.

MATERIALS AND METHODS

Plant Culture. Sunflower seeds (*Helianthus annuus* L. var. Mammoth Russian) were germinated in filter paper-lined beakers for a period of 5 days. In experiments in which only nitrate was to be utilized as a nitrogen source, pairs of seedlings were transferred to 600-ml beakers containing culture solution and were supported with roots immersed in solution by double-grooved plastic beaker covers. Culture solution for these plants was changed daily. For the experiment in which ammonium was provided after a period of nitrate culture, 5-day-old seedlings were transferred to the continuous flow apparatus previously described (29), and culture solution was passed over the roots for the remainder of the 20-day growth period. Under these conditions of growth, culture solutions experienced no more than a decrease of 0.1 pH unit during a 24-hr period.

Following the transfer to either 600-ml beakers or to the continuous flow apparatus, all plants were treated with culture solution lacking in nitrogen (25) for a period of 7 days. The cotyledons were then removed from the 12-day plants, and culture solution containing 10 mM KNO_3 was provided. All plants were grown in a controlled temperature chamber (26–27 C day and 20–21 C night) under artificial light on a 14-hr photoperiod.

In order to induce changes in the energy charge of root tissue, the 12-day sunflower plants were subjected to four different regimens: A: 10 mM KNO_3 for 9 days; B: 10 mM KNO_3 for 7 days followed by 10 mM NH_4Cl for 2 days: C: 10 mM KNO_3 for 5 days followed by culture solution lacking nitrogen for 5 days; D: 10 mM KNO_3 and the regular 14-hr photoperiod for 5 days followed by 10 mM KNO_3 in continuous darkness for 5 days.

Methods of Analysis. Previous analyses for glutamine synthetase in this laboratory have been performed on acetone powders (27), while analysis for the content of adenine nucleotides has utilized freeze-dried tissue (28). A comparison of the results obtained by ion exchange chromatography of acetone powder preparations and of freeze-dried tissue makes it clear that acetone powders yield data for AMP, ADP, and ATP in root tissue that differs in no significant way from that obtained with freeze-dried tissue. It was thus possible to perform both glutamine synthetase and adenine nucleotide determinations on the same root tissue preparation. Acetone powders were prepared by the method of Loomis (17) and, when maintained in a vacuum desiccator at −10 C, these preparations showed less than a 10%

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decrease in transferase activity and adenine nucleotide content after 1 year.

Glutamine synthetase and glutamyl transferase activities were determined by the methods, with some modification, of Hubbard and Stadtman (10). The activity of the synthetic reaction proved to be very low for all tissues and is reported only for the nitrate (regimen A) and ammonium-treated (regimen B) series. Transferase activity was vigorous in a reaction mixture consisting of 5 mg of acetone powder and 1 ml of solution containing 20 mM imidazole (pH 7); 3 mM $MnCl_2 \cdot 4H_2O$; 20 mM sodium arsenate; 300 mM hydroxylamine-HCl (pH 7); 4 mM ADP (pH 7); and 100 mM glutamine. Controls lacking glutamine were included in all assays. Following an incubation period of 5 min at 38 C, the reaction was stopped by the addition of 2 ml of a solution containing 0.83% $FeCl_3 \cdot 6H_2O$ and 2% trichloroacetic acid in 0.5 N HCl. The mixture was filtered, and the absorbance of the red chelated complex of glutamyl hydroxamate with ferric ion was measured at 540 nm. Protein in the enzyme preparation was estimated by the method of Lowry *et al.* (18).

With the procedural modifications previously described (28), adenosine mono-, di-, and triphosphate were determined on samples of root acetone powder by the ion exchange chromatography method of Keys (13).

Free glutamic acid, glutamine, and alanine were determined by two-dimensional paper chromatography of extracts prepared from fresh root tissue (2).

Glutamine synthetase, glutamyl transferase, and adenine nucleotide analyses were performed on tissues obtained from two separate experiments. For each experiment, mean values were computed after enzyme analyses were performed in triplicate and nucleotide analyses in duplicate. Only minor variations occurred between the two experiments, and the results reported are of one trial.

