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AMINO ACID SYNTHESIS IN PHOTOSYNTHESIZING SPINACH CELLS. EFFECTS OF AMMONIA ON POOL SIZES AND RATES OF LABELING FROM $^{14}CO_2$

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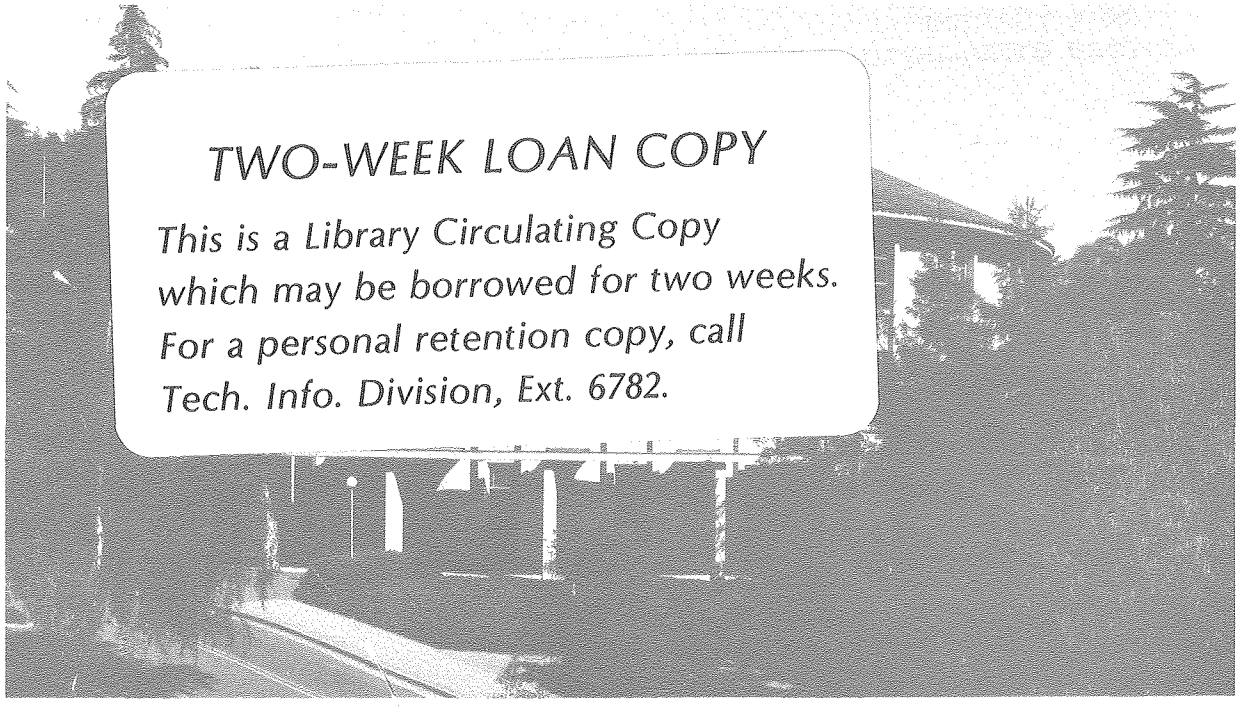

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DISCLAIMER

AMINO ACID SYNTHESIS IN PHOTOSYNTHESIZING SPINACH CELLS. EFFECTS OF AMMONIA ON POOL SIZES AND RATES OF LABELING FROM $^{14}CO_2$ [1]

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Footnotes

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3. Abbreviations: Mops (3-(N-morpholino)ethanesulfonic acid)

ABSTRACT

Isolated cells from leaves of Spinacea oleracea have been maintained in a state capable of high rates of photosynthetic CO_2 fixation for more than 60 h. The incorporation of $^{14}CO_2$ under saturating CO_2 conditions into carbohydrates, carboxylic acids, and amino acids, and the effect of ammonia on this incorporation have been studied. Total incorporation, specific radioactivity and pool size have been determined as a function of time for most of the protein amino acids and for γ-aminobutyric acid. The measurements of specific activities and of the approaches to ^{14}C "saturation" of some amino acids indicate the presence and relative sizes of metabolically active and passive pools of these amino acids.

Added ammonia decreased carbon fixation into carbohydrates and increased fixation into carboxylic acids and amino acids. Different amino acids were, however, affected in different and highly specific ways. Ammonia caused large stimulatory effects in incorporation of ^{14}C into glutamine (a factor of 16). No effect or slight decreases were seen in glycine, serine, phenylalanine, and tyrosine labeling. In the case of glutamate, ^{14}C-labeling decreased, but specific activity increased. The production of labeled γ-aminobutyric acid was virtually stopped by ammonia.

The results indicate that added ammonia stimulates the reactions mediated by pyruvate kinase and phosphoenolpyruvate carboxylase, as seen with other plant systems. The data on the effects of added ammonia on total labeling, pool sizes, and specific activities of several amino acids provides a number of indications about the intracellular sites of principal synthesis from carbon skeletons of these amino acids and the selective nature of effects of increased intracellular ammonia concentration on such synthesis.

INTRODUCTION

In recent years, the production of photosynthetically active cells from leaf tissue has been reported for a number of plant species, including Papaver somniferum (opium poppy)(21), spinach (29), and cotton (27). Such cells afford possibilities for studying plant metabolism in a simple but intact system. Effects on poppy cell metabolism of ammonia (23), sulfite (22), and 2,4-D (24) have been reported from this laboratory, and others have described effects of ammonia and other nitrogen compounds on metabolism in isolated spinach cells (29,30).

The present paper reports on the effects of ammonia added to photosynthetically active cells from spinach (Spinacea oleracea L.) leaves. Studies with spinach cells can be compared with results obtained with the widely used spinach chloroplasts. Other isolation procedures for spinach cells are available in the literature but the cells have not been reported to retain their activity over long periods of time (3,12,20,29).

This paper describes the photosynthetic [^{14}C]bicarbonate fixation by the spinach cells into major metabolites. In particular, incorporation into amino acids has been studied to provide information about amino acid biosynthesis and the provision of carbon skeletons for this purpose. For the amino acids occurring in the free state, values have been obtained for total ^{14}C-labeling, specific radioactivities, and, by combination of these data, pool sizes. The methods used have permitted some measurements for all the protein amino acids except cysteine/cystine and methionine, and thus provide a more detailed and complete picture of amino acid formation from CO_2 in leaf cells than previously available.

