Energy-dependence of the Assimilatory Nitrate Uptake in *Azotobacter chroococcum*

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Nitrate assimilation by suspensions of *Azotobacter chroococcum*, as determined by the disappearance of the ion from the external medium, displayed saturation kinetics, was inhibited by nitrite, and exhibited an affinity for nitrate higher than that of nitrate reductase. This suggests that the entry of nitrate into the cell is mediated by a specific transporter. Nitrate assimilation required a readily utilizable carbon source and aerobic conditions and was blocked by the uncouplers carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 2,4-dinitrophenol (DNP) but not by *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of ATPases. The inhibition of nitrate assimilation in the absence of an appropriate carbon source was overcome by the non-physiological energy source ascorbate plus *N*-methylphenazinium methylsulphate (PMS), a substrate combination that allowed respiration. Though an ATP-dependent nitrate uptake mechanism cannot be ruled out, these data suggest that transport of nitrate into the cell is directly dependent on the proton electrochemical gradient across the cytoplasmic membrane.

INTRODUCTION

The process of nitrate assimilation comprises at least three consecutive steps: uptake, followed by reduction of nitrate and incorporation of the reduced nitrogen into carbon skeletons. In micro-organisms that accumulate nitrate, for example the diatoms *Phaeodactylum tricornutum* (Cresswell & Syrett, 1981) and *Skeletonema costatum* (Serra *et al.*, 1978) and the fungus *Neurospora crassa* (Schloemer & Garrett, 1974), nitrate uptake and its subsequent metabolism can be distinguished by measuring both nitrate disappearance from the medium and intracellular nitrate. It has been proposed that transport of nitrate into these cells is an ATP-dependent process (Cresswell & Syrett, 1981; Falkowski, 1975).

In unicellular micro-organisms that lack storage vacuoles, and are thus unable to accumulate nitrate, separation of nitrate uptake from its subsequent assimilatory reduction is not possible unless the short half-life isotope ^{13}N is used. Studies of *Klebsiella pneumoniae* (Thayer & Huffaker, 1982) and *Pseudomonas fluorescens* (Betlach *et al.*, 1981) have shown, using $^{13}\text{NO}_3^-$, that cells accumulate nitrate internally and that, in the case of *K. pneumoniae*, one of the two transport systems must be active. However, the energy requirements of the uptake process were not described in either case.

We have previously reported on the conditions required for the development of assimilatory nitrate uptake in the strictly aerobic, heterotrophic bacterium *Azotobacter chroococcum* (Revilla

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PMS, *N*-methylphenazinium methylsulphate.

et al., 1985). We now present data indicating the existence in this organism of an active transport mechanism for nitrate which seems to be directly dependent on the proton electrochemical gradient across the cytoplasmic membrane.

METHODS

Chemicals. MOPS and PMS were purchased from Sigma; FCCP was from Boehringer; DNP, DCCD and all other chemicals were of analytical grade and were acquired from Merck.

Organism and culture conditions. *A. chroococcum* (strain ATCC 4412 from the Valencia University Collection) was used throughout. Maintenance of stock cultures and preparation of liquid cultures with nitrate as the nitrogen source were as previously described (Paneque *et al.*, 1982). Where indicated, mannitol (the carbon and energy source) was replaced by 0.5% (w/v) glucose or succinate, or by 1% (w/v) pyruvate, ethanol or acetate.

Measurement of nitrate uptake. Mid-exponential phase cells (OD_{650} approximately 0.5) were used for each experiment. Cultures were harvested by centrifugation at 8000 *g* for 10 min at 4 °C, washed twice with 25 mM-MOPS/KOH buffer (pH 7.5), and resuspended to 0.4–0.5 mg cell protein ml⁻¹ in the same buffer (50 mM) supplemented with 2% (w/v) mannitol. Where indicated, mannitol was replaced by the specified substrate as the energy source. Assays were done with continuous shaking at 27 °C in conical flasks open to the air or, for anaerobic assays, in closed flasks containing Ar as the gas phase. The experiments were started by the addition of KNO₃ (0.3–0.5 mM final concentration) and, at intervals thereafter, nitrate uptake was assayed by following the disappearance of nitrate from the medium after rapid removal of bacteria by filtration in a Millipore system with Whatman glass microfibre paper. All the results are representative of three separate experiments on different batches of bacteria.

Analytical procedures. Nitrate reductase activity was determined *in situ* as follows: 5 ml cell suspension was centrifuged at 9750 *g* for 5 min at 4 °C, washed once with 25 mM-MOPS/KOH buffer (pH 7.5), and resuspended to 2–4 mg cell protein ml⁻¹ in 1 ml of the same buffer (125 mM) supplemented with 0.25 mM-EDTA. Toluene (20 µl) was added and the mixture vigorously shaken for 2 min. A sample of this suspension was added to the reaction mixture for nitrate reductase activity with dithionite-reduced methyl viologen as the electron donor (Guerrero *et al.*, 1973). Activity units correspond to µmol nitrite produced min⁻¹. Glutamine synthetase was determined in cells permeabilized with alkyltrimethylammonium bromide mixture (Cejudo *et al.*, 1984) by the γ-glutamyl transferase assay at pH 7.5 (Shapiro & Stadtman, 1970). Nitrate was determined spectrophotometrically at 210 nm in acid solution (Cawse, 1967) after eliminating, when necessary, ascorbate by heating at 80 °C for 10 min with alkali (Kato, 1963). Nitrite was estimated by the method of Snell & Snell (1949). Oxygen consumption by *A. chroococcum* cells was followed with a Hansatech oxygen electrode equipped with a Varian recorder.

RESULTS AND DISCUSSION

Effect of external nitrate concentration on nitrate assimilation

To study whether nitrate uptake in *A. chroococcum* is mediated by an active transport process, the effect of various external nitrate concentrations on the rate of nitrate uptake was investigated. The rate remained constant within the range 0.1–1 mM and was inhibited at higher concentrations (not shown). When the initial external nitrate concentration was 0.1 mM the rate of uptake was constant until the concentration of nitrate in the medium reached approximately 40 µM (Fig. 1). Hence the half-saturation constant of the nitrate assimilation system must be lower than 40 µM.

Since the consumption of nitrate by *A. chroococcum* depends on the entry of the ion into the cell and on its subsequent reduction and assimilation of the ammonium produced, the simplest way to interpret the results shown in Fig. 1 is that assimilation of nitrate by this bacterium displays saturation kinetics and has an affinity for nitrate higher than that estimated *in vitro* (0.25 mM) for nitrate reductase (Guerrero *et al.*, 1973). As discussed by Hayashi & Lin (1965), this difference in affinity is presumptive evidence for the existence of an active transport.

The K_m value for nitrate (0.25 mM) of *A. chroococcum* nitrate reductase was obtained with viologen dyes as the reducing substrate, and recently Morpeth & Boxer (1985) have reported a K_m for nitrate of the purified nitrate reductase from *Escherichia coli* of 2 µM when quinols (analogues of the physiological reductant) are oxidized, as compared to 0.42 mM with reduced viologen dyes as the electron donor. Therefore, *A. chroococcum* nitrate reductase could exhibit a higher affinity for nitrate *in vivo*. Although not accurately measured, the half-saturation concentration for nitrate of the uptake process in *A. chroococcum* approaches those of other

Fig. 1. Utilization of limiting amounts of nitrate by *A. chroococcum*. Cell suspensions ($0.4 \text{ mg protein ml}^{-1}$) were assayed for nitrate uptake as described in Methods except that the initial concentration of KNO_3 was 0.1 mM .

micro-organisms, which lie within the range $0.1\text{--}10 \text{ }\mu\text{M}$ (Syrett, 1981; Ullrich, 1983; Thayer & Huffaker, 1982; Betlach *et al.*, 1981).

Effect of external nitrite concentration on nitrate assimilation

Nitrite, which was previously found to act as an inducer of assimilatory nitrate uptake (Revilla *et al.*, 1985), strongly inhibited nitrate assimilation (79% inhibition at 0.3 mM -nitrite increasing to a maximum of 96% at 0.5 mM -nitrite). Nitrite was taken up by the cells irrespective of the presence of nitrate (as measured by following nitrite disappearance from the medium), at a rate which was practically the same as that of nitrate assimilation [$20\text{--}30 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$]. The decrease in nitrate utilization caused by nitrite was partly counteracted by nitrite consumption, the total amount of inorganic nitrogen taken up being almost the same in the presence of nitrate alone (290 nmol) as with nitrate plus nitrite (53 and 204 nmol , respectively). By contrast, ammonium (0.3 mM) totally inhibited nitrate assimilation (Revilla *et al.*, 1985). Nitrite inhibition of nitrate uptake was not prevented by L-methionine-DL-sulphoximine, a glutamine synthetase inhibitor which releases nitrate assimilation from ammonium inhibition (Revilla *et al.*, 1985).

From these results we propose that the passage of nitrate across the cell envelope is mediated by a protein with a high degree of specificity for the ion. In algae (Ullrich, 1983), as well as in *Neurospora crassa* (Schloemer & Garrett, 1974), nitrate uptake and nitrite uptake do not proceed via the same carrier.

Effect of energy source on nitrate assimilation

The requirement of a readily usable carbon source for assimilatory nitrate uptake was investigated. When the cells were incubated in the nitrate uptake assay mixture that lacked a carbon source, there was no assimilation of nitrate (Table 1). Under anaerobic conditions, nitrate was not taken up even when a carbon source was available. In general, cells consumed nitrate at the highest rates when assayed in the presence of the carbon compound used for growth (Table 1). However, when cells that had been grown with mannitol were assayed in a medium with a different carbon source, nitrate uptake was either very low or absent (Table 1). Glucose was a striking exception, being more efficient than mannitol itself. No significant change in nitrate and nitrite reductase activities was detected when the cells grown in the standard mannitol/salts medium were suspended in media with different carbon sources (data not shown). Metabolic energy is therefore required for the assimilation of nitrate in *A. chroococcum*. When transferred to a new substrate, the slow induction or derepression of the appropriate enzyme(s) may be the cause of the low rates of nitrate uptake observed. In favour of this interpretation is the fact that cells grown on mannitol were unable to respire aerobically using succinate. Since enzymes for glucose utilization are constitutive, glucose was always the better substrate after transfer of the cells from mannitol.

Fig. 2. Effect of metabolic inhibitors on nitrate uptake by *A. chroococcum*. Cells ($0.5 \text{ mg protein ml}^{-1}$) were preincubated in the absence of additions (●) and in the presence of $25 \text{ } \mu\text{M}$ -FCCP (△), 1 mM -DNP (○) or $100 \text{ } \mu\text{M}$ -DCCD (▲) for 10 min before the nitrate (0.5 mM-KNO_3 added at zero time) utilization assay.

Table 1. *Effects of growth conditions and of energy source on nitrate uptake rate by washed suspensions of A. chroococcum*

A. chroococcum cells were grown in media containing 0.5% (w/v) mannitol (A), and 0.5% (w/v) glucose, 0.5% (w/v) succinate, 1% (w/v) pyruvate, 1% (w/v) ethanol or 1% (w/v) acetate (B). Cells were harvested, washed and suspended as described in Methods, in the presence of the indicated energy source, and then incubated for 10 min before the nitrate uptake assay. The energy source concentration in the nitrate uptake assay was 20% of that used in the growth medium. The nitrate uptake rates are expressed as percentages of the rate of nitrate uptake by cells grown and assayed with mannitol as the carbon and energy source. A rate of 100% corresponded to $30 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$.

Growth medium	Energy source during assay	Relative rates of nitrate uptake (%)
A. Mannitol	None	0
	Mannitol (anaerobically)	0
	Mannitol	100
	Glucose	182
	Succinate	0
	Pyruvate	18
	Ethanol	0
	Acetate	0
B. Glucose	Glucose	278
	Succinate	77
	Pyruvate	42
	Ethanol	53
	Acetate	50

Additional evidence on the dependence of assimilatory nitrate uptake upon metabolic energy was obtained from studies with the uncouplers of oxidative phosphorylation FCCP (Padan *et al.*, 1976) and DNP, the ATPase inhibitor DCCD (Harold *et al.*, 1969) and the respiratory chain inhibitors azide and cyanide. Both FCCP (at $25 \text{ } \mu\text{M}$ final concentration) and DNP (1 mM) hindered the cells from utilizing nitrate, whereas DCCD ($100 \text{ } \mu\text{M}$) caused only a slight decrease (Fig. 2). To check whether the effect of FCCP and DNP on nitrate consumption was due to inhibition of the nitrate-reducing enzymic system, nitrate and nitrite reductase activities were determined *in situ* at zero time and 25 min after initiation of the nitrate uptake assay described in Fig. 2. FCCP, DNP or DCCD caused no significant change in either activity. With $100 \text{ } \mu\text{M}$ -DCCD, we observed a complete inhibition of nitrogen fixation, a process strictly dependent on ATP. Nitrate uptake was also totally inhibited by 1 mM -azide or cyanide, the latter being an inhibitor of nitrate reductase activity both *in vitro* and *in situ* (Guerrero *et al.*, 1973; Llobell *et al.*, 1984).

The utilization of nitrate by the cyanobacterium *Anacystis nidulans* was hindered by 10 μM -FCCP or DCCD, compounds that are inhibitors of ATP-requiring reactions in *Anacystis nidulans* (Flores *et al.*, 1983). Nitrate uptake by the photosynthetic bacterium *Rhodospseudomonas capsulata* was completely and rapidly inhibited by 0.46 μM -FCCP (Jackson *et al.*, 1981). In *A. chroococcum* nitrate consumption was much less sensitive to these inhibitors, with inhibition ranging from 25–35% for 10 μM -FCCP, 85–95% for 25 μM -FCCP, and 10–20% for 100 μM -DCCD. FCCP at 25 μM acted as an uncoupler since aerobic respiration increased in its presence.

In general, inhibitors which decrease assimilatory nitrate uptake are those which affect energy metabolism. Inhibition of nitrate assimilation by uncouplers has been described in a variety of micro-organisms (Hofman, 1972; Schloemer & Garrett, 1974; Jackson *et al.*, 1981; Flores *et al.*, 1983; Cresswell & Syrett, 1981; Ullrich, 1974, 1983; Larsson & Andersson, 1982). Since DCCD had no effect on nitrate uptake whereas the proton-conducting uncouplers FCCP and DNP were strongly inhibitory, the energy required for nitrate uptake may not depend directly on ATP hydrolysis but rather on a proton electrochemical gradient generated by the respiratory chain. In fact, the uncoupler-sensitive assimilatory nitrate uptake by the photosynthetic bacterium *R. capsulata* (Jackson *et al.*, 1981), referred to above, was dependent on a proton electrochemical gradient across the plasma membrane. Alternatively, the uncouplers may inhibit indirectly by preventing ATP synthesis. Cresswell & Syrett (1981) have interpreted the inhibition of nitrate uptake by CCCP in the diatom *P. tricornutum* to indicate that uptake requires a supply of ATP. Except for the lack of an effect by DCCD, our results do not rule out that nitrate transport is dependent on hydrolysis of ATP. In this context, the report by Falkowski (1975) of a membrane-bound nitrate-stimulated ATPase in *S. costatum* lends support to a direct dependence of nitrate transport on ATP hydrolysis.

Effect of ascorbate/PMS on nitrate assimilation

Since ascorbate plus PMS can serve as a respiratory substrate in *E. coli*, thus generating an energized state of the cytoplasmic membrane (Wilson, 1974), nitrate uptake by *A. chroococcum* was measured in assay mixtures lacking a carbon source but containing the ascorbate/PMS electron-donating system as the energy source. Oxygen uptake occurred at a rate of 264 nmol (mg protein)⁻¹ min⁻¹, 24% of that observed with mannitol [1108 nmol (mg protein)⁻¹ min⁻¹], and was strictly dependent on the artificial electron donor system, since neither ascorbate nor PMS alone supported any oxygen consumption. Nitrate uptake by *A. chroococcum* was observed in the presence of ascorbate/PMS (Table 2). Thus this non-physiological substrate could fulfil the energy requirement of the cell for nitrate uptake from the external medium. These results indicate that electron transport from ascorbate/PMS to oxygen generates an electrochemical gradient that can be used for the assimilatory nitrate uptake. Reduced PMS was unable to act as an electron donor for spinach nitrate reductase (Paneque *et al.*, 1965) and probably behaves similarly towards the bacterial enzyme. The ascorbate/PMS-supported nitrate uptake by *A. chroococcum* did not take place under anaerobic conditions or in the presence of the uncoupler FCCP. Therefore if nitrate was reduced by the electron transport chain using reducing equivalents from the artificial substrate, it is clear that the energy required for the uptake process was used to transport nitrate into the cell.

A. chroococcum glutamine synthetase is very sensitive to oxygen radical species such as hydroxyl radicals (Paneque *et al.*, 1982), and Levine *et al.* (1981) have described inactivation of both *E. coli* and *K. pneumoniae* glutamine synthetase by an activated oxygen species. Since oxidation of reduced PMS with oxygen gives rise to oxygen radical species (Nishikimi *et al.*, 1972), we examined the effect of the ascorbate/PMS pