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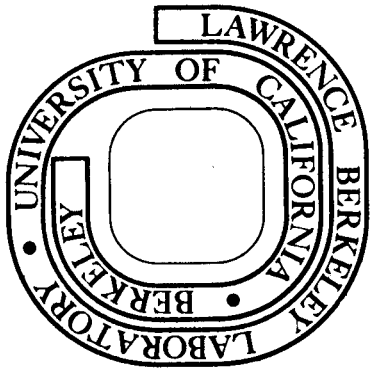

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Title: Ammonia Regulation of Carbon Metabolism in Photosynthesizing Leaf Discs[1]

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4. Abbreviations: α-KG: α-ketoglutarate; OAA: oxaloacetate; PEPA: phosphoenolpyruvate; RPP: reductive pentose phosphate; TCA: tricarboxylic acid; UDPG: uridine diphosphoglucose.

ABSTRACT

Alfalfa (Medicago sativa L., var. El Unico) leaf discs, floating on buffer containing NH_4Cl and photosynthesizing with $^{14}CO_2$, produced substantially 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. We 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 anapluerotic reactions was apparently stimulated. The probable physiological significance of the ammonia effect is discussed.

Nitrate reduction and the initial incorporation of ammonia in higher plant leaves (along with the role of light in those processes) have been subjects of appreciable recent research (5,6,13,14,20). Given the requirement for incorporated ammonia in protein production, it is of interest to determine whether regulatory mechanisms in leaves controlling the flow of carbon to end products involve that substance. The production of amino acids and protein during photosynthesis, the production of protein by chloroplasts, and the incorporation of ammonia during photosynthesis are well established (3,4,6,19,22). The intracellular NH_4^+ level could control leaf photosynthetic carbon metabolism 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 $^{14}CO_2$ has indicated that ammonia brings about increased amino acid synthesis in part due to stimulation of pyruvate kinase (11). We have now investigated whether a similar regulatory mechanism is active in the leaves of higher plants. The plant chosen for investigation was alfalfa because of its potential as a source of leaf protein for direct human consumption (8).

In our experiments we have used the techniques of kinetic tracer analysis of steady state photosynthesis (2,25). Labeled carbon dioxide fixation by alfalfa leaf discs in the presence and absence of NH_4Cl was examined. Analysis of the labeled products formed in the discs as a function of time indicates that ammonia is 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 3000 ft-c and 15 C with a 9-hr light period and a 15-hr dark period. Plants were fertilized with modified Hoagland's solution. Second through fourth unfolded leaves were excised after 1 hr of light (with their petioles under water) from six-week old plants. The leaves were placed on a rubber sheet and 1 cm diameter leaf discs were cut out with a cork borer.

Steady State Gas Circulation System and Leaf Disc Exposure Flasks. The closed gas circulation system described previously was employed (25). The levels of $^{12}CO_2$, $^{14}CO_2$, and oxygen in the circulating gas stream were continuously recorded. A small diaphragm pump was used to recirculate the gas. Leaf discs were exposed to carbon dioxide in twelve 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. For experimental purposes a disc placed in each holder was floated on buffer solution (see below). The flasks were mounted in the temperature controlled shaking device described for chloroplast experiments (10) and were attached to the steady state gas circulation system through a manifold. The flasks were illuminated (2400 ft-c) through the transparent bottom of the shaker device water bath.

Photosynthetic Carbon Dioxide Fixation. Immediately after being cut, the leaf discs were floated on 4 ml of 0.05 M phosphate at pH 7.4 and 0 C in the disc exposure flasks. Twelve flasks were utilized; each contained five discs comprising a single sample. The flasks were transferred onto the shaking device (temperature regulated at 27 C $\pm$ 1 C) and attached to

the gas circulation system. Gas flow was commenced with 0.04% CO_2 in air (20% O_2). 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 flasks to give a final concentration of 0.005 M NH_4Cl. Photosynthesis with $^{12}CO_2$ was observed for 17 min. At that time the unlabeled carbon dioxide in air was replaced by 0.038% $^{14}CO_2$ (specific radioactivity 30.2 μCi/μmole) in air (20% O_2). Samples of control and ammonium-treated leaf discs were removed at the time intervals shown in the RESULTS. As each sample was removed, the leaf discs composing it were immediately frozen in liquid nitrogen.

