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Amino Acid Synthesis in Photosynthesizing Spinach Cells[1]

EFFECTS OF AMMONIA ON POOL SIZES AND RATES OF LABELING FROM $^{14}CO_2$

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ABSTRACT

Isolated cells from leaves of *Spinacia oleracea* have been maintained in a state capable of high rates of photosynthetic CO_2 fixation for more than 60 hours. 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 radio-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 21), aspartate, asparagine, valine, alanine, arginine, and histidine. 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 radioactivity 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 radioactivities 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.

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) (22), spinach (31), and cotton (28). Such cells afford possibilities for studying plant metabolism in a simple but intact system. Effects on poppy cell metabolism of ammonia (24), sulfite (23), and 2,4-D (25) have been reported from this laboratory, and others have described effects of ammonia and other nitrogen compounds on metabolism in isolated spinach cells (31, 32).

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The present paper reports on the effects of ammonia added to photosynthetically active cells from spinach (*Spinacia 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, 21, 31).

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 (27), poppy cells (24), cotton cells (28), and spinach cells (31, 32). 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 (*Spinacia oleracea* L., Burpee Hybrid no. 7) was grown in a growth chamber with 16-h dark and 8-h light periods. The light intensity was 2×10^4 lux or 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ and temperature was maintained at 17 C. The plants were grown in vermiculite and watered with Hoagland solution.

Cell Isolation. Leaves were selected from plants 6 to 8 weeks old. Four leaves weighing approximately 1 g each were washed in distilled H_2O 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 2 times for 1 min 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 cheese cloth 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 nylon filter. Then, 5 ml of wash medium was added to the leaf pieces

Table I. *Media Compositions for Isolation of Spinach Cells*

	Macerating	Wash	Storage and Assay
Sorbitol	0.5 M	0.5 M	0.5 M
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	20 mM		
Hepes			50 mM
KNO_3	1 μM	1 μM	5 mM
KH_2PO_4	0.2 μM	0.2 μM	0.5 mM
$MgSO_4$	0.1 μM	0.1 μM	1 mM
$CaCl_2$	1 mM	1 mM	2 mM
KCl	1 μM	1 μM	
$CuSO_4$	0.01 μM	0.01 μM	0.01 mM
pH	5.8	6.8	7.8

[a] Obtained from Calbiochem.

and the procedure was repeated until 30 to 40 ml of cell suspension was collected. The cells were washed by centrifugation (1,000 rpm for 1.5 min) twice with wash media and resuspended in assay medium (Table I) with a resulting Chl concentration of 30 to 40 μg Chl/ml. The cells were observed under the microscope at this time. Chl 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 (250 $\mu E \cdot m^{-2} \cdot s^{-1}$) 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 microfernbach flask. The flasks were placed on a rotary shaker and shaken at 50 rpm in a Plexiglas water bath maintained at 22 C. The bath was illuminated from below by fluorescent lamps with a light intensity of 600 $\mu E \cdot m^{-2} \cdot s^{-1}$. The assay was initiated by the addition of $H^{14}CO_3^-$ at a specific radioactivity of 12.5 mCi/mmol 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 5 min before the addition of $H^{14}CO_3^-$. 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 radioactivity of the $^{14}CO_3^-$ used was 49 mCi/mmol.

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 × 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 (Bio-Rad 50 × 8, 200–400 mesh, 0.4 × 3 cm, Richmond, CA). The column was flushed with 4 × 0.5 ml of water to give an effluate containing neutral compounds (carbohydrates), carboxylic acids, and phosphate esters. The column was then washed with 6 × 1 ml of water and eluted with 4 × 1 ml of 1 M aqueous pyridine to give the fraction containing acidic and neutral amino acids, and was finally eluted with 4 × 1 ml of 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 (26). After location by radioautography, the labeled areas of paper were cut into small pieces and shaken for 1 h with 2.5 ml of water in scintillation vials and 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-P-glycerate, P-enolpyruvate, 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% of the total for the acid and neutral amino acids, 50% for the basic amino acids) was subjected to two-dimensional paper chromatography on Whatman No. 1 paper: (a) solvent: butanol: acetic acid:water (12:3:5, v/v/v); (b) solvent: butanol:methylethylketone:NH_4OH:water (5:3:1:1, v/v/v/v) (compare [30]). 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

[3] Abbreviations: Mops, (3-(*N*-morpholino)ethanesulfonic acid).

