



Lawrence Berkeley Laboratory

UNIVERSITY OF CALIFORNIA

CHEMICAL BIODYNAMICS DIVISION

Submitted to Plant Physiology

EFFECTS OF CARBON DIOXIDE AND OXYGEN ON THE
REGULATION OF PHOTOSYNTHETIC CARBON METABOLISM
BY AMMONIA IN SPINACH MESOPHYLL CELLS

Arthur L. Lawyer, Karen L. Cornwell, Peder O. Larsen,
and James A. Bassham

February 1981

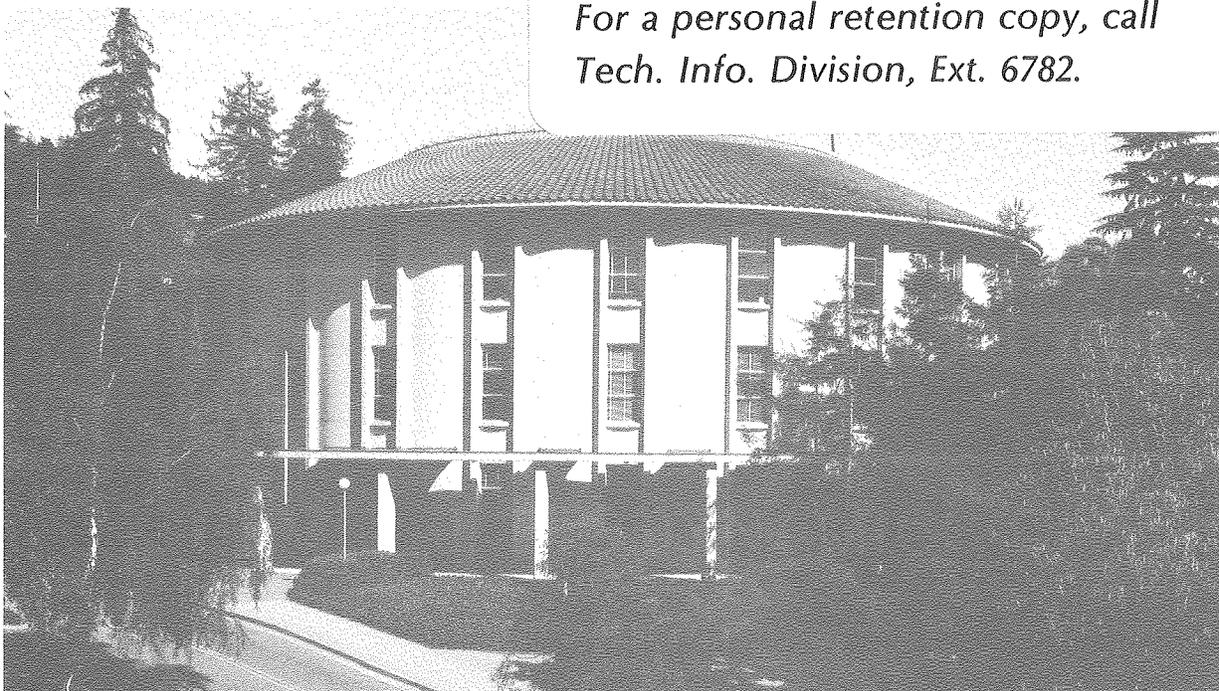
RECEIVED
LAWRENCE
BERKELEY LABORATORY

MAR 5 1981

LIBRARY AND
DOCUMENTS SECTION

TWO-WEEK LOAN COPY

*This is a Library Circulating Copy
which may be borrowed for two weeks.
For a personal retention copy, call
Tech. Info. Division, Ext. 6782.*



LBL-12276
c.2

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

Effects of Carbon Dioxide and Oxygen on the Regulation of Photosynthetic
Carbon Metabolism by Ammonia in Spinach Mesophyll Cells¹

Arthur L. Lawyer, Karen L. Cornwell, Peder O. Larsen², and
James A. Bassham

Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory,
University of California, Berkeley, California 94720

¹This work was supported by the Division of Biological Energy Conversion and Conservation, Office of Basic Energy Sciences, U.S. Department of Energy under Contract No. W-7405-ENG-48, by ^aRockefeller Foundation Postdoctoral Fellowship to A.L.L., and by the Danish National Science Research Council 511-15546 to P.O.L.

²Present Address: Chemistry Department, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871, Copenhagen V, Denmark.

³Abbreviations: PR: photorespiratory; NPR: nonphotorespiratory; DHAP: dihydroxyacetonephosphate; OG: 2-oxoglutarate; PEP: phosphoenolpyruvate

ABSTRACT

Photosynthetic metabolism of isolated spinach mesophyll cells was characterized under conditions favoring photorespiratory (PR; 0.04% CO₂ and 20% O₂) and nonphotorespiratory (NPR; 0.2% CO₂ and 2% O₂) metabolism, as well as intermediate conditions. Comparisons were made between the metabolic effects of extracellularly supplied NH₄⁺ and intracellular NH₄⁺, produced primarily via PR metabolism. The metabolic effects of ¹⁴C₂ fixation under PR conditions were similar to perturbations of photosynthetic metabolism brought about by externally supplied NH₄⁺; both increased labeling and intracellular concentrations of glutamine at the expense of glutamate and increased the anaplerotic synthesis of 2-oxo-glutarate. The metabolic effects of added NH₄⁺ during NPR fixation were greater than those during PR fixation, probably due to lower initial NH₄⁺ levels during NPR fixation. During PR fixation, addition of ammonia caused decreased pools and labeling of glutamate and serine and increased glycolate, glyoxylate, and glycine labeling. The glycolate pathway was thus affected by increased rates of carbon flow and decreased glutamate availability for glyoxylate transamination, resulting in increased usage of serine for transamination. Sucrose labeling decreased with NH₄⁺ addition only during PR fixation, suggesting that higher photosynthetic rates under NPR conditions can accommodate the increased drain of carbon toward amino acid synthesis while maintaining sucrose synthesis. The perturbation of photosynthetic metabolism by extracellular addition of ammonia thus appears to resemble natural physiological regulation by intracellular NH₄⁺, primarily in its role in photorespiratory metabolism.

High carbon dioxide and/or low oxygen concentrations increase net photosynthesis in C_3 plants (11,20,21,23). Increased CO_2 pressure increases CO_2 fixation rates directly by supplying substrate CO_2 at a higher concentration and possibly by increasing the activity of ribulose-bis-P carboxylase through regulatory activation (5). At the same time, increased binding of CO_2 due to higher concentrations may decrease competitive binding of O_2 . Oxygen binding can also be decreased by lowering the O_2 concentration. Increased incorporation of photosynthetically fixed ^{14}C into glycolate, glycine, and serine are characteristics of photosynthesis under decreased partial pressures of CO_2 or increased O_2 levels (11,20,21).

The photorespiratory metabolism of glycolate results in both CO_2 release and production of NH_4^+ through the activity of glycine synthase associated with mitochondrial conversion of glycine to serine (22). The rate of NH_4^+ production via NO_3^- assimilation in C_3 plants is often insignificant compared to the rate of photorespiratory NH_4^+ release (8,25). Plants with higher photorespiratory rates might therefore be expected to have metabolic responses which are in part similar to the effects of adding extracellular NH_4^+ to cells. The pathway of carbon and nitrogen flow involving the release of NH_4^+ from glycine, its re-fixation back to glutamate via glutamine synthetase and glutamine: 2-oxoglutarate amidotransferase (GOGAT) activities, and transamination of glyoxylate with glutamate has been termed the photorespiratory nitrogen cycle (8).

The effects of NH_4^+ on photosynthetic carbon metabolism have been characterized in various systems including alfalfa leaf discs (19), isolated leaf cells of poppy (4,16) and spinach (9,26), and *Chlorella* (7). Ammonia's effects on higher plants have been recently reviewed by Givan (3).

This report describes the effects of NH_4^+ addition on the metabolism of isolated spinach mesophyll cells carrying out photosynthesis under differing concentrations of CO_2 and O_2 . Internally produced NH_4^+ from photorespiration and externally supplied NH_4^+ are compared with respect to their effects on photosynthetic carbon metabolism and intracellular concentrations of amino acids.

MATERIALS AND METHODS

Plant Materials. Spinach (Spinacea oleracea L., Burpee Hybrid #7) was grown in a 17 C growth chamber in vermiculite and watered 3 times per week with Hoagland's solution. Light (300 to 400 $\mu\text{E m}^{-2} \text{s}^{-1}$, 400-700 nm) was supplied 8 h per day by a combination of fluorescent tubes and incandescent bulbs.

Cell Isolation and Storage. Cells were isolated as previously described (9) and stored in assay media without nitrate. Cells used for the nonphotorespiratory, NPR³, experiment were stored in the dark at 6 C for 20.5 h, placed on a rotary shaker at 50 rpm in the light (200 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 17 C for 4.5 h, and then placed in the dark at 6 C for 0.5 h before the experiment. Cells used for the photorespiratory, PR, experiment were stored in the same manner except that the initial dark period was 23 h.

Photosynthetic $^{14}\text{CO}_2$ Incorporation. The experimental system consisted of a rotary shaker-water bath illuminated from below (650 $\mu\text{E m}^{-2} \text{s}^{-1}$) (6) with serum stoppered 5 ml Fernbach flasks fitted with inlet and outlet ports. These flasks were connected to a closed steady-state gas circulation system as previously described (18).

Gas composition for the nonphotorespiratory, NPR, experiment was 2.75% O_2 and 0.234% $^{14}\text{CO}_2$ (specific radioactivity, 9.3 $\mu\text{Ci}/\mu\text{mol}$). The O_2

concentration was 20.39% for the photorespiratory, PR, experiment with the $^{14}\text{CO}_2$ level at 0.0476% (specific radioactivity, $12.8 \mu\text{Ci}/\mu\text{mol}$). Experiments with $[^{14}\text{C}]$ bicarbonate were carried out simultaneously with both the NPR and PR experiments as controls. The bicarbonate flasks were not connected to the steady-state apparatus and contained $8 \text{ mM NaH}^{14}\text{CO}_3$ (specific radioactivity $16.36 \mu\text{Ci}/\mu\text{mol}$).

The experiment detailed in Fig. 1, in which fixation under 4 different combinations of O_2 and CO_2 gases was carried out, was technically similar to the PR and NPR experiments. The concentrations and specific radioactivities of the gases used in that experiment are detailed in the legend to Fig. 1.

With the gas circulating, $680 \mu\text{l}$ of assay medium (9) and 200 units of carbonic anhydrase (Sigma Chemical Co.) in $20 \mu\text{l}$, was added to the steady-state flasks. The closed control-flasks had $50 \mu\text{l}$ of $[^{14}\text{C}]$ bicarbonate and $650 \mu\text{l}$ of assay medium added. After 10 min, $700 \mu\text{l}$ of 2-times concentrated cells ($90 \mu\text{g chl/ml}$) were added to all flasks, initiating $^{14}\text{CO}_2$ fixation. Chlorophyll content was determined by the method of Arnon (1).

Samples of $200 \mu\text{l}$ were removed from the flasks and injected into methanol (80% v/v final concentration), 15 and 30 min after initiating $^{14}\text{CO}_2$ fixation. At 33 min, $50 \mu\text{l}$ of $(\text{NH}_4)_2\text{SO}_4$ (0.5 mM final concentration) or $50 \mu\text{l}$ H_2O were added to the flasks and $200 \mu\text{l}$ samples were withdrawn and injected into methanol as above at 36, 45, and 65 min. Total $^{14}\text{CO}_2$ incorporation was measured as previously described (9).

Analysis of $^{14}\text{CO}_2$ -Metabolites. Samples were fractionated by cation exchange chromatography and 2-dimensional paper chromatography as described by Larsen et al. (9) with the following modifications. To every sample, $0.5 \mu\text{mol}$ of Ca glycolate and 2-oxoglutarate were added. The order of the ethanol extractions was reversed and the ether extraction was omitted. Incorporation

of $^{14}\text{CO}_2$ into glycolate was determined by 2-dimensional paper chromatography (17) using a portion of the effluent. Analysis of 2-oxoacids was performed by conversion to their 2,4-dinitrophenylhydrazones using the method of Bachelard (2) following addition of 10 μg each of hydroxypyruvate and glyoxylate to aliquants amounting to 70% of the neutral and organic acid eluates. The derivatives were separated using paper chromatography (19). Incorporation of ^{14}C was determined by liquid scintillation counting.

