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RELATIONSHIPS BETWEEN NITROGEN METABOLISM AND PHOTOSYNTHESIS

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

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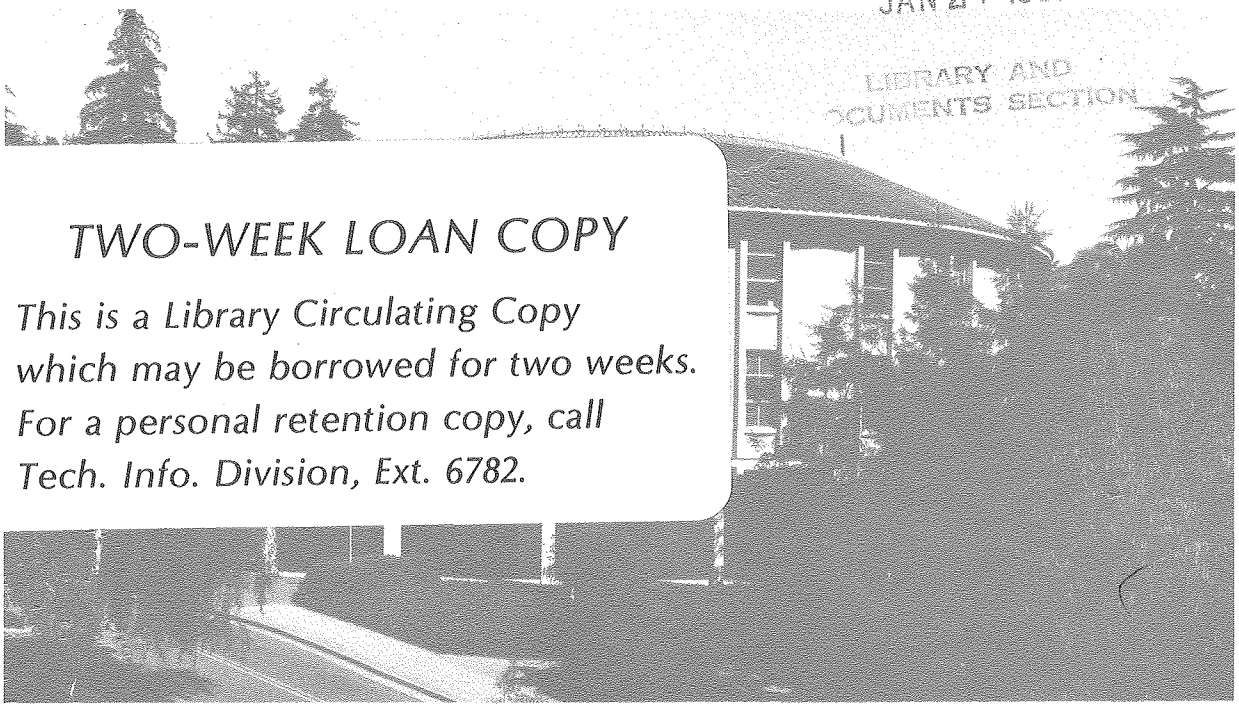

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James A. Bassham, Peder O. Larsen*,

Arthur L. Lawyer and Karen L. Cornwell

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

*Chemistry Department
Royal Veterinary and Agricultural
University
40, Thorvaldsensvej - DK 1871
Copenhagen V. DENMARK

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INTRODUCTION

Photosynthetic green cells generate reducing power from the oxidation of water to O_2, and use the reducing power for the reduction of CO_2, nitrate and sulfate. The principal products of green cells then are oxygen, sucrose and other carbon compounds, amino groups of amino acids, and sulfhydryl groups of acids.

Even if the biosynthetic pathways leading to these various organic compounds were separate after the initial act of reduction, regulation would be required to allocate the reducing power and appropriate organic precursors to the required biosynthetic pathways. Because of photorespiration and its consequent required carbon pathways for the recovery of reduced carbon, flows of nitrogen and carbon are further intertwined at the conversions of glycolate, glyoxylate, glycine, glutamate, serine, and hydroxypyruvate. The glycolate pathway of photorespiration operates between the chloroplasts, peroxisomes, mitochondria, and cytoplasm. Thus, various specific transport mechanisms are involved, and a careful regulation of these transport mechanisms is required.

There is also the question of biosynthetic capabilities of the various organelles and compartments in green cells. For example, which keto acid skeletons can the chloroplast make for itself from CO_2 for amino acid synthesis, and which must be imported?

These issues are being addressed by papers that precede and follow this one, so that considerable overlap is

expected. At the same time, the range of issues is so broad that it is doubtful that one person could hope to deal adequately with them all. This discussion will be limited, therefore, to three issues under which investigators in our laboratory can provide some information. These issues are first, the keto acid and amino acid biosynthetic capabilities of the chloroplast; second, the bidirectionality of the glycine-serine interconversion associated with photorespiration or its absence; and third, the complex issue of the regulation of carbon flow from photosynthesis to biosynthesis of starch and sucrose as compared with synthesis of amino acids in the photosynthetic cell. In particular, we will discuss the profound regulatory effects of the addition of low levels of ammonium ion to cell media. These were seen first with *Chlorella pyrenoidosa*, and then with spinach leaf discs, poppy cells and now with isolated spinach leaf cells.

