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Ammonia Regulation of Carbon Metabolism in Photosynthesizing Leaf Discs[1]

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ABSTRACT

Alfalfa (*Medicago sativa* L., var. El Unico) leaf discs, floating on buffer containing NH_4Cl and photosynthesizing with $^{14}CO_2$, produced more labeled amino acid and less sucrose than did control discs (no added NH_4Cl). The level of pyruvate increased and that of phosphoenolpyruvate decreased. These and other changes in levels of labeled compounds led us to conclude that pyruvate kinase was activated by ammonia, resulting in increased transfer of photosynthetically incorporated carbon to synthesis of amino acid skeletons at the expense of sucrose synthesis. Carbon flow through enzymes catalyzing the anaplerotic reactions was apparently stimulated.

The over-all process of nitrate reduction and ammonia incorporation into amino acids in higher plant leaves (along with the role of light in that process) has been a subject of recent research (3, 10, 12). Given the production of amino acids as well as sucrose during photosynthesis (16), it is of interest to determine whether leaf regulatory mechanisms involve ammonia. The intracellular NH_4^+ level could influence leaf carbon metabolism during photosynthesis with respect to sucrose synthesis *versus* the amino acid synthesis necessary for protein production and leaf growth. Kinetic studies of ^{14}C-labeled compounds formed in the unicellular green alga *Chlorella pyrenoidosa* during photosynthesis with $^{14}CO_2$ have indicated that ammonia brings about increased amino acid synthesis in part due to stimulation of pyruvate kinase (8). We have now investigated whether a similar regulatory mechanism is active in the leaves of higher plants. The specific plant chosen for investigation was alfalfa because of its potential as a source of leaf protein for direct human consumption (5).

In our experiments we have used the techniques of kinetic tracer analysis of steady-state photosynthesis (2, 19). Labeled CO_2 fixation by alfalfa leaf discs in the presence and absence of NH_4Cl was examined. Analysis of the labeled products formed as a function of time indicates that ammonia can function as a regulatory agent in alfalfa leaves.

MATERIALS AND METHODS

Plant Material. Alfalfa (*Medicago sativa* L., var. El Unico) seeds were planted in 12 cm of vermiculite. The plants were grown at 3,000 ft-c and 15 C with a 9-hr light period and a 15-hr dark period. Plants were fertilized with modified Hoagland solution. Second through fourth unfolded leaves (numbered from the plant apex; not fully expanded) were excised after 1 hr of light from 6-week-old plants. The leaves were placed on a rubber sheet and 1-cm-diameter leaf discs were cut out with a cork borer.

Photosynthetic Carbon Fixation. The closed steady-state gas circulation system described previously was employed (19). Leaf discs were exposed to CO_2 in 4.5-cm-diameter flasks made from ground-glass joints and having transparent upper and lower surfaces. Each flask was fitted with gas inlet and outlet tubes, a serum stopper (allowing for addition of reagents during experiments), and five glass leaf disc holders (serving to prevent leaf overlap). Immediately after being cut, the leaf discs were floated on 4 ml of pH 7.4 buffer (0.05 M K_2HPO_4, pH adjusted with HCl) in the disc exposure flasks. Twelve flasks were utilized; each contained five discs comprising a single sample. The flasks were placed in a temperature-regulated shaking device (previously described for chloroplast experiments) (7) and attached to the closed gas circulation system through manifolds. Gas flow was commenced with 0.04% CO_2 in air (20% O_2); flask temperature was 27 C. After 12 min of preincubation in the dark, the lights were turned on and ammonium chloride in the pH 7.4 buffer was injected into six of the flasks to give a final concentration of 5 mM NH_4Cl. Photosynthesis with $^{12}CO_2$ was observed for 17 min. The unlabeled CO_2 in air was then replaced by 0.038% $^{14}CO_2$ (30.2 μCi/μmol) in air (20% O_2). Samples of control and ammonium-treated leaf discs were removed at the time intervals shown under "Results." As each sample was removed, the leaf discs were immediately frozen in liquid N_2.

Analysis of ^{14}C-labeled Products. Each sample was ground and successively extracted with 80% ethanol (v/v), 20% ethanol, and then water essentially as described previously (20). The extracts of each sample were combined. The residue from each sample was collected on filter paper, washed with 3 drops of formic acid, and dried in a vacuum desiccator over silica gel and KOH. The residue was then combusted (Packard automatic

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combustion apparatus) to give data on $^{14}CO_2$ fixation into insoluble materials. Sample extracts were analyzed by liquid scintillation for fixation into total soluble metabolites.