RESULTS AND DISCUSSION

The recognition of glutamine as a major cell metabolite has led to extensive efforts aimed at gaining an understanding of the mechanisms involved in the regulation of glutamine synthetase activity. As a preliminary to the analysis of the role of energy charge, it may be of value to examine the data of this study as they relate to regulatory mechanisms that have been suggested as operative in other cell types and tissues. In *Escherichia coli,* for example, both the synthetic and transferase activity of the enzyme have been demonstrated as sensitive to feedback inhibition by end products of glutamine metabolism (31). Inhibition of this type appears to be dependent upon the degree to which subunits of the enzyme are in the adenylylated state (22). In the presence of end product inhibitors, the adenylylated enzyme of *E. coli* is characterized *in vitro* by low synthetic-high transferase activity (14). A similar difference in synthetic and transferase activity is displayed in the present study by enzyme preparations from nitrate-supplied and ammonium-supplied roots (Fig. 1, Table IA) Therefore, consideration must be given to the possible existence of an adenylylation-deadenylylation control mechanism in the sunflower roots under examination. Analysis of other data does not indicate any failure of *in vivo* glutamine synthesis in either nitrate or ammonium-supplied roots. In previous studies with nitrate-supplied sunflower roots showing low synthetic-high transferase activity *in vitro,* the large amounts of glutamine transported from root to shoot make it clear that the roots are actively engaged in the synthesis of glutamine (26, 27). Similar evidence is provided by the present study. It will be noted that despite the limited *in vitro* synthetic activity of enzyme preparations from nitrate plants treated for 2 days with ammonium, glutamine concentration of the tissue increased sharply as a result of ammonium treatment and is correlated with the increased activity of the enzyme as determined by the transferase reaction (Tables IA and IIB). It may be concluded that the *in vivo* synthesis of glutamine is indeed an active process in both nitrate-supplied and ammonium-supplied roots and that the low synthetic-high transferase data cannot be interpreted as indicating the presence of an inhibited glutamine synthetase induced by an adenylylating control mechanism. Rather, it is strongly indicated that transferase activity provides an approximately close estimate of the capacity of the enzyme, in the presence of required substrate, to carry on the synthetic reaction *in vivo.* The failure in this study to detect significant synthesizing activity *in vitro* probably arises out of some inadequacy of the reaction mixture that is specific for the sunflower root enzyme. The same reaction mixture has yielded clear synthesizing activity with acetone powder preparations of soybean roots (unpublished results) and with extracts of *Chlorella* (11).

In addition to the probable absence of an adenylylation-deadenylylation control mechanism, regulation of the sunflower root enzyme appears to differ in another respect from that in *E. coli.* In this microorganism (31) and in *Chlorella pyrenoidosa* as well (7), the synthesis of glutamine synthetase has been reported as subject to inhibition by ammonia. Others have interpreted the decline in enzyme activity in the presence of ammonia as due to a conversion of the enzyme to the adenylylated state (9). With regard to the present study, the increase in activity that develops after the transfer of nitrate-supplied plants into an ammonium culture solution (Table IA) suggests that the glutamine synthetase of sunflower roots is subject neither to ammonium inhibition of enzyme synthesis nor to inhibition of activity by the adenylylation process. A similar stimulation of glutamine synthetase activity by ammonia has been reported for pea roots (7).

It is of interest to consider the enzyme as it exists in species of *Bacillus* and some other gram-positive bacteria that have been investigated. In these microorgamisms, unlike *E. coli,* adenylylation plays no role in the control of glutamine synthetase (6, 8). As is the case for *E. coli*, however, the enzyme in *Bacillus* species is sensitive to inhibitory action by the end products of glutamine metabolism (10). The data of the present study suggest that the sunflower root enzyme is similar to that of *Bacillus* species in that it lacks an adenylylation control mechanism but, as will be indicated below, is sensitive to end product inhibition.