CHLOROPLAST SYNTHESIS OF KETO ACIDS AND AMINO ACIDS

The pathway of nitrogen assimilation in plants and especially in chloroplasts, has been reviewed recently by Lea and Miflin (1), who in 1974 first discovered the key enzyme in glutamate synthesis in chloroplasts (2). This enzyme, glutamine-oxyglutarate aminotransferase (GOGAT) utilizes reduced ferredoxin from the light reactions in the chloroplast to bring about the conversion of one molecule of oxoglutarate and one molecule of glutamine to two molecules of glutamate. In green cells this reaction appears to occur mostly, if not exclusively, in the chloroplast. The synthesis of glutamine, requiring ATP, ammonia, and glutamic acid and mediated by glutamine synthetase, occurs partly in the chloroplast and partly in the cytoplasm. In pea cells about half of the leaf glutamine synthetase is located in the chloroplast with the remainder in the cytoplasm (3).

The synthesis of glutamate in the chloroplast may occur in the green cell on a scale far in excess of the needs of biosynthetic pathways for glutamate. Keys et al. (4)

proposed that there is a photorespiratory nitrogen cycle required to recapture the ammonia liberated by the conversion of two glycine molecules to one serine molecule in that part of the glycolate pathway which occurs in the mitochondria. The ammonia is recaptured by conversion of glutamate to glutamine with ATP in the glutamine synthetase reaction. Keys et al. believe that this ammonia incorporation occurs in the cytoplasm since it would be there that ammonia first encounters glutamine synthetase activity. The glutamate for glutamine synthesis is formed by the action of GOGAT in the chloroplast (Fig. 1). The other glutamate formed by the GOGAT reaction is utilized in the peroxisome to provide an amino group for transamination of glyoxylate to glycine, releasing oxoglutarate in the process which is then recycled to the chloroplasts for the GOGAT reaction.

Although the major amount of glutamine synthesis may be occurring via the photorespiratory nitrogen cycle, any net synthesis of glutamate for subsequent biosynthesis, including protein synthesis, requires that there be a net conversion of oxoglutarate to glutamate. Presumably oxoglutarate is derived from anaplerotic reactions occurring in the mitochondria. As we shall discuss later, the flow of carbon from photosynthesis into the TCA cycle in the mitochondria can be stimulated by the addition of small amounts of ammonia external to the photosynthetic cell. In the light both the conversion of phosphoenylpyruvic acid (PEP) to pyruvate and the carboxylation of PEP to give oxaloacetate are increased in the presence of added ammonium ion. Consequently, an increased supply of ketoacid skeletons becomes available in the form of oxoglutarate for the synthesis of glutamate.

Mitchell and Stocking (5) have shown that isolated chloroplasts can assimilate ammonia into glutamine when provided with glutamate. Conversion of oxoglutarate to glutamate primarily occurs via GOGAT while ammonia incorporation occurs via glutamine synthetase (1). Glutamate dehydrogenases are present in plant cell chloroplasts and

mitochondria, but are considered to be involved only in degradative reactions because of their high K_m values for ammonia (see ref. 1, for example).

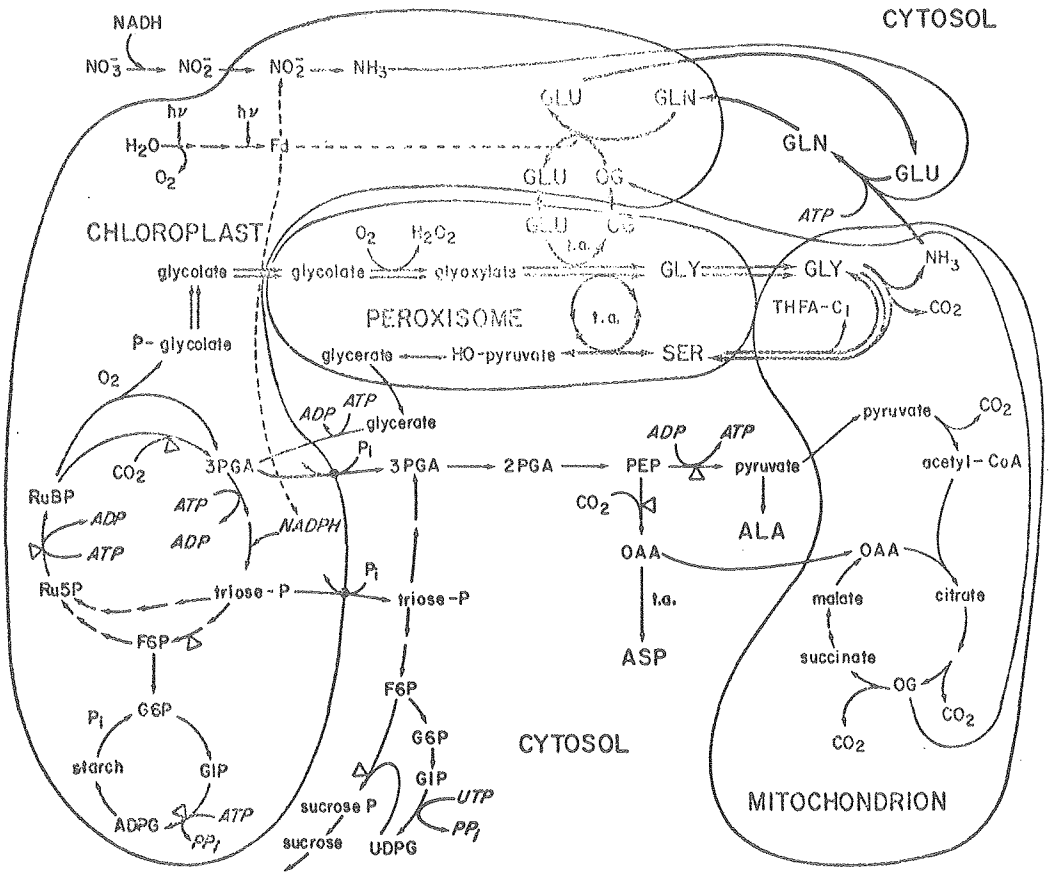

Figure 1. Carbon and nitrogen photosynthetic and photorespiration pathways. Abbreviations: RuBP, ribulose-1,5-bisphosphate; Ru5P, ribulose-5-phosphate; PGA, 3-phosphoglycerate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; ADPG, adenosine diphosphoglucose; UDPG, uridine diphosphoglucose; PEP, phosphoenolpyruvic acid; OG, 2-oxoglutaric acid; standard amino acid abbreviations. The reactions of the photorespiratory nitrogen cycle (adapted from Keys et al., ref. 4) are indictaed by heavier lines.