Fixation of $^{14}CO_2$ into most soluble metabolites was determined from chromatograms developed for 24 hr and also 48 hr in each direction using, with one modification, the solvent system of Pedersen *et al.* (17). The pH of the phenol-water-acetic acid-EDTA solvent used in the 24-hr development was adjusted to pH 4.2 (18). Glycolate, glycine, and serine were isolated as described earlier (18). Radioactive areas were located and major labeled metabolites identified as described earlier (17-19). Radioactivity in each compound was determined by elution with water, followed by liquid scintillation counting.

Samples were analyzed for α-ketoacids by first preparing and purifying the 2,4-dinitrophenylhydrazone derivatives (1). Unlabeled sodium pyruvate was added to aliquots of each sample prior to reaction with 2,4-dinitrophenylhydrazine. The mixture of hydrazones was analyzed on Whatman 1 paper; chromatograms were first developed for 24 hr with 1-butanol-ethanol-1 N NH_4OH (13:2:5) (11) and then in the second dimension for 19 hr with butanol-propionic acid-water (17). Labeled materials were located by autoradiography (17). The pyruvic and α-ketoglutaric acid derivatives (yellow in color) were identified by coincidence with added unlabeled carrier. The OAA[4] derivative was identified by its mobility in butanol-ethanol-1 N NH_4OH (11). The α-KG and OAA derivative spots were excised from the paper prior to development in the second dimension. The locations of the derivatives of hydroxypyruvate and glyoxylate were determined by chromatography of standards and were found to be separated well from the pyruvate, α-KG, and OAA derivative spots. Several of the derivatives gave double spots on the chromatograms as has been long noted (11) apparently due to formation of syn-anti isomers. Radioactivity was determined by automatic Geiger counter (17).

All fixation results were expressed on the basis of microgram atoms $^{14}C/dm^2$ of leaf area. The microgram atoms of ^{14}C were calculated by dividing the radioactivity of the product, in μCi, by the specific radioactivity of the entering $^{14}CO_2$.

RESULTS

The presence of ammonium chloride did not affect the total rate of leaflet photosynthesis (11.2 mg $CO_2/hr \cdot dm^2$), nor the rate of incorporation of ^{14}C into total soluble products, but it did bring about a decrease in sucrose labeling (Fig. 1). Decreased tracer incorporation into sucrose was accompanied by sharply increased labeling of glutamate and aspartate (Fig. 2). Glutamine labeling, which was extremely low in the absence of NH_4Cl, was quite high in its presence (Fig. 2). Labeling of other identified amino acids (alanine, glycine, serine) also increased (Fig. 3). Tricarboxylic acid cycle intermediates varied in their response to NH_4^+: citrate labeling increased while malate labeling was unchanged (Fig. 4). α-KG labeling decreased while OAA labeling was unchanged. Neither compound reached saturation with label (Fig. 5). The decline in α-KG labeling (Fig. 5) was substantially less than the increased labeling of glutamate and glutamine (Fig. 2).

Differences between the two experimental conditions in the levels of pyruvate and PEP were of particular interest given the location of these metabolites between the RPP cycle and probable pathways of carbon utilization, particularly those of amino acid synthesis. Pyruvate labeling increased, while the steady-state level of PEP decreased, when the discs were exposed to

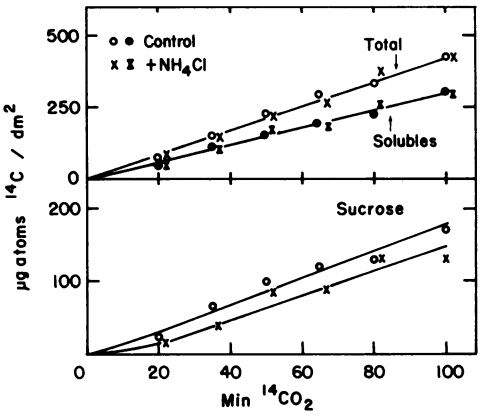

FIG. 1. Effect of NH_4^+ on total photosynthetic ^{14}C incorporation, ^{14}C incorporation into solubles, and ^{14}C incorporation into sucrose by alfalfa leaf discs exposed to 0.038% $^{14}CO_2$ (30.2 μCi/μmol) in air (20% O_2) at 2,400 ft-c. (○ ●): control; (× ⧗): 5 mM NH_4^+.

