BBA 12153

THE INHIBITION OF NITRATE ASSIMILATION BY AMMONIUM IN CHLORELLA

P. J. SYRETT AND I. MORRIS

Botany Department, University College, London (Great Britain)

(Received August 10th, 1962)

SUMMARY

Chlorella vulgaris growing with ammonium nitrate as nitrogen source preferentially assimilates ammonium. Nitrate assimilation ceases completely when ammonium is added and recommences as soon as ammonium has disappeared. Ammonium does not inhibit nitrate reduction by cells unable to assimilate ammonium because they lack a carbon source. Thus the inhibition is not due to ammonium *per se* but is connected with its assimilation. The inhibition is not thought to result from competition for reduced pyridine nucleotide because nitrate reductase of Chlorella is specific for DPN while glutamic dehydrogenase is specific for TPN. Nitrite addition also inhibits nitrate assimilation completely but ammonium only partially inhibits nitrite assimilation.

INTRODUCTION

It has often been observed that algae growing with ammonium nitrate as nitrogen source assimilate ammonium ions preferentially, nitrate not being utilized until ammonium has almost disappeared[1-3]. Several fungi behave similarly[4]. It is clear from studies with ^{15}N-labelled substrate[5,6] that this behaviour does not result from an equilibrium reaction involving nitrate and ammonium which maintains the level of nitrate constant until ammonium has disappeared. Rather must it result from an inhibition of one stage in nitrate reduction. MORTON AND MACMILLAN[4] observed that while ammonium completely inhibited the assimilation of nitrate by the fungus *Scopulariopsis brevicaulis*, it had little effect on the assimilation of nitrite. They therefore suggested that ammonium acted by inhibiting the reduction of nitrate to nitrite. In further studies with fungi, MORTON[5] concluded that the inhibition by ammonium did not occur unless it was assimilated.

In this paper some effects of ammonium on nitrate assimilation by the alga *Chlorella vulgaris* are described and it is shown that ammonium inhibits nitrate assimilation as it does in the fungi studied by MORTON AND MACMILLAN[4]. Moreover, the evidence suggests that the inhibitor is a product of ammonium assimilation which inhibits the reduction of nitrate to nitrite. In another paper[7] a further effect of ammonium will be discussed, namely its repressive effect on the synthesis of the enzyme nitrate reductase.

METHODS

Growth of organism

Pure cultures of *Chorella vulgaris* (Pearsall's strain) were grown in a medium containing, per litre glass-distilled water, 0.2 g anhydrous $MgSO_4$, 7.76 g KH_2PO_4, 2.32 g K_2HPO_4, 1 ml A_5 trace-element solution[9] and 10 mg Fe as Fe-EDTA (see ref. 9). 280 mg nitrogen per litre was supplied as either $(NH_4)_2SO_4$, KNO_3 or NH_4NO_3. The pH of the medium, after autoclaving, was 6.1.

Cultures were normally grown for 4 days in wash-bottles at 25° with a light intensity of 600 ft-candles at the surface of the cultures. The cultures were vigorously aerated with air containing 0.5% (v/v) carbon dioxide[10]. When the cells were growing exponentially the generation time was 8 h.

Carbohydrate-starved cells were prepared by aerating cultures for 18 h in darkness before harvesting. Nitrogen-starved cultures were prepared by removing the cells by centrifugation at 400 × *g*, washing and resuspending them in full growth medium without a nitrogen source. The cultures were then illuminated and aerated with air containing 0.5% (v/v) carbon dioxide for 16 h before being harvested for experimentation[10].

Before the experiments, the cells were harvested by centrifugation at 400 × *g*, washed and suspended in nitrogen-free growth medium. The cell density was adjusted turbidometrically to 3–8 mg/ml depending on the experiment.

Gas exchange

This was measured by conventional Warburg manometry at 25°. For light experiments, the flasks were illuminated with fluorescent tubes giving a light intensity in the flasks of about 800 ft-candles.

Ammonium-N

This was determined colorimetrically by a Nessler method[11] after separation from the cells and medium by the method of CONWAY[12].

Nitrate-N

This was determined in samples of the medium after removal of the cells by centrifugation. A phenol-disulphonic acid colorimetric method was used[13]. The light absorption of the coloured compound was read at 440 mμ and compared with that of freshly prepared standards.

Nitrite-N

This was determined colorimetrically with Griess-Ilosway reagents[13] on samples of the medium after removal of the cells. The colour was read at 530 mμ, 20 min after the addition of the reagents.

Preparation of cell-free extracts

Cells were harvested, washed and resuspended in ice-cold 0.1 M Tris buffer (pH 7.4). The cell density was 6–10 mg/ml. The cell suspension was stored at −14° for 24–28 h. After thawing, the cell suspension was passed through a French press[14] to

smash the cells. During this process the temperature rose to 17–20° but the suspension leaving the press was immediately immersed in ice. The suspension was centrifuged at 13 000 × *g* for 20 min at 2°. The clear, green, supernatant was decanted, stored at −14° and used for measurements of enzyme activity.

Assay of nitrate reductase activity

Nitrate reductase activity was measured by estimating the nitrite formed after incubation of the cell-free extract with KNO_3 and DPNH. Full details are given in a later paper[7].

Measurement of pyridine nucleotide oxidation by fluorimetry

Both nitrate reductase and glutamic dehydrogenase activities of cell-free extracts were determined by following the oxidation of reduced pyridine nucleotide fluorimetrically. The reagents were contained in about 1 ml in a quartz cuvette in a 'Locarte' fluorimeter. The primary filter was LF/2 and the secondary LF/3/2. Details of the assay mixtures are given in the legend to Fig. 6.

The protein content

The protein content of cell-free extracts was determined by the method of LOWRY *et al.*[15] using bovine-albumin standards.

RESULTS

1. The preferential assimilation of ammonium

Fig. 1 shows the disappearance of ammonium nitrogen and nitrate nitrogen from cultures of *C. vulgaris* growing with ammonium nitrate as nitrogen source.

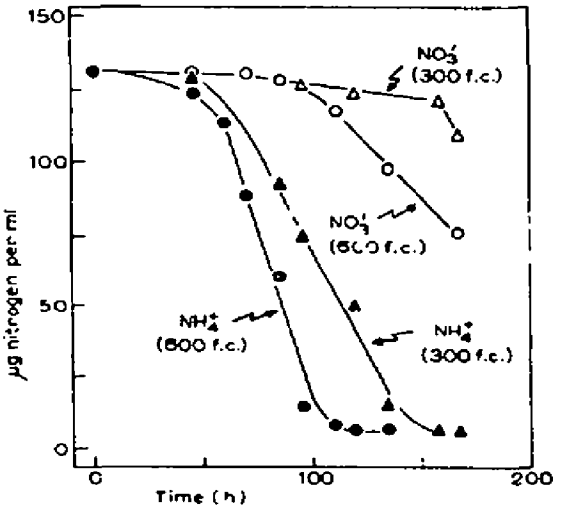

Fig. 1. The assimilation of ammonium and nitrate by cultures of *C. vulgaris* growing at 300 or 600 ft-candles light intensity with ammonium nitrate as nitrogen source. Note that nitrate is not assimilated until almost all the ammonium has disappeared. Temperature 25°. Cultures aerated with 0.5% (v/v) CO_2 in air.

Cultures were grown at two different light intensities. Growth is faster at 600 ft-candles than at 300 ft-candles and ammonium nitrogen disappears more rapidly. In neither culture is nitrate utilized until the ammonium-N has been assimilated.

2. The inhibition of nitrate assimilation by the addition of ammonium-N

Fig. 2a shows the result of adding a small quantity of ammonium-N to a suspension which is assimilating nitrate in darkness with glucose as carbon source. Nitrate assimilation ceases as soon as ammonium-N is added. The ammonium-N is rapidly assimilated. As soon as it has all gone, nitrate assimilation commences again.

Fig. 2b shows the similar behaviour of cells assimilating nitrate in light with carbon dioxide as carbon source.

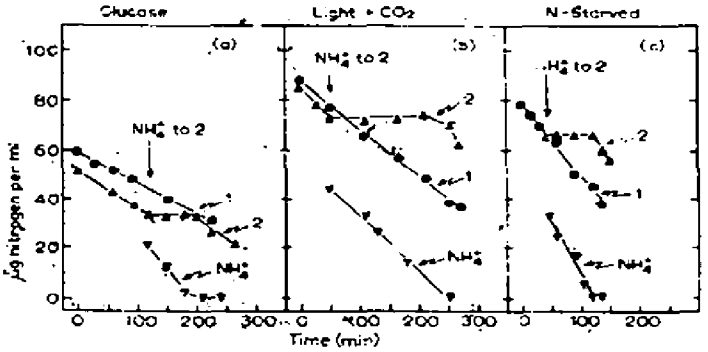

Fig. 2. The effect of ammonium addition on nitrate assimilation by (a) nitrate-grown cells in dark in presence of 1% (w/v) glucose, (b) nitrate-grown cells in light in presence of 5% (v/v) carbon dioxide in air, (c) nitrogen-starved cells in dark in presence of 1% (w/v) glucose. ●—●, nitrate-N in culture 1 which does not receive ammonium; ▲--▲, nitrate-N in culture 2 which receives ammonium at time indicated; ▼—▼, ammonium-N in culture 2. Cell density, 5.0 mg dry wt./ml. Light intensity, 800 ft-candles. The figures represent μg nitrate or ammonium-N per ml for Expts. a and b, and μg N per 0.5 ml for Expt. c. Temperature, 25°.

URHAN[16] reported that nitrogen-starved cells of Chlorella and Scenedesmus assimilated ammonium and nitrate simultaneously. However, under the conditions of our experiments, ammonium inhibits nitrate assimilation by nitrogen-starved cells just as it does that of normal cells (Fig. 2c)

3. The effect of nitrite on nitrate assimilation

Fig. 3a shows the effect of the addition of nitrite on nitrate assimilation. It can be seen that its effect is similar to that of ammonium addition, *i.e.* it produces a complete inhibition of nitrate assimilation which persists until all the nitrite has been assimilated.

4. The effect of ammonium on nitrite assimilation

The addition of ammonium-N also inhibits nitrite assimilation but the inhibition is not complete (Fig. 3b). In three experiments of this type the percentage inhibition

of nitrite assimilation was 75, 84 and 85% giving a mean value of about 80% inhibition.

5. *The dependence of inhibition upon the assimilation of ammonium*

In the experiments described above, the inhibition of nitrate assimilation by ammonium could be due either to the presence of ammonium or to the ammonium assimilation which occurs immediately after ammonium is added. The results described in this section suggest that the inhibition is not due to the presence of ammonium *per se* but to ammonium assimilation.

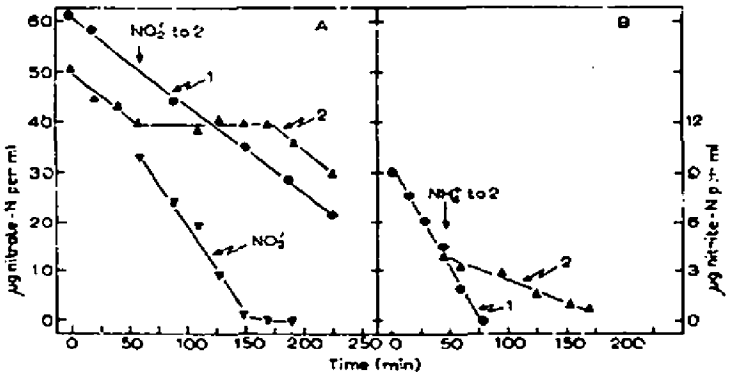

Fig. 3. A. The effect of nitrite on nitrate assimilation by nitrate-grown cells. The cultures contained 5.5 mg dry wt. cells per ml and 1% (w/v) glucose. ●—●, nitrate-N in culture 1 which received no nitrite; ▲—▲, nitrate-N in culture 2 which received 10 μg nitrite-N per ml at time indicated; ▼—▼, nitrite-N in culture 2. B. The effect of ammonium on nitrite assimilation by nitrate-grown cells. The cultures contained 3 mg dry wt. cells per ml and 1% (w/v) glucose. Culture 2 received 22 μg ammonium-N per ml at the time indicated.

Experiments with cell-free extracts containing nitrate reductase (section 6) show that ammonium sulphate in a final concentration of $3 \cdot 10^{-2}$ M has no effect at all on the activity of nitrate reductase. This ammonium concentration is thirty times that which inhibits nitrate assimilation by intact cells and the absence of inhibition with cell-free extracts suggests that ammonium alone does not inhibit nitrate reduction.