It is generally accepted that net synthesis of glutamate and glutamine in chloroplasts depends on five-carbon ketoacid skeletons derived from the mitochondria. Similarly, there is abundant evidence that in both C_4 and C_3 plants, oxaloacetate formed by PEP carboxylation in the

cytoplasm can be utilized in the chloroplast for the synthesis of aspartic acid and asparagine. These important four- and five-carbon amino acids can then serve as substrates for the synthesis of a variety of other amino acids in the chloroplasts. Given these possibilities, what is the relative importance of the biosynthesis of amino acids in the chloroplasts as compared with that in the green cell as a whole? There has been much discussion of the biochemical autonomy of green cell chloroplasts (see for example review by Givan and Leach (6)). The generally accepted view appears to be that chloroplasts can form most if not all required amino acids from the appropriate ketoacid skeletons, but that some of these ketoacid skeletons may have to be imported from other parts of the cell in a manner similar to that just discussed for four- and five-carbon skeletons. There has been some interest, therefore, in the capability of isolated leaf cell chloroplasts to carry out the synthesis of amino acids from $^{14}CO_2$ during photosynthesis.

Relatively crude chloroplast preparations such as those used by Jensen and Bassham (7) produced very little labeled amino acids compared to the major products of $^{14}CO_2$ photosynthesis. This absence or near absence of appreciable amounts of labeled amino acids from photosynthesizing crude chloroplast preparations has led us in the past to believe that amino acid biosynthesis in chloroplasts is indeed heavily dependent on external supply of three-, four-, and five-carbon ketoacid skeletons. Kirk and Leach (8), investigating amino acid biosynthesis by isolated chloroplasts during photosynthesis, found significant labeling of aspartate, alanine, glycine, serine, threonine and lysine, with the greater part of the label being in aspartate. More recently, Buchholz et al. (9) investigated amino acid biosynthesis during isolated chloroplast photosynthesis using unpurified intact chloroplasts as well as chloroplasts purified by the method of Larsson and Albertson (10). These authors found considerably more incorporation of ^{14}C into amino acids in their "unpurified" chloroplasts than we

normally do, but the amount of incorporation of ^{14}C into amino acids was reduced to very low levels in the purified chloroplast. Of that found, the largest fixation was into alanine with lesser amounts into the four- and five-carbon amino acids, and serine and glycine. Addition of leaf peroxisomes restored glycine synthesis and addition of leaf mitochondria resulted in serine synthesis. On the other hand, the rate of aromatic amino acid synthesis in the purified chloroplast was not enhanced by the addition of other organelles. This supports the proposition that the shikimic acid pathway of biosynthesis of aromatic compounds is mainly located in the chloroplast (11).

Recently we reinvestigated the question of isolated chloroplast competence for amino acid biosynthesis. The results are preliminary and generally confirm existing views as just stated, but there are several interesting aspects to the recent results. The spinach chloroplasts were prepared according to the Jensen and Bassham method (7) but were washed twice with buffer solution to remove most cytoplasmic impurities. Nevertheless, this procedure leaves the chloroplast contaminated with peroxisomes. This system, while not representing the situation of pure chloroplasts, was interesting particularly when the experiment was carried out as it was with the steady-state apparatus (12) which provided for $^{14}CO_2$ fixation at air levels of CO_2 and O_2. Parallel experiments were carried out with isolated whole spinach cells also photosynthesizing with air levels of CO_2 and O_2. Various additions of nitrite, oxoglutarate, glutamate and glutamine either alone or in combination were made to the isolated chloroplasts

The details of the investigations will be published elsewhere, but a few points are worth mentioning.

The chloroplasts contaminated with peroxisomes incorporated about 20% as much ^{14}C into glycine as did the whole cells (Table 1). Addition of 0.1 mM glutamate increased glycine formation in the chloroplast preparations to 140% of that of whole cells, while addition of 0.1 mM oxoglutarate

decreased it to 13%. Addition of both oxoglutarate and glutamine, which would be expected to produce glutamate by the GOGAT reaction, increased glycine formation to 31%. All of these effects are confirmatory of the scheme of nitrogen cycling shown in Fig. 1. It is interesting that this much glycine synthesis can be accomplished by isolated chloroplasts contaminated with peroxisomes. One might expect considerable dilution of the glycolate produced by photorespiration if it goes into the suspending medium before finding its way into the peroxisomes. Peroxisomes in green leaf cells are, in fact, frequently seen to be tightly appressed to chloroplasts (see, for example, Frederick and Newcomb (13)). It certainly is possible that in leaf cells there could be a direct flow of glycolate from chloroplasts into peroxisomes.

Table 1. Incorporation of ^{14}C into amino acids in isolated spinach Chloroplasts (+ peroxisome contamination) compared with whole isolated spinach cells (expressed as percent).

Medium	Glycine	Alanine	Serine	Valine	Phenylalanine
Control	20	2.8	3.5	110	18
+ glutamate	140	7.6	3.3	90	29
+ oxoglutarate	13	1.2	0.4	--	14
+ glutamine	25	6.4	1.3	120	5.4
+ NO_2^-	11	3.9	1.4	60	6.0
+ oxoglutarate + glutamine	31	2.8	2.3	--	24

Chloroplasts were isolated by procedure of Jensen and Bassham (7). Chloroplast photosynthesis with $^{14}CO_2$ was for 40 min. Spinach cells were isolated by a modification of method of Paul and Bassham (24) and photosynthesized for 30 min (Larsen et al., to be published).