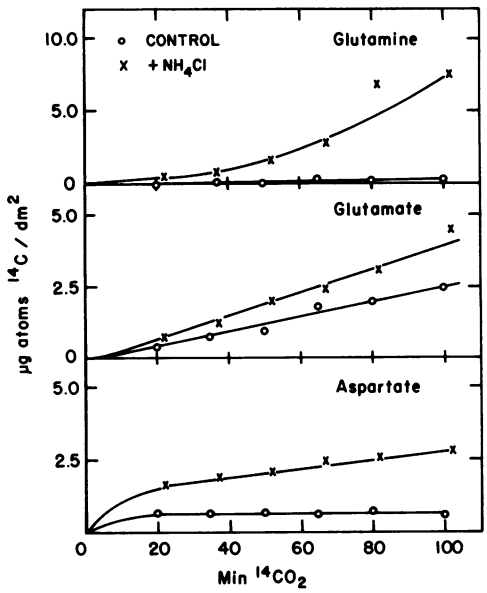

FIG. 2. Effect of NH_4^+ on the labeling of glutamine, glutamate, and aspartate in alfalfa leaf discs photosynthesizing with $^{14}CO_2$ under the conditions described in Figure 1. (○): control; (×): 5 mM NH_4^+.

NH_4Cl (Fig. 6). The ratio of pyruvate to PEP doubled (Fig. 6). The shape of the pyruvate-labeling curve, in which there is an initial rapid rise followed by a prolonged slower increase, may indicate two slowly equilibrating pools of pyruvate, only one of which is rapidly turning over.

In the presence of NH_4^+, active steady-state pool sizes of several metabolites including glycolate, phosphoglyceric acid, and the total sugar diphosphates (as measured by their ^{14}C content following label saturation) were unchanged from the control values, while the total steady-state pool size of the sugar monophosphates was slightly lower than the control value (data not shown). UDPG labeling increased in the presence of ammonium ion (Fig. 7).

DISCUSSION

It might be expected that greater intracellular ammonia would result in increased amino acid synthesis for a brief period of time, due to mass action. However, for increased amino acid synthesis to occur for an extended period without a comparable decrease in the level of α-ketoacids, it is necessary that an increase occur in the rate of flow of carbon through rate-limiting

[4] Abbreviations: α-KG: α-ketoglutarate; OAA: oxaloacetate; PEP: phosphoenolpyruvate; RPP: reductive pentose phosphate; UDPG: uridine diphosphoglucose.

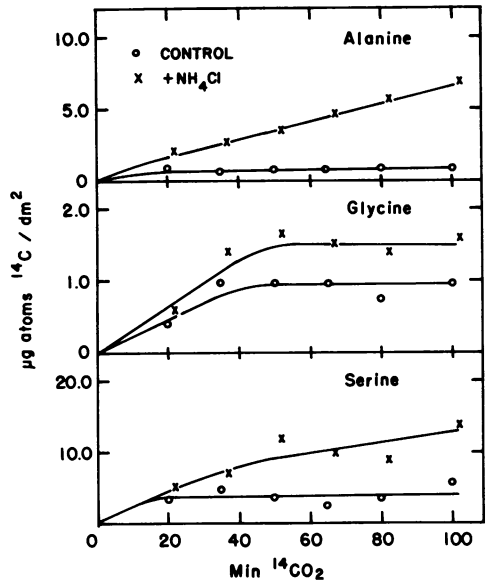

FIG. 3. Effect of NH_4^+ on the labeling of alanine, glycine, and serine in alfalfa leaf discs photosynthesizing with $^{14}CO_2$ under the conditions described in Figure 1. (O): control; (×): 5 mM NH_4^+.

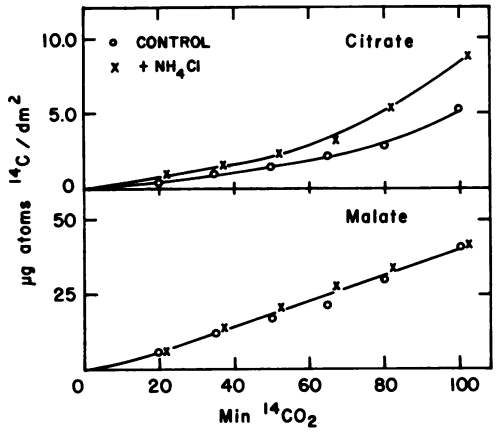

FIG. 4. Effect of NH_4^+ on the labeling of malate and citrate in alfalfa leaf discs photosynthesizing with $^{14}CO_2$ under the conditions described in Figure 1. (O): control; (×): 5 mM NH_4^+.

steps. Our data show that the latter occurred upon addition of ammonia to the photosynthesizing leaf discs.