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 Airhart *et al.* (2) with ^{3}H-labeled dansyl chloride (5-dimethylamino-l-naphthalenesulfonyl 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 × 2 cm) of the neutral polystyrene resin Porapak Q (obtained from Alltech Associates, Arlington Heights, IL) (17). The Porapak material was swelled overnight in ethanol (20) and after the columns were poured, they were flushed with 0.1 N HCl. The dansylation reaction mixture with 0.4 ml 0.1 N HCl added was applied to the column, which was then washed with 3 × 0.5 ml N HCl. Subsequently, most of the dansyl hydroxide formed was eluted with 5 × 1 ml 5% v/v aqueous acetic acid and the dansyl derivatives were eluted with 3 × 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 and Schuell, Keene, NH) (2, 17). Combinations of the solvent systems described in the lieterature 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 (Permafluor 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/mg Chl. Figures for specific radioactivities are presented as per cent saturation, that is, as the percentage 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 nmol/mg Chl.

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, 22).

Rates of photosynthetic fixation for isolated spinach cells ranging from 40 to 130 μg-atom ^{14}C fixed (mg·Chl·h)$^{-1}$ was 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 h in the light. This schedule was designed to deplete the pool of inorganic nitrogen and to avoid the initial "stress response" reaction previously observed in poppy

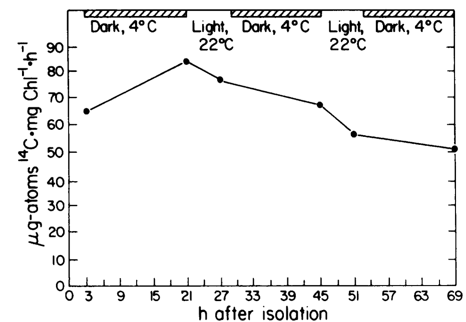

FIG. 1. Total photosynthetic $^{14}CO_2$ incorporation by isolated spinach cells. Incubation was performed for 30 min in light.

cells (22). The planned depletion of the pool of inorganic nitrogen does not seem to have been accomplished, since the values obtained in the experiments with no N source were identical with those obtained in the NO_3 experiments. To simplify the presentation of the results, the experiments with no N source are therefore not included in the Tables and Figures. 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 (31), 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 1 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 tryptophan 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 ^{14}C incorporation into the protein fraction in parallel experiments is low and remains the same for the control and ammonia experiments (data not shown).

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 radioactivities could be measured. Finally, for proline, the total incorporation in all experiments was too low to provide reliable results, whereas specific radioactivities could be measured and used for calculation of the ammonia effect.

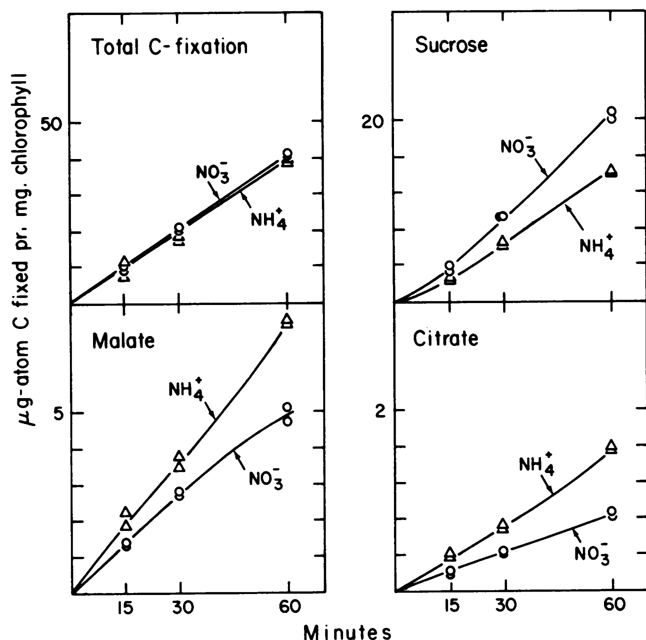

FIG. 2. Total photosynthesis and incorporation of $^{14}CO_2$ into sucrose, malate, and citrate in spinach cells 27 h after isolation (22 h in dark, 5 h in light) with NO_3^- or NH_4^+ as N source.

Table II. *Effect of NH_4^+ on Incorporation of ^{14}C into Carbohydrates and Carboxylic Acids in Spinach Cells in the Light*

Experimental time was 60 min.