The amount of ^{14}C incorporated into most other major amino acids in the chloroplast preparations was in the range of a few percent compared with whole cell incorporation. Alanine and serine serve as examples of this incorporation (Table 1). Whether these low amounts of biosynthesis from $^{14}CO_2$ of common amino acids by isolated chloroplasts represent chloroplastic synthesis or synthesis due to contamination cannot be said from these experiments, but there is other evidence in the literature to suggest that small amounts of these amino acids can in fact be synthesized by chloroplasts (8). In contrast to these small amounts are the relatively large amounts of valine and phenylalanine found with the crude preparations of chloroplasts. As already mentioned, phenylalanine may be labeled by synthesis via the shikimic acid pathway which occurs in chloroplasts.

The synthesis of almost the entire cellular requirement of valine by isolated chloroplasts (Table 1) provides further strong evidence for the presence in chloroplasts of adequate levels of pyruvate and "active acetaldehyde" derived from pyruvate since these are the precursors in valine biosynthesis. The fact that only a small part of the cell's alanine synthesis occurs in the chloroplasts must therefore be due to the presence of active synthesis of alanine outside the chloroplast rather than to the absence of pyruvate in the chloroplast.

Addition of oxoglutarate had a generally negative effect on synthesis of amino acids by the isolated chloroplasts, presumably by shifting the equilibrium of glutamate aminotransferases in the direction of glutamate formation. By the same token, added glutamate greatly increased the synthesis of some amino acids, notably glycine.

In summary, it appears that chloroplasts do indeed have a nearly complete capability for the synthesis of all amino acids, provided only that there is an external supply of oxoglutarate and oxaloacetate. The chloroplasts do apparently play a prominent role in the synthesis of certain amino acids for the cell. These include amino acids such as

phenylalanine and tyrosine that are formed by the shikimic acid pathway and valine which is formed from pyruvate. Given the apparently intimate association between peroxisomes and chloroplasts, it would not be surprising if the chloroplasts obtained their complement of glycine from the peroxisomes, but the possibility of chloroplast synthesis of small amounts of glycine cannot be excluded. It should be noted that, in general, developed green leaf cells devote only a relatively small proportion of their total photosynthate to the synthesis of amino acids for the purpose of subsequent protein, pigment, and nucleotide biosynthesis. The major amino acid biosynthetic activity of green cells has to do with the binding of the ammonia which is formed by the reduction of nitrate to nitrite in the cytoplasm and nitrite to ammonia in the chloroplast. This binding results in the synthesis of glutamate, glutamine, and perhaps asparagine. Amino-containing compounds are then translocated to other organs of the plant.

BIDIRECTIONALITY OF GLYCINE-SERINE INTERCONVERSION

Formation of glycine and serine in C_3 plants is generally attributed to the glycolate pathway sequence: glycolate to glycine to serine to glycerate, believed to result from the process of photorespiration as shown in Fig. 1 (14,15). An alternative pathway is the reverse sequence from 3-phosphoglycerate (PGA) to glycerate to serine to glycine (16). The glycolate to glycerate pathway is expected under conditions of photorespiration such as exist with C_3 plants in air with bright illumination and warm temperatures. The reverse pathway is generally expected to occur under conditions where there is little or no photorespiration such as might exist with elevated CO_2 concentration, for example.

Platt et al. (17) found that in alfalfa leaflets photosynthesizing under steady-state conditions, elevated concentrations of $^{14}CO_2$ greatly decreased the amount of label in glycine and glycolate, as would be expected with diminished photorespiration. They also found, however, that pool sizes

of serine and glycerate reflected the size of the PGA pool under steady state conditions with either air levels or high levels of CO_2. While recognizing that these results may be consistent with the reversibility of the pathway, they suggested that the parallel between serine and PGA pool sizes under both conditions might indicate a predominent formation of serine from PGA even with air levels.

In view of this and other uncertainties with respect to the magnitude and direction of flow between serine and glycine, it was fortunate that Lawyer and Zelitch (18) found that with tobacco callus tissue, glycine hydroxamate acts as a rather specific inhibitor of the conversion of glycine to serine and CO_2 in the glycolate pathway. This inhibitor has now been used by Lawyer et al. (to be published) to investigate the bidirectionality of flow between glycine and serine in isolated spinach cells photosynthesizing under steady-state conditions with several levels of O_2 and CO_2. For comparison, experiments were also carried out with 7 mM bicarbonate (saturating CO_2). As expected (Fig. 2), the highest rates of spinach cell photosynthesis were obtained with high CO_2 and low O_2 as well as with bicarbonate in air. Nearly as high rates were obtained with high CO_2 and high O_2, although there was a lag at the beginning of the fixation period. Much lower rates of photosynthesis were seen with air level CO_2 and with low O_2. Similarly, low rates of photosynthesis were seen with air level CO_2 and with high O_2, and in this case there was again a lag at the beginning of the fixation period. Thus the effect of CO_2 pressure is on the long-term rate of photosynthesis whereas the effect of oxygen pressue appears mainly in the time required to achieve the full rate of steady-state photosynthesis.

