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PATHWAY OF AMMONIA ASSIMILATION IN ILLUMINATED AND DARKENED *CHLAMYDOMONAS REINHARDII*

JULIE V. CULLIMORE and ANTHONY P. SIMS

School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, U.K.

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Key Word Index—*Chlamydomonas reinhardii*; Chlorophyceae; algae; light NH_3 assimilation; dark NH_3 assimilation; glutamine synthetase; glutamate dehydrogenase; glutamate synthase cycle, sole operation of; control by glutamine synthetase.

Abstract—Evidence is presented which shows that NH_3 assimilation in *Chlamydomonas* occurs exclusively via the glutamate synthase cycle in illuminated and darkened cells and those in which the internal level of NH_3 is elevated. This result indicates that glutamate dehydrogenase probably plays a catabolic rather than anabolic role in the N nutrition of the alga. Glutamine synthetase and glutamate dehydrogenase were characterized and their kinetic properties shown to be consistent with these proposals. It is suggested that reversible activity modulations of glutamine synthetase regulate the operation of the glutamate synthase cycle in the light but the availability of reductant and ATP limits its activity in darkened cells. The possible involvement of the two glutamate synthase enzymes in both light and dark assimilation is discussed.

INTRODUCTION

Over the past decade the widely held view that glutamate dehydrogenase (GDH)* plays a unique role in the assimilation of NH_3 has had to be substantially revised following the discovery, in plants and micro-organisms, of an alternative route of glutamate biosynthesis involving the concerted action of glutamine synthetase (GS) and glutamate synthase (the glutamate synthase cycle [1]).

Surprisingly, few detailed studies have been carried out on the status of these two pathways in green algae and much of the work had been centred on establishing the presence of the enzymes of the two pathways [1, 2]. It is not yet known, for example, whether green algae assimilate N exclusively by means of the glutamate synthase cycle or whether, depending upon conditions of light or N availability, the GDH pathway may also function. There is some support for this latter suggestion since it has been shown that *Chlorella* growing on NH_3 possesses high levels of an inducible cytoplasmic NADPH-dependent GDH with a high affinity for NH_3 as well as a constitutive enzyme with a non-specific pyridine nucleotide requirement [3, 4]. The observation that the NADPH-specific enzyme undergoes a rapid inactivation following transfer to NO_3 [3, 5] coupled with the detection, on NO_3, of a ferredoxin-dependent (Fd) glutamate synthase [6] is suggestive of a system of control modulated by N availability, and similar to that found in some bacteria [7].

In this paper we have quantitatively investigated the pathways of light and dark NH_3 assimilation in the green alga *Chlamydomonas reinhardii*. This organism can grow both phototrophically and heterotrophically and is known to possess, in addition to GDH [8], both a Fd- and a NADH-specific glutamate synthase [9].

RESULTS

Some properties of GS and the NADPH-dependent activity of GDH in algae growing on NH_3 and NO_3 as sole sources of N are shown in Table 1. Unlike *Chlorella*, NADPH–GDH can be detected in cells grown on NO_3 as well as NH_3. A number of lines of evidence suggest that there is only a single enzyme form with a non-specific requirement for pyridine nucleotide (see also [8]). The behaviour of the two activities during sucrose density gradient centrifugation and gel filtration is identical and exhibits a constant NADH/NADPH ratio of 1:7. Moreover, both activities increase in parallel following a transfer of NH_3- and NO_3-grown cells to a medium lacking N (data not included). GDH exhibited positive co-operativity with NH_3 and 2-oxoglutarate. Both NADPH and NADH activities yielded an identical value of $s_{0.5}$ with NH_3 which was 80-fold higher than that determined for GS. In the oxidative deaminating direction activity with NADP was only about 15% of that observed with NAD at the pH optimum of 9.0, and using NAD as oxidant a K_m for glutamate of 1.5 mM was obtained.

Several experiments were carried out with phototrophically and mixotrophically grown algae to measure the rate of light and dark NH_3 assimilation by *Chlamydomonas* and to establish the pathway by which it is assimilated by using a number of metabolic inhibitors (Fig. 1). In Fig. 1a, measurements of $^{15}NH_3$ incorporation into intact cells and cells extracted with 60% ethanol to remove free NH_3

* Abbreviations: GS = glutamine synthetase; GDH = glutamate dehydrogenase; Fd-(ferredoxin) glutamate synthase; NADH-glutamate synthase; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DSPD = disalicylidene propanediamine; MSO = L-methionine-D-L-sulfoximine; MFA = monofluoroacetate.

Table 1. Some kinetic properties and hydrodynamic characteristics of GS and GDH (NADPH activity-aminating)

Properties	GS	GDH
Stokes' radius (nm)	6.35	4.82*
$s_{20,w}$	14.6	10.9*
Molecular weight	380000	210000
$s_{0.5}$ for NH_3 (mM)	0.20	16*
Hill coefficient for NH_3	1.2	2.0
K_m for glutamate (mM)	0.42	—
$s_{0.5}$ for 2-oxoglutarate (mM)	—	1.25
Hill coefficient for 2-oxoglutarate	—	1.3
pH optimum	7.1	7.8

* Identical values were obtained when NADH was used as reductant in the assay.

Partially purified extracts of phototrophic cells grown on NH_3 were used in these experiments.

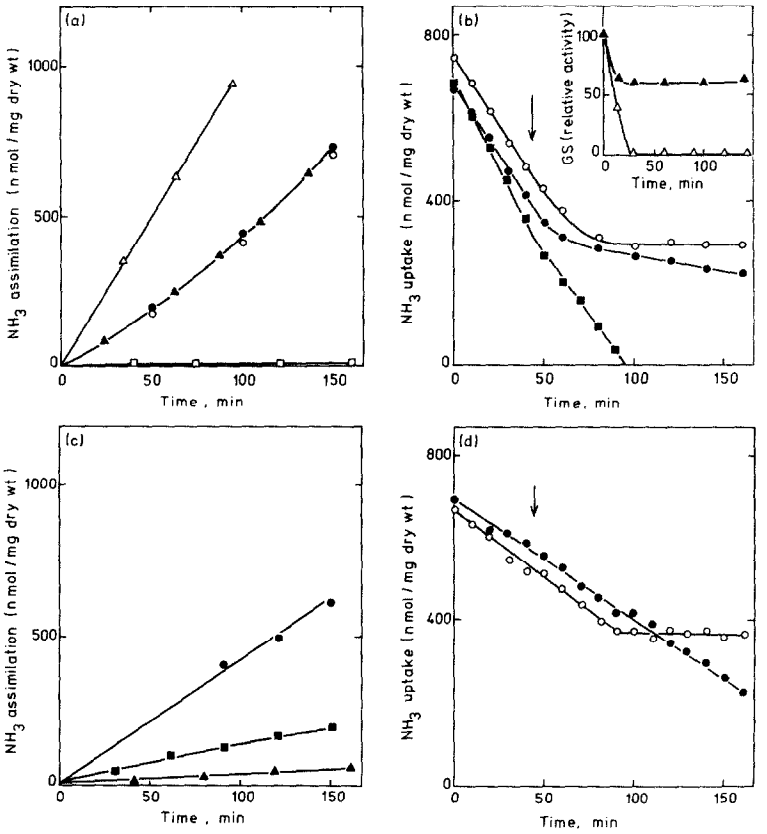

Fig. 1. The effect of inhibitors on light and dark NH_3 assimilation in phototrophically and mixotrophically grown cells. (a) Uptake and assimilation in the light and the effect of MSO. Estimates of phototrophic assimilation were calculated from measurements of ^{15}N incorporation into intact cells (●) and 60% EtOH-extracted cells (○), and from measurements of NH_3 uptake from the medium (▲). Phototrophic assimilation (based on ^{15}N measurements) following 2 hr pretreatment with 0.2 mM MSO (□). Mixotrophic assimilation (△). (b) Effect of DCMU and DSPD on assimilation in the light. Response of phototrophic (●) and mixotrophic (■) assimilation to 6 μM DCMU and of phototrophic assimilation to 1 mM DSPD (○). Inset: Effect on GS activity of phototrophic cells following addition of DCMU (▲) and DSPD (△) to the culture. (c) Assimilation in the dark (based on ^{15}N measurements) and the effect of MSO. Assimilation in mixotrophically cultured cells, (●) and cells pretreated with 0.2 mM MSO for 2 hr (■). Assimilation in phototrophically grown cells (▲). (d) Effect of DCMU (●) and DSPD (○) on dark assimilation in mixotrophically grown cells. Throughout, ↓ denotes time of addition of inhibitors; GS transferase activity was measured unless otherwise stated. In (b) and (d) and in Figs. 4 and 5, NH_3 uptake is shown as loss from the medium.