Compound	% Incorporation Obtained in Cells with NO_3^- as N Source	μg ^{14}C/mg Chl/h	
		+ NO_3^-	+ NH_4^+
Sucrose	70	21.0	14.6
Maltose	100	1.03	1.01
Glucose	90	0.341	0.309
Fructose	70	0.194	0.144
Malate	150	4.92	7.47
Citrate	190	0.856	1.58
Glycerate	140	0.126	0.179
Hexosemonophosphates	90	0.600	0.542
Phosphoglycerate	90	0.306	0.271
Phosphoenolpyruvate	110	0.0262	0.0283

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, as mentioned under "Results," only insignificant differences were observed 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 5-h, 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 across the chloroplast membrane (1, 8, 9, 11).

The ^{14}C labeling of several other amino acids, including aspartate, serine, and arginine (without NH_4^+) approached saturation by 60 min $^{14}CO_2$ photosynthesis (Fig. 3). The levels of saturation were lower than for alanine. For the remaining amino acids, no approach to saturation was observed, but this may be because of the low labeling rates.

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 its relatively unimportant role in intracellular metabolism as compared with its important role in intercellular 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 P-enolypyruvate) 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 (31, 32) and with *Chlorella* (13, 14), poppy cells (24), alfalfa leaf discs (27), cells from bean and discs from cow pea (19), and from cotton cells (28). 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 glutamine. The glutamine pool size was also greatly increased. Only a small fraction of the ammonia in the