Analysis of ^{14}C-Labeled Products. Each sample was ground at liquid nitrogen temperature in a tissue grinder, and then with 80% ethanol (v/v) in a dry ice-acetone bath. The leaflet powder was successively extracted with 6 ml of 80% ethanol (dark, 1.5 hr, 20 C), 2 ml of 20% ethanol (1 hr, 20 C), and then with 2 ml water (0.5 hr, 70 C). The extracts of each sample were combined. The pellet from each sample was collected on filter paper, washed with 3 drops of formic acid and dried in a vacuum dessicator over silica gel and KOH. The pellets were then combusted (Packard Automatic 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 individual soluble metabolites was analyzed by two-dimensional paper chromatography. Several chromatograms were developed for each sample. Most metabolites were 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. (23). The pH of the phenol-water-acetic acid-EDTA solvent used in the 24 hr development was adjusted to pH 4.2 (24). Quantitative glycolate isolation and glycine and serine isolation

were accomplished on two sets of chromatograms (24). Radioactive areas were located and major labeled metabolites identified as described earlier (23-25). 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 essentially as described by Bachelard (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 using a recently devised solvent system for two-dimensional paper chromatography. Chromatograms were first developed for 24 hr with n-butanol-ethanol-1N NH_4OH (13:2:5) (15) and then in the second dimension for 19 hr with butanol-propionic acid-water (23). Labeled materials were located by radioautography (23). The pyruvic and α-ketoglutaric acid derivatives (yellow in color) were identified by coincidence with added unlabeled carrier. The oxaloacetic acid derivative was identified by its mobility in butanol-ethanol-1 N NH_4OH (15). The α-KG[4] 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 well separated from the pyruvate, α-KG, and OAA derivative spots. Several of the derivatives gave double spots on the chromatograms as previously noted (15,16). Radioactivity was determined by automatic Geiger counter (23).

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·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 substantial in its presence (Fig.2). Labeling of other identified amino acids (alanine, glycine, serine) also increased (Fig. 3). Tricarboxylic acid intermediates varied in their response to NH_4^+: Citrate labeling increased while malate labeling was unchanged (Fig. 4). Alpha-ketoglutarate labeling decreased while oxaloacetate labeling was unchanged (data not shown). These changes result from interactions between several paths of carbon flow into and out of the TCA cycle (see DISCUSSION).

Differences between the two experimental conditions in the levels of pyruvate and phosphoenolpyruvate 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 PEPA decreased when the discs were exposed to NH_4Cl (Fig. 5). The ratio of pyruvate to phosphenol-pyruvate doubled (Fig. 5).

In the presence of NH_4^+, steady-state pool sizes of several metabolites including glycolate, glycerate, phosphoglyceric acid, and the total sugar diphosphates 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. 6).

DISCUSSION

The data indicate that ammonia acted as a regulatory agent in the photosynthesizing leaf discs. The large increases in amino acid labeling (Figs.2 and 3) are evidence for increased flow of recently fixed carbon from the RPP cycle to production of amino acid carbon skeletons. One site of regulation is concluded to be the pyruvate kinase-mediated reaction. Our observation of increased pyruvate level and decreased PEPA pool size in the presence of ammonia (Fig. 5) is evidence for activation of that enzyme. Greater carbon flow to pyruvate and the increased pool size of that metabolite provided for the observed increase in alanine labeling (presumably formed from pyruvate by transamination (9,11,12)). The carbon skeleton for glutamate (α-KG) presumably arises from the operation of several TCA cycle reactions (18). Increased labeling of citrate (Fig. 4), glutamate and glutamine (Fig. 2) required a greater rate of acetyl CoA synthesis (as well as increased C-4 acid synthesis, see below). An increase in the rate of acetyl CoA synthesis in the presence of a lower steady state pool size of PEPA is further evidence for pyruvate kinase activation. Decreased α-KG labeling in the presence of buffer containing NH_4^+ was apparently due to its increased withdrawal for use in glutamate and glutamine synthesis exceeding the greater influx of carbon into the α-KG pool. The increased formation of glutamine suggests that newly fixed carbon was used to increase the size of the glutamate pool used in incorporating ammonia into glutamine (13), as well as that put to other metabolic uses.

