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REGULATORY EFFECTS OF AMMONIA ON CARBON METABOLISM IN PHOTOSYNTHESIZING *CHLORELLA PYRENOIDOSA*

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SUMMARY

Addition of ammonia to *Chlorella pyrenoidosa*, photosynthesizing under steady-state conditions, causes changes in the metabolism which are due not only to the increased availability of NH_4^+ for reductive amination but also to regulation of controlled enzymes. One such effect is an increased rate of the reaction which converts phosphoenolpyruvate to pyruvate *in vivo*. This regulatory effect was revealed by kinetic tracer studies with $^{14}CO_2$, paper chromatography and radioautographic analysis, which showed that upon addition of NH_4^+ (1) the levels of both 3-phosphoglycerate and phosphoenolpyruvate drop, with the ratio of 3-phosphoglycerate/phosphoenolpyruvate increasing, (2) the level of labeled pyruvic acid increases and the rate of formation of alanine increases rapidly, while the rate of formation of serine is unaffected, (3) the rate of flow of carbon into the tricarboxylic acid cycle acids, malate and citrate, increases along with the increased rates of formation of glutamate, glutamine and aspartate and (4) the rate of labeling of lipids increases. The increased flow of carbon into amino acids is mostly at the expense of sucrose synthesis; starch synthesis decreases only slightly. The interruption of sucrose synthesis apparently is due to stopping the reaction between UDP-glucose and fructose 6-phosphate. The rate of conversion of fructose 1,6-diphosphate to fructose 6-phosphate is also decreased upon NH_4^+ addition.

INTRODUCTION

Kinetic studies of photosynthesis with labeled carbon in green cells and isolated chloroplasts[1-3] revealed regulatory mechanisms controlling the flow of carbon within and out of the chloroplasts and the relations between photosynthetic carbon reduction and biosynthetic metabolism.

Comparison of the patterns resulting from $^{14}CO_2$ incorporation during photosynthesis in *Chlorella pyrenoidosa*, at various stages in the cell life cycle, showed that the smallest cells (which had recently divided in the dark) incorporate ^{14}C much more rapidly into sucrose, and much more slowly into amino acids and acids of the tricarboxylic acid cycle, than growing cells[4]. Cells at different stages of division differ in their ability to reduce NO_3^-. This was revealed by a consequent difference in the rates of flow of carbon from the photosynthetic carbon reduction cycle into the amino

acids and other compounds. The growing cells could use added nitrogen in the form of NO_3^-, NO_2^-, or NH_4^+ for amino acid synthesis, but the newly divided cells which had been in the dark for 12 h were able to utilize NH_4^+ only (ref. 5).

With NH_4^+ present, either added directly or formed by the reduction of NO_3^- or NO_2^-, there was an increased rate of ^{14}C labeling of several amino acids, as well as malate and citrate, and lipid. At the same time, there was a marked decrease in the rate of labeling of sucrose. These effects of NH_4^+ were attributed not only to the increased supply of NH_4^+ for reductive amination and subsequent transamination reactions, but also tentatively to a stimulation of the conversion of phosphoenolpyruvate to pyruvate, probably mediated by pyruvate kinase (EC 2.7.1.40).

We report here kinetic tracer experiments with *C. pyrenoidosa* photosynthesizing under steady-state conditions, in which concentrations of several metabolites were followed before and after addition of NH_4Cl. In order to locate with some certainty the possible regulatory site of NH_4^+, we have paid particular attention to the kinetics of labeling of 3-phosphoglycerate, phosphoenolpyruvate, and pyruvate as well as alanine and serine. Also $^{32}P_i$ was used to permit measurement of levels of ATP, UDPG, and PP_i.

EXPERIMENTAL

C. pyrenoidosa was cultured aseptically using the turbidostat[6] at 25° with a stream of air *plus* 4 % CO_2 (v/v). Cell density was kept constant at 0.3 % by adding culture medium (modified Myers medium[4]) automatically.