Of the amino acids, ^{14}C -incorporation into histidine, lysine, arginine, methionine, and cysteine was not determined. Specific radioactivities of ^{14}C -amino acids were determined by formation of their [^3H]dansyl chloride derivatives as described previously (9). For a few determinations, concentrated [^3H]dansyl chloride (24.4 mM, as opposed to 2 mM) of the same specific radioactivity (8.15 $\mu\text{Ci}/\mu\text{mol}$) was used.

RESULTS

Effects of CO_2 and O_2 Concentrations on $^{14}\text{CO}_2$ Fixation. Photosynthetic incorporation of $^{14}\text{CO}_2$ into spinach cells was characterized under two concentrations of CO_2 (0.04% and 0.2%) and two concentrations of O_2 (20% and 2%) (Fig. 1). The fixation into control cells in 8 mM bicarbonate, carried out in parallel to the experiment, is also presented.

Detailed analysis was carried out on the effects of adding NH_4^+ to spinach cells incorporating $^{14}\text{CO}_2$ under the two extreme combinations of CO_2 and O_2 gases (low CO_2 and high O_2 , PR; high CO_2 and low O_2 , NPR). Under NPR conditions for $^{14}\text{CO}_2$ fixation, total incorporation was twice the amount under PR conditions (Fig. 2). Cells photosynthesizing under saturating bicarbonate levels incorporated $^{14}\text{CO}_2$ at even faster rates (Fig. 2).

Distribution of ^{14}C -Metabolites Under Photorespiratory and Nonphotorespiratory Conditions. Incorporation of ^{14}C into most compounds increased in proportion to the increases in total $^{14}\text{CO}_2$ fixation under NPR conditions. Such labeling increases occurred for sucrose (accounting for up to 40% of total incorporation), glucose, fructose, maltose, hexose-mono-P, pentose-mono-P, glycerate, citrate, dihydroxyacetone-P, pyruvate, glutamate, γ -aminobutyric acid, proline, threonine, isoleucine, alanine, valine, leucine, and phenylalanine. Compounds which were about equally labeled under PR and NPR conditions were 2-oxoglutarate, fumarate, 3-P-glycerate, hydroxypyruvate, and aspartate. A few compounds were more labeled under PR conditions despite the slower total incorporation rate (Fig. 2). These include photorespiratory pathway intermediates (glycolate, glyoxylate, glycine, and serine), compounds directly affected by NH_4^+ (glutamine and asparagine), and compounds involved in the anaplerotic formation of C_5 amino acids (phosphoenolpyruvate and malate).

For some compounds, the metabolically active pool size could be estimated due to the saturation of labeling of the compounds during the experiment. Compounds for which metabolically active pool sizes increased under PR conditions were glycine, serine, and glyoxylate (Fig. 2). Glycerate, pyruvate, and dihydroxyacetone-P had smaller active pool sizes under PR conditions.

The intracellular concentrations of most amino acids were unaffected by fixation under PR and NPR conditions. The amino acids with concentrations which were affected are listed in Table I. Alanine and glutamate concentrations were lower in cells incorporating CO_2 under PR conditions. The concentrations of glutamine, glycine, and serine were higher. Glutamine pool sizes increased after initiation of $^{14}\text{CO}_2$ fixation under both conditions

(data not shown). This suggests that low glutamine levels existed prior to the experiment due to the storage of cells in media lacking NO_3^- and/or pretreatment of the cells with light (depleting their amino acids via protein synthesis).

Effects of NH_4^+ on $^{14}\text{CO}_2$ Fixation under Photorespiratory and Nonphotorespiratory Conditions. The labeling of a number of compounds was affected by the addition of NH_4^+ during $^{14}\text{CO}_2$ fixation under PR and NPR conditions (Figs. 2 and 3). Differences in labeling were observed for intermediates of photorespiratory carbon metabolism (glycolate, glyoxylate, glycine, and serine), photorespiratory nitrogen metabolism (glutamine, glutamate, and 2-oxoglutarate) and other inter-related pathways (phosphoenolpyruvate, malate, fumarate, citrate, asparagine, aspartate, alanine, and valine).

The rate of incorporation into sucrose under PR and NPR conditions was proportional to total fixation. The rate of sucrose labeling was unaffected by NH_4^+ addition in cells during NPR incorporation but was decreased by two-thirds upon addition of NH_4^+ under PR conditions (Fig. 3). The decrease in sucrose labeling under PR conditions can quantitatively account for the observed decrease in total fixation after addition of NH_4^+ (Fig. 2).

Net ^{14}C -incorporation into amino acids was unaffected by addition of NH_4^+ under PR conditions but increased under NPR conditions (data not shown). This increase in amino acid labeling (4 $\mu\text{g-atom } ^{14}\text{C}/\text{mg Chl}$ by 65 min) was almost completely accounted for by the net increase resulting from changes in glutamine (Fig. 2) and glutamate (Fig. 3) labeling. The increased labeling of the amino acid fraction can also account for the observed increase in total fixation upon the addition of NH_4^+ under NPR conditions (Fig. 2).

Most amino acid concentrations were unaffected by addition of NH_4^+ . As listed in Table I, the intracellular concentrations of glutamine increased and glutamate decreased in the presence of NH_4^+ . Glycine, serine, and alanine were relatively unaffected by NH_4^+ .

DISCUSSION

In C_3 plants, intracellular ammonia is produced primarily by the oxidative conversion of two glycine molecules to one each of serine, CO_2 , and NH_3 as part of the photorespiratory pathway (8). Lowering the rate of photorespiration by either raising CO_2 or lowering O_2 concentrations should, therefore, decrease the flux and perhaps the intracellular concentration of NH_4^+ . The addition of NH_4^+ extracellularly during NPR fixation might have a greater effect on carbon metabolism than the addition of NH_4^+ under PR conditions. The pronounced effects would be due to the larger absolute change in internal NH_4^+ concentration caused by NH_4^+ addition under NPR conditions.

The amount of nitrogen available to our spinach cells during $^{14}\text{CO}_2$ fixation was minimized by isolation and storage of the cells for over 24 h prior to the experiments in media lacking any nitrogen source. In addition, during part of the storage period, the cells were illuminated in an attempt to increase protein synthesis and amino acid utilization, thereby further decreasing their available nitrogen content. Control assays have indicated that the absence of NO_3^- in the storage media and the light preincubation had only limited effects on $^{14}\text{CO}_2$ fixation and on the regulation by NH_4^+ .