Previous work from this and other laboratories has demonstrated that ammonia influences photosynthetic rates and the distribution of fixed $^{14}CO_2$

between major photosynthetic products in algae (13,14), alfalfa leaf discs (26), poppy cells (23), cotton cells (27), and spinach cells (29,30). The detailed analysis of labeling and pool sizes of individual amino acids in photosynthesizing spinach cells provides more insight into the mechanism of the ammonia effect and its influence on amino acid biosynthesis.

MATERIALS AND METHODS

Plant Material. Spinach (Spinacea oleracea L., Burpee Hybrid #7) was grown in a growth chamber with 16 hour dark and 8 hour light periods. The light intensity was 5 x 10^4 lux or 700 $\mu E \cdot m^{-2} \cdot sec^{-1}$ and temperature was maintained at 17°C. The plants were grown in vermiculite and watered with Hoagland's solution weekly.

Cell isolation. Leaves were selected from plants six to eight weeks old. Four leaves weighing approximately one gram each were washed in distilled water and cut into 1 mm squares with a razor. The midrib was discarded. Leaf pieces were placed in a 125 ml sidearm flask in 50 ml of macerating medium (Table I), vacuum-infiltrated two times for one minute each, and placed on a rotary shaker at 120 rpm in the dark at room temperature for 30 min. At this time the macerating medium, containing chloroplasts and broken cells, was discarded and 50 ml of fresh macerating medium was added. The flasks were again placed on the shaker and incubated for 60 min.

The leaf pieces were then collected on cheesecloth and the macerating medium was discarded. The cheesecloth with the leaf pieces was gathered into a small bag, placed in a small Petri dish, and 5 ml of wash medium (Table I) was added. The cells were isolated by gently rubbing the cheese cloth against the leaf pieces. The green medium was then filtered through

150 μm mesh. Then, 5 ml of wash medium was added to the leaf pieces and the procedure was repeated until 30 to 40 ml of cell suspension was collected. The cells were washed by centrifugation (1000 rpm for 1.5 min) twice with wash media and resuspended in assay medium (Table I) with a resulting chlorophyll concentration of 30-40 μg chl/ml. The cells were observed under the microscope at this time. Chlorophyll was measured according to the method of Arnon (4).

Long term storage. Seven ml of cell suspension was placed in a rectangular plastic flask with 25 cm^2 of growth area (Falcon 3013, Falcon Plastics, Oxnard, CA). Such flasks are specially treated by the supplier to have a charge so that the cells do not break on contact with the plastic. Furthermore, storage in these flasks provides for more surface area for gas exchange. The cells were stored at 4 C in the dark for the first 21 h after isolation. Thereafter, they are kept 8 h at 25 C in the light (700 $\mu E \cdot m^{-2} \cdot sec^{-1}$) on a rotary shaker at 50 rpm, alternating with 16 h at 4 C in the dark.

Total photosynthetic $H^{14}CO_3$-incorporation. One ml of cell suspension was placed in each serum-stoppered 5 ml microfernbac flask. The flasks were placed on a rotary shaker and shaken at 50 rpm in a plexiglass water bath maintained at 22 C. The bath was illuminated from below by fluorescent lamps with a light intensity of 1850 $E \cdot m^{-2} \cdot sec^{-1}$. The assay was initiated by the addition of $H^{14}CO_3^-$ at a specific activity of 12.5 mCi/mM and a final concentration of 8 mM. Samples were removed at 15 and 30 min and killed by addition to methanol (80% final concentration). Total $^{14}CO_2$ incorporation was measured by removing aliquots of the methanol samples, acidifying to remove $H^{14}CO_3^-$, and counting by liquid scintillation.

Experiments for analysis of photosynthetic carbon-flow and for investigation of the effect of ammonia. The cell isolation was performed as above with minor modifications. The cells were stored without KNO_3 and the macerating and wash media were prepared without KCl, $MgSO_4$, KH_2PO_4, KNO_3, and $CuSO_4$. Also instead of the Hepes buffer used in the storage and assay systems, a Mops[3] buffer system of the same total concentration was used. This change was necessary because the ampholytic properties of Hepes caused the buffer to elute with the amino acid fraction during cation exchange fractionation (see below). These large quantities of Hepes impaired the subsequent chromatographic and analytic steps. The change of buffer had no effect on total photosynthetic rates.

The cells used for the experiments were stored for 22 h in the dark followed by 5 h in the light before incubation Temperatures and light intensity were maintained as before. The incubation with $H^{14}CO_3^-$ was performed as above (again with Mops buffer) but in a total volume of 1.5 ml. Six parallel experiments were carried out, two including 5 mM KNO_3 in the medium, two including 0.5 mM $(NH_4)_2SO_4$, and two with no N-source added. 200 µl samples were removed after 15,30 and 60 min incubation, and 600 µl after 100 min. All the removed samples were killed in four volumes of methanol and total photosynthetic rate was determined as described above. The 15, 30, and 60 min samples were further analyzed as described below. The specific activity of the $^{14}CO_3^-$ used was 49 mCi/mM.

Fractionation and analysis of samples. The samples were centrifuged and the residue washed with 1 ml of 80% ethanol, 1 ml of 20% ethanol, and 1 ml of water, centrifuging between washes. The combined supernatants were extracted with 2 x 5 ml of ether and the aqueous phase was concentrated to about half the initial volume by use of a stream of dry nitrogen. This

volume reduction was performed to remove most ethanol and methanol, thus avoiding losses of glutamic acid in the subsequent ion-exchange fractionation (15). The concentrated solution was applied to a column of cation-exchange resin (Biorad 50 x 8, 200-400 mesh, 0.4 x 3 cm, Richmond, CA). The column was flushed with 4 x 0.5 ml of water to give an efflu containing neutral compounds (carbohydrates), carboxylic acids and phosphate esters. The column was then washed with 6 x 1 ml of water and eluted with 4 x 1 ml 1 M aqueous pyridine to give the fraction containing acidic and neutral amino acids, and was finally eluted with 4 x 1 ml 3 M aqueous ammonia to give the fraction containing basic amino acids (and amines).

The radioactivity in the different fractions was measured by liquid scintillation counting. Part of the effluent was subjected to two-dimensional paper chromatography in the standard system previously described (25). After location by radioautography, the labelled areas of paper were cut into small pieces and shaken for 1 h with 2.5 ml of water in scintillation counting after the addition of 15 ml of water in scintillation vials. The radioactivity was then determined by liquid scintillation counting after the addition of 15 ml of scintillation solution. Values of ^{14}C labeling were obtained for hexosemonophosphates, 3-phosphoglycerate, phosphoenolpyruvate, citrate, malate, glycerate, sucrose, maltose, glucose and fructose.