The cells were harvested, washed once with 0.1 mM KH_2PO_4 and resuspended (1 %) in this medium. 60 ml of the suspension and 2 mC of $^{32}P_i$ were poured into the steady-state apparatus. After 30 min of photosynthesis at 20° with 1.5 % CO_2, $^{14}CO_2$ was added from a small loop to the closed system. At nearly the same time CO_2 with $^{14}CO_2$ in another reservoir was added in order to increase the air volume and thus lessen the change in CO_2 concentration during the experiment. Samples (approx. 1 ml) were taken into 4 ml of methanol at intervals as indicated in Figs. 1–7. At 21 min from the introduction of $^{14}CO_2$ into the system, 1 ml of 0.05 M NH_4Cl was injected into the suspension and the sampling was continued for another 20 min. The pH was kept constant at 5.5. When a nitrogen source was not included, adjustment of the pH was unnecessary, and after the injection of NH_4Cl it was corrected automatically by adding 0.1 M NH_4OH. The partial pressure of CO_2 at the start of the experiment was 1.6 % (with specific activity of 12.4 μC/μmole), and was 1 % at the end of 41 min of the experiment. The detailed method of the steady-state experiment has been described elsewhere[3,7].

Two 500-μl portions of each sample were analyzed by two-dimensional paper chromatography, first with phenol–acetic acid–water with EDTA, and second with butanol–propionic acid–water[8]. One of the portions was run 24 h in each direction for analysis of the secondary products of photosynthesis. The other portion was chromatographed 48 h in each direction in order to separate the intermediates of the photosynthetic carbon reduction cycle and other phosphorylated compounds.

A 1-ml portion of the sample was defatted, hydrolyzed for 1 h at 100°, and an aliquot (corresponding to 25 μl of the original) was chromatographed two dimensionally (each 24 h) for starch glucose analysis[4].

A 25-ml portion of each sample was chromatographed with the first solvent for 20 h and the fastest moving spot was counted and reported as total lipid.

Location of the labeled metabolites was detected by radioautogram and the content of ^{14}C and ^{32}P was determined semi-automatically, as was described in detail[3].

For analysis of pyruvate, a 2-ml portion of the sample with 0.5 μmole of authentic pyruvate was diluted to 5 ml with water and coupled with 2,4-dinitrophenylhydrazine. The coupling and extraction were essentially similar to those described by BACHELARD[9]. The extract of the hydrazone was paper chromatographed 20 h using *n*-butanol–ethanol–0.5 M NH_4^+ (7:1:2, by vol.) in the dark and the radioactivity of the yellow spot was counted as described above.

RESULTS

Upon addition of NH_4Cl to the photosynthesizing cells, there is a rapid increase in the rates of labeling of alanine and glutamine (Fig. 1), and of aspartic acid (Fig. 2). The rate of labeling of glutamic acid decreases momentarily and then is increased to several times the rate before addition (Fig. 1). Threonine labeling, not detected before addition of NH_4Cl, becomes appreciable after addition (Fig. 2), while the rate of labeling of serine is virtually unchanged by the addition of NH_4Cl.

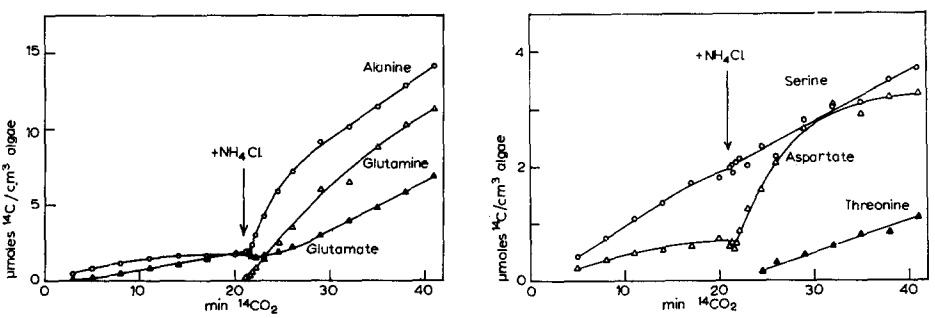

Fig. 1. Labeling of alanine, glutamate, and glutamine in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+. O, alanine; △, glutamine; ▲, glutamate.