In parallel with each of the experiments shown in Fig. 2, duplicate flasks contained spinach cells to which up to 3.8 mM glycine hydroxamate had been added. In all cases the cells were killed and analyzed for levels of amino acids and other metabolites by two-dimensional paper chromatography following a preliminary ion exchange separation of neutral,

acidic, and basic amino acids from other components. As expected (Table 2), the level of labeled glycine was highest in the control with low CO_2 and high O_2 (air, photorespiratory conditions), and this high level was further increased in the flask with added glycine hydroxamate. In both intermediate cases (low CO_2, low O_2 and high CO_2, high O_2) addition of glycine hydroxamate also increased the level of glycine indicating flow in the direction from glycine to serine. Under the condition where the least photorespiration would be expected (high CO_2, low O_2) addition of glycine hydroxamate decreased the level of labeled glycine by 73%.

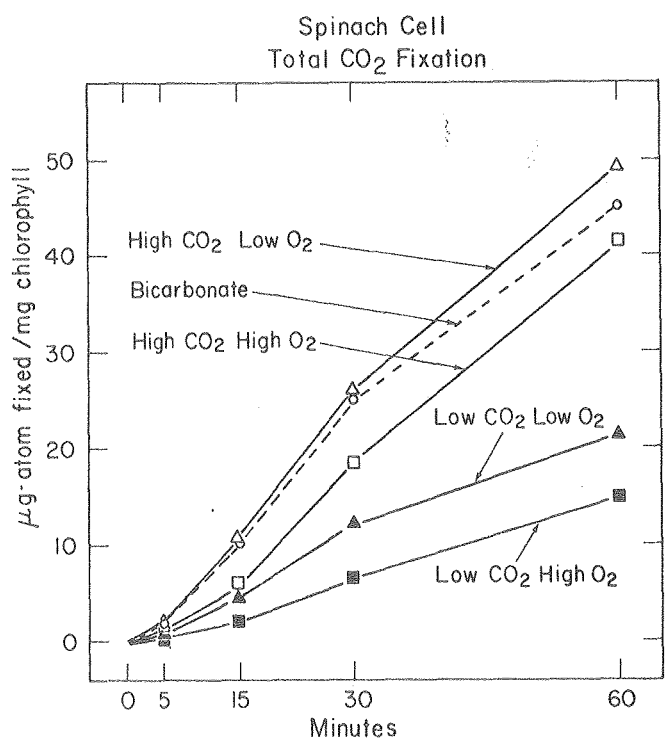

Figure 2. Total $^{14}CO_2$ fixation into isolated spinach mesophyll cells at 22°C and 580 $\mu E \cdot m^{-1} \cdot sec^{-1}$ under the following conditions: △, 0.21% CO_2 and 2.3% O_2; □, 0.04% CO_2 and 2.4% O_2; ▲, 0.20% CO_2 and 20.0% O_2; ■, 0.038% CO_2 and 20.2% O_2; ○, 8 mM $H^{14}CO_3^-$.

In this case, where we would expect glycine to be formed from serine, it appears that glycine hydroxamate inhibits serine to glycine conversion. Not all steps in the serine-

glycine interconversions are truly reversible, but it appears that some key step in both directions is inhibited by glycine hydroxamate. In the two intermediate cases (low CO_2, low O_2, and high CO_2, high O_2) despite very different overall photosynthetic rates (Fig. 1), addition of glycine hydroxamate increased the level of glycine by about 60% suggesting that in these cases, the net flow of carbon was from glycine to serine.

Table 2. ^{14}C labeling of glycine in isolated spinach cells after 1 h photosynthesis with $^{14}CO_2$ - effect of glycine hydroxamate

	μg-atoms $^{14}C \cdot mg\ Chl^{-1} \cdot hr^{-1}$			
	High CO_2 Low O_2	Low CO_2 Low O_2	High CO_2 High O_2	Low CO_2 High O_2
Control	0.12	0.12	0.061	0.29
+ glycine hydroxamate	0.032	0.20	0.097	0.35

Isolated spinach mesophyll cells were allowed to photosynthesize with $^{14}CO_2$ with or without 3.8 mM glycine hydroxamate in the medium at 22 C and 580 $\mu E \cdot m^{-2} \cdot sec^{-1}$. The four gas mixtures were: 0.20% CO_2 and 2.3% O_2; 0.035% CO_2 and 2.6% O_2; 0.19% CO_2 and 18.8% O_2; and 0.044% CO_2 and 20% O_2. Total CO_2 fixation rates ($\mu g\text{-atoms} \cdot mg\ Chl^{-1} \cdot hr^{-1}$) were 20.1 and 23.5; 15.4 and 15.3; 26.0 and 21.1, and 16.9 and 9.9 without and with glycine hydroxamate, respectively.

The changes in serine induced by the inhibitor are more complex (Table 3). Under conditions of low photorespiration, the level of serine was unchanged. Under all of the other three conditions, the level of serine was increased significantly. The explanation for this lies in the fact that the glycine hydroxamate, in blocking the flow of carbon from glycine to serine, also blocks the release of ammonia. As a consequence of this blockage, glycine increases dramatically as already mentioned. This increase in glycine

concentration leads to a large shift in the equilibrium mediated by serine-glyoxylate aminotransferase in the direction of serine and glyoxylate formation. This glyoxylate plus new glyoxylate coming from photorespiration uses up more glutamic acid to make more glycine and oxoglutaric acid so that the net result is the conversion of amino groups from glutamate to the pools of serine and glycine. As a consequence of the decreased glutamate pool, considerably less fixation of carbon is seen in amino acids other than glycine and serine.

Table 3. ^{14}C labeling of serine in isolated spinach cells after 1 h photosynthesis with $^{14}CO_2$ - effect of glycine hydroxamate

	μg atoms $^{14}C \cdot mg\ Chl^{-1} \cdot hr^{-1}$			
	High CO_2 Low O_2	Low CO_2 Low O_2	High CO_2 High O_2	Low CO_2 High O_2
Control	0.15	0.21	0.17	0.32
+ glycine hydroxamate	0.13	0.57	0.39	0.98

Conditions were the same as described for Table 2.