Regulation of Photosynthetic $^{14}\text{CO}_2$ Incorporation by CO_2 and O_2 Concentrations. As expected, rates of photosynthetic $^{14}\text{CO}_2$ fixation increased with increasing CO_2 or decreasing O_2 levels (Fig. 1). The effect

of changing CO_2 from 0.04 to 0.2% was greater than the effect of decreasing O_2 levels from about 20 to 2%. Increasing the O_2 levels decreased the initial fixation rates (before 30 min) but had little effect on incorporation rates after 30 min (Fig. 1). This temporary effect of oxygen on photosynthesis in our spinach cells could be indicative of an efficient refixation of the CO_2 respired by the cells. Efficient refixation of CO_2 , produced by light and/or dark respiration, would be expected to result in an initial lag in the rate of photosynthetic $^{14}\text{CO}_2$ incorporation during the time period necessary to bring the intracellular CO_2 pools up to the specific radioactivity of the $^{14}\text{CO}_2$ supplied. The extent of this lag period would therefore be increased by the higher initial rates of photorespiratory $^{12}\text{CO}_2$ release under conditions of high O_2 (Fig 1).

The labeling of most compounds was proportional to the total $^{14}\text{CO}_2$ incorporation rates. The notable exceptions were compounds related to photorespiratory glycolate metabolism, compounds directly involved in the assimilation of NH_4^+ , and intermediates in the anaplerotic reactions leading to synthesis of C_5 -amino acids.

Fixation under PR conditions increased the total labeling into the glycolate pathway intermediates; glycolate, glyoxylate, glycine, and serine (Fig. 2). Previous studies with leaves of tomato (11), beans (24), and alfalfa (20) have noted that fixation into glycolate and glycine increased with lowered CO_2 or raised O_2 levels. Serine labeling has often been relatively unaffected. The intracellular concentrations of both glycine and serine were higher under PR conditions with our spinach cells (Table I) whereas Lee and Whittingham (11) found that the effect of CO_2 levels on glycine and serine pool sizes varied between experiments. The sensitivity of these pool sizes to experimental treatment might, therefore, explain some of

the variability between the published effects on labeling patterns.

Labeling of hydroxypyruvate and glycerate was not greater under PR conditions (data not shown). The two compounds are intermediates in the photorespiratory conversion of serine to 3-P-glycerate and might be expected to be labeled proportionately to photorespiratory flow. However, both compounds are also intermediates in the alternative pathway for synthesis of serine via 3-P-glycerate (12). The labeling patterns of hydroxypyruvate and glycerate, therefore, indicate that metabolism via 3-P-glycerate in the reverse pathway of photorespiration may be predominant over labeling via photorespiratory glycolate synthesis for these compounds.

Both the intracellular concentration and labeling rate of glutamine were higher under PR conditions (Table I, Fig. 2). Glutamate pool sizes and labeling were both lower (Table I, Fig. 3). It is likely that increases in the rate of photorespiratory NH_4^+ release, coupled with the high affinity of glutamine synthetase for ammonia (15) were responsible for the effects on glutamine and glutamate. Since the photorespiratory nitrogen pathway is cyclic, the level of glutamine would tend to increase at the expense of the glutamate level until the rate of glutamine: oxoglutarate amidotransferase reactions using glutamine would equal the rate of glutamine formation, thus establishing a steady-state. It appears (Figs. 2 and 3) that such a steady-state is not reached in one-half hour, although it is possible that active pools reach a steady-state and glutamine is accumulated elsewhere; for example in vacuole. C_5 skeletons thus removed from the cycle might be replaced by accelerated de novo synthesis of glutamate (see below).

Fixation under conditions favorable to photorespiration also increased the labeling into asparagine (Fig. 2); a compound not directly related to photorespiratory metabolism but directly involved in NH_4^+ assimilation.

Increased asparagine labeling was probably the result of increased activity of glutamine-dependent and/or ammonia-dependent asparagine synthetase (10). Both glutamine and NH_4^+ levels were higher under PR conditions. The intracellular concentration of asparagine, unlike that of glutamine, was unaffected by PR fixation conditions, due, possibly, to the small size of the metabolically active asparagine pools relative to the total intracellular asparagine level.

The overall level of glutamate labeling (Fig. 3) was proportional to the rates of $^{14}\text{CO}_2$ under PR and NPR conditions. This suggests that despite the faster turnover rates of the photorespiratory nitrogen cycle (8) and the drain of carbon into glutamine due to NH_4^+ release, cells were able to synthesize the carbon-skeletons of the C_5 -amino acids at the increased rates necessary. The cellular response to the increased demand for C_5 -skeletons for glutamine synthesis during PR fixation was indicated by the faster labeling rates of phosphoenolpyruvate and malate (Fig. 2). The increased anaplerotic activity is also observed in the equivalent rates of labeling, under both PR and NPR conditions, of 2-oxoglutarate, fumarate, and aspartate (Fig. 3). Increased photorespiratory metabolism, therefore, results in carbon demands which are provided by anaplerotic synthesis of glutamate from 2-oxoglutarate supplied from the tricarboxylic acid cycle and increased phosphoenolpyruvate carboxylase activity.

Effects of Ammonia Addition on $^{14}\text{CO}_2$ Incorporation Under Photorespiratory Conditions. The addition of NH_4^+ under PR conditions increased fixation of ^{14}C into some amino acids and decreased sucrose labeling; observations similar to earlier studies with isolated poppy (16) and spinach (9) mesophyll cells and alfalfa leaf discs (19). The primary effect of NH_4^+ on our spinach cells under PR conditions was to increase glutamine (Fig. 2) and decrease

glutamate (Fig. 3) labeling by equivalent amounts largely due to the corresponding changes in the pools of these amino acids (Table I), but not due to increased de novo synthesis of C₅ skeletons (see discussion below). Glutamine increases have been observed by others (4,7,9,16,19,26). The effect of NH₄⁺ on glutamine and glutamate is likely due to the high affinity of glutamine synthetase for NH₄⁺ (15). The sensitivity of glutamine synthetase to added NH₄⁺ was observed despite the already high internal levels of NH₄⁺ present under PR conditions. Poppy cells also increased glutamine labeling in response to NH₄⁺ addition at air levels (16).