Fig. 2. Labeling of serine, aspartate, and threonine in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+. O, serine; △, aspartate; ▲, threonine.

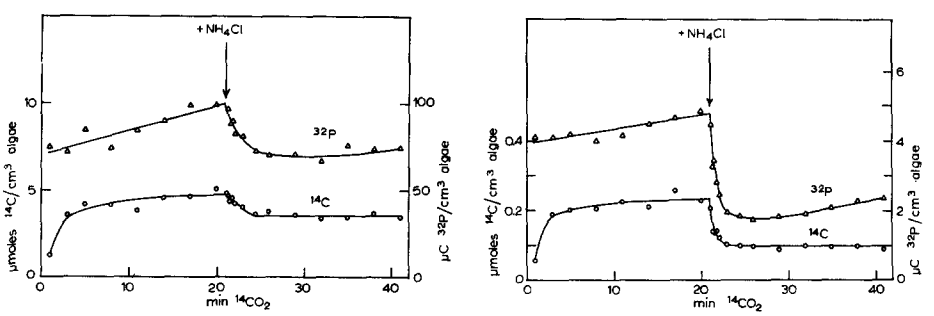

Fig. 3. Labeling of 3-phosphoglycerate in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+. O, ^{14}C label; △, ^{32}P label.

Fig. 4. Labeling of phosphoenolpyruvate in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+. O, ^{14}C label; △, ^{32}P label.

The ^{14}C- and ^{32}P-labeling curves of 3-phosphoglycerate (Fig. 3) and of phosphoenolpyruvate (Fig. 4) show that the relative transient change in phosphoenolpyruvate is faster than that of 3-phosphoglycerate. The steady-state level of phosphoenolpyruvate declines about 60% upon NH_4Cl addition, while that of 3-phosphoglycerate declines only 30%. Although the ^{32}P labeling continues to rise during the course of the experiment (see below), the relative pool sizes of 3-phosphoglycerate and of phosphoenolpyruvate are about the same when determined by either isotope, and the size of the changes in steady-state level are also about the same.

Addition of NH_4Cl to the photosynthesizing cells causes a rapid increase in the level of labeled pyruvate (Fig. 5). It appears that the pyruvate pool was not saturated with ^{14}C label prior to addition of NH_4^+, due presumably to the slowness of the conversion of phosphoenolpyruvate to pyruvate.

In the case of ^{32}P-labeled compounds in this experiment (see Figs. 3, 4 and 6), there is a "pseudo-saturation" of the metabolic pools with labeled phosphate. The metabolic pool label may be at any given time about the same specific radioactivity as the metabolic pool of P_i within the cells. However, this inorganic pool and other metabolically active pools of P_i inside the cell are slowly increasing in specific radioactivity due to a limited rate of entry of labeled P_i into the cells and to slow turnover of larger, less active pools, perhaps polyphosphates[10]. In any event, the addition of

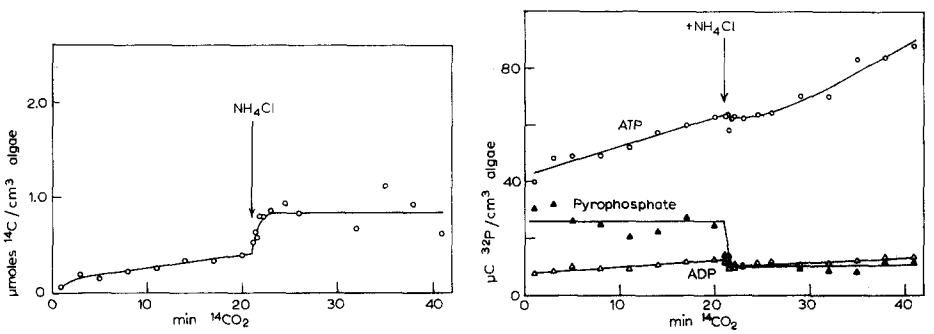

Fig. 5. Labeling of pyruvate in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+.

Fig. 6. Labeling of ATP, ADP, and PP_i in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+. O, ATP; △, ADP; ▲, PP_i.