Apparently, the pathway from PGA to glycerate to hydroxypyruvate can supply carbon to serine under these conditions when the flow of carbon from glycine to serine is blocked. The level of glycerate was highest in the photorespiratory condition (Table 4, low CO_2 and high O_2), and was decreased 60% by the presence of glycine hydroxamate. In the intermediate conditions glycerate levels were lower, but nonetheless were decreased upon addition of glycine hydroxamate, presumably due to conversion to hydroxypyruvate

which in turn is converted to serine as a consequence of glycine buildup.

Table 4. ^{14}C labeling of glycerate in isolated spinach cells after 1 hr photosynthesis with $^{14}CO_2$ - effect of glycine hydroxamate

	μg atoms $^{14}C \cdot mg\ Chl^{-1} \cdot hr^{-1}$			
	High CO_2 Low O_2	Low CO_2 Low O_2	High CO_2 High O_2	Low CO_2 High O_2
Control	0.070	0.066	0.097	0.136
+ glycine hydroxamate	0.058	0.056	0.048	0.027

Conditions were the same as described for Table 2.

These results add further support for the already well accepted pathway of glycine to serine conversion during conditions of photorespiration. They also support the existence of the reverse pathway from serine to glycine under conditions where photorespiratory formation of glycine is minimal. In terms of overall steady state photosynthesis rate, CO_2 concentration appears to be considerably more important than O_2 concentration in the ranges tested. With 0.2% CO_2, the rate of photosynthesis during the second half hour was as high with 20% O_2 as it was with 2% O_2 despite the fact that inhibitor data indicate a photorespiratory formation of glycine at 20% O_2 though at a much lower level than with air. Even with air levels of CO_2, the long-term rate of photosynthesis is unaffected by the difference between 20% O_2 and 2% O_2 at least with these isolated spinach cell preparations. Conceivably, cells exhibiting higher photosynthetic rates could show a greater dependency on oxygen concentration. In any event, the present

experiments clearly demonstrate the bidirectionality of glycine-serine interconversion.

REGULATION OF CARBON FLOW FROM PHOTOSYNTHESIS TO AMINO ACID BIOSYNTHESIS

Since mature leaf cells are primarily devoted to the synthesis of sucrose for export to the rest of the plant, the direct synthesis of amino acids and other cellular constituents is normally limited in amount, though of great importance to the cell's metabolism. Because of this and other reasons, it is somewhat easier to examine regulation of photosynthetic carbon metabolism in unicellular algae which are undergoing a complete cell cycling through growth and division. Ten years ago, Kanazawa et al. (19) established the pattern of change in secondary metabolism in synchronously growing Chlorella pyrenoidosa during the cycling of cells from recently divided cells through the growth stage, predivision and division. Profound shifts in the allocation of carbon to sucrose, proteins and amino acids and nucleic acids were seen over the course of the cell cycle. Accompanying the shifts were changes in the rate of reduction of nitrate to amino acids (20). Since these changes in the rate of reduction of nitrates to ammonia resulted in changes in supplies of ammonia, the effects of ammonia itself on the regulation of carbon metabolism were investigated (21,22).

Not surprisingly, the addition of small amounts of ammonia (1 mM) to the medium of the photosynthesizing Chlorella resulted in considerable stimulation of the synthesis of certain amino acids, especially glutamine, glutamic acid, alanine, and aspartate. Further investigation revealed that this stimulation was not the result solely of an increased supply of ammonia for amino groups, but was in fact a consequence of an accelerated flow of carbon from photosynthesis into intermediates of the TCA cycle such as citrate with a concurrent decrease in the flow of carbon into starch and especially sucrose. Besides the increased

flow of carbon into amino acids, there was an increased rate of flow of carbon from photosynthesis into the synthesis of fatty materials. A principal cause of this latter increase was found to be a stimulation of the conversion of phosphoenolpyruvic acid (PEP) to pyruvic acid, presumably through a stimulation of the activity of pyruvate kinase. Since increased synthesis of amino acids derived from the TCA cycle requires anaplerotic reactions, it was assumed that carboxylation of PEP to produce oxaloacetic acid also increased (21,22).

Investigation of the effects of adding ammonia to respiring Chlorella pyrenoidosa (in the dark following a period of photosynthesis to label the carbon intermediates) showed that the effects of ammonia could be seen equally well in the dark and were generally interpretable as effects occurring outside of the chloroplast and in the cytoplasm. When the light was turned off following a period of photosynthesis by Chlorella which had been starved for nitrogen, the formation of sucrose stopped, but sucrose was not utilized for respiration, whereas the store of starch, presumably in the chloroplast, became utilized for respiratory reactions as indicated by a steady decline in the level of labeled material. When the light was turned off, there was a temporary drop in the level of ATP, but the level was then restored to the same level in the dark as in the light, presumably by the operation of oxidative phosphorylation. The light to dark change did not in this condition result in any increase in the flow of carbon into amino acid biosynthesis (22).

When ammonia was subsequently added to the medium in the dark, the rate of starch utilization remained constant and the level of sucrose began immediately to decline and continued to do so at a constant rate. Thus, both sucrose and starch were now contributing carbon to respiratory and biosynthetic metabolism in the cells. Also, with the addition of ammonium ion, the steady-state level of ATP declined momentarily and then rose again but to a lower steady-

state level than before. This is surprising because ammonium ion, while known to have an effect on photophosphorylation in the chloroplast, would not be expected to affect oxidative phosphorylation in the mitochondria. With the addition of ammonia to the respiring Chlorella, the steady-state level of PEP dropped and the steady state level of pyruvate rose, clearly indicating an acceleration of the pyruvate kinase reaction. Other increases were seen in the labeling of citrate and the amino acids, glutamate, glutamine, alanine and some others. Thus, it was clear that the presence of 1 mM ammonium ion in the medium of the Chlorella had produced a profound shift in metabolism from sucrose to amino acid and fatty acid biosynthesis in the light and an accelerated utilization of sucrose in the dark accompanied by an increased amino acid synthesis. The regulatory mechanism for pyruvate kinase is not known but could be related to the change in the levels of ATP (22).