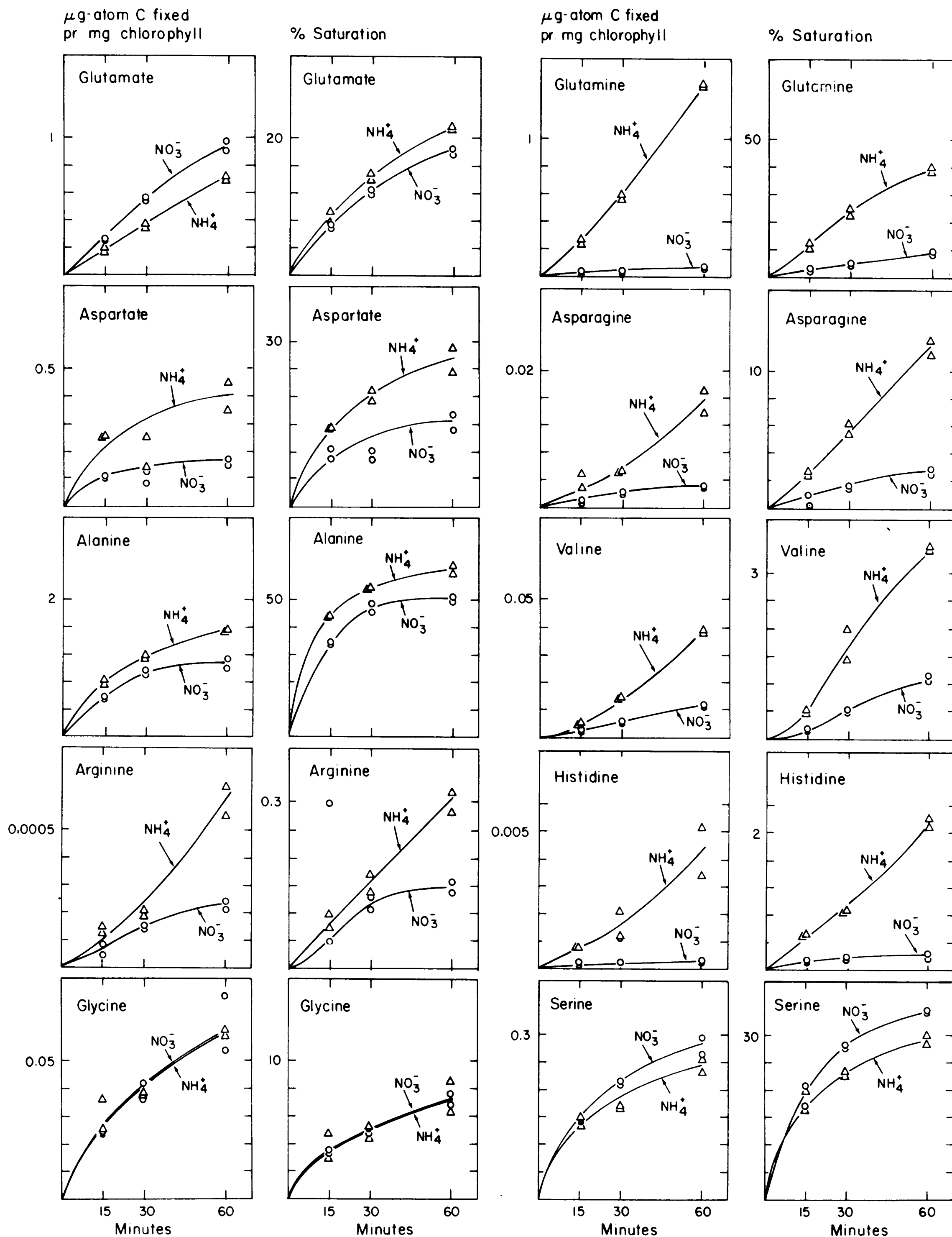

FIG. 3. Total incorporation of $^{14}CO_2$ and specific radioactivities of amino acids in spinach cells (conditions as in Fig. 2).

medium was taken up during the course of the experiment, however. The ^{14}C labeling of glutamate decreased, but its pool size decreased even more, so that its specific radioactivity increased.

Keys *et al.* (16) have described an amino acid cycle of C_5 compound interconversions involving glutamate, glutamine, and α-ketoglutarate and involving the enzymes glutamine synthetase and glutamine α-ketoglutarate amidotransferase. In the present study, as expected, the increase in intracellular ammonia shifted the steady-state to increase glutamine concentration at the expense of glutamate. The synthesis of glutamine, requiring ATP, ammonia, and glutamate and mediated by glutamine synthetase, occurs partly in the chloroplast and partly in the cytoplasm (29). 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 in the chloroplast mediated by glutamine oxoglutarate amidotransferase. In studies with *Chlorella* (14) and poppy (24), the level of labeled glutamate first declined when ammonia was added due to more rapid conversion to glutamine, and then rose due to a more than compensating increased flow of ^{14}C into the tricarboxylic acid cycle. This in-

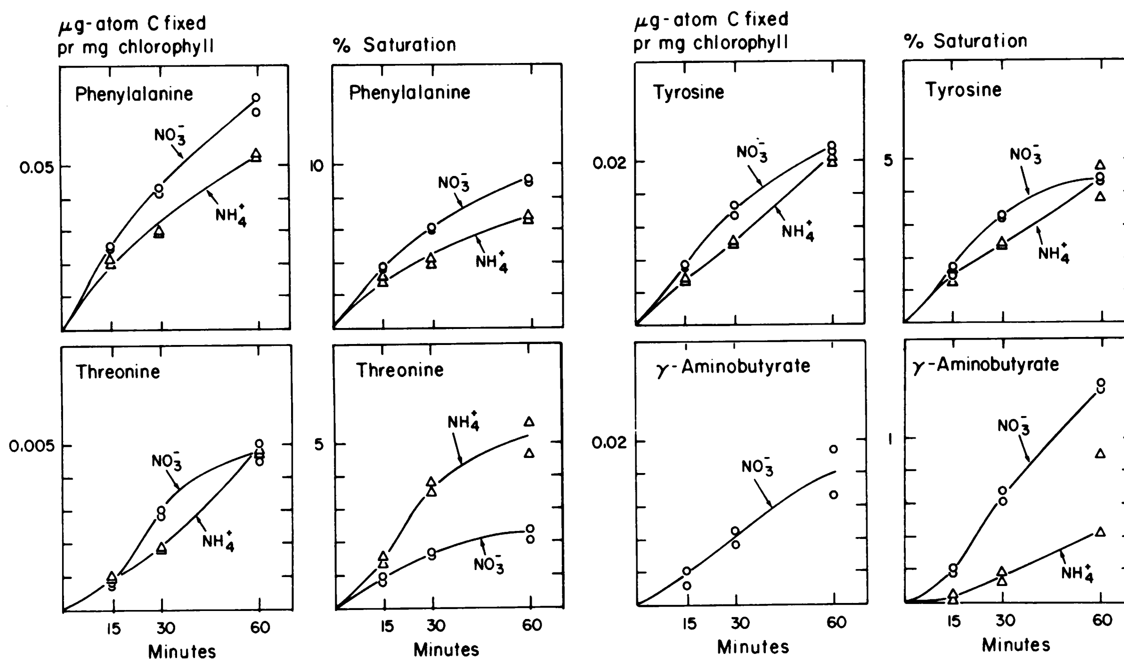

FIG. 3. Continued.