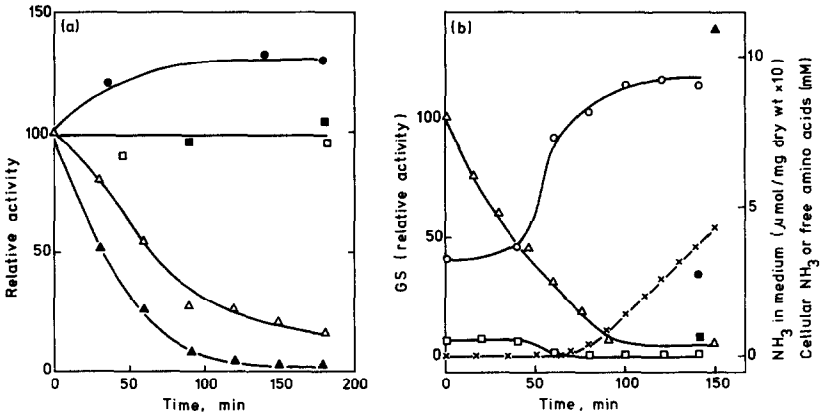

Fig. 2. Effect of MSO on enzymes, and the levels of free amino acids and NH_3 in the cell. (a) Enzymes of phototrophic cells: GS (▲), GDH (□), Fd (■) and NADH (●) glutamate synthase. GS of mixotrophic cells (△). (b) NH_3 and total soluble amino acids of phototrophic cells. Cellular NH_3 (○), amino acids (□), GS (△) and excretion of NH_3 into medium (×), control values (closed symbols). The cells used here were previously grown with NH_3 as sole source of N and were transferred to a medium lacking N and containing 0.2 mM MSO at zero time. The initial activities of the enzymes measured in this experiment were similar to those shown in Table 2.

were made and compared with estimates of assimilation based on NH_3 uptake from the medium. The rates determined by uptake and assimilation into intact cells were identical; the slight difference in the amount of N incorporated into extracted cells could be accounted for by a soluble fraction of N comprised of a percentage of the total N of amino acids (0.009%), soluble protein (5.0%) and chlorophyll a and b (2.4%). Free NH_3 represented only 0.73% and therefore NH_3 uptake can be used as a simple but reliable measure of assimilation. Consequently in many experiments N assimilation has been estimated from measurements of NH_3 disappearance from the medium. Adaptation of phototrophic cells to acetate increased both the growth and N assimilatory rate of the alga.

The extent to which GS was involved in NH_3 assimilation was investigated using MSO, an inhibitor of GS. The specificity of the inhibitor in the alga was established in experiments where its effect on the level and activity of a number of N assimilatory enzymes and the level of free NH_3 and amino acids in the cell was investigated (Fig. 2). MSO (5 mM) did not affect the activity of GDH or Fd- and NADH-dependent glutamate synthase in enzyme extracts (data not included) and treatment of intact cells with MSO actually produced a small increase in the level of NADH-glutamate synthase and no significant change in the levels of GDH or Fd-glutamate synthase (Fig. 2a). The virtually complete deactivation of GS observed in this experiment was associated with the total abolition of ^{15}N assimilation by the cell (Fig. 1a) and this suggests that under phototrophic conditions the GS-dependent glutamate synthase cycle represents the sole pathway of assimilation. In Fig. 1b the effects of DCMU (a photosystem II inhibitor) and DSPD (an inhibitor reported to block the photoreduction of Fd and its dependent reactions) on light-dependent NH_3 assimilation and the level of GS are shown. The extent to which DCMU inhibited NH_3 assimilation depended on the conditions of culture; only 22% inhibition was observed in mixotrophic cells catabolizing acetate whereas this occurred to nearly 90% in phototrophic cells. DSPD totally abolished NH_3 assimilation in phototrophic cells 20 min after its addition, but unlike DCMU, it could have produced its effect directly by completely deactivating GS.

Some experiments to measure the rate of dark assimilation in growing cultures of the alga were undertaken. Rates of assimilation calculated from ^{15}N incorporation into cellular N established that cells utilizing exogenous acetate were able to sustain dark assimilation above 40% of the light rate although incorporation of N into darkened phototrophic cells represented only about 10% of the value found in the light. The effect of MSO on mixotrophically grown cells assimilating NH_3 in the dark was different to that described for phototrophic cells such that although some inhibition of assimilation was observed, appreciable rates occurred throughout the experiment (Fig. 1c). These results suggest that GS is partly responsible for dark assimilation, but the incomplete deactivation of the enzyme at this time (Fig. 2) does not distinguish between the glutamate synthase cycle being the sole pathway of assimilation or GDH playing some role in this process. Confirmation for the sole operation of the glutamate synthase cycle for dark assimilation was obtained in an experiment in which the rates of assimilation were measured at different levels of GS during the deactivation of the enzyme with MSO (Fig. 3). Synthetase activity was estimated using physiological substrates since MSO was found to effect transferase and synthetase activities differentially (Fig. 3a). Addition of MSO to the culture led initially to an increase in both synthetase activity and the rate of NH_3 assimilation to be followed by a parallel decline in these two functions. The correlation between enzyme activity and NH_3 assimilation is shown in Fig. 3b where it can be seen that dark assimilation is totally dependent on GS; following the transfer of cells from light to dark, GS activity was initially in excess but subsequent deactivation of the enzyme produced a stoichiometric relationship between assimilation and enzyme level which

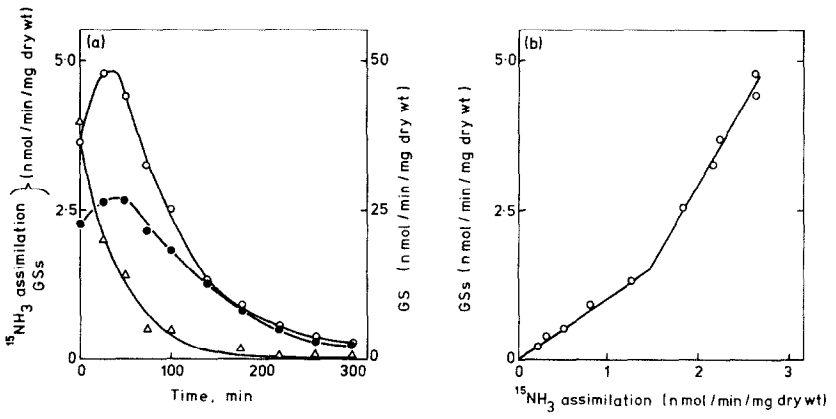

Fig. 3. Effect of MSO on NH_3 assimilation and GS level in darkened, mixotrophically grown cells. (a) NH_3 assimilation (based on ^{15}N measurements) (●), GS_t (○) and GS (△) activities. (b) Correlation of rate of assimilation and GS activity at different times (○). GS = synthetase.

extrapolated through the origin. This observation completely rules out any possibility of the operation of a GDH pathway in the dark. This conclusion was confirmed by the observation that DSPD, which completely deactivated GS under these conditions, also eliminated dark NH_3 assimilation (Fig. 1d). The absence of any effect of DCMU on dark assimilation shows that this inhibitor must act indirectly in phototrophic cells and that under these conditions it does not affect the activity of enzymes of the glutamate synthase cycle.

An experiment investigating the development of dark acetate supported NH_3 assimilation in cells previously grown under phototrophic conditions is shown in Fig. 4. The results reveal a close association between the development of an acetate transport system and the increase in dark assimilation; the appearance of both processes was inhibited by cycloheximide and MFA. Measurements of a number of N assimilatory and associated enzymes established that the activity of all these enzymes throughout the experiment was sufficient to support the highest rates of dark assimilation observed and that with the exception of GS their activities did not markedly change in response to the addition of acetate. This suggests that dark assimilation could be limited by the availability of appropriate substrates rather than by the lack of a component enzyme for the dark pathway.

A method of increasing dark assimilation in phototrophic cells entailing the build-up of internal reserves of carbohydrate following N starvation has been described [10]. Measurements of the rates of N assimilation and the levels of N assimilatory and associated enzymes in algal cells treated this way are shown in Table 2 together with those obtained from exponentially dividing cultures growing in the light on NH_3, with and without acetate.

Activities are expressed in such a way that a direct comparison with the rate of assimilation can be made. A number of general points are evident from these

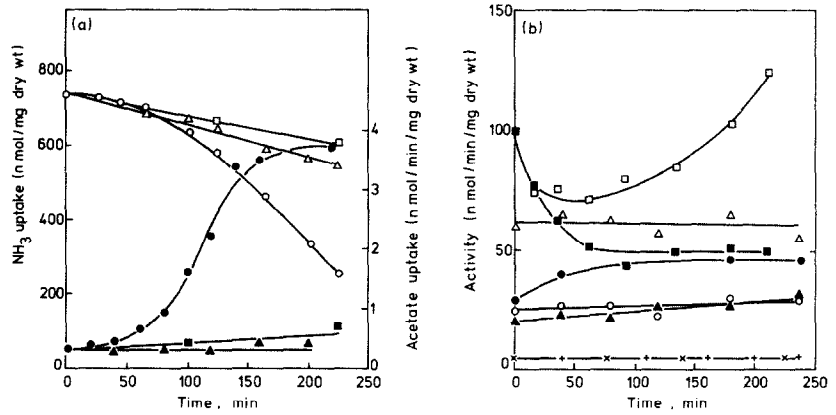

Fig. 4. The increase of dark NH_3 assimilation following the addition of exogenous acetate to phototrophic cells. (a) The increase in acetate transport (closed symbols) and NH_3 assimilation (open symbols) and their prevention by cycloheximide and MFA. Darkened control culture (■, □), with acetate (2 mM) (●, ○), with acetate and cycloheximide (5 μg/ml) (▲, △) (identical results were obtained with MFA). (b) Alteration of the levels of certain enzymes following addition of acetate to darkened cells. GS_t (□), GDH (×), Fd (○) and NADH (●) glutamate synthase; isocitrate dehydrogenase (▲), glucose-6-phosphate dehydrogenase (△), isocitrate lyase (+). GS in darkened control culture (■). Phototrophic cells were darkened and acetate added at zero time.

Table 2. Comparison of the rates of NH_3 assimilation and the activities of N assimilatory enzymes in NH_3-grown phototrophic, mixotrophic and starved cells

	NH_3 assimilation or enzyme activity (nmol/min/mg dry wt)		
	Phototrophic cells*	Mixotrophic cells*	N-starved cells†
NH_3 assimilation in light	4.5	5.0	16.7
NH_3 assimilation in dark	0.5	2.0	10.6
GS_x	4.9	5.2	16.8
NADH-glutamate synthase	21.3	8.7	108.6
Fd-glutamate synthase	17.1	5.6	61.6
GDH (NADPH)	1.8	2.2	11.9
Glucose-6-phosphate dehydrogenase	90	63	248
Isocitrate dehydrogenase	78	105	95

* Enzyme levels and rates of assimilation were measured in exponentially dividing cultures.

† Phototrophic cells were transferred to a medium lacking N for 16 hr and the enzyme activities were measured at this time. Rate of NH_3 assimilation was determined following re-addition of NH_3 to the culture. The enzyme activities shown are corrected for full substrate saturation.

measurements. (1) The N starvation treatment greatly increased the activity of all the N assimilatory enzymes but did not markedly alter their relative proportions. (2) Both NADH- and Fd-glutamate synthase activities were in excess of the measured rates of assimilation under all these conditions. (3) GS activity very closely matched the rate of assimilation in all the treatments and this suggests that the first member of the glutamate synthase cycle could control assimilation.

An experiment was carried out to measure NH_3 assimilation and to monitor changes in the cell of the level of a number of key enzymes following alterations to the light and N regime of a phototrophic culture. It can be seen (Fig. 5) that the reduction in NH_3 assimilation following darkening of the culture was associated with a 25% loss of GS activity whereas the levels of all the other enzymes measured remained constant. On re-illumination of the culture, extensive reactivation of GS preceded the increase in NH_3 assimilation, an observation that further suggests that GS may regulate assimilation by the glutamate synthase cycle.

It has been established [9] that the NADH-glutamate synthase in *Chlamydomonas* is completely unreactive with NADPH. However, in Table 3 it is shown that under some conditions glutamate biosynthesis may be achieved using NADPH, providing that ferredoxin is present; this