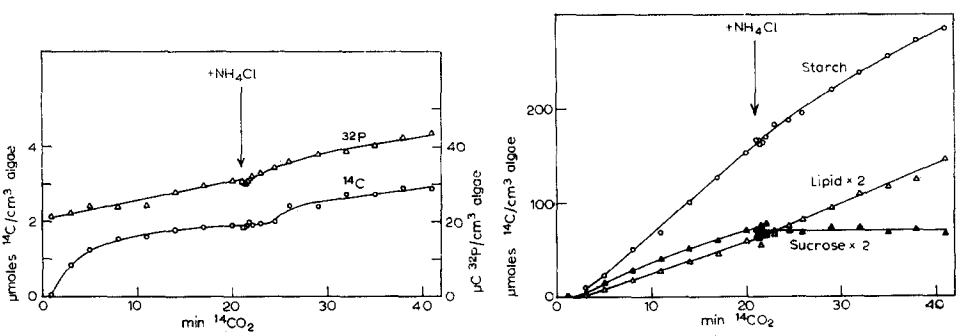

Fig. 7. Labeling of UDP-glucose in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+. O, ^{14}C label; △, ^{32}P label.

Fig. 8. Labeling of starch, lipid, and sucrose in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+. O, starch; △, lipid; ▲, sucrose.

NH_4Cl causes only a minor transient change in the levels of labeled ATP and ADP (Fig. 6), after which the level of ATP increases slightly.

In contrast to the effects of NH_4Cl addition on ATP and ADP, there is a sudden drop in the level of PP_i to a new steady-state level less than half that existing before the addition of NH_4Cl (Fig. 6). Surprisingly, considering the drop in sucrose labeling rate and in PP_i level, the labeling of UDPG (Fig. 7) was only slightly affected by the addition of NH_4^+. After 5 min the pool size seems to increase about 20 % and then drifts upward (based on ^{14}C labeling). Small transient changes apparently occurred in the levels of most of the sugar phosphate compounds of the carbon reduction cycle just after NH_4^+ addition. Since these immediate changes were within the range of experimental error, they are not shown. The initial changes were followed by a general decline in the levels of these metabolites. The steady-state levels just before NH_4^+ addition, and 20 min later, are shown in Table I.

The rate of formation of lipid is slightly, albeit significantly increased (about 25 %) by NH_4^+ addition (Fig. 8), while the rate of starch formation is decreased approx. 40 %. The rate of increase in labeled sucrose drops to zero. The rates of flow of ^{14}C into the tricarboxylic acid cycle intermediates, malate and citrate (Fig. 9), increase immediately on addition of NH_4^+.

TABLE I

LEVELS OF LABELED SUGAR PHOSPHATES JUST BEFORE, AND 20 min AFTER NH_4^+ ADDITION

Compound	*Levels of labeled ^{14}C (μmoles/cm^3 algae)*	
	Before NH_4^+	*After NH_4^+ (20 min)*
Fructose 6-phosphate	6.39	4.38
Glucose 6-phosphate	11.64	6.89
Sedoheptulose 7-phosphate	4.41	3.47
Sedoheptulose 1,7-diphosphate	0.15	0.12
Fructose 1,6-diphosphate	0.72	0.98
Glucose 1,6-diphosphate	0.32	0.22
Ribulose 1,5-diphosphate	1.40	0.83
Pentose 5-phosphate (includes xylulose 5-phosphate, ribose 5-phosphate, and ribulose 5-phosphate)	0.55	0.60

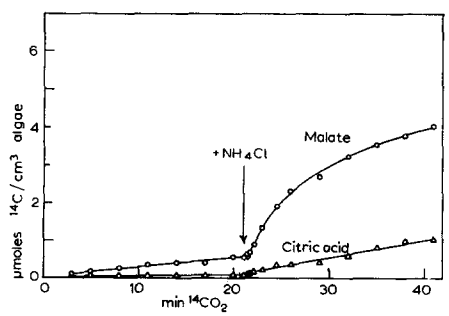

Fig. 9. Labeling of citrate and malate in photosynthesizing *C. pyrenoidosa* before and after addition of NH_4^+. O, malate; △, citrate.