Almost all other effects of NH₄⁺ under PR conditions can be attributed to the primary effects of increasing glutamine synthesis. Increases in glutamine and decreases in glutamate labeling brought about a temporary decrease in the rate of 2-oxoglutarate labeling (Fig. 3). This temporary decrease was probably the result of increased use of oxoglutarate by glutamine:2-oxoglutarate amidotransferase (GOGAT), which has high affinities for both substrates. The drain of carbon through oxoglutarate was also indicated by decreased labeling in malate (Fig. 2) and citrate (Fig. 3). These results suggest that the available activities of the anaplerotic reactions responsible for oxoglutarate formation were inadequate due to low rates of CO₂ fixation under PR conditions. The decreased labeling of glutamate was also probably responsible for decreased proline and γ -aminobutyric acid labeling (data not shown).

The addition of NH₄⁺ under PR conditions also effected photorespiratory carbon metabolism. Increased photorespiratory activity, as indicated by the faster labeling into glycolate, glyoxylate, and glycine after addition of NH₄⁺ (Fig. 2) could be the result of decreased glutamate concentrations (Table I). Glutamate has been shown to inhibit glycolate synthesis and

photorespiration in tobacco (14) and, therefore, decreased glutamate concentrations could have stimulated glycolate pathway labeling under PR conditions. Glyoxylate has also been characterized as an inhibitor of glycolate synthesis (13). Increased labeling of glyoxylate was observed upon addition of NH_4^+ , however, the absolute changes in glyoxylate labeling (about $0.01 \mu\text{g-atom } ^{14}\text{C/mg Chl}$, Fig. 2) were insignificant compared to the observed decreases in glutamate concentrations (about $0.84 \mu\text{mol/mg Chl}$, Table I). Decreases in glutamate concentrations upon addition of NH_4^+ were probably also responsible for the observed decreases in serine labeling (indicative of the metabolically active pool size due to ^{14}C -saturation) (Fig. 2) and serine concentrations (Table I). These observations suggest that serine was being increasingly used for glyoxylate transamination via serine: glyoxylate aminotransferase due to decreased availability of glutamate for glutamate: glyoxylate aminotransferase and increased rates of glycolate pathway metabolism.

Effect of Ammonia Addition on $^{14}\text{CO}_2$ Incorporation Under Nonphoto-respiratory Conditions. The faster photosynthetic rates during NPR fixation were able to overcome the drain of carbon into C_5 amino acids that resulted from the addition of NH_4^+ (Fig. 3), Sucrose labeling which was inhibited upon NH_4^+ addition during PR fixation was unaffected during NPR fixation suggesting that the drain of carbon away from sucrose synthesis was not necessary during NPR fixation due to higher fixation rates. Woo and Calvin (26) also found no effect by NH_4^+ on sucrose labeling in their isolated spinach cells. Their cells were assayed under saturating CO_2 concentrations ($4 \text{ mM NaH}^{14}\text{CO}_3$) and high temperatures. The resulting high rates of $^{14}\text{CO}_2$ fixation were apparently adequate to accommodate increased carbon flow into amino acids due to the presence of NH_4^+ , without decreasing sucrose synthesis.

Many perturbations brought about by NH_4^+ under NPR conditions were similar to but of greater magnitude than those observed during PR fixation. Under NPR conditions, glutamine and glutamate were the compounds most affected by NH_4^+ addition. They were the only amino acids analyzed in which intracellular concentrations were affected by NH_4^+ ; glutamate decreasing and glutamine increasing by about $0.80 \mu\text{mol/mg Chl}$ (Table I).

Under NPR fixation NH_4^+ increased glutamine labeling by a greater amount than under PR conditions. In contrast to the PR experiment, increased glutamine labeling ($1.0 \mu\text{g-atom } ^{14}\text{C/mg Chl}$) could not be quantitatively accounted for in decreased glutamate labeling ($0.25 \mu\text{g-atom } ^{14}\text{C/mg Chl}$) under NPR conditions. The increased production of glutamine via glutamine synthetase was therefore carried out by net flow of carbon into C_5 -skeletons, presumably through anaplerotic formation of 2-oxoglutarate. Under PR conditions, the rate of glutamate labeling decreased to less than half its original value in the presence of NH_4^+ . Oxoglutarate labeling returned to its initial labeling rate after a 10 min lag. Under NPR conditions, however, the labeling of both oxoglutarate and glutamate returned to their initial rates after a short cessation of labeling (Fig. 3). In addition to the help of faster photosynthetic rates in overcoming the carbon drain into glutamine under NPR conditions, glutamate was not needed for transamination of glyoxylate due to low rates of photorespiration. The decrease in glutamate:glyoxylate aminotransferase activity is significant to glutamate metabolism in that the enzyme can use up nearly half the glutamate produced by glutamine:oxoglutarate amidotransferase under PR conditions (8).

The ability of anaplerotic reactions to accommodate the increased demand for carbon skeletons of C_5 -amino acids under NPR conditions is further evidenced by the increased labeling of malate, citrate, fumarate,

phosphoenolpyruvate and aspartate upon addition of NH_4^+ (Figs. 2 and 3). Compounds such as phosphoenolpyruvate and malate, which were more rapidly labeled before NH_4^+ addition under PR conditions were again raised by external NH_4^+ additions under the low intracellular NH_4^+ conditions present during NPR fixation.

Asparagine labeling was slower under NPR fixation due to low internal NH_4^+ levels. Upon addition of NH_4^+ asparagine labeling increased up to the levels present in PR fixing cells (Fig. 2). After 65 min, asparagine labeling was nearly equivalent in PR conditions and under both PR and NPR conditions after addition of NH_4^+ . This suggests that maximal rates of asparagine synthesis had been obtained under these conditions of high internal NH_4^+ content.

The total labeling of amino acids increased with NH_4^+ addition during NPR fixation. Under PR fixation, no net change was observed. In addition to glutamine and asparagine, the labeling of alanine, valine, and aspartate (Fig. 3), and threonine and proline were increased under NPR conditions. Only glutamate labeling decreased. Asparagine and glutamine increases could be explained by their direct dependence on NH_4^+ and/or glutamine concentrations. No increases in the intracellular concentrations of the amino acids, other than glutamate and glutamine, were observed. Increased labeling could be indicative of a faster flow of carbon through some of these amino acids. For example, increased labeling of aspartate after addition of NH_4^+ under NPR conditions (Fig. 3) was probably due to the accelerated flow of carbon through aspartate resulting from increased asparagine formation.