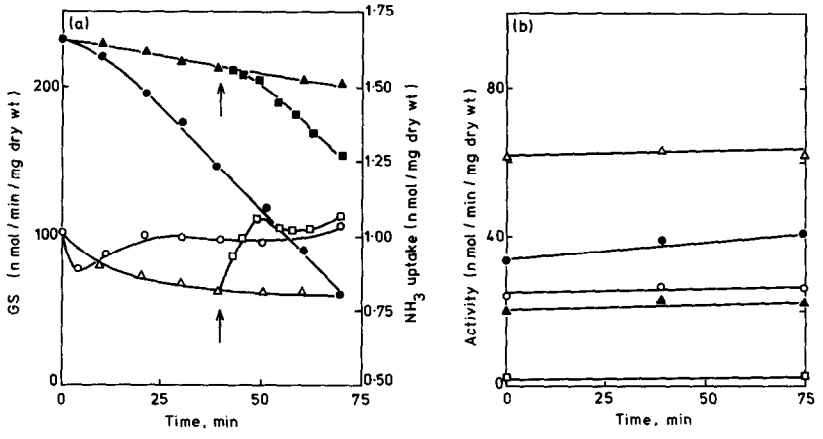

Fig. 5. Alterations in enzyme levels and NH_3 assimilation in response to changes of illumination following the addition of NH_3 to phototrophic cells grown on NO_3. (a) NH_3 assimilation (closed symbols) and GS activity (open symbols) in light (●, ○), dark (▲, △) and following re-illumination (■, □). 0.2 mM NH_4Cl was added at zero time and the darkened culture was re-illuminated at a time denoted by an arrow. (b) Levels of GDH (□), Fd (○) and NADH (●) glutamate synthase, isocitrate dehydrogenase (▲), glucose-6-phosphate dehydrogenase (△) following darkening of the culture.

Table 3. The occurrence and requirements of an NADPH-supported Fd-glutamate synthase activity

Ferredoxin assay	Activity (nmol/min/mg dry wt)
Complete	13.6
+AZA	0.49
−DT + NADPH	0.70
−DT + NADPH (degassed)	2.34
−DT + NADPH + AZA (degassed)	0.86
−DT + NADPH + PP (degassed)	1.31
−DT + NADPH + isocitrate (degassed)	1.56

AZA = azaserine; DT = sodium dithionite; PP = pyrophosphate.

These determinations were carried out using algae grown mixotrophically on NH_3 and permeabilized in liquid nitrogen, which extensively inactivates the NADH-specific glutamate synthase [9].

occurred only in the absence of O_2 and was sensitive to pyrophosphate as well as azaserine. Moreover, the addition of NADP and isocitrate also supported a significant synthesis of the amino acid in the assay.

DISCUSSION

Evidence presented in this paper has unequivocally established that the assimilation of NH_3 by *Chlamydomonas* occurs exclusively via the glutamate synthase cycle and that GDH does not play any anabolic role in the light, the dark or under conditions where the cellular concentration of NH_3 is greatly elevated. Although measurements of enzyme levels and rates of assimilation (Table 2) indicated that the activity of GDH was insufficient to support the total rate of assimilation observed in the light, these data by themselves did not rule out a possible exclusive functioning of GDH in the dark or a partial contribution to assimilation in the light. Certainly the properties of GDH, of exhibiting positive co-operativity with NH_3 and 2-oxoglutarate as substrates (see Table 1), were not inconsistent with it playing such an anabolic role. The $s_{0.5}$ values for these substrates were, however, more than 10-fold greater than the values established respectively for GS (Table 1) and the two glutamate synthases [9] and this indicated, in view of the low cellular GDH activity present, that this pathway would be relatively inefficient for the assimilation of NH_3 at low substrate concentrations. It has been claimed, however [11, 12], that GDH in higher plants may have an (exclusive) anabolic function when the cellular concentration of NH_3 is elevated and that under these circumstances it provides a more economical route for assimilation than the glutamate synthase cycle. The experimental evidence shown in Figs. 1 and 2, however, completely rules out this possibility for *Chlamydomonas*. Even under conditions where NH_3 accumulates at sufficient concentrations to saturate GDH appreciably, following the deactivation of GS, no sustained ^{15}N incorporation into cells occurred either in the light or the dark. The exhaustion of the soluble amino acid pools under these conditions (Fig. 2b) supports this conclusion. Moreover, the high affinity for glutamate and specificity for NAD indicate that GDH is well suited to function as a catabolic enzyme.

The absolute dependence of light and dark NH_3 assimilation on a functional GS was established in a number of studies employing MSO and DSPD. In phototrophic cells the extensive deactivation of GS by MSO was accompanied by a virtual abolition of assimilation (Fig. 1a); the interdependence of these two functions in the light was independently confirmed using DSPD (Fig. 1b). Moreover, although it was shown that deactivation of GS was incomplete when MSO was supplied to darkened algae using acetate, following a loss of about 50% GS activity, a one-to-one relationship extrapolating through zero existed between synthetase activity and dark NH_3 assimilation (Fig. 3b). This observation established that, as well as the glutamate synthase cycle being the sole pathway for NH_3 assimilation in the dark, under these conditions GS was the rate-limiting step in this process. Evidence that GS can regulate assimilation in normal illuminated algae can be seen from Table 2 and Fig. 5. A very close agreement was found between GS activity and the rate of assimilation in cells grown phototrophically and mixotrophically on NH_3. In algae previously starved of N (Table 2) the activities of the two glutamate synthases were greatly in excess in all these conditions, an observation consistent with GS activity limiting assimilation.