This suggestion was supported by experiments carried out with intact cells which had been previously starved of carbohydrate. BONGERS[17] found that carbohydrate-starved cells of Scenedesmus would reduce nitrate to ammonium quantitatively in light in the absence of carbon dioxide. Such cells presumably lack carbon reserves with which to make carbon skeletons for ammonium assimilation.

Fig. 4A(i) shows that in light, in the absence of carbon dioxide, carbohydrate-starved cells of *C. vulgaris* reduce nitrate to ammonium which accumulates. The addition of 0.005 M ammonium has no effect on this reduction. However, when either carbon dioxide or glucose is present to provide a carbon source (Fig. 4Aii and iii) no ammonium accumulates since it is assimilated and, under these conditions, the addition of ammonium inhibits nitrate disappearance. In fact, when the carbon source is 5% (v/v) carbon dioxide in the gas phase, ammonium addition inhibits

nitrate reduction completely (Fig. 4Aiii). The effect of the presence of glucose is of interest. When glucose is added, in the absence of carbon dioxide, *i.e.* the Warburg flasks in which the experiments are carried out contain caustic potash, the inhibition of nitrate reduction by ammonium is present but incomplete (Figs. 4Aii and 4Bii). If caustic potash is omitted from the Warburg flasks the inhibition in the presence of glucose is much more pronounced (Fig. 4Biii). This may be a reflection of the fact that ammonium assimilation to amino acids is accompanied by considerable carbon dioxide fixation[18]; it might, however, indicate a more direct relationship between ammonium, carbon dioxide and the inhibitor of nitrate reduction. Fig. 4B also

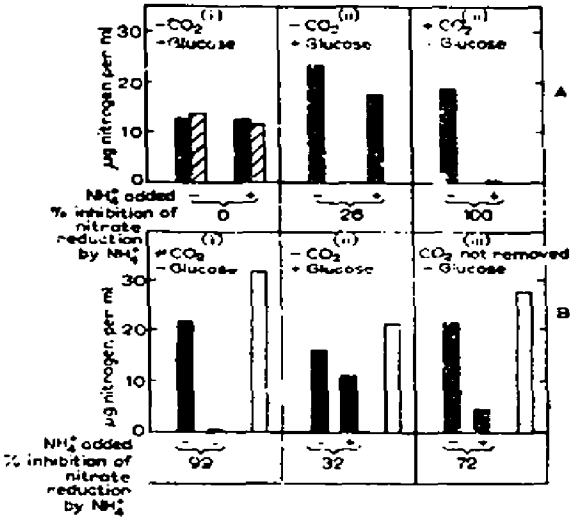

Fig. 4. The effect of ammonium on nitrate reduction and assimilation by carbohydrate-starved cells in light. The plus and minus signs below the column denote the presence or absence of added ammonium-N (70 μg/ml). The experiments were carried out in Warburg flasks, each containing 2.0 ml cell suspension (3 mg dry wt. cells/ml). The initial concentration of nitrate-N was 70 μg/ml. The flask contents were analysed 2 h after the addition of nitrate (and/or ammonium). Light intensity, 800 ft-candles. Temperature, 25°. $-CO_2$ indicates that the Warburg flasks contained caustic potash in the centre wells. In experiment 4B (iii), this was omitted; $+CO_2$ indicates manometers flushed with 5% CO_2 in air. When glucose was present, its final concentration was 1% (w/v). Solid column, nitrate-N disappearing from suspension in 2 h; cross-hatched column, ammonium-N appearing in suspension in 2 h; open column in lower figure, ammonium-N disappearing from suspension in 2 h in absence of nitrate. The percentage inhibition of nitrate reduction by presence of ammonium is shown.

shows the amount of ammonium assimilation carried out by the cells in the absence of nitrate. It is greatest when 5% CO_2 is the carbon source (i) and least with glucose in the absence of carbon dioxide (iii). It is clear that the inhibitory effect of ammonium on nitrate reduction is most pronounced under those conditions which most favour ammonium assimilation.

The experiments illustrated in Fig. 4 were carried out in Warburg manometers, the flask contents being analysed at the end of the experiment. The gas exchange which occurs during an experiment of this type is shown in Fig. 5. In light, nitrate reduction is accompanied by a considerable evolution of oxygen[19,20]. Thus gas exchange is a sensitive indicator of the rate of nitrate reduction. It can be seen from

Fig. 5a, that, in the absence of glucose and carbon dioxide, the addition of NH_4^+ has no effect on gas exchange in the presence of nitrate. This result is in sharp contrast to that shown in Fig. 5c where the addition of NH_4^+ to cells assimilating nitrate in the presence of 5% (v/v) carbon dioxide, lowers the rate of gas output to the level of that of cells with NH_4^+ alone; this corresponds to the almost complete inhibition of nitrate reduction shown in Figs. 4A (iii) and 4B (i). In the presence of glucose and absence of carbon dioxide, the gas-exchange measurements indicate an incomplete inhibition of nitrate reduction by NH_4^+ (Fig. 5b) corresponding to that shown in Figs. 4A (ii) and 4B (ii).

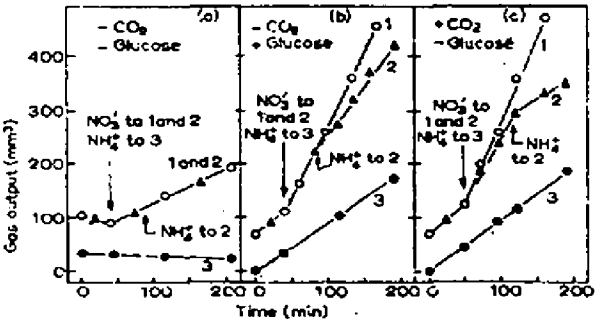

FIG. 5. Gas exchange accompanying nitrate assimilation by carbohydrate-starved cells in light. Light intensity, 800 ft-candles. Temperature, 25°. Nitrate-grown cells starved of carbohydrate for 16 h. Cell suspension contained 3.0 mg dry wt. cells per ml. (a) and (b), flasks contained 2.0 ml cell suspension. Caustic potash in centre well, in (b) 1% (w/v) glucose was added. 84 μg NO_3-N or 96 μg NH_4^+-N added at times indicated. (c), Flasks contained 1.0 ml cell suspension. Gas phase, 5% (v/v) CO_2 in air. 104 μg NO_3-N or 57 μg NH_4^+-N added at times indicated.

These experiments, then, suggest that it is ammonium assimilation rather than ammonium itself which inhibits nitrate assimilation. MORTON[6] came to a similar conclusion for different reasons.

6. The pyridine nucleotide specificity of nitrate reductase and glutamic dehydrogenase in C. vulgaris

The presence of both nitrate reductase and glutamic dehydrogenase was demonstrated in cell-free extracts of cells grown with KNO_3 as nitrogen source (Fig. 6a). Nitrate reductase required DPNH as electron donor and does not utilize TPNH. The reverse is true for glutamic dehydrogenase (Fig. 6b).

7. Nitrate assimilation by cells grown with NH_4NO_3 as nitrogen source

Fig. 7 shows the time course of nitrate assimilation of cells grown with NH_4NO_3 as nitrogen source in contrast to those grown with KNO_3. Whereas the cells grown on KNO_3 assimilate nitrate immediately after it is added, the cells grown on NH_4NO_3 show a long period of 3 h before nitrate assimilation starts. This long period is considerably shortened if the cells are aerated for 3 h in a N-free medium before nitrate is added. That the difference in behaviour of these cultures is not due to differences