The amino acid fractions were concentrated to dryness in a stream of nitrogen. The residues were dissolved in water and an aliquot (5%/for the of the total acid and neutral amino acids, 50% for the basic amino acids) was subjected to two-dimensional paper chromatography on Whatman No. 1 paper: 1. solvent: butanol-acetic acid-water (12:3:5 v/v), 2. solvent: butanol-methylethyl-

ketone-NH_4OH-water (5:3:1:1 v/v) (compare (28)). The development times used for the neutral and acidic amino acids were 20 and 28 h, for the basic amino acids, 40 and 48 h, respectively. Carrier amounts (about 20 µg of each) of the neutral and acidic protein amino acids plus γ-aminobutyric acid but not aspartic acid, tyrosine (because of solubility problems), methionine, nor cysteine were added to paper chromatogram origins to facilitate the subsequent location and identification (see below). Carrier amounts of arginine, histidine, and lysine were added to the chromatograms for basic amino acids.

The solvent system used accomplishes complete separation of aspartic acid, glutamic acid, asparagine, glutamine, glycine, serine, threonine, alanine, proline, tyrosine, phenylalanine, tryptophan, γ-aminobutyric acid, isoleucine, and leucine. Valine and methionine are partially overlapping, but most or all the methionine present was oxidized to methionine sulfoxide prior to the paper chromatography. Methionine sulfoxide appears close to glycine. Cysteine and cystine appear very near the point of origin but were not included in the analysis. The three basic amino acids were completely separated by the chromatographic system.

After autoradiography the chromatograms were sprayed with o-phthalaldehyde to produce UV-fluorescent spots for the amino acids added as carriers (except proline)(7). The spots were cut out and radioactivity determined as described above.

In the experiments with ammonia added it could be observed that limited degradation of glutamine to give 5-oxoproline (pyroglutamic acid) took place during development with the first solvent system. The 5-oxoproline found contained less than 2.5% of the radioactivity found in glutamine (compare Kasai and Larsen, 1979 (15)).

Determination of specific radioactivities of the amino acids. These determinations were performed as previously described by Airharta et al. (2) with ^{3}H-labeled dansyl chloride (5-dimethylamino-1-napthhalenesulfonyl chloride, Amersham). The specific radioactivity of the dansyl chloride used was determined by reaction with standard samples of ^{14}C-leucine and ^{14}C-proline. Half of the amino acid fraction from each sample in 20 µl of $NaHCO_3$-buffer at a/ pH of about 8.5 was mixed with 20 µl of 2 mM dansyl chloride in acetone and reacted at 37 C for 1.5 h. The resulting dansyl derivatives of amino acids were purified on a column (0.4 x 2 cm) of the neutral polystyrene resin Porapak Q (obtained from Alltech Associates, Arlington Heights, IL)(16). The Porapak material was swelled overnight in ethanol (19) and after the columns were poured, they were flushed with 0.1 N HCl / after having been poured. The dansylation reaction mixture with 0.4 ml 0.1 N HCl added was applied to the column, / which was then washed with 3 x 0.5 ml N HCl. Subsequently, most of the dansyl hydroxide formed was eluted with 5 x 1 ml 5% v/v aqueous acetic acid and the dansyl derivatives were eluted with 3 x 1 ml 80% v/v aqueous acetone. The acetone eluate was taken to dryness in a stream of nitrogen, dissolved in an appropriate volume of 1% triethylamine in ethanol, and a fraction (about 50%) subjected to TLC on polyamide plates (F 1700 from Schleicher & Schuell, Keene, NH)(2,16). Combinations of the solvent systems described in the literature permitted separation of the derivatives of all the amino acids studied. The spots were located in UV light, cut out, and placed in counting vials. To each vial 0.5 ml of tissue solubilizer (Protosol from New England Nuclear), 15 ml of scintillation solution (Permafluro 1 from Packard in toluene) and 2 drops of glacial acetic acid (to avoid chemiluminescence) were added, and the ^{3}H/^{14}C-ratio was determined by liquid scintillation counting.

Display of results. All figures for total incorporation of ^{14}C are presented as µg-atoms carbon fixed per mg of chlorophyll. Figures for specific activities are presented as percent saturation, that is, as the percent of the specific radioactivity of the $H^{14}CO_3^-$ (taking into account the number of carbon atoms in each individual amino acid). Pool sizes are presented as nmoles per mg of chlorophyll.

RESULTS

Under the microscope, the cells appear regularly shaped with the chloroplasts arranged around the sides of the walls. The chloroplasts are highly refractile and have a yellow-green color. Broken or damaged cells have chloroplasts that appear dark green. Storage of cells under a schedule of light and dark periods similar to those of the growth chamber where the plants are grown appears to have resulted in the retention of activity by the cells over a longer period of time than in the case of cells stored under different regimes of light and dark (compare Paul and Bassham, 21).

Rates of photosynthetic fixation for isolated spinach cells ranging from 40 to 130 µg-atom ^{14}C fixed $(mg \cdot chl \cdot hr)^{-1}$ were observed. These rates could be obtained even after the cells had been stored as above for 3 days (Fig. 1). The photosynthetic fixation rate remained constant for the first 60 min of fixation.

In the experiments with various nitrogen sources and with detailed analysis of the ^{14}C incorporation into metabolites, cells were used after 22 h dark and 5 hr in the light. This schedule was designed to deplete the pool of inorganic nitrogen (although this does not seem to have been accomplished) and to avoid the initial "stress response" reaction previously

observed in poppy cells (21). Incubation with $^{14}CO_2$ initiated at other times in the cycle of light and dark periods in some cases showed somewhat different incorporation patterns. This suggests that isolation and storage conditions must be very precisely defined in order to obtain reliable and reproducible results. Thus the effects of environmental factors capable of exerting metabolic regulation could vary with the chosen conditions.

An example of this variability with environmental conditions may be the modulation of the effect of ammonia on metabolism by the prior storage conditions. When the cells were incubated with $^{14}CO_2$ after 22 h dark and 5 h light, there was no increase in the total photosynthetic rate, despite the occurrence of other shifts in metabolism associated with ammonia. As reported by Woo and Canvin (29), the total fixation rate by freshly prepared cells is increased by ammonia treatment. With freshly prepared cells we also found as much as 40% increase in total rate.