Although the primary photosynthetic reactions and their regulation and chloroplast metabolism may be rather similar between Chlorella pyrenoidosa and green cells of higher plants, there is no reason to think that subsequent biosynthetic pathways and their regulation in the cytoplasm and plant cell organelles would be the same, particularly considering the fact that unicellular algae apparently do not exhibit the same glycolate pathway for conversion of glycine to serine with its consequent large release of ammonia and recycling of ammonia within the cell. In the case of the unicellular algae Chlorella, it did appear that the reduction of nitrate to nitrite and the nitrite to ammonia, and the consequent rate of ammonia formation might play a rather significant role in the overall regulation of carbon metabolism. It was of interest to see what effect, if any, small amounts of ammonium ion added to leaves or cells of leaves in higher plants might have on the regulation of carbon metabolism in such cells.

When alfalfa leaf discs were floated on a buffer containing ammonium chloride (5 mM), photosynthesis with

$^{14}CO_2$ produced more labeled amino acid and less sucrose than in the control disc without ammonia (23). The level of pyruvate increased and that of PEP decreased. These and other changes in labeling of secondary metabolites led to the conclusion that pyruvate kinase was activated in the presence of ammonia resulting in an increased transfer of photosynthetically incorporated carbon to synthesis of amino acid skeletons at the expense of sucrose synthesis.

Given the limitations of leaf discs in terms of uniform penetration of added chemicals to all the cells, it became desirable to carry out experiments with isolated leaf cells. Methods were developed for maintaining cells isolated from poppy plants (Papaver somniferum L.)(24) and these cells were used in studies with added 1 mM and 2 mM ammonium ion (25). Addition of ammonia to leaf cells caused an immediate rapid increase in the level of pyruvate and of alanine which is formed from pyruvate by transamination. The ratio pyruvate:PEP rose immediately and rapidly upon the addition of ammonia, clearly indicating the stimulation of the flow of carbon via the pyruvate kinase reaction. At the same time, there was an even more dramatic sudden increase in the ratio of aspartate:PEP. Since the levels of labeled oxoglutarate and glutamate were also measured, it was possible to calculate, via the oxaloacetate-glutamate transamination reaction, that oxaloacetate labeling had rapidly increased upon addition of ammonia, and that consequently the PEP carboxylase step had been dramatically stimulated by the addition of the ammonium ion.

The increased flow of carbon from photosynthesis into the TCA cycle could also be seen by a large stimulation in the rates of labeling of both malate and citrate. The level of glutamate itself dropped momentarily, presumably due to the stimulation of glutamine formation from glutamate by the added ammonia, but then rose more rapidly than the control despite the fact that there was a general stimulation of amino acid synthesis which would be using up glutamate via the transaminase catalyzed reaction. After 25 min with

cells in the presence of ammonia, the sucrose pool was about 16% less than in the control. Starch synthesis in this case was largely unaffected.

From these results it may be concluded that in these cells isolated from leaves of higher plants, as in the case of the Chlorella, the addition of ammonium ion resulted in a shift in metabolism from the formation of sucrose to the formation of increased amounts of amino acids and that this shift was produced not only by the availability of ammonia for amino group formation but by also regulatory phenomenon resulting in increased flow of carbon from photosynthetic products to the TCA cycle. That the magnitude of the shift away from sucrose synthesis was not as great in this case of leaf cells might be attributed to the less complete nitrogen starvation prior to the beginning of the experiment. Another difference from the effects on Chlorella was noted when the studies with the poppy cells were extended to addition of ammonium in the dark following a period of photosynthesis (26). The stimulation of PEP carboxylation resulting in increased TCA cycle intermediates and a lower level of PEP was again clearly seen, but the level of pyruvate kinase was not significantly stimulated in this case, or if it was, then the utilization of pyruvate increased by the like amount so that no net increase in pyruvate was observed.

The fact that both in Chlorella and in isolated cells from poppy leaves addition of ammonium ion to cells respiring in the dark sometimes results in a rapid increase in glutamate labeling (in some cases there is an initial decrease followed by an increase) might seem to suggest that there must be a dark reaction for glutamate synthesis separate from the GOGAT reaction in the chloroplast which requires light. Probably, this labeling is due to increased availability of four- and five-carbon ketoacid skeletons labeled with $^{14}CO_2$ due to PEP carboxylation and transamination in which the amino group comes from pools of unlabeled amino acids and protein degradation.

Recently we have been able to isolate mesophyll cells from the leaves of Spinacea oleracea in such a way to allow high photosynthetic rates to be obtained even after the isolated cells have been maintained for as long as 60 hours. The incorporation of $^{14}CO_2$ into carbohydrates, carboxylic acids and amino acids in these cells and the influence of ammonia on that incorporation have been studied. The total incorporation, the specific radioactivity, and the pool sizes of most of most of the protein amino acids and gamma amino butyric acid have been determined as a function of time. By measuring specific activities and saturation effects, it has been possible to determine the presence and relative sizes of the metabolically active and passive pools for the various amino acids (Larsen et al. to be published). In general, the effects of added ammonium are similar to those seen earlier with Chlorella pyrenoidosa, with spinach leaf discs, with poppy cells, and with isolated spinach cells as reported recently by Woo and Canvin (27, 28). Reduced carbon from photosynthetic fixation is diverted away from sucrose synthesis and into the synthesis of amino acids. The labeling of malate, citrate, and in this case, of glycerate are considerably enlarged (Table 5).