The increases in the labeling of some other amino acids seemed in contrast to the lower glutamate concentrations available after NH_4^+ addition under NPR conditions (Table I). For example, increased labeling of alanine

and valine was observed (Fig. 3). Both are derived from pyruvate, labeled temporarily lower after addition of NH_4^+ (data not shown). Glutamate, even though lower in total intracellular levels (Table I), could be more available for transaminations in certain subcellular locations. Much of the glutamate used for alanine and valine synthesis could be produced in the mitochondria via glutamate dehydrogenase. Glutamate dehydrogenase, despite a low affinity for NH_4^+ (12), could produce glutamate under conditions of high NH_4^+ production or extracellular NH_4^+ addition, particularly with the increased rate of tricarboxylic acid cycle metabolism observed during NPR fixation.

There was no effect of NH_4^+ on the labeling of photorespiration pathway intermediates during NPR fixation. This greatly contrasted the results under PR fixation and was indicative of the low rates of photorespiratory metabolism.

Summary. The perturbation of photosynthetic carbon metabolism by added ammonia, which has been characterized in many biological systems (4,7,9,16,19,26), appears to be similar to a physiological regulatory phenomenon and is not merely a cellular response to the toxic effects of the ion. The effects of photorespiratory NH_4^+ production on metabolism and amino acid concentrations were nearly equivalent to the effects of adding external NH_4^+ to the cells. The overall response of cells to externally supplied NH_4^+ was accentuated in cells whose internal NH_4^+ content was low due to fixation under NPR conditions. Addition of NH_4^+ to cells during photosynthesis therefore just leads to an extreme version of a naturally occurring regulation carried out by plants.

The ammonia response was instantaneous. Not only were cellular responses to externally added NH_4^+ observed within 3 min, but the differences

between PR and NPR fixation brought about by internal NH_4^+ production (such as increased glutamine and asparagine labeling) were also observed in the earliest time points analyzed.

The results indicate that the partitioning of photosynthate away from sucrose upon addition of NH_4^+ only occurs when the photosynthetic rates are inadequate to accommodate the increased synthesis of glutamine, a hypothesis discussed by Larsen *et al.* (9). Sucrose labeling was unaffected in spinach cells fixing $^{14}\text{CO}_2$ at high rates under NPR conditions and in spinach cells fixing $^{14}\text{CO}_2$ with rapid rates under saturating CO_2 (26). We also have observed that faster rates of fixation can accommodate accelerated glutamine formation via increased anaplerotic reactivity without a concomitant decrease in sucrose synthesis.

Acknowledgement. The capable technical assistance of Sherry L. Gee throughout these experiments is most gratefully acknowledged.

This work was supported by the Division of Biological Energy Conversion and Conservation, Office of Basic Energy Sciences, U.S. Department of Energy under Contract No. W-7405-ENG-48, by a Rockefeller Foundation Postdoctoral Fellowship to A.L.L., and by the Danish National Science Research Council 511-15546 to P.O.L.

LITERATURE CITED

1. Arnon DJ 1949 Copper enzymes in isolated chloroplasts. polyphenol-oxidase in Beta vulgaris. Plant Physiol 24:1-15
2. Bachelard HS 1965 Glucose metabolism and α -ketoacids in rat brain and liver in vivo. Nature 205:903-904
3. Givan CV 1979 Metabolic detoxification of ammonia in tissues of higher plants. Phytochemistry 18:375-382
4. Hammel KE, KL Cornwell, JA Bassham 1979 Stimulation of dark CO₂ fixation by ammonia in isolated mesophyll cells of Papaver somniferum L. Plant Cell Physiol 20:1523-1529
5. Jensen RG, JT Bahr 1977 Ribulose 1,5-bisphosphate carboxylase-oxygenase. Ann Rev Plant Physiol 28:379-478
6. Jensen RG, JA Bassham 1966 Photosynthesis by isolated chloroplasts. Proc Nat Acad Sci USA 56:1095-1101
7. Kanazawa T, MR Kirk, JA Bassham 1970 Regulatory effects of ammonia on carbon metabolism in photosynthesizing Chlorella pyrenoidosa. Biochim Biophys Acta 205: 401-408
8. Keys AJ, IF Bird, MJ Cornelius, PJ Lea, RM Wallsgrave, BJ Mifflin 1978 Photorespiratory nitrogen cycle. Nature 275:741-743
9. Larsen, PO, KL Cornwell, SL Gee, JA Bassham 1981 Amino Acid synthesis in photosynthesizing spinach cells. Effects of ammonia on pool sizes and rates of labeling from ¹⁴CO₂. Plant Physiol (in press)
10. Lea PJ, L Fowden 1975 Asparagine metabolism in higher plants. Biochem Physiol Pflanzen 168:3-14

11. Lee RB, CP Whittingham 1975 The influence of partial pressure of carbon dioxide upon carbon metabolism in the tomato leaf. *J Expt Bot* 25:277-287
12. Mifflin BJ, PJ Lea 1977 Amino acid metabolism. *Ann Rev Plant Physiol* 28:299-329
13. Oliver DJ, I Zelitch 1977 Increasing photosynthesis by inhibiting photorespiration with glyoxylate. *Science* 196:1450-1451
14. Oliver DJ, I Zelitch 1977 Metabolic regulation of glycolate synthesis, photorespiration, and net photosynthesis in tobacco by L-glutamate. *Plant Physiol* 59:688-694
15. O'Neal D, KW Joy 1974 Glutamine synthetase of pea leaves. divalent cation effects, substrate specificity, and other properties. *Plant Physiol.* 54:773-779
16. Paul JS, KL Cornwell, JA Bassham 1978 Effects of ammonia on carbon metabolism in photosynthesizing isolated mesophyll cells from Papaver somniferum L. *Planta* 142:49-54
17. Platt SG, JA Bassham 1977 Separation of ^{14}C -labeled glycolate pathway metabolites from higher plant photosynthate. *J Chromatography* 133:396-401
18. Platt, SG, Z Plaut, JA Bassham 1976 Analysis of steady state photosynthesis in alfalfa leaves. *Plant Physiol* 57:69-73
19. Platt, SG, Z Plaut, JA Bassham 1977 Ammonia regulation of carbon metabolism in photosynthesizing leaf discs. *Plant Physiol* 60:739-742
20. Platt, SG, Z Plaut, JA Bassham 1977 Steady-state photosynthesis in alfalfa leaflets. effects of carbon dioxide concentrations. *Plant Physiol* 60:230-234