DISCUSSION

The increased rate of labeling of amino acids and the increased rate of flow of carbon into malic and citric acids upon addition of NH_4^+, as well as the accompanying drop in levels of 3-phosphoglycerate and phosphoenolpyruvate and the decrease in the rate of labeling of sucrose have been noted before[5,11]. The results of the present study provide clear evidence for the sites of the regulatory effects of NH_4^+. A primary site of such regulation is the conversion of phosphoenolpyruvate (and ADP) to pyruvate (and ATP), presumably mediated by pyruvate kinase (EC 2.7.1.40). This conclusion is supported by the sudden increase in level of pyruvate and sudden decrease in phosphoenolpyruvate on addition of NH_4^+. The decrease in phosphoenolpyruvate is faster and relatively larger than that of 3-phosphoglycerate; therefore, the regulated site is not between 3-phosphoglycerate and phosphoenolpyruvate. The pyruvate increase is relatively faster than that of alanine. Thus, the increased flow of carbon into alanine is due to increased pyruvate level and not solely the consequence of an increased rate of transamination resulting from an enlarged pool of glutamate.

The relatively slow rate at which glutamate increases upon addition of NH_4^+, together with earlier findings that reductive amination of α-ketoglutaric acid is the primary route of incorporation of NH_4^+ in Chlorella[7], shows that regulatory effects of NH_4^+ on carbon metabolism are responsible for the observed immediate increases in ^{14}C labeling of other amino acids. The fact that glutamate labeling initially dips slightly (Fig. 1) upon addition of NH_4^+ shows that the increased demand upon the glutamate pool is initially greater than the increased rate of reductive amination forming glutamate. Only after 4 min does the increased flow of carbon in the tricarboxylic acid cycle reach the point of synthesis of glutamate.

The unchanged rate of serine labeling (Fig. 2) is further evidence that the increased labeling of alanine, aspartate, *etc.* is due to increased levels of carbon skeleton precursors. In Chlorella, supplied with 1 % CO_2, serine is probably derived from 3-phosphoglycerate (which decreases in level, Fig. 3) by several steps not involving phosphoenolpyruvate. Upon addition of NH_4^+, the carbon precursor of serine is probably decreased. This change, together with the increased level of glutamate coming from the addition of NH_4^+, leads to an unchanged rate of serine synthesis.

The increase in pyruvate level with NH_4^+ addition is not due to a decreased rate of oxidative decarboxylation, since lipid labeling (Fig. 8) slightly increases, indicating an increased rate of conversion of pyruvate to acetyl-CoA. Furthermore, there is an immediate increase in the rates of flow of ^{14}C into malate and citrate (Fig. 9). The increased citrate formation could be a reflection of a higher level of acetyl-CoA.

The increased rate of labeling of malate requires some additional explanation. One possibility would be that reductive carboxylation of pyruvate to give malate (with malate enzyme, EC 1.1.1.40) occurs and is increased by the increased level of pyruvate. Also, the malic enzyme from *E. coli* is reported to be stimulated by NH_4^+ (ref. 12). It is also possible that increased malate formation could result from condensation of acetyl-CoA with glyoxylate.

The observed changes in carbon metabolism in this experiment are clearly not dependent on a change in the level of ATP and ADP (Fig. 6). This is consistent with

other findings[1] that major shifts in metabolic pathways occurring in Chlorella with the shift from light to dark are not accompanied by significant changes in the steady-state levels of ATP and of ADP.

The sudden drop in level of PP_i suggests that the reaction in which it is formed with UDPG from glucose 1-phosphate and UTP may have decreased in rate. The fact that PP_i level does not go to zero, even though sucrose synthesis stops, indicates that PP_i is still produced by other biosynthetic routes (for example, starch synthesis and amino acid activation).