Activation of pyruvate kinase in higher plants by ammonia is supported by work with the partially purified enzyme. NH_4^+ stimulates pea- and cotton-seed (17,30) as well as carrot (30) pyruvate kinase. Activation in alfalfa is also consistent with the earlier conclusion drawn from Chlorella pyrenoidosa photosynthesis data (11). It could be argued that pyruvate kinase was only

indirectly activated by ammonia; the direct effector being either glutamate or glutamine. We do not believe this to be the case. Glutamate and glutamine have been found to not affect the activity of partially purified higher plant pyruvate kinase (7,21). Furthermore, in Chlorella (where it was also concluded that NH_4^+ activated pyruvate kinase) more immediate labeling changes occurred in pyruvate and PEPA than in the two amino acids when ammonia was added (11). It can be noted that pyruvate kinase appears to be a generally important regulatory enzyme (28).

Increased labeling of glutamate, glutamine, aspartate (Fig. 2), and citrate (Fig. 4), in the presence of NH_4^+ suggests that oxaloacetate was formed at an increased rate and that a stimulation of anapluerotic carbon flow had occurred. Oxaloacetate is presumably synthesized in the cytoplasm by phosphoenolpyruvate carboxylase (12) and perhaps also in the mitochondria through oxidation of malate produced by malic enzyme (11). Malic enzyme or phosphoenolpyruvate carboxylase may have been activated by NH_4^+. It is also possible that malic enzyme was simply responding to the increased level of its substrate pyruvate. The absence of an increase in the empirically determined level of OAA was due to the increased carbon skeleton withdrawal for amino acid production made possible by exogenous ammonia.

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 the photosynthetic cycle was well regulated. 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 (32). The decrease in sucrose labeling (Fig. 1), given an increased level of UDPG (Fig. 6), suggests an inhibition by NH_4^+ of sucrose phosphate synthetase the enzyme which catalyzes that reaction.

The same reaction was apparently inhibited by ammonia in algae (11). 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. Hence, it appears 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 particular interest as the carbon skeletons for those two amino acids are formed by different paths from those for aspartate and glutamate. Glycine and serine production in higher plants is generally thought of as proceeding from glycolate formation (29), but evidence also exists for serine production more directly from PGA under certain conditions in some species including alfalfa (26,29,31). It is therefore evident that ammonia in higher plants can act to increase amino acid production by many metabolic paths. Regulation by ammonia is therefore well designed to provide for greater protein synthesis.

Our data, and the data on isolated higher plant pyruvate kinase, suggests an actual physiological regulatory role for ammonia in amino acid synthesis. However, while nitrate reduction occurs in the cytoplasm, nitrite reduction and concommitant ammonia formation and incorporation in leaves apparently occurs in the chloroplast (13,14,18). Regulation by means of ammonia requires an interaction with pyruvate kinase and possibly other enzymes producing amino acid carbon skeletons, enzymes which are apparently located mainly outside the chloroplast (9,12,18). 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 (18) 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 (27). 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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LEGENDS FOR FIGURES

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/ μmole) in air (20% O_2) at 2400 ft-c. o, control; x, 0.005 M 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 Fig. 1. o, control; x, 0.005 M NH_4^+.

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 Fig. 1. o, control; x, 0.005 M 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 Fig. 1. o, control; x, 0.005 M NH_4^+.

Fig. 5. Effect of NH_4^+ on the labeling of pyruvate, PEPA, and the ratio of labeled pyruvate to PEPA in alfalfa leaf discs photosynthesizing with $^{14}CO_2$ under the conditions described in Fig. 1. o, control; x, 0.005 M NH_4^+.

Fig. 6. Effect of NH_4^+ on the labeling of UDPG in alfalfa leaf discs photosynthesizing with $^{14}CO_2$ under the conditions described in Fig. 1. o, control; x, 0.005 M NH_4^+.

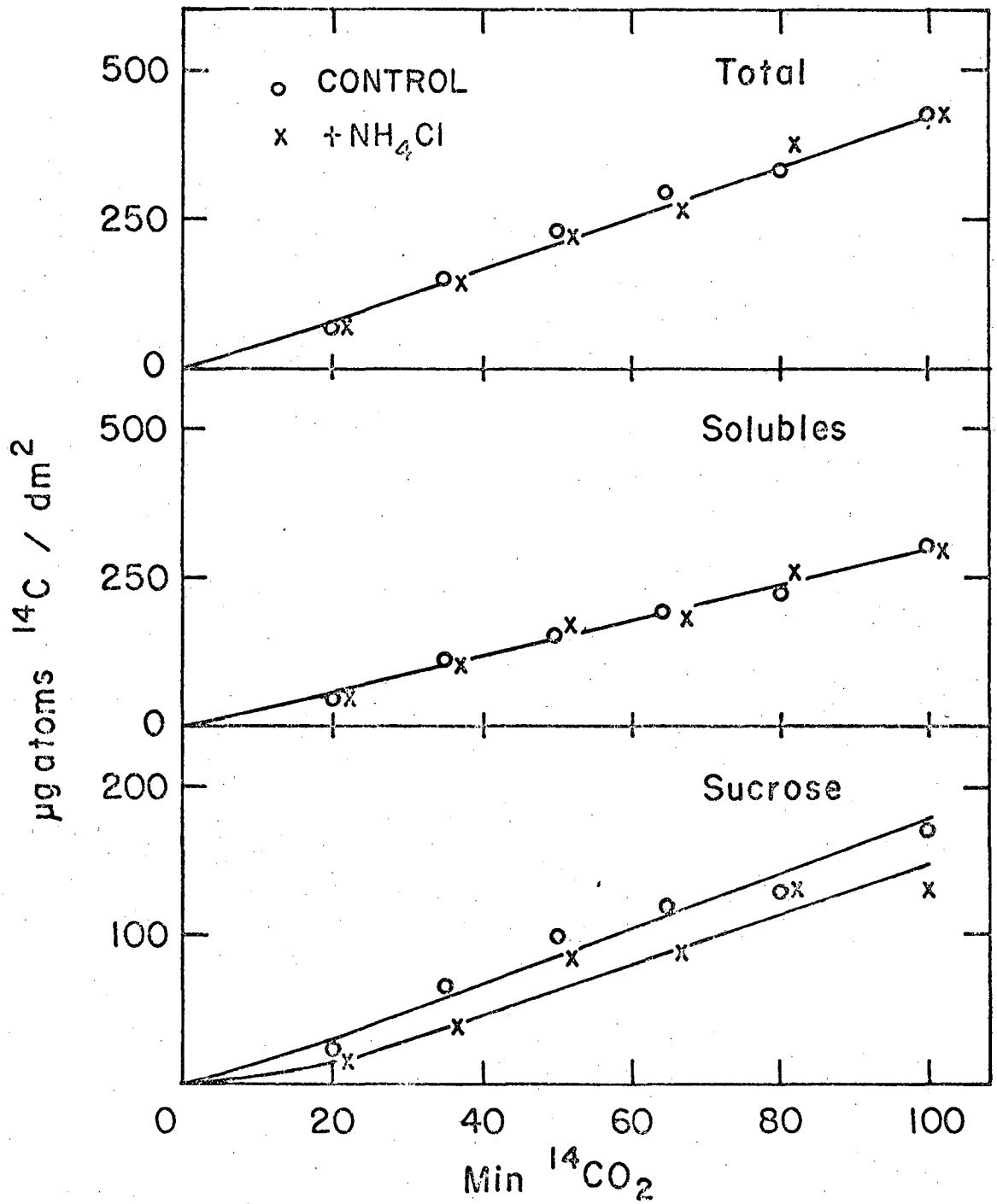
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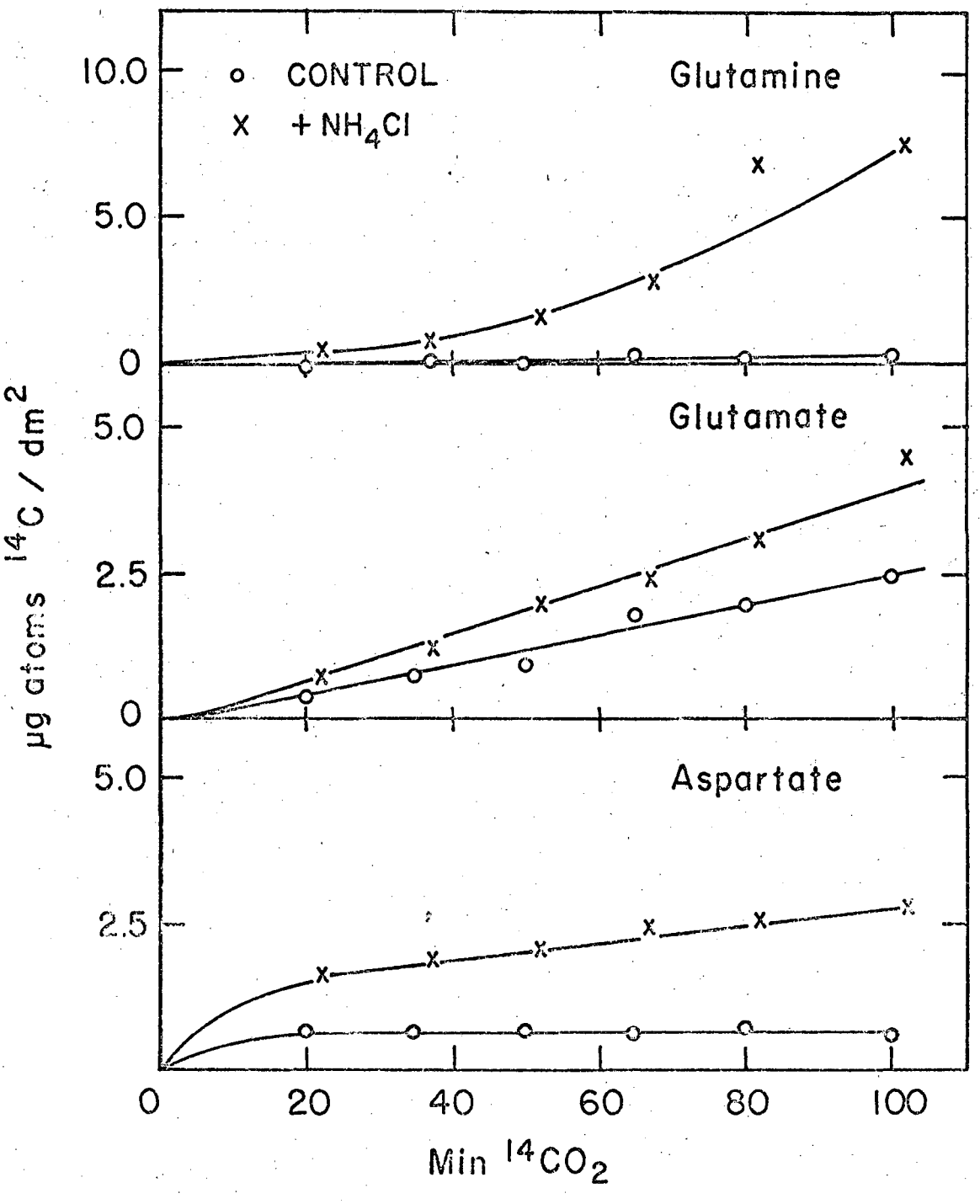
Figure 1



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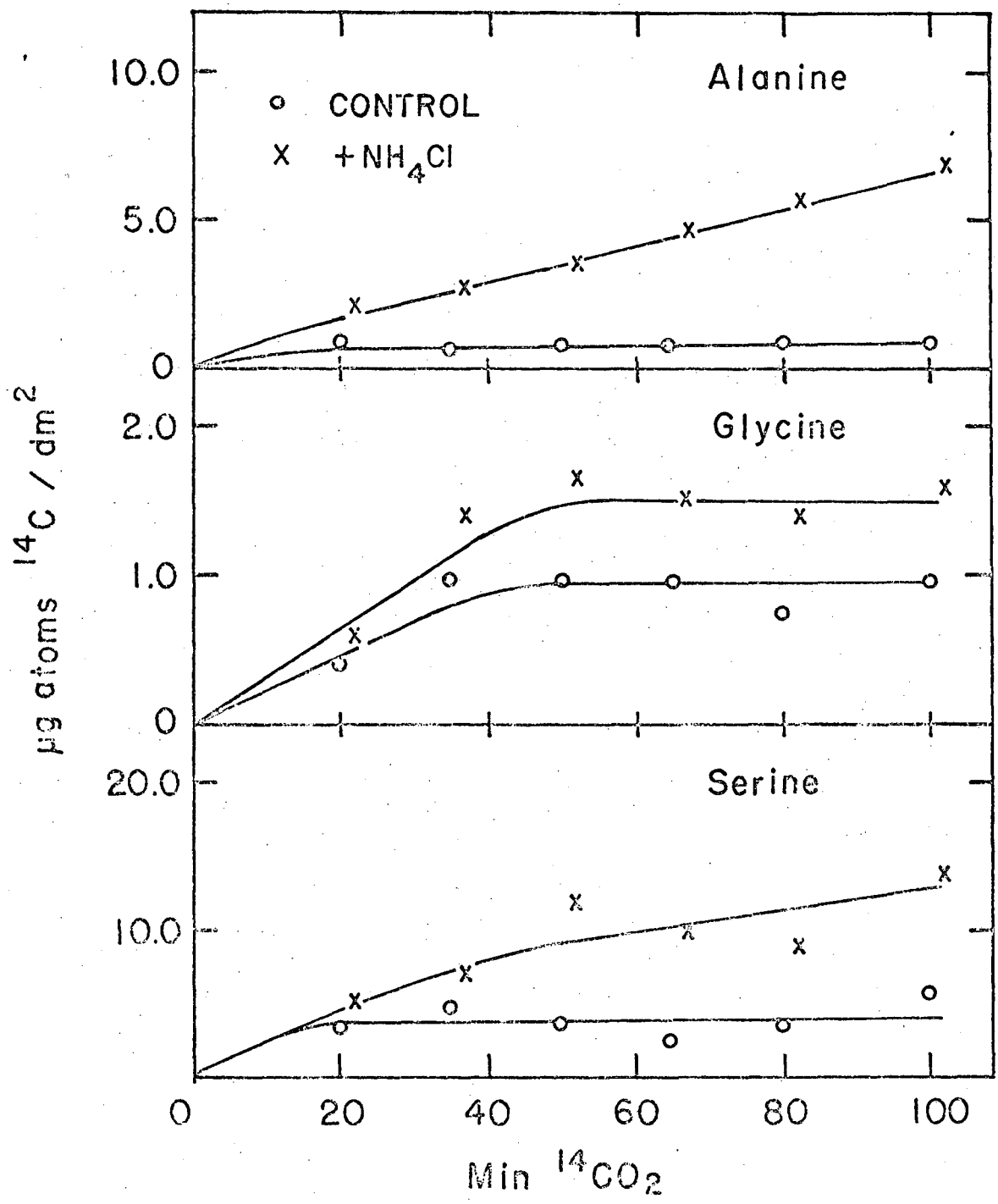

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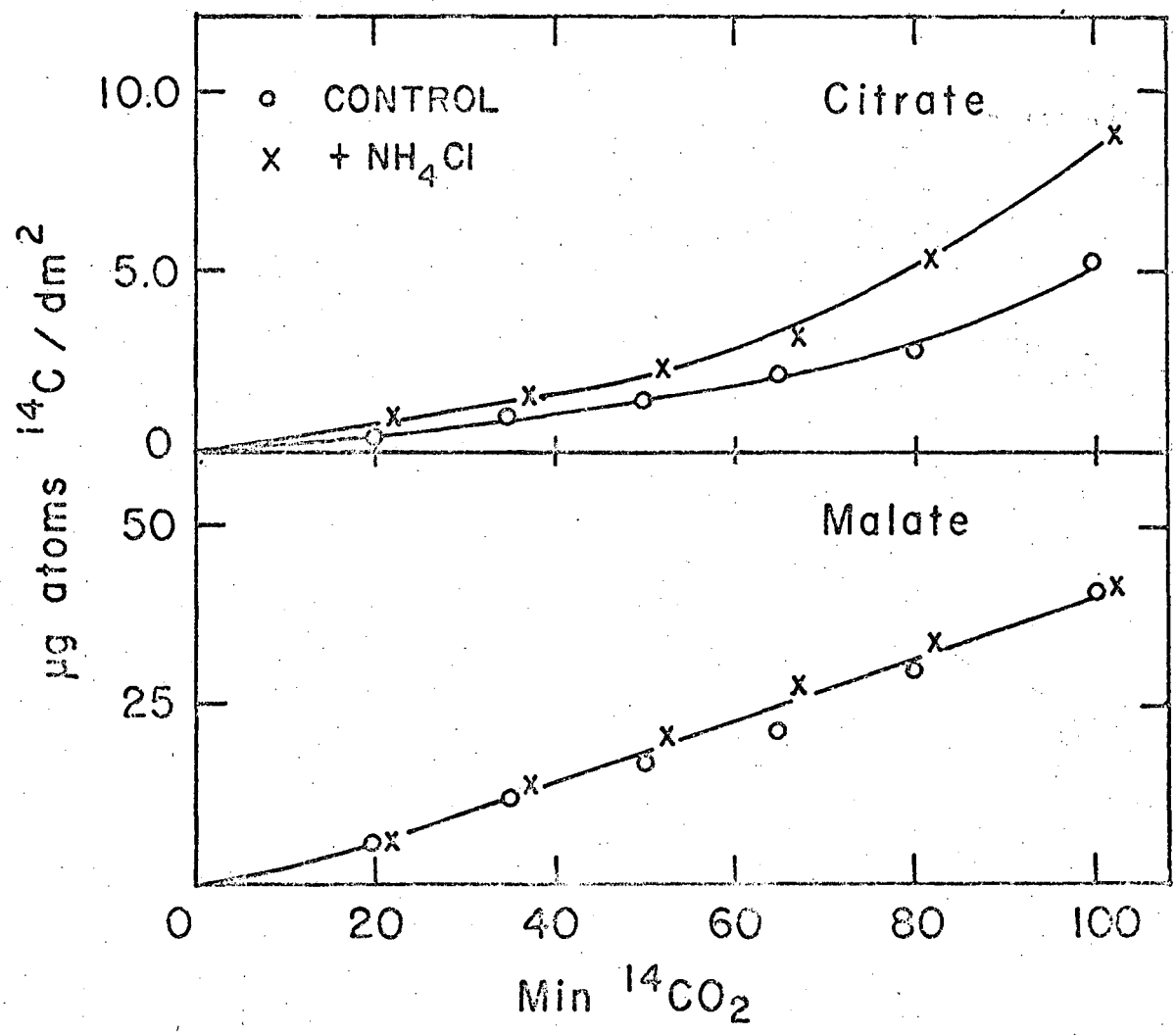

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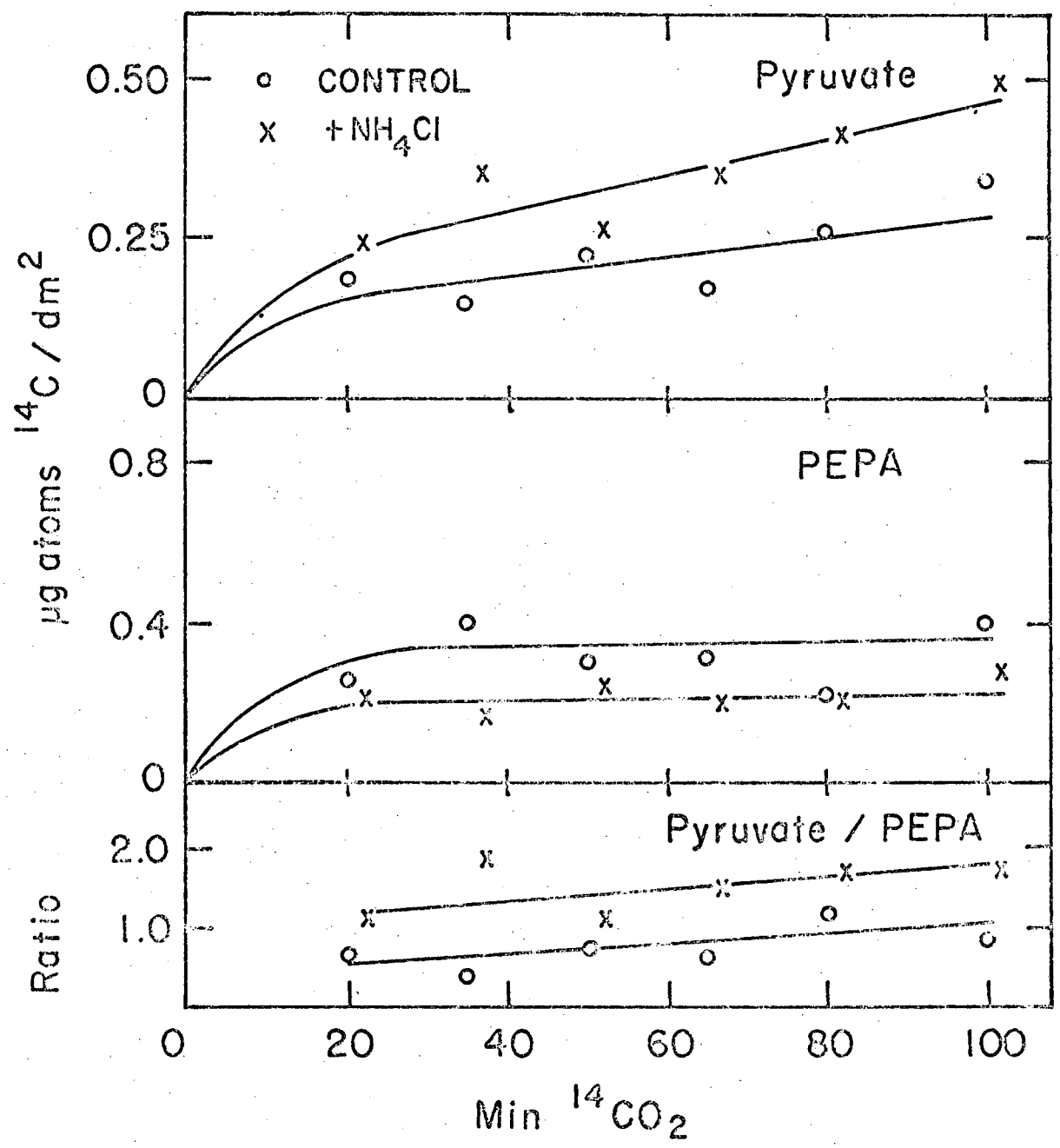