21. Snyder FW, NE Tolbert 1974 Effect of CO₂ concentration on glycine and serine formation during photorespiration. *Plant Physiol* 53:514-515
22. Tolbert NE 1979 Glycolate metabolism by higher plants and algae. In M Gibbs, E Latzko eds, *Photosynthesis II. Encyclopedia of Plant Physiology*, Springer Verlag, New York, Vol 6, pp 338-352
23. Viil J, T Pärnik 1974 Influence of oxygen upon photosynthetic carbon metabolism at high CO₂ concentrations and saturating irradiance. *Photosynthetica* 8:208-215
24. Voskresenskaya NP, YA Wiil, GS Grishina, TR Parnik 1970 Effect of oxygen concentration and light intensity on the distribution of labelled carbon in photosynthesis products in bean plants. *Photosynthetica* 4:1-8
25. Wallsgrave RM, AJ Keys, IF Bird, MJ Cornelius, PJ Lea, BJ Mifflin 1980 The location of glutamine synthetase in leaf cells and its role in the reassimilation of ammonia released in photorespiration. *J Exp Bot* 31:1005-1007
26. Woo KC, DT Canvin 1980 Effect of ammonia on photosynthetic carbon fixation in isolated spinach leaf cells. *Can J Bot* 58:505-510

Table I. Effect of Ammonia on Free Amino Acid Concentrations Under Photorespiratory and Nonphotorespiratory Conditions

The intracellular concentrations of [^{14}C]amino acids were determined by analyzing their [^3H]dansyl chloride derivatives as described under "Materials and Methods". The values shown are the final concentrations calculated after 65 min of $^{14}\text{CO}_2$ fixation; 35 min after either NH_4^+ or H_2O was added to the cell suspensions. Conditions for $^{14}\text{CO}_2$ fixation were the same as those described in Fig. 2.

	INTRACELLULAR CONCENTRATIONS			
	Photorespiratory		Nonphotorespiratory	
	Conditions		Conditions	
	H_2O	NH_4^+	H_2O	NH_4^+
	$\mu\text{mol/mg Chl}$			
Gln	0.253	0.754	0.134	1.02
Glu	1.74	0.898	2.00	1.16
Gly	1.06	1.29	0.584	0.488
Ser	0.537	0.399	0.350	0.379
Ala	0.503	0.542	1.04	1.32