The presence of ammonia during photosynthesis by these isolated spinach cells has profound effects on the pool sizes and specific activity of various amino acids (Table 6). However, detailed analysis of ^{14}C incorporation into individual amino acids showed that the ammonia effect is highly specific. Analysis of these effects in the light of present understanding of pathways of amino acid biosynthesis provides a better insight into the mode of action of ammonia, and the flow of nitrogen into the various amino acids.

Glutamine labeling increases 21-fold in cells treated with ammonia, while glutamate labeling decreases 30%, and at the same time there is a 5-fold increase in glutamine pool size and a 40% reduction in glutamate pool size (Table 6). The 4-fold increase in specific activity of glutamine

is nearly matched by a similar increase in that of asparagine, but the pool size of asparagine remains constant while that of glutamine has increased 5-fold. Particularly dramatic is the 16-fold increase of ^{14}C incorporation into histidine accompanied by an increase of 10-fold in specific activity but only a modest change in pool size.

Table 5. Effect of NH_4^+ on incorporation of ^{14}C into carbohydrates and carboxylic acids in isolated spinach cells in the light.

Compound	Percent 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

Isolated mesophyll spinach cells were allowed to photosynthesize for 60 min (1850 $\mu E \cdot m^{-2} \cdot sec^{-1}$) at 20 C in 7 mM $H^{14}CO_3^-$.

In contrast, added ammonia has no effect on incorporation of ^{14}C into glycine, serine, phenylalanine and tyrosine. The lack of effect on glycine and serine is understandable in terms of their origin in the peroxisomes, where carbon ketoacid skeletons are dependent on glycolate formation or glycerate formation in the chloroplasts. These carbon skeleton origins bypass the reactions in the cytoplasm that are stimulated by ammonia addition. Phenylalanine and tyrosine may be less affected because of their dependence on the shikimic acid pathway located in the chloroplasts. Valine appears also to be formed in chloroplasts, as discussed in the first section, but for this

amino acid, there is a strong stimulation in rate of labeling with added ammonia.

Table 6. Effect of NH_4^+ on biosynthesis of amino acids in spinach cells in the light

	% of value obtained with NO_3^- as N-source		
Amino Acid	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	100	90
Serine	90	90	100
Phenylalanine	80	80	110
Tyrosine	100	100	100
Threonine	100	230	50
Isoleucine	120	220	60
Proline	--	50	--
γ-Aminobutyrate	--	50	--

Conditions were as described for Table 5. Amino acids were separated by cation exchange chromatography followed by two-dimensional paper chromatography, radioautography and scintillation counting to determine ^{14}C incorporation. Portions of the samples were reacted with tritiated dansyl chloride after ion exchange chromatography, and the resulting derivatives were separated by polyamide thin-layer chromatography. Tritium/^{14}C ratios were then determined to obtain specific ^{14}C radioactivity after which pool sizes were calculated from total ^{14}C content and specific radioactivity. Detailed procedure to be published.

With all this evidence for a general shift in metabolism caused by external addition of ammonia, the next problem is to determine the mechanisms. This shift results

in relatively greater synthesis of amino acids and less carbohydrates, especially sucrose. When there is a stimulation in CO_2 fixation, the extra fixed carbon is used in forming carbon skeletons for amino acid synthesis (28,29). In other cases, sucrose synthesis stops, is diminished, or sucrose is metabolized to provide carbon for amino acid synthesis (19,20,21,22,25). The shift seems to be somewhat independent of photosynthesis *per se*, since evidence is seen in the dark for stimulation of pyruvate kinase (and by inference PEP carboxylase)(20,22) with respiring *Chlorella pyrenoidosa* and for PEP carboxylase with respiring poppy cells (26).

The mechanism whereby ammonia added to the medium could cause these changes is difficult to determine. Given the low K_m for ammonium ion of glutamine synthetase, the level of free ammonia in cells would be expected to be kept very low. Nevertheless, the observation of a simultaneous shift in several rate-limiting steps linking photosynthetic carbon metabolism and amino acid synthesis seems meaningful. Perhaps ammonium ion in the medium induces internal changes that mimic an important regulatory process.

Externally applied ammonia might act in some indirect way. It could, for example, cause a change in the pH of the cytoplasm and this in turn could alter the activity of PEP carboxylase (26). In fact, Davies (29) proposed that PEP carboxylase acts as a cytoplasmic pH stat, increasing in activity with increasing pH, thus producing acid via carboxylation. If the signal (rise in pH) were caused by ammonia, the PEP carboxylation would supply oxaloacetate and oxoglutarate for glutamate synthesis and consequently for glutamine synthesis, using up the excess ammonia. The metabolic branch point in the cytoplasm for allocation of carbon to sucrose synthesis or to PEP formation *in the light* seems likely to be at triose phosphate coming from the chloroplast. The export of triose phosphate from the chloroplast is controlled by the level of P_i in the cyto plasm (30,31). The conversion of triose phosphate to hexose

and low O_2 (2%). With inhibition of the glycine to serine conversion by glycine hydroxamate, diminished ammonia evolution results in accumulation of amino groups in glycine and serine and a decrease in glutamate and other amino acids.

Ammonia added to a variety of photosynthetic cells in liquid suspension shifts biosynthetic metabolism away from carbohydrate (especially sucrose) synthesis and towards synthesis of certain (but not all) amino acids. A fuller understanding of this shift may shed light on the way plant cells control the flow of photosynthate to biosynthesis in response to physiological conditions.

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