With cells stored 22 h in the dark and 5 h in the light, ammonia caused increased ^{14}C incorporation into citrate and malate and decreased incorporation into sucrose (Fig. 2), in agreement with several previous studies of the effects of ammonia on the metabolism of photosynthetic cells. After one h with ammonia, there is a general decrease in incorporation of ^{14}C into carbohydrates, an increase into carboxylic acids (including glycerate), and no change for the phosphate esters analyzed (Table II).

The ^{14}C incorporation into most of the common amino acids was determined (Fig. 3). Unfortunately, the ^{14}C labeling of leucine, lysine, and tryptophane was too low to provide reliable figures. For isoleucine, the figures were also low and hence not very precise, but have been used to provide some estimate of pool size.

The pool sizes of a number of the amino acids have been calculated (Table III) as a function of time of incubation with $^{14}CO_2$ and with or without ammonia treatment. The pool sizes of these various amino acids were obtained by combination of total ^{14}C incorporation with specific radioactivities, measured by the labeled dansyl chloride method. The effect of ammonia treatment on ^{14}C incorporation, specific ^{14}C-labeling and total pool size have been measured or calculated for a number of the amino acids (Table IV). For γ-aminobutyric acid the values for total incorporation in the ammonia experiments were close to zero. However, the specific activities could be measured. Finally, for proline the total incorporation in all experiments was too low to provide reliable results whereas specific activities could be measured and used for calculation of the ammonia effect.

DISCUSSION

Choice of storage and incubation conditions. The use of green cells isolated from leaves for metabolic studies has several advantages such as uniformity of sampling, ease of administration of chemicals, etc. The choice of conditions used in the preparation, storage, and incubation of the cells does, however, considerably affect the results obtained, and some compromises are required. Since freshly prepared cells can exhibit a shift towards respiratory type metabolism similar to some of those expected from the application of ammonia, we chose to use cells stored for a day and then allowed to photosynthesize for 5 h in air to decrease the supply of intracellular inorganic nitrogen (though results subsequently obtained showed little difference between cells in the presence and in the absence of nitrate in the media during this period of photosynthesis).

That this pretreatment of storage and light preincubation results in no stimulation of $^{14}CO_2$ incorporation upon addition of ammonia may be due either to the accumulation of photosynthetic products, especially sucrose, during the five hours, or to recovery of the cells from the postulated "stress response" in which freshly prepared cells may divert photosynthate from sucrose synthesis into amino acid synthesis.

Amino acid labeling rates, pool sizes and specific activities. The present study provides considerable additional information about intracellular pool sizes of amino acids and their rates of labeling and specific radioactivities, as well as the effects of added ammonia on the formation and pool sizes of most common amino acids.

Among the amino acids, alanine had the highest specific radioactivity and was "saturated" at 50% after 30 min. In other words, the ^{14}C labeling of alanine (in cells with nitrate, Fig. 3) was no longer increasing, having leveled off at a ratio of labeled carbon to total carbon 50% of that of the $^{14}CO_2$ administered from 30 to 60 min after the start of the incubation. This suggests that part of the total intracellular pool of free alanine is a metabolically active pool and part is one or more inactive pools. It is possible that the inactive pool could be in the vacuole, whereas the active pool would be the total of alanine in the cytosol, chloroplasts, mitochondria, and other organelles. It has been reported that the cytosol and vacuole of leaf cells constitute separate compartments with an effective permeability barrier between them at least for some amino acids (1). At the same time, relatively rapid transport of amino acids is reported to occur accross the chloroplast membrane (1,8,9,11).

The ^{14}C labeling of several other amino acids, including arginine, glycine, serine, phenylalanine, tyrosine, and threonine approached "saturation" by 60 min $^{14}CO_2$ photosynthesis (Fig. 3). The levels of "saturation" were lower than for alanine, and were only about 0.2% for arginine and 2.5% for threonine. For the remaining amino acids no approach to saturation was observed, but this may be because of the low labeling rates, even for the metabolically-active pools.

The major labeling of amino acids occurred with alanine, glutamate, aspartate, and serine. Labeling of glycine was small as would be expected with saturating CO_2 where photorespiratory pathways should be minimized. The total ^{14}C labeling of glutamine was small (without added ammonia) but the specific radioactivity (approaching 9% saturation at 60 min) was substantial and comparable to that of glutamate (which was around 20%). Both compounds are central to nitrogen metabolism in photosynthetic cells, and the active pools should be turning over rapidly, even with minimal photorespiration. Very low labeling of asparagine conforms with the absence of an important role in intracellular metabolism (as compared with its important role in extracellular nitrogen transport).

The low level of ^{14}C labeling of the remaining amino acids probably reflects the fact that they are primarily synthesized for subsequent protein synthesis which occurs at a low rate in mature leaf mesophyll cells. The comparatively higher labeling of phenylalanine and to a lesser extent tyrosine may be because they are made from primary products of photosynthetic CO_2 reduction (erythrose-4-P and phosphoenolpyruvate) as well as the fact that the aromatic amino acids are precursors for a number of secondary plant products.

Effects of ammonia in the medium. The shifts in carbon metabolism away from carbohydrate synthesis and to amino acid synthesis were in general agreement with previous studies with spinach cells (29,30) and with Chlorella (13,14), poppy cells (23), alfalfa leaf discs (26), cells from bean and discs from cow pea (18), and from cotton cells (27). The present study, by providing more detailed information about pool sizes and additional amino acids gives a more complete picture of this dramatic shift in metabolism.

The ammonia effects on the incorporation of ^{14}C into amino acids and on their pool sizes and specific radioactivities were highly selective. As expected, the most dramatic increase was in the ^{14}C labeling of lutamine. The glutamine pool size was also greatly increased. Only a small fraction of the ammonia in the medium was taken up during the course of the experiment, and the increased glutamine pool size could account for only 10% of the ammonia supplied. In contrast, ^{14}C labeling of glutamate decreased, but its pool size decreased even more, so that its specific radioactivity increased.

In the cycle of C_5 compound interconversions involving glutamate, glutamine and oxoglutarate, the increase in intracellular ammonia shifted the steady state to increase glutamine concentration at the expense of glutamate. At the same time, increased flow of ^{14}C from sugar phosphates into the tricarboxylic acid cycle provided more carbon skeletons for glutamate synthesis via the reaction mediated by glutamine oxoglutarate aminotransferase. In studies with Chlorella (14) and poppy (23), the level of labeled glutamate first declined when ammonia was added due to more rapid converison to glutamine, and then rose due to a more than compensating increased flow of ^{14}C into the tricarboxylic acid cycle. This increased

flow was attributed to accelerated reactions mediated by phosphoenolpyruvate carboxylase and pyruvate kinase. Given the accelerated formation of C_5 carbon skeletons and the increased rate of conversion of glutamate to glutamine in the presence of ammonia, it is understandable that the result is sometimes an increase in labeled glutamate (29) and sometime a decrease (27).