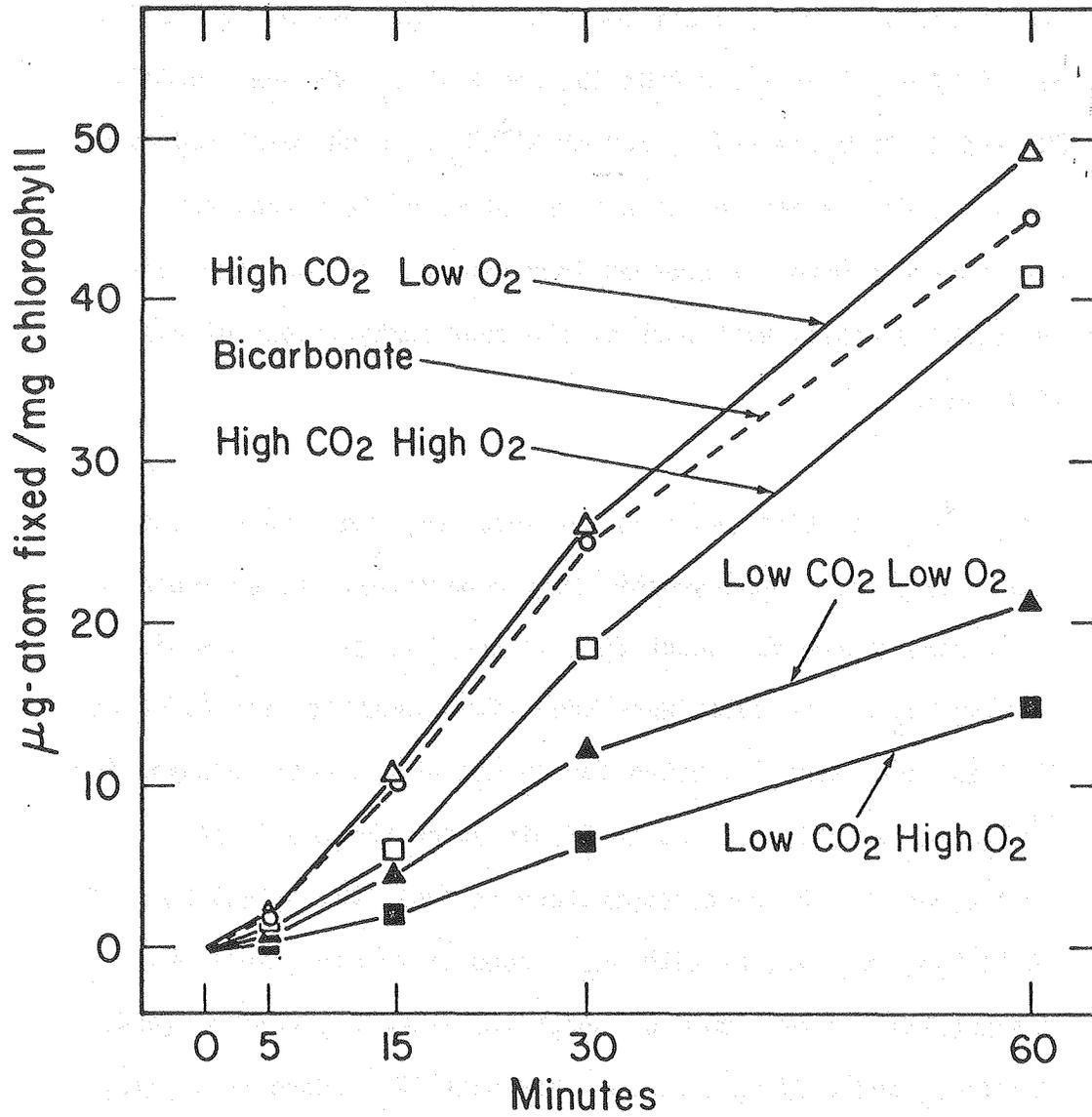
FIGURE LEGENDS

Fig. 1. Total $^{14}\text{CO}_2$ fixation by spinach cells under various atmospheres. Fixation was at 22 C and $650 \mu\text{E m}^{-2} \text{sec}^{-1}$ under the following conditions: Δ — Δ , 0.21% CO_2 and 2.3% O_2 , \square — \square , 0.20% CO_2 and 20.0% O_2 ; \blacktriangle — \blacktriangle , 0.040% CO_2 and 2.4% O_2 ; \blacksquare — \blacksquare , 0.038% CO_2 and 20.2% O_2 ; \circ --- \circ , 8.0 mM $\text{H}^{14}\text{CO}_3^-$. Each point represents duplicate determinations with a variation of less than 20%. Bicarbonate controls represent four separate assays, one carried out simultaneously with each of the four combinations of gas mixtures.

Fig. 2 Total $^{14}\text{CO}_2$ fixation and fixation into compounds under photorespiratory and nonphotorespiratory conditions. After isolation, cells were stored for about 22 h in the dark at 6 C in media lacking NO_3^- . The cells were then light-incubated for 4.5 h at 17 C and then dark incubated for 30 min at 6 C until assayed for $^{14}\text{CO}_2$ incorporation for up to 65 min under the following conditions: \circ --- \circ , photorespiratory control (PR, 0.048% CO_2 and 20.4% O_2); Δ --- Δ , PR with NH_4^+ added at 33 min (final NH_4^+ concentration 1 mM); \bullet — \bullet nonphotorespiratory control (NPR, 0.23% CO_2 and 2.8% O_2); \blacktriangle — \blacktriangle , NPR with NH_4^+ added at 33 min; \star --- \star , $\text{H}^{14}\text{CO}_3^-$ control.

Fig. 3. Fixation from $^{14}\text{CO}_2$ into compounds under photorespiratory and nonphotorespiratory conditions. Conditions were as described in Fig. 2.

Figure 1.



XBL 806-4239

Figure 2.

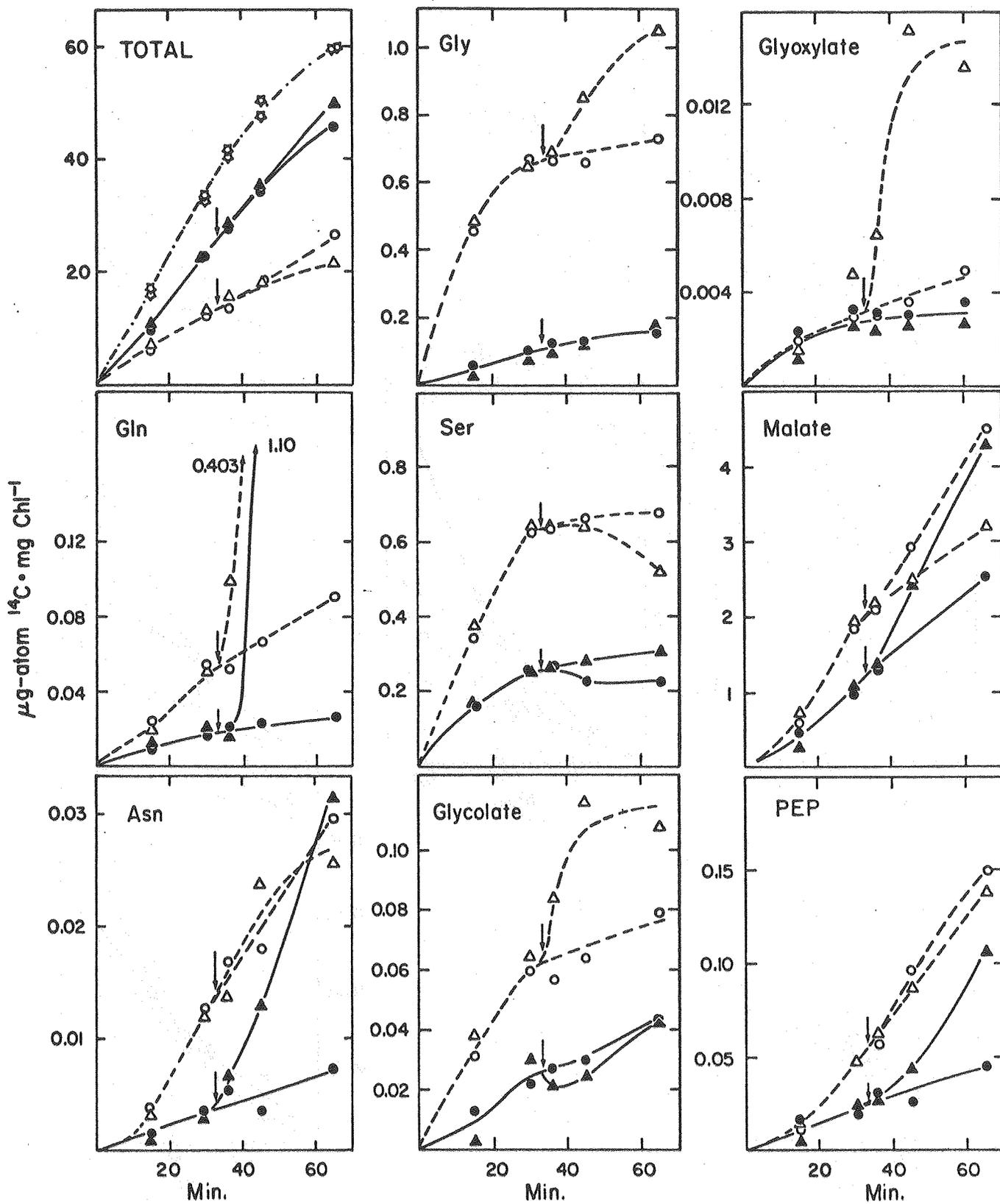


Figure 3.