In Fig. 5 GS was shown to undergo rapid and reversible activity changes in response to an alteration of N, C and reductant supply. Deactivation of the enzyme was achieved by addition of NH_3 to a darkened, NO_3-grown culture and following its re-illumination the increase in NH_3 assimilation that occurred could be exactly correlated to an increase of GS activity. In the dark, however, the level of GS was substantially greater than the observed assimilatory rate, a situation which is also found in darkened, mixotrophically grown cells (Fig. 3 and Table 2). These observations indicate that although activity modulation of GS may regulate NH_3 assimilation in the light, in the dark assimilation is limited by the availability of either energy, reductant or C substrates. This view was supported and developed in an experiment in which (Fig. 4) NH_3 assimilation, acetate uptake and the

levels of a number of N assimilatory and associated enzymes were measured following the addition of acetate to a darkened, phototrophically grown culture. The increase in NH_3 assimilation observed coincided with the *de novo* synthesis of an acetate transport system and significantly at this time the levels of the enzymes of the glutamate synthase cycle were all in excess. Furthermore, MFA has been shown to inhibit extensively both acetate utilization and NH_3 assimilation on transfer of mixotrophically grown cells to the dark (unpublished observations) relative to a control culture and this inhibitor also completely prevented the development of increased acetate-supported NH_3 assimilation in darkened, phototrophic cells (Fig. 4). Taken together, these results indicate that the development of dark assimilation is more dependent on the increased availability of C and energy that accompanies the development of an acetate transport system than the *de novo* synthesis of any enzyme necessary to support the operation of the glutamate synthase cycle.

It has been shown that *Chlamydomonas* possesses both a Fd- and NADH-specific glutamate synthase ([9] and see also Table 2) and it was suggested that these enzymes could be associated with pathways concerned exclusively with light and dark assimilation (separate glutamate synthase cycles). Certainly the observation that DCMU virtually abolished NH_3 assimilation in phototrophic cells (Fig. 1b) could be reconciled to the sole operation of the (photosystem II dependent)Fd-specific enzyme in the light.

A number of observations reported here, however, suggest that such an explanation may be oversimplified. For example, the finding that DCMU does not reduce NH_3 assimilation in illuminated algae growing on acetate to its rate in the dark can be explained if the pathway functioning under these conditions (the NADH system if we accept the earlier interpretation of Fig. 1) can utilize the ATP still being generated by cyclic phosphorylation. If this explanation is correct it means at least that the supply of ATP to the two glutamate synthase cycles is not rigidly compartmentalized. Similar considerations may also apply to the supply of reductant to the pathways. Several lines of evidence suggest that extensive dark assimilation of NH_3 occurs under conditions where the capacity of the alga to synthesize NADPH is increased (Table 2). For example, dark enhancement in phototrophic algae starved of N was associated both with an elevation of carbohydrate reserves [10] and a specific increase in the NADP-specific glucose-6-phosphate dehydrogenase, a member of the pentose phosphate pathway. Similarly in *Chlamydomonas* provided with acetate, extensive selective synthesis of the NADP-specific isocitrate dehydrogenase was found (Table 2) as well as enzymes of the glyoxylate cycle [13]. However, it is clear that if NADPH is involved in the dark assimilation of NH_3 it must achieve its effect indirectly since it has been shown that the NADH-glutamate synthase is completely unreactive with NADPH as reductant [9]. One possible way of achieving such a link is shown in Table 3 where it can be seen that a coupling of electrons between NADPH and the Fd-glutamate synthase can occur (at least in permeabilized cells). Moreover, the rate of synthesis measured in this experiment was sufficient to account for dark assimilation. The dependence of glutamate biosynthesis in this couple on Fd, and its sensitivity to O_2 and inhibition by pyrophosphate argues that Fd and Fd-NADP reductase (EC 1.18.1.2) are involved in this process. It is possible, therefore, that under some conditions the Fd enzyme may also contribute to dark assimilation and that within the cell a flexible alternation of the two cycles or their coordinate operation both in the light and dark may occur, depending on the supply of reductant or ATP. This may be particularly necessary to maintain (as suggested in [9]) a balanced synthesis of amino acids in the chloroplast and the cytosol under a wide range of environmental conditions. The actual contributions of Fd- and NADH-glutamate synthase to NH_3 assimilation under different conditions of N, C and light supply, and the mechanisms of providing reductant for dark assimilation are at present under investigation.

EXPERIMENTAL

Organisms and culture. Chlamydomonas reinhardii (Dangeard) CCAP 11/32a was grown phototrophically and mixotrophically (on 24 mM NaOAc) as described in ref. [9].

Assays of enzyme activities. Enzymes were assayed either in partially purified cell extracts or in permeabilized cells [9, 14].

Glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3). The enzyme was assayed in cell-free extracts by following NAD(P)H oxidation. The reaction mixture contained in 1.2 ml: 120 μmol Na-Pi buffer, pH 7.8; 72 μmol NH_4Cl; 7.2 μmol 2-oxoglutarate; 0.017 μmol NAD(P)H; 100 μl enzyme extract.

Enzyme activity was also determined radiochemically in cells permeabilized by freezing in liquid N_2 by measuring the formation of Glu from 2-oxoglutarate. The reaction mixture contained in 1.5 ml: 75 μmol imidazole-acetate buffer, pH 7.8; 90 μmol NH_4Cl; 6 μmol 2-oxoglutarate containing 7.4 kBq (0.2 μCi); 0.45 μmol NAD(P)H. After assay cell debris was removed and 1.0 ml applied to 1.7 × 0.5 cm Dowex AG 50W-X8 (H^+) and washed with 3 ml H_2O. Glu was eluted with 4 ml 0.4 M KOAc and its ^{14}C content determined.

Glutamine synthetase (L-glutamate:ammonia ligase (ADP), EC 6.3.1.2). Transferase activity was determined as in ref. [14]. Synthetase activity in cells permeabilized by freezing in liquid N_2 was determined radiochemically by measuring the formation of L-Gln from L-Glu. Reaction mixture contained in 1.5 ml: 75 μmol imidazole acetate buffer, pH 7.2; 0.75 μmol EDTA; 6.0 μmol ATP; 60 μmol $MgCl_2$; 6.0 μmol NH_4Cl; 4.5 μmol Glu containing 7.4 kBq (0.2 μCi). After assay cell debris was removed and 1.0 ml applied to a 3.0 × 0.5 cm Dowex AG1-X8 (Ac) column and Gln was eluted by washing with 3 ml 50 mM HOAc and its ^{14}C content determined.

Glutamate synthase activities. L-glutamate:NAD$^+$ oxidoreductase (transaminating) (EC 1.4.1.14) and L-glutamate:ferredoxin oxidoreductase (transaminating) (EC 1.4.7.1) were measured exactly as described in ref. [9].

Isocitrate lyase (*threo*-D$_s$-isocitrate:glyoxylate-lyase, EC 4.1.3.1.). The enzyme was assayed in cells permeabilized by freezing in liquid N_2. Reaction mixture contained in 1.5 ml: 75 μmol imidazole acetate buffer, pH 7.9; 2 μmol L-cysteine; 4.5 μmol Na_3-D,L-isocitrate; 1.5 μmol $MgCl_2$. After assay cell debris was removed and glyoxylate was determined in 1 ml by the addition of 0.5 ml 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl followed by incubation at 20° for 5 min. 1 ml 95% EtOH and 2.5 ml 1.5 N NaOH were then added and the A measured immediately at 458 nm.

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP$^+$ 1-oxidoreductase, EC 1.1.1.49) and isocitrate dehydrogenase (*threo*-D$_s$-isocitrate:NAD$^+$ oxidoreductase (decarboxylating) EC 1.1.1.41) were measured as in ref. [15].

Analytical procedures. Methods used for determining ^{15}N abundance, NH_3 and protein are described in ref. [16]. Mean cellular concns of free NH_3 and amino acids were estimated in samples of cells extracted with 60% EtOH (1.8 mg dry wt cell/ml). 1 ml extract was applied to 1 × 0.5 cm column of Dowex-50 (Na^+) equilibrated in 0.1 M Na-citrate buffer, pH 4 and neutral amino acids were eluted using 2 ml of the same buffer. Basic amino acids and NH_3 were eluted with 5 ml 0.2 M Na-Pi buffer, pH 7.5 and the fractions were assayed for amino acids [17] and NH_3 [16]. A vol./dry wt ratio of 3 was used in the calculation. The uptake of acetate was measured essentially as described in ref. [18].

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