The blocking of sucrose synthesis when NH_4^+ is added could occur either in the condensation of UDPG with fructose 6-phosphate to give sucrose phosphate or in the subsequent hydrolysis of sucrose phosphate. The position of sucrose phosphate on the two-dimensional paper chromatograms developed with phenol–water and butanol–propionic acid–water is known to be either coincident with the hexose monophosphate area[13] or to be just below it. We could find no evidence for sucrose phosphate in the area below the hexose monophosphate area, since faint spots in that area gave no sucrose, glucose, or fructose upon treatment with phosphatase and rechromatography. Our present chromatographic techniques give two usually well separated hexose phosphate areas. The upper one gives almost entirely fructose after treatment with phosphatase, while the lower one, which may contain sucrose phosphate, gives mostly sedoheptulose and glucose *plus* a trace of fructose. The amount of fructose is sometimes increased when the two hexose phosphate spots are not well separated, but the smaller amount which is nearly always found may be due to the presence of sucrose phosphate in the original lower hexose phosphate spot and to its subsequent hydrolysis by phosphatase and contaminant invertase. This trace of labeled fructose from the lower hexose phosphate spot did not increase after the addition of NH_4^+. Thus, there is no detectable increase in sucrose phosphate labeling to indicate that the step mediated by sucrose phosphatase is inhibited by the addition of NH_4^+.

A slight increase in UDPG (Fig. 6), at a time when the levels of fructose 6-phosphate and glucose 6-phosphate decline (Table I), and the sudden drop in PP_i thus suggest a negative effect of NH_4^+ addition on the condensation of UDPG with fructose 6-phosphate. The enzyme mediating this reaction in wheat germ, sucrose phosphate synthetase (uridine diphosphoglucose: D-fructose-6-phosphate-2-glucosyl transferase, EC 2.4.1.14) has been found to exhibit allosteric properties[14]. The activity of that enzyme showed a sigmoidal dependence on the concentration of fructose 6-phosphate and was increased at high (22 mM) levels of Mg^{2+}. The smallness of the increase in UDPG accompanying the apparent stopping of sucrose synthesis in the present study is probably due to the decline in levels of glucose 6-phosphate and fructose 6-phosphate.

Besides the increase in pyruvate kinase activity and the decrease in sucrose phosphate synthetase activity, which result in increasing the flow of carbon from the cycle at one point and decreasing the flow from the cycle at another point, there is a small but perhaps significant, regulatory effect evident in the carbon reduction cycle itself. From Table I, we see that the level of fructose 1,6-diphosphate has increased, while the level of fructose 6-phosphate decreased. Thus, the activity of fructose-1,6-diphosphatase (EC 3.1.3.11), one of the regulated enzymes of the photosynthetic carbon reduction cycle[1–3], has decreased. In Chlorella, with the light–dark transition[1,3],

with fatty acids[8], and with different stages in the cell life cycle[4], the activity of this enzyme often changes in the same direction as that of ribulose-diphosphate carboxylase (EC 4.1.1.39). With the addition of NH_4^+, however, we see (Table I) that the level of ribulose 1,5-diphosphate has declined. Perhaps this is due to too large a drain of carbon from the cycle following the addition of 1 mM NH_4^+.

Calculation of free energy changes (ΔG^s) accompanying reactions of steady-state in photosynthesizing *C. pyrenoidosa* (rates of energy dissipation as heat accompanying the reactions) showed that steps known to be metabolically regulated were those with large negative free energy changes[15]. The physiological standard free energy change (all reactants at unit activities, except that the pH is taken as 7.0) for the pyruvate kinase reaction was calculated to be $\Delta G' = -5.6$ kcal[15]. From the data of McQUATE AND UTTER[16], the logarithm of the equilibrium constant (not including H^+) at pH 7.7 is calculated to be 4.28. From the present study, the ratio [pyruvate]-[ATP]/[phosphoenolpyruvate][ADP] can be estimated as 10 before addition of NH_4^+, and 40 after addition of NH_4^+. Thus $\Delta G^s_{7.7} = -1.363\,(4.28 - \log 10) = -4.47$ kcal, before NH_4^+ addition, and $\Delta G^s_{7.7} = -3.65$ kcal, after NH_4^+ addition. Corresponding ratios of forward to back reaction rates are thus 2130 and 826, before and after NH_4^+ addition.

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