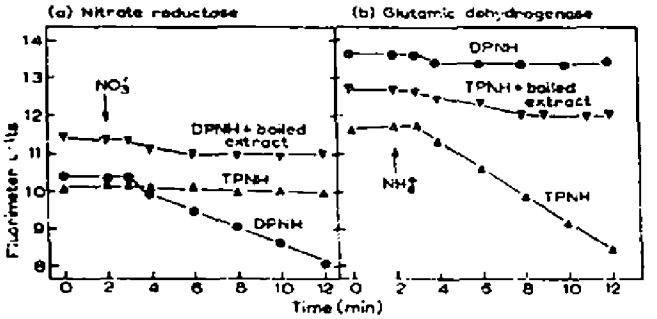

Fig. 6. The pyridine nucleotide specificity of (a) nitrate reductase and (b) glutamic dehydrogenase; the oxidation of reduced pyridine nucleotide was measured fluorimetrically. The assay mixtures contained 0.5 ml cell-free extract containing 0.15 mg protein, 0.2 ml 0.1 M Tris buffer (pH 7.4); 0.1 ml 0.001 M (approx.) DPNH or TPNH. A stream of oxygen-free nitrogen was bubbled through the mixture between readings; this reduced the endogenous oxidation of reduced pyridine nucleotide. In (a) 20 μmoles KNO_3 were added at the time indicated; in (b) 20 μmoles α-oxoglutarate were included in the reaction mixture and 20 μmoles $(NH_4)_2SO_4$ added at the time indicated. Temperature, 31°. 10 fluorimeter units approx. 40 mμmoles PNH.

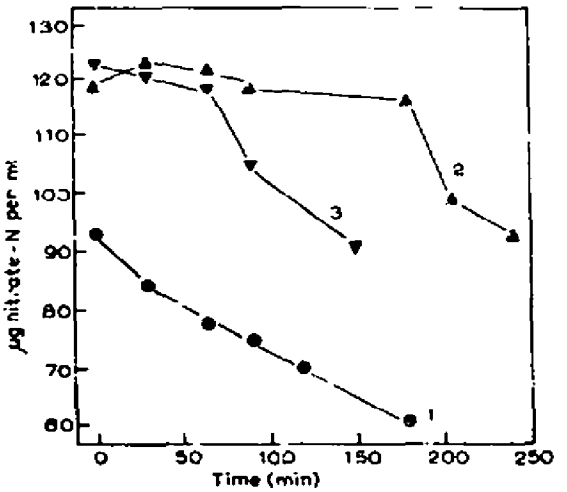

Fig. 7. Nitrate assimilation by cells grown with ammonium nitrate as N-source. Culture 1 contained 13 ml cell suspension grown in potassium nitrate; the cells assimilate nitrate as soon as it is added. Cultures 2 and 3 each contain 8 ml cell suspension grown in ammonium nitrate but culture 3 was shaken for 3 h in nitrogen-free medium before nitrate was added. Note that culture 2 only assimilates the added nitrate after a lag of about 3 h; in culture 3 the lag has been considerably reduced. All flasks contained Chlorella suspension, 7 mg dry wt./ml, with glucose added to give a final concentration of 1% (w/v). The flasks were shaken in darkness at 25°. All received 100 μmoles KNO_3 at zero time. Samples of each type of cell were passed through a French press and nitrate reductase activity assayed. The enzyme levels are shown below. Note that the nitrate reductase level of the ammonium nitrate-grown cells is unchanged by 3 h aeration in nitrogen-free medium.

Nitrate reductase activity in cell-free extracts for	*mμmoles nitrite formed per min per mg protein*
KNO_3-grown cells	15.6
NH_4NO_3-grown cells	4.0
NH_4NO_3-grown cells after 3 h in N-free medium	4.8

in the amount of nitrate reductase present is shown by the data included in Fig. 7. It is clear that the cells grown on NH_4NO_3 posses nitrate reductase (although less than that in the KNO_3 cells) and that their nitrate reductase content does not alter appreciably during the 3-h period of nitrogen starvation. Nevertheless the period of nitrogen starvation considerably increases their ability to assimilate nitrate.

DISCUSSION

Nitrate assimilation by *C. vulgaris* is completely inhibited as soon as a small quantity of ammonium is added and the inhibition is relieved as soon as all the ammonium has been assimilated. Since ammonium only partially inhibits the assimilation of nitrite and since no appreciable quantity of nitrite ever accumulates in our cultures, ammonium must inhibit the first step of nitrate assimilation, namely the reduction of nitrate to nitrite. The addition of nitrite also inhibits nitrate assimilation; this effect may well be a result of the conversion of nitrite to ammonium so that both act in a common manner. In many respects our results resemble those of MORTON AND MACMILLAN[4] with fungi. They, too, found that ammonium completely inhibited nitrate assimilation and partially inhibited nitrite assimilation, but in their work, nitrite apparently had little effect on the reduction of nitrate.

Ammonium appears to inhibit nitrate assimilation only when it is itself assimilated. Thus ammonium has no effect on nitrate reduction by carbohydrate-starved cells unless a carbon source is added which enables the cells to assimilate ammonium. MORTON[6], too, suggested that ammonium must be assimilated before it can inhibit nitrate reduction.