The decrease in specific activities of the glutamate-derived proline and γ-aminobutyrate and the decrease in the total incorporation into γ-aminobutyrate may be due to decreased pool size of some metabolically active glutamate pool. A decrease in ^{14}C incorporation into products derived from the carbon skeletons of glutamate would be expected if the specific activity of the active metabolic pool of glutamate was already near saturation without ammonia addition, but the size of this active pool decreased substantially, perhaps to one-half or one-third, in the presence of added ammonia, and if the rate of formation of the products from glutamate is dependent on glutamate concentration.

With ammonia present, no decrease in labeling of arginine was seen even though it is also derived from glutamate. In this case, one of the nitrogen atoms is derived directly from ammonia and a second one from aspartate, while the carbon atom in the guanidino group comes directly from CO_2. Increased arginine labeling thus may be due to the increased level of intracellular ammonia.

The pool size of asparagine was unaffected by ammonia, but total labelling and specific activity increased several-fold (Table IV). Incorporation of ^{14}C was in any case rather small, in keeping with the minor role of asparagine in the intracellular metabolism of leaf cells as compared with its role in nitrogen transport.

Total ^{14}C incorporation and specific activity of alanine and of aspartate increased with ammonia, and there was some increase in pool sizes (29). As discussed earlier, the data in Figure 3 suggest an inactive pool of alanine and one or more metabolically active pools which by 60 min is "saturated with ^{14}C. The increased ^{14}C incorporation and specific activity of the total pool can be interpreted as an expansion of the active pool which is about half the total pool. Similarly for aspartate, increases in ^{14}C incorporation, specific activity, and pool size could be attributed mainly to expansion of an active pool approximately a fifth of the total pool of aspartate.

There were only slight decreases in the ^{14}C incorporation into serine and glycine and no effects on total pool size. Under the conditions of saturating CO_2 (as bicarbonate) used in these experiments, little photorespiratory formation of these amino acids would be expected. Rather, they would be formed by the reverse pathway, through phosphoglycerate, glycerate, and hydroxypyruvate, to serine, and glycine. The small decrease in labeling might be expected due to the decreased size of the active pool of glutamate, as required for the transamination of hydroxypyruvate to form serine.

Labeling of phenylalanine declined only slightly with ammonia, and tyrosine labeling was unaffected (Table IV). The shikimic acid pathway of aromatic amino acid biosynthesis requires erythrose-4-phosphate and phosphoenolpyruvate, both of which can be formed in the chloroplasts, and synthesis of both aromatic amino acids by isolated and purified chloroplasts has been reported (5).

The ^{14}C labeling of threonine was unchanged with ammonia, and for isoleucine only slightly increased (Table IV), but the total pool sizes were halved and the specific activities roughly doubled. These amino acids

are derived from aspartate, with the biosynthesis taking place in the chloroplasts (17). This decrease in pool sizes could be due to decreased pool size of glutamate in the chloroplasts,

Besides the effects on glutamine, by far the most dramatic effect of ammonia was the sixteen-fold increase in labeling of histidine. Only a 60% increase in pool size occurred, but specific activity increased ten-fold (Table IV). While this large effect is of interest, no interpretation can be advanced due to the paucity of information about the pathways of biosynthesis of histidine in higher plants.

A three-fold increase in labeling and specific activity of valine, without much increase in pool size suggests increased availability of carbon skeletons derived from photosynthetic CO_2 reduction in the presence of ammonia, together with a more rapid turnover of the active pool of valine. Valine is derived from pyruvate so that correlation with the changes in alanine, also derived from pyruvate, might be expected. The ^{14}C incorporation into leucine (only total incorporation was determined) increased 70%. Leucine is derived from valine (or the corresponding oxo-acid) and acetyl CoA, so that some similarity of ammonia effects to those seen for alanine and valine is expected.

The different effects of ammonia on ^{14}C incorporation, pool sizes, and specific radioactivities of various amino acids can in general be explained by the following hypotheses:

1. Addition of ammonia to the medium results in an increase in the intracellular level of ammonia resulting in:

2. Increased conversion of glutamate to glutamine and hence decreased glutamate pool size and increased glutamine pool size,

3. Stimulation of carbon flow from triose phosphates formed by photosynthetic CO_2 reduction into pyruvate and the tricarboxylic acid cycle,

providing more carbon skeletons for the synthesis of amino acids, where such synthesis occurs outside the chloroplasts. Carbon skeletons receiving more labeling by this flow include those required of synthesis of alanine, aspartate, glutamate, glutamine, asparagine, valine, arginine, histidine, and leucine. Rate-limiting steps in the flow of carbon stimulated directly or indirectly by ammonia include those mediated by pyruvate kinase and phosphoenolpyruvate carboxylase. Possible mechanisms of increased rates of these and of other steps between triose phosphates and phosphoenolpyruvate are discussed below.

4. Amino acids synthesized primarily inside the chloroplast do not become more rapidly labeled since there is no increase in the rates of formation of their carbon skeletons inside the chloroplasts. For example, aromatic amino acids formed in the chloroplasts from phosphoenolpyruvate (and erythrose-4-P) are not more rapidly labeled, while such amino acids as alanine formed from pyruvate outside the chloroplasts are. Threonine and isoleucine, believed to be formed inside the chloroplast from aspartate, are formed with higher specific activity because aspartate labeling has increased, but pool sizes are decreased, perhaps because of the decreased pool size of glutamate in the chloroplasts.

A possible interpretation of the mechanism whereby ammonia affects carbon flow from photosynthesis to oxo-acids synthesis can be based on expected effects of ammonia entry into the cells. Primary effects would be an increase in pH of the cytosol and a decrease in the adenylate energy charge due to greatly increased conversion of glutamate to glutamine (mediated by glutamine synthetase) requiring the expenditure of ATP.

An increase in pH could result in increased activity of phosphoenolpyruvate carboxylase, as was suggested in the case of effects of ammonia on poppy cells (10). A regulatory role for this enzyme, mediated by pH has been proposed by Davies (6).

A substantial decline in the energy charge in the cytosol (but probably not in the chloroplasts) could affect several steps in the flow of carbon from triose phosphates to pyruvate and oxalacetate. The observed increased rate of conversion of phosphoenolpyruvate to pyruvate could be due at least in part to lower energy charge. Other possible effects might include increased rate of conversion of triose phosphate to phosphoglycerate through stimulation of phosphoglycerate kinase, and decreased rates of conversion of fructose 1,6-diphosphate to fructose 6-P, due to regulatory inhibition of fructose diphosphatase. Perhaps also, lower energy charge could cause lower UTP levels, diminishing the rate of sucrose phosphate synthesis by lowering the steady-state concentration of uridine diphosphoglucose. A combination of such effects could result in a shift in metabolism away from glucose formation and towards oxo-acid formation.

The detailed examination of the effects of ammonia on the labeling, pool sizes and specific activity of most of the common amino acids shows that ammonia effects are specific and selective, and that ammonia does not appear to induce a generalized stimulation of protein synthesis, at least on the time scale examined in these experiments. Results from isolation of the protein fractions in these experiments (not shown) indicate also that there was no difference in the labeling of the protein amino acids on ammonia treatment. Clearly, there is a substantial difference between these studies with leaf cells, where the results throughout indicate we were unable to "starve" the cells for inorganic nitrogen, and earlier

studies with Chlorella (13,14) where such nitrogen depletion prior to adding ammonia was possible, and increased protein synthesis resulted.

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Table I. Media Compositions for Isolation of Spinach Cells

	Macerating	Wash	Storage and Assay
Sorbitol	0.5M	0.5M	0.5M
Potassium Dextran-Sulfate	1% w/v	-	-
Macerase[a]	2% w/v	-	-
Bovine serum albumin	0.2% w/v	0.2%	0.2%
Polyethylene glycol	-	5.0% w/v	5.0%
Succinate	20mM	-	-
Hepes	-	-	50mM
KNO_3	1μM	1μM	5mM
KH_2PO_4	0.2μM	0.2μM	0.5M
$MgSO_4$	0.1μM	0.1μM	1mM
$CaCl_2$	1mM	1mM	2mM
KCl	1μM	1μM	-
$CuSO_4$	0.01μM	0.01μM	0.01mM

[a] Obtained from Calbiochem

Table II. Effect of NH_4^+ on Incorporation of ^{14}C into Carbohydrates and Carboyxylic acids in spinach cells in the Light. Experimental Time 60'. For experimental conditions and analytical methods see Text.

Compound	% of incorporation obtained in cells with NO_3^- as N-source
Sucrose	70
Maltose	100
Glucose	90
Fructose	70
Malate	150
Citrate	190
Glycerate	140
Hexosemonophosphates	90
Phosphoglycerate	90
Phosphoenolpyruvate	110

Table III. Sizes of Amino Acids in Spinach Cells in the Light with NO_3^- or NH_4^+ as N-source. Each value represents the average of determinations from two separate experiments where not otherwise indicated. For experimental conditions and analytical methods see Text.

Amino acid	NO_3^-			NH_4^+		
	Time after start of Experiment					
	15'	30'	60'	15'	30'	60'
	nmoles per mg chlorophyll					
Glutamate	750	920	1060	480	490	650
Glutamine	180	150	150	450	500	730
Aspartate	300	280	280	450	250	370
Asparagine	60	40	30	40	30	30
Alanine	570	660	710	610	710	850
Valine	240	200	210	200	180	220
Arginine	10	20	30	30	20	30
Histidine	20	20*	20	20	30	30
Glycine	390	410	450	400	390	420
Serine	270	260	270	270	250	280
Phenylalanine	80	80	90	80	90	90
Tyrosine	60	50	60	50	50	60
Threonine	30	50	60	20	10	30
Isoleucine	-	420	450	-	-	250
γ-Aminobutyrate	-	320	310	-	-	-

TABLE IV. Effect of NH_4^- on Biosynthesis of Amino Acids in Spinach Cells in the Light. For experimental conditions and analytical methods see Text.

Amino acid	% of value obtained with No_3^- as N-source		
	Total Incorporation of ^{14}C	Specific activity	Pool size
Glutamate	70	120	60
Glutamine	2100	430	500
Aspartate	230	170	140
Asparagine	540	420	100
Alanine	150	120	120
Valine	330	310	110
Arginine	270	200	130
Histidine	1600	1000	160
Leucine	170	-	-
Glycine	90	90	100
Serine	90	90	100
Phenylalanine	80	80	110
Tyrosine	100	100	100
Treonine	100	230	50
Isoleucine	120	220	60
Proline	-	50	-
γ-Aminobutyrate	-	-	-

LEGENDS TO FIGURES

Fig. 1. Total Photosynthetic $^{14}CO_2$ incorporation by isolated spinach cells. Incubation was performed for 30 min in light.

Fig. 2. Total photosynthesis and incorporation of $^{14}CO_2$ into sucrose, malate, and citrate in spinach cells 27 h after isolation (22 h in dark, 5 h in light) with NO_3^- or NH_4^+ as N-source.

Fig. 3. Total incorporation of $^{14}CO_2$ and specific activities of amino acids in spinach cells (conditions as in Fig. 2).

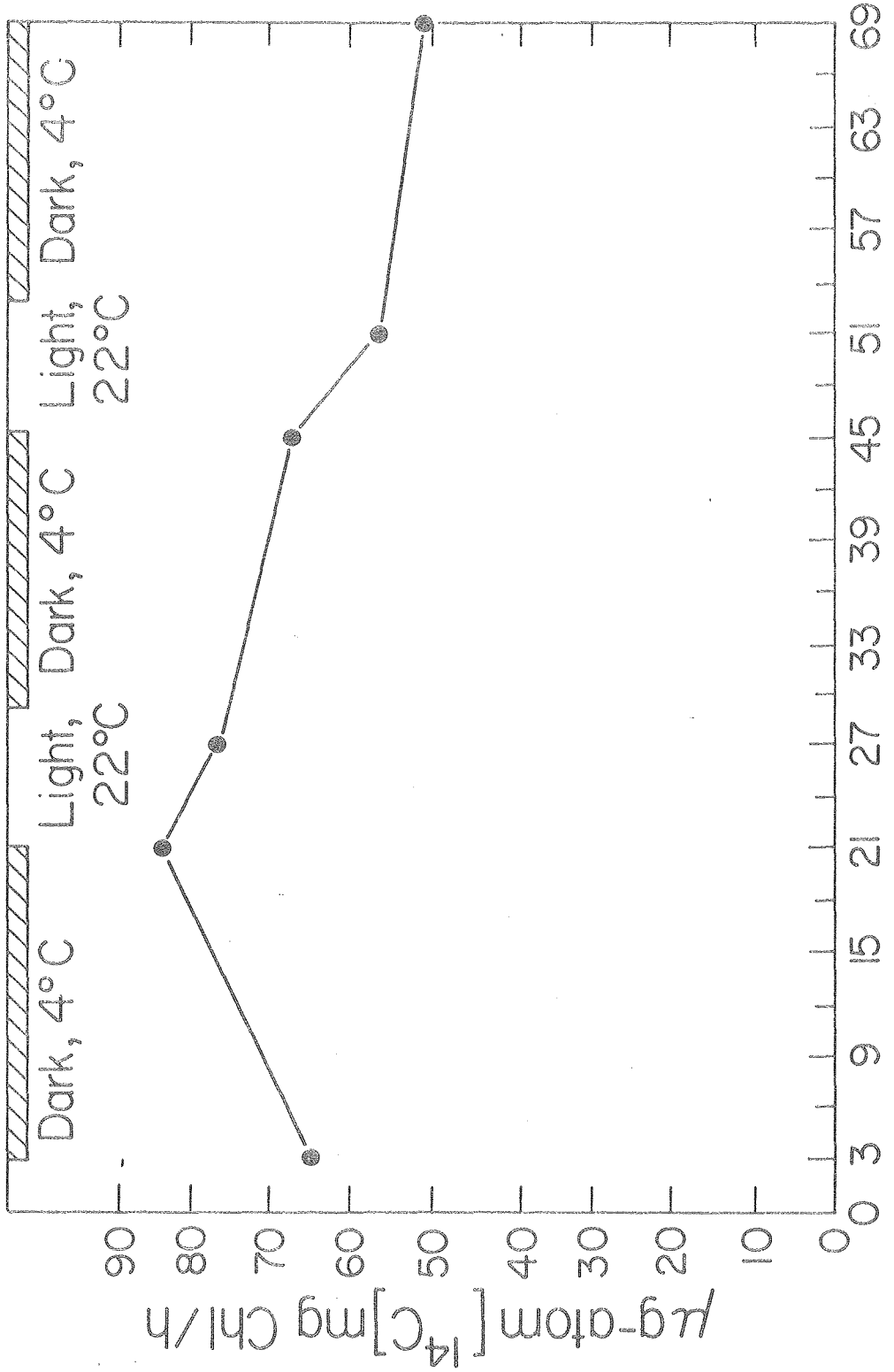

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Fig. 1

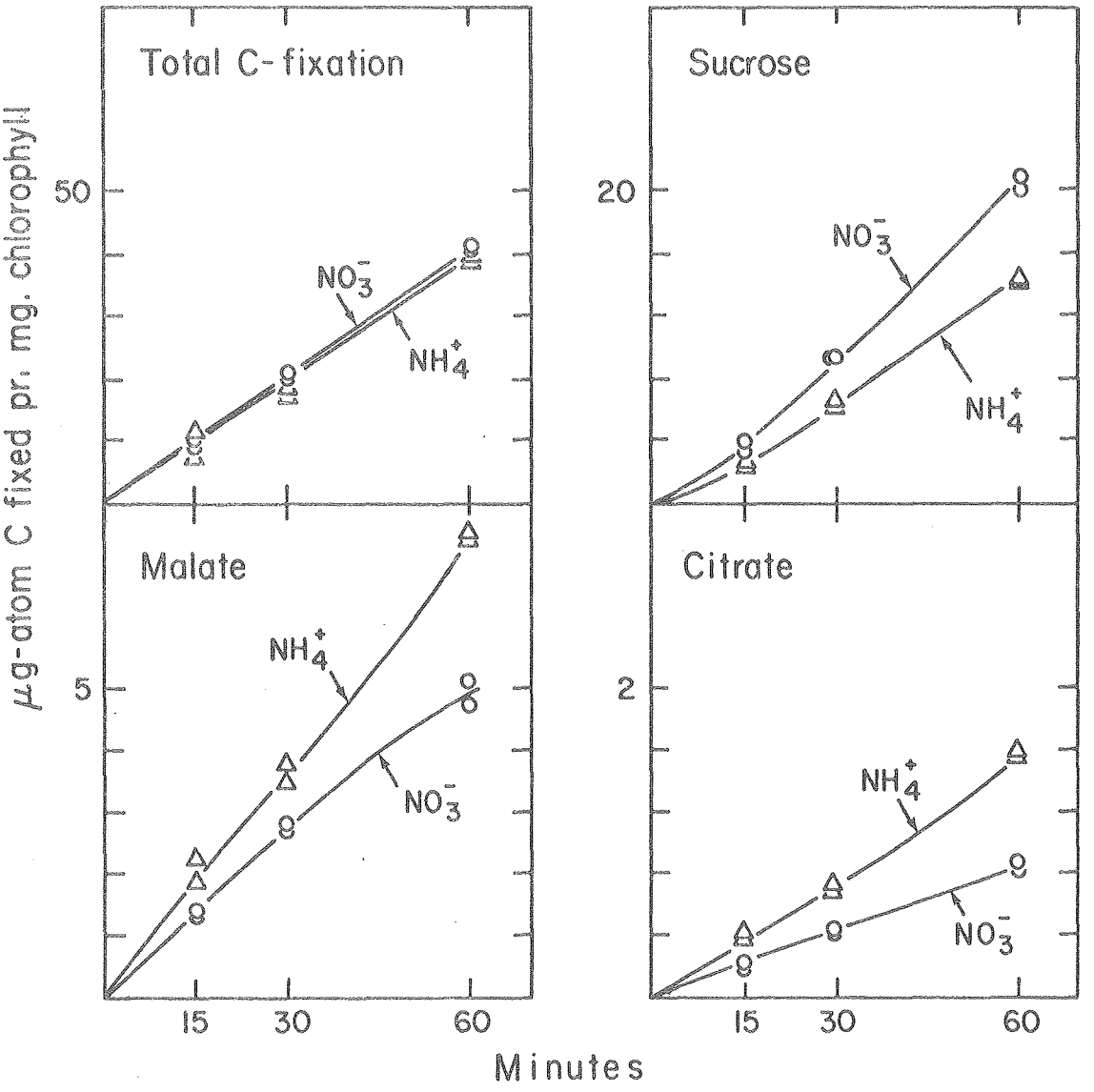

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Fig. 2