Greatly increased alanine labeling (Fig. 3) in the presence of NH_4^+ required more utilization of pyruvate, the α-ketoacid from which it is presumably synthesized by transamination (6, 9). α-KG, the carbon skeleton for glutamate and glutamine, is made by anaplerotic reactions of the tricarboxylic acid cycle. Increased synthesis of α-KG requires both increased OAA from C_3 carboxylation, and acetyl-CoA from pyruvate oxidation. Increased synthesis of glutamate and glutamine (Fig. 2) in the absence of a comparable decline in α-KG (Fig. 5), as well as increased citrate synthesis (Fig. 4), also required more utilization of pyruvate. If greater labeling of alanine, glutamate, and glutamine was solely due to mass action effects of NH_4^+ the level of pyruvate would have decreased. In fact, pyruvate labeling increased even though the steady-state level of its precursor PEP was lower (Fig. 6). Our observation of increased pyruvate level and decreased PEP pool size in the presence of ammonia is therefore a clear indication of activation of pyruvate kinase.

Alternative pathways from PEP to pyruvate are conceivable: for example, through reactions mediated by PEP carboxylase, malic dehydrogenase, and malic enzyme. The malate-labeling curve (Fig. 4) was differently shaped from those of PEP and pyruvate (Fig. 6), and its rate of labeling was unchanged by the presence of NH_4^+. Malate thus seems unlikely as an intermediate between PEP and pyruvate. Activation of pyruvate kinase, an enzyme widely recognized to be of importance in metabolic regulation (4, 22), is the simplest conclusion consistent with our data.

Direct activation of pyruvate kinase in higher plants by ammonia is supported by work with the partially purified

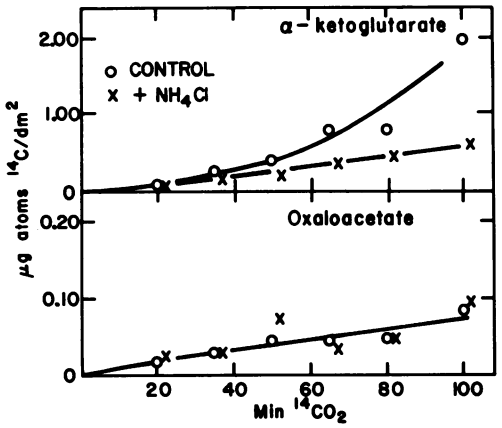

FIG. 5. Effect of NH_4^+ on the labeling of α-KG and OAA in alfalfa leaf discs photosynthesizing with $^{14}CO_2$ under the conditions described in Figure 1. (O): control; (×): 5 mM NH_4^+.

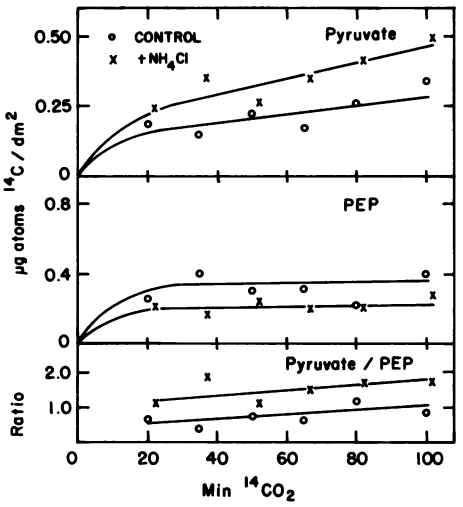

FIG. 6. Effect of NH_4^+ on the labeling of pyruvate, PEP, and the ratio of labeled pyruvate to PEP in alfalfa leaf discs photosynthesizing with $^{14}CO_2$ under the conditions described in Figure 1. (O): control; (×): 5 mM NH_4^+.