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Figure 5

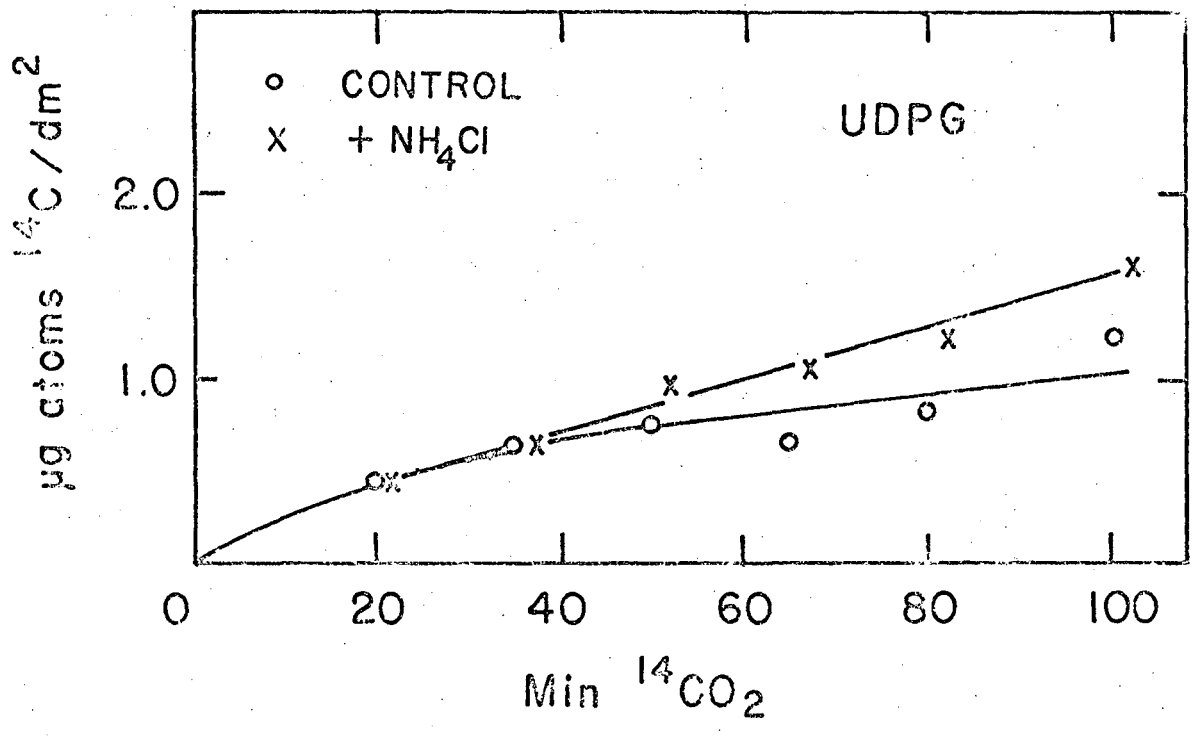

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Figure 6

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