The major postulate of the energy charge concept as it pertains to enzymes utilizing ATP in biosynthetic reactions is that any tendency for the energy charge to rise or fall will lead to concomitant increased or decreased activity of the enzyme (1). According to this view and to a similar concept developed by Bomsel and Pradet (4), the level of enzyme activity is a means of maintaining the energy economy of the cell. The previously expressed hypothesis (28) that such a control mechanism regulates the activity of glutamine synthetase of sunflower roots is supported by the present study in which a consistent correlation between energy level and *in vitro* glutamine synthetase activity (transferase reaction) was demonstrated for each of the environmental conditions to which the plants were subjected. When 12-day-old sunflower plants were decotyledonized (0 days in nitrate) and then provided with culture solution containing nitrate for a period of 9 days, both energy charge and enzyme activity fell sharply after decotyledonization and then increased in a near congruent manner up to the 9th day in nitrate (Fig. 1). The observation that energy charge and glutamine synthetase activity were greater in roots supplied with ammonium for 2 days (after 7 days in nitrate) than in roots supplied only with nitrate provides further evidence of a correlation between these two parameters (Table IA). In a third experimental procedure, plants grown with nitrate for 5 days and then placed on a zero nitrogen regimen displayed an increase in energy charge of the root tissue after 3 days without nitrogen followed by a decline in charge after 5 days. These changes were accompanied by an increase and then a decrease in enzyme activity after 3 and 5 days, respectively (Table IB). A final demonstration of correlation between energy charge and glutamine synthetase activity is the similar decline in

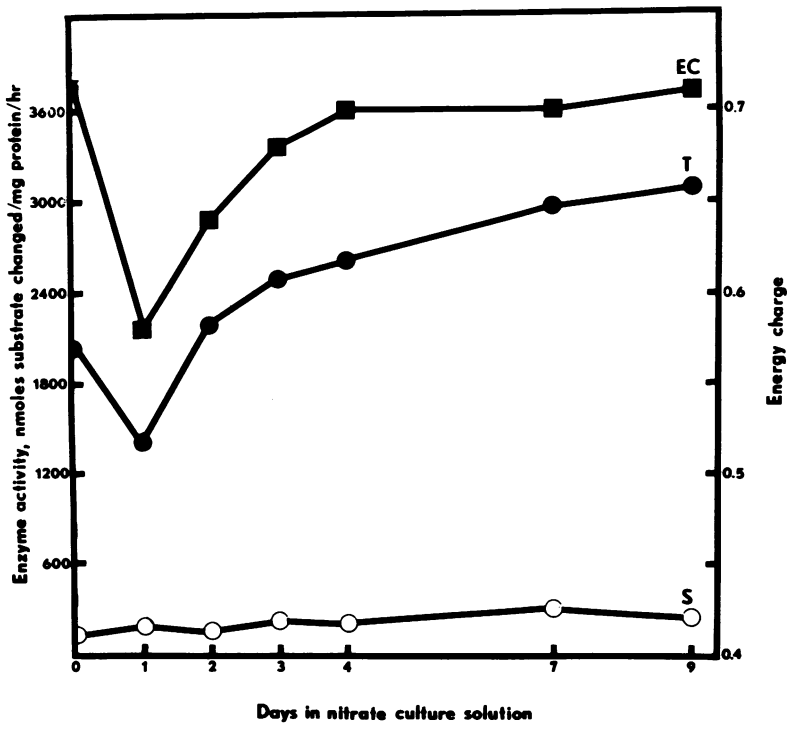

FIG. 1. Glutamine synthetase activity and energy charge of sunflower root tissue during 9 days of growth in culture solution containing nitrate. Synthetic activity (S, O—O); transferase activity (T, ●—●); energy charge (EC, ■—■).

Table I. *Glutamine Synthetase Activity, Adenine Nucleotides, and Energy Charge of Sunflower Roots*

A: Effect of 2 days in nitrate or 2 days in ammonium after 7 days in nitrate; B: effect of 3 days and 5 days in zero nitrogen solution after 5 days in nitrate; C: effect of 3 days and 5 days in darkness (D) after 5 days in light (L).

	Synthetic reaction	Transferase reaction	AMP	ADP	ATP	Energy Charge
	nmoles substrate changed per mg protein per hr		nmoles per gram tissue			
A. 7:NO_3	255	2360	160	1052	1077	0.70
7:NO_3+2:NO_3	261	2896	156	1264	1186	0.70
7:NO_3+2:NH_4	297	4970	150	865	1453	0.77
B. 5:NO_3	a	3280	91	930	741	0.69
5:NO_3+3:0-N	a	5560	118	942	1283	0.75
5:NO_3+5:0-N	a	4880	180	1409	753	0.62
C. 5:L	a	3560	108	982	773	0.68
5:L+3:D	a	2540	118	1129	443	0.60
5:L+5:D	a	1720	145	1120	439	0.59

[a] Synthetic activity not determined.

Table II. *Protein, Glutamic Acid, Glutamine, and Alanine in Sunflower Roots*

A: Effect of 9 days in nitrate (1:NO_3-9:NO_3) after removal of cotyledons (O:NO_3); B: effect of 2 days in nitrate or 2 days in ammonium after 7 days in nitrate; C: effect of 3 days and 5 days in zero nitrogen solution after 5 days in nitrate; D: effect of 3 days and 5 days in darkness (D) after 5 days in light (L).

	Protein	Glutamic acid	Glutamine	Alanine
	µg per mg powder	µg	per gram fresh weight	
A. 0:NO_3	368	38	9	12
1:NO_3	302	45	7	25
2:NO_3	224	55	12	45
3:NO_3	296	90	13	74
4:NO_3	222	110	18	214
7:NO_3	198	138	18	286
9:NO_3	198	140	78	328
B. 7:NO_3	234	138	22	314
7:NO_3+2:NO_3	214	144	69	336
7:NO_3+2:NH_4	198	97	263	410
C. 5:NO_3	178	94	20	310
5:NO_3+3:0-N	196	26	20	225
5:NO_3+5:0-N	186	57	5	62
D. 5:L	178	100	15	265
5:L+3:D	170	33	3	25
5:L+5:D	158	3	3	0

these parameters when nitrate-supplied plants were subjected to a 5-day period of darkness (Table IC).

Indicative of the high order of correlation between energy charge and enzyme activity is the rank order coefficient of 0.72 calculated from all of the data of the four experiments in which each experiment was repeated twice. A graphic display of this high order of correlation is obtained if one plots, for each tissue, enzyme activity against energy charge (Fig. 2). The data fall into two distinct groupings, each yielding a curve with a slowly increasing slope at low energy charge and rapidly increasing slope at higher energy charge. Such a curve is reminiscent of those obtained by Atkinson as a result of *in vitro* studies of other ATP-utilizing biosynthetic enzymes in *E. coli;* it was suggested that

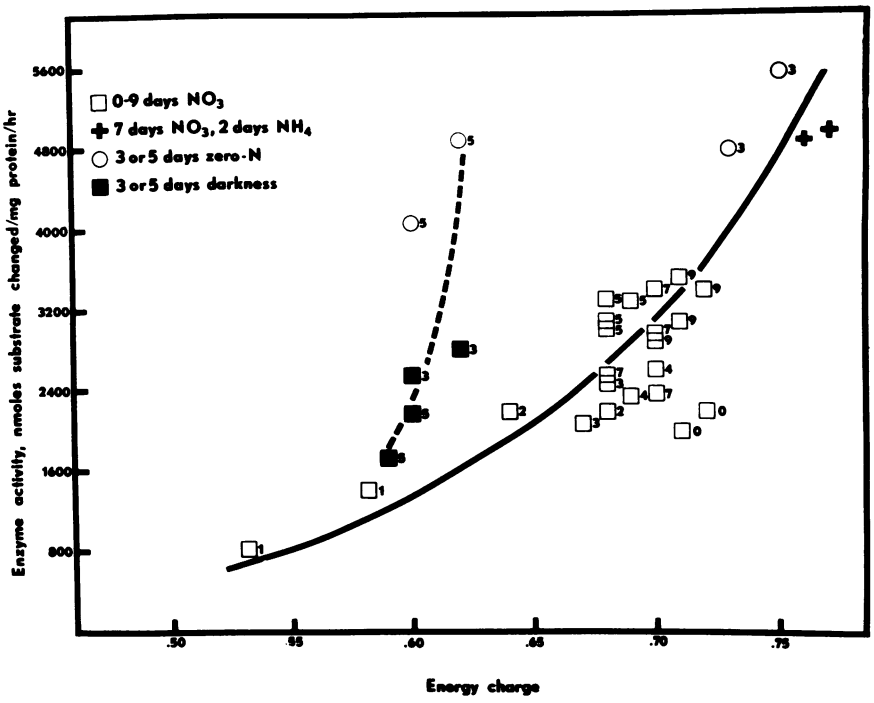

FIG. 2. Glutamine synthetase activity (transferase reaction) as a function of energy charge. High end product curve (—); low end product curve (----). Nitrate series, 0 to 9 days (□); ammonium series (+); zero nitrogen series (○); dark series (■).

such a relationship would provide the cell with a highly sensitive control mechanism under conditions in which there was occurring too rapid consumption of the adenylate energy supply (1). Further examination of Figure 2 indicates that one of the enzyme activity-energy charge curves is composed of data derived from the nitrate series, the ammonium-treated plants, and the 3-day zero nitrogen plants while the other curve is composed of data from the dark-treated series and the 5-day zero nitrogen plants. A similar divergence into separated curves relating enzyme activity to energy charge has been obtained with *E. coli* through the use of end products of glutamine metabolism acting in the presence of a constant energy charge (15). The regulation of ATP-utilizing biosynthetic enzymes by energy charge appeared to be modulated by the concentration of end product inhibitors. It therefore becomes of interest to determine whether the tissues composing the two enzyme activity-energy charge curves do indeed differ in their concentrations of glutamine end products. Various amino acids have been identified as end products of glutamine metabolism in bacteria and mammalian tissue (22) but in the present study only alanine could be detected in quantities sufficient for analysis. It is not yet clear how much of the total alanine in the root is a product of glutamine metabolism but the views of Lea and Miflin (16) on the role of glutamate synthase, if confirmed and extended to root tissue, provide a mechanism for the utilization of glutamine amide-N in alanine synthesis. At any rate, it can be seen from Table II that alanine is present in relatively high concentration in roots of the nitrate series, in roots of ammonium-treated plants, and in plants subjected to 3 days of nitrogen starvation. These are the same plants that are grouped together in a specific enzyme activity-energy charge relationship (Fig. 2); their root glutamine synthetase can be viewed as an ATP-utilizing enzyme subject to control by energy charge but modulated in its effect by a high concentration of end product inhibitor. On the other hand, roots of dark-treated plants and of 5-day zero nitrogen plants, containing as they do only limited amounts of alanine (Table II), appear to possess a glutamine synthetase regulated by energy charge but without the modulating effect of end products. From this point of view only the enzyme of zero days nitrate plants (before decotyledonization) appears out of place on Figure 2. The enzyme activity-energy charge position of this root system is suggestive of an enzyme controlled by energy charge but modulated by end products. Yet, the alanine content of the root is quite low. It may be that the tissue possesses a high concentration of some other, as yet undetected, end product or combination of end products acting as a modulator of energy charge regulation.

Knowledge of the factors that control the level of the adenine nucleotides in plant tissue is very incomplete. Only tentative hypotheses can be offered in explanation of the significant changes in proportions of AMP, ADP, and ATP, and the resultant changes in energy charge observed in this study. From Figure 3 it can be determined that the previously noted decline in charge following decotyledonization is the result of a sharp decline in the percentage of ATP. A concomitant effect is a decline in total adenine nucleotide. These changes have probably been induced by the sudden insufficiency of nitrogen and organic carbon compounds, and the resultant decline in nucleotide synthesis and respiratory phosphorylation, that decotyledonization has imposed upon the root tissue. The return of total adenine nucleotide and ATP to pre-decotyledonization levels after 4 days (Fig. 3) suggest that the expanding photosynthetic shoot and the supply of exogenous nitrate are capable of providing the root system with the metabolites required for nucleotide synthesis and phosphorylation reactions.

Probably another factor of importance in establishing the AMP:ADP:ATP ratio in roots is the form of nitrogen in the culture solution. Although nitrate may act as a stimulus to glycolysis (30), the overall effect of the reduction process to ammonia, both by redirection of electrons into the reduction of nitrate to nitrite rather than towards terminal oxidation (3) and by the stimulation of the pentose phosphate pathway (5, 24) with its reduced ATP-producing capacity, is to place a limit on ATP production and thus on energy charge. If nitrate were to be replaced by ammonium as the source of nitrogen, it may be expected that these limitations on ATP production would be removed. This hypothesis is supported by the sharp increase in ATP and decline in ADP observed in the roots of nitrate plants of this study following their transfer to an ammonium culture

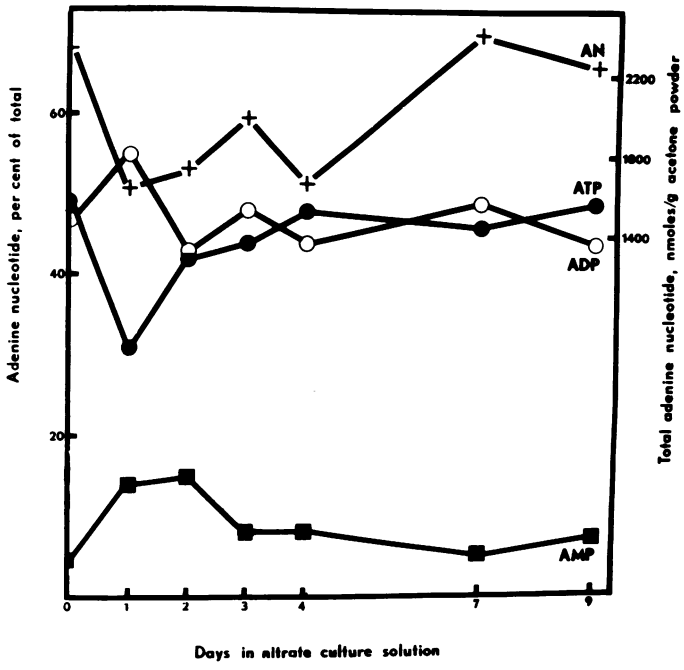

FIG. 3. Total adenine nucleotide and AMP, ADP, and ATP as percentages of total in sunflower root tissue during 9 days of growth in culture solution containing nitrate. Total adenine nucleotide (AN, +—+); AMP (■—■); ADP (○—○); ATP (●—●).

solution (Table IA). For the plants placed in darkness, it is probably the limited phosphorylation of ADP in the carbohydrate-starved roots that accounts for the decline in ATP and increase in ADP observed in this tissue (Table IC). More difficult to understand is the manner in which energy charge first rises and then falls in root tissue of plants that were deprived of external nitrogen over a 5-day period. The increase in ATP level during the first 3 days of nitrogen deficiency (Table IB) may have been caused by a conservation of ATP under conditions of limited protein synthesis. After 2 additional days in a nitrogen-free culture solution, ATP declined to only 59% of its previous concentration, a change accompanied by proportionate increases in ADP and AMP. Changes of this magnitude are suggestive of a breakdown, under conditions of nitrogen starvation, in the complex enzymic mechanism responsible for the maintenance of the normal energy economy of the cell.

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