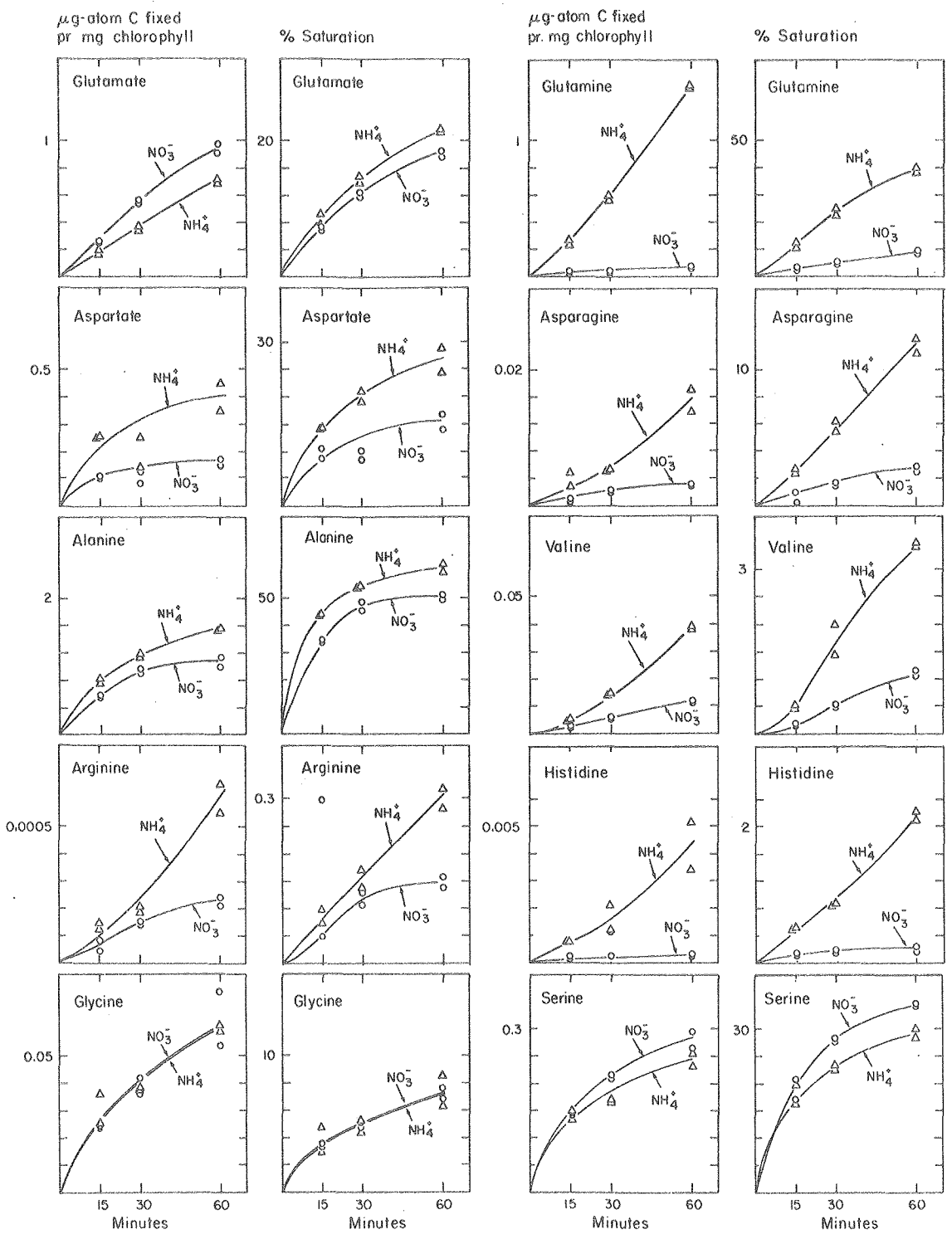

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Fig. 3

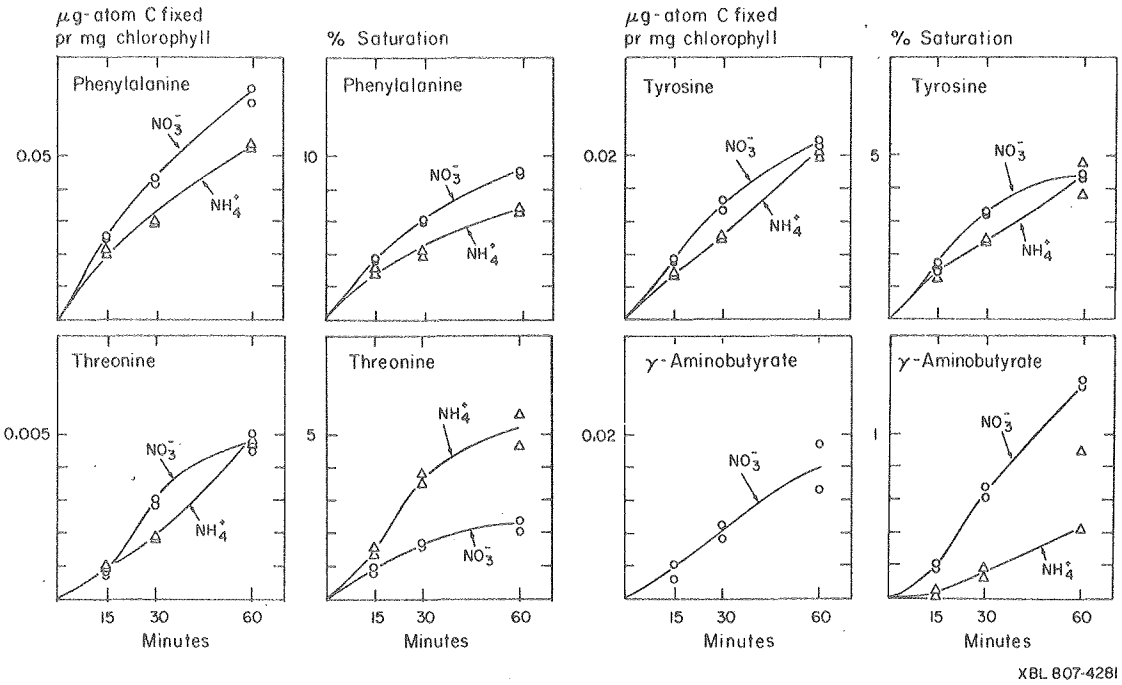

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Fig. 3 (cont'd)