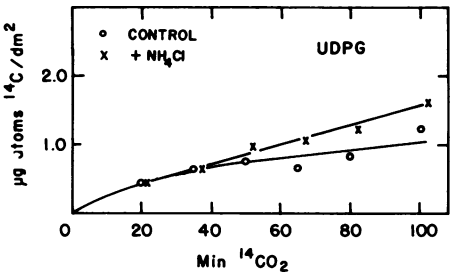

FIG. 7. Effect of NH_4^+ on the labeling of UDPG in alfalfa leaf discs photosynthesizing with $^{14}CO_2$ under the conditions described in Figure 1. (O): control; (×): 5 mM NH_4^+.

enzyme. NH_4^+ stimulates pea and cotton seed as well as carrot pyruvate kinase (13, 23). Indirect activation by ammonia with the direct effector being either glutamate or glutamine is unlikely as those two amino acids have been found not to affect the activity of the partially purified higher plant enzyme (4, 15). Furthermore, in *Chlorella* (where it was also concluded that pyruvate kinase was activated in the presence of ammonia) more immediate labeling changes occurred in pyruvate and PEP than in glutamate and glutamine when ammonia was added (8).

Increased labeling of glutamate, glutamine, aspartate (Fig. 2), and citrate (Fig. 4), in the presence of NH_4^+, suggests that OAA was formed and utilized at an increased rate and that a stimulation of anaplerotic carbon flow had occurred. OAA is presumably synthesized in the cytoplasm by PEP carboxylase (9) and perhaps also in the mitochondria through oxidation of malate produced by malic enzyme (8). Malic enzyme may have been simply responding to the increased level of its substrate pyruvate. It is possible that PEP carboxylase or malic enzyme was also activated by NH_4^+.

The absence of major change in alfalfa steady-state levels of RPP cycle intermediates, when the discs were supplied with NH_4Cl-containing buffer, indicates that reactions of the photosynthetic cycle were not affected by changes in the proportions of the compounds made later. Increased carbon withdrawal to yield amino acids (Figs. 2 and 3) was compensated for by the decreased rate of carbon withdrawal to form sucrose (Fig. 1).

Condensation of fructose-6-P and UDPG is possibly the major path of sucrose synthesis in leaves (24). The decrease in sucrose labeling (Fig. 1), given an increased level of UDPG (Fig. 7), suggests an inhibition by NH_4^+ of sucrose phosphate synthetase, the enzyme which catalyzes the rate-limiting step in sucrose synthesis. The same reaction was apparently inhibited by ammonia in algae (8). However, in alfalfa (in contrast to the situation in algae), the production of sucrose did not completely cease, but only declined when NH_4^+ was supplied. It seems that in higher plants a balance is maintained between amino acid synthesis and sucrose synthesis, and one process is not completely inhibited to provide for greater operation of the other. This satisfies the continuing need for sucrose export from leaves to nonphotosynthetic tissue in higher plants.

The observed increases in labeling of glycine and serine (Fig. 3) are of special interest. The carbon skeletons for those two amino acids are formed by different paths from those for alanine, aspartate, glutamate, and glutamine. We have concluded that in alfalfa serine is made from 3-P-glycerate, while glycine is made from glycolate (20). It is evident that ammonia in higher plants can act to increase amino acid production by many metabolic paths thereby providing for greater protein synthesis.

Our data, and the data on isolated higher plant pyruvate kinase, support a regulatory role for ammonia in leaf amino acid synthesis. However, while nitrate reduction occurs in the cytoplasm, nitrite reduction and concomitant ammonia formation and incorporation in leaves apparently occur in the chloroplast (10, 12, 14). Regulation by means of ammonia requires an interaction with pyruvate kinase and possibly other enzymes producing amino acid carbon skeletons. Those enzymes are apparently located mainly outside the chloroplast (6, 9, 14). For ammonia produced within that organelle, as well as ammonia of extrachloroplastic origin, to regulate amino acid synthesis requires a relationship between cytoplasmic and chloroplastic NH_4^+ levels. Since ammonia apparently penetrates the chloroplast membrane to some extent (14) such a relationship is not unexpected. Furthermore, in additional experiments similar to that we now describe (but conducted at low O_2 pressure) we have found that exogenously supplied NO_3^-, as well as NH_4^+, resulted in activation of pyruvate kinase and increased amino acid formation during photosynthesis with $^{14}CO_2$ in alfalfa leaf discs (21). That activation is most simply interpreted as being the result of an increase in intracellular ammonia level resulting from nitrate reduction followed by nitrite reduction.

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