Table III. *Pool 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.

Amino Acid	NO_3^-			NH_4^+		
	Minutes after start of experiment					
	15	30	60	15	30	60
	nmol/mg Chl					
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[a]	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	ND[b]	420	450	ND	ND	230[a]
γ-Aminobutyrate	320[a]	320	310	ND	ND	ND

[a] One result discarded.
[b] Not detectable.

Table IV. *Effect of NH_4^+ on Biosynthesis of Amino Acids in Spinach Cells in the Light*

Experimental time was 60 min.

Amino Acid	Ratio of Level in the Presence of NH_4^+ Compared to the Level in the Presence of NO_3^-		
	Total incorporation of ^{14}C	Specific radioactivity	Pool size
Glutamate	0.7	1.2	0.6
Glutamine	21.0	4.3	5.0
Aspartate	2.3	1.7	1.4
Asparagine	5.4	4.2	1.0
Alanine	1.5	1.2	1.2
Valine	3.3	3.1	1.1
Arginine	2.7	2.0	1.3
Histidine	16.0	10.0	1.6
Leucine	1.7		
Glycine	0.9	0.9	1.0
Serine	0.9	0.9	1.0
Phenylalanine	0.8	0.8	1.1
Tyrosine	1.0	1.0	1.0
Treonine	1.0	2.3	0.5
Isoleucine	1.2	2.2	0.6
Proline	ND[a]	0.5	ND
γ-Aminobutyrate	ND	0.5	ND

[a] Not detectable.

creased flow was attributed to accelerated reactions mediated by P-enolpyruvate carboxylase (10) and pyruvate kinase (24). 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 (31) and sometime a decrease (28).

The decrease in specific radioactivities 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 radioactivity 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 guanidine 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 labeling and specific radioactivity increased several-fold (Table IV). Incorporation of ^{14}C was in any case rather small, in keeping with the minor role of asparagine carbon skeleton turnover in the intracellular metabolism of leaf cells as compared with its role in nitrogen transport.

Total ^{14}C incorporation and specific radioactivity of alanine and of aspartate increased with ammonia, and there was some increase in pool sizes (31). 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 radioactivity 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 radioactivity, 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 P-glycerate, glycerate, and hydroxypyruvate, to serine, and glycine. The small decreases 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-P and P-enolyruvate, 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 radioactivities roughly doubled. These amino acids are derived from aspartate, with the biosynthesis taking place in the chloroplasts (18). 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 16-fold increase in labeling of histidine. Only a 60% increase in pool size occurred, but specific radioactivity increased 10-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 3-fold increase in labeling and specific radioactivity 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 keto-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: (a) addition of ammonia to the medium results in flow of ammonia into the cells resulting in: (b) increased conversion of glutamate to glutamine and hence decreased glutamate pool size and increased glutamine pool size, (c) 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 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 P-enolpyruvate carboxylase (10, 13, 24). The mechanism whereby the presence of ammonia in the medium causes changes in pyruvate kinase activity and perhaps in other rate-limiting steps in carbon metabolism leading to ketoacid skeletons is unknown. We considered the possibility of a decrease in ATP level energy charge due to increased utilization of ATP in the reaction mediated by glutamine synthetase. The total increased ammonia incorporation into all amino acids (estimated as increasing from 0.7 to 1.2 μmol N/mg Chl·h, based on Table III data) seems to be too small, however, to have caused a serious change in ATP level.

An increase in pH could result in increased activity of P-pyruvate 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).

Amino acids synthesized inside the chloroplasts and partly or wholly from carbon skeletons provided primarily by the chloroplasts (*e.g.* Phe + Tyr) themselves do not become more rapidly labeled since there is no increase in the rates of formation and availability of these carbon skeletons. For example, aromatic amino acids formed in the chloroplasts from P-pyruvate and erythrose-4-P are not more rapidly labeled, while such amino acids as alanine and valine formed from pyruvate are. Threonine and isoleucine, believed to be formed inside the chloroplast from aspartate, are formed with higher specific radioactivity because aspartate (and malate) labeling has increased.

The detailed examination of the effects of ammonia on the labeling, pool sizes, and specific radioactivity 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. Clearly, there is a substantial difference between these studies with leaf cells, where the results throughout indicate we apparently 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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