There are at least two ways in which ammonium assimilation might inhibit nitrate reduction. Firstly, the two processes might compete for the available supply of reduced pyridine nucleotide in the cells. Secondly, ammonium might be assimilated to an organic nitrogenous compound which inhibits nitrate reduction in some way. The second of these possibilities appears the more likely. It is true that the primary steps in both ammonium and nitrate assimilation require reduced pyridine nucleotide, in the first, for the reductive amination of α-oxoglutarate to glutamic acid and, in the second, for the reduction of nitrate to nitrite. But in *C. vulgaris*, the pyridine nucleotide specificity of the enzymes catalysing these two reactions appears to differ. Glutamic dehydrogenase requires TPNH while nitrate reductase requires DPNH. Thus a direct competition appears unlikely although it cannot be completely ruled out. Moreover, the experiment with cells grown on ammonium nitrate suggests that something inhibiting nitrate assimilation accumulates in cells which are assimilating ammonium. These cells, although possessing nitrate reductase, do not begin to reduce nitrate until they have been aerated for 3 h in an ammonium-free medium. It looks as if the inhibitor is removed during this period. Presumably, in the experiments in which rather a small quantity of ammonium was added, the quantity of inhibitor formed would be small and would disappear soon after all the ammonium had been assimilated.

If such an inhibitor is formed during ammonium assimilation it might inhibit nitrate reduction in one of two ways. Firstly it might, in some way, prevent the entry of nitrate into the cell or interfere with its access to the enzyme, nitrate reductase. Secondly it might act by inhibiting nitrate reductase itself. We prefer the

second of these possibilities and have some evidence to support it[7]. We have failed to find evidence of any effect of ammonium assimilation on the entry of nitrate into the cells but since the quantity of nitrate in Chlorella is always extremely low the results are inconclusive. We have also examined the effect of a variety of nitrogenous compounds on the nitrate reductase activity of cell-free extracts; the results are presented in a later paper[7].

ACKNOWLEDGEMENT

We should like to thank the Department of Scientific and Industrial Research for a grant to one of us (I.M.).

REFERENCES

1 C. A. LUDWIG, *Am. J. Botany*, 25 (1938) 448.
2 R. PRATT AND J. FONG, *Am. J. Botany*, 27 (1940) 735.
3 J. F. SCHULER, V. M. DILLER AND H. J. KERSLEN, *Plant Physiol.*, 28 (1953) 299.
4 A. G. MORTON AND A. MACMILLAN, *J. Expll. Bot.*, 5 (1954) 232.
5 C. C. DELWICHE, *J. Biol. Chem.*, 189 (1951) 167.
6 A. G. MORTON, *J. Expll. Bot.*, 7 (1956) 97.
7 I. MORRIS AND P. J. SYRETT, in preparation.
8 D. I. ARNON, *Am. J. Botany*, 25 (1938) 322.
9 L. JACOBSON, *Plant Physiol.*, 26 (1951) 411.
10 P. J. SYRETT, *Ann. Botany (London)*, 17 (1953) 1.
11 O. FOLIN AND W. DENIS, *J. Biol. Chem.*, 26 (1916) 473.
12 E. J. CONWAY, *Micro-diffusion analysis and volumetric error*, Crosby Lockwood and Son, London, 1947.
13 F. D. SNELL AND C. T. SNELL, *Colorimetric Methods of Analysis*, 3rd ed., Vol. II, Van Nostrand, 1949, p. 785–807.
14 H. W. MILNER, N. S. LAWRENCE AND C. S. FRENCH, *Science*, 111 (1950) 633.
15 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDAL, *J. Biol. Chem.*, 193 (1951) 265.
16 O. URHAN, *Jahrb. wiss. Botan.*, 75 (1932) 1.
17 L. H. J. BONGERS, *Mededel. Landbouwhogeschool Wageningen*, 56 (1950) 1.
18 P. J. SYRETT, *Physiol. Plantarum*, 9 (1956) 165.
19 O. WARBURG AND E. NEGELEIN, *Biochem. Z.*, 110 (1920) 66.
20 J. MYERS, in J. FRANCK AND W. E. LOOMIS, *Photosynthesis in Plants*, Iowa State College Press, Ames, Iowa, 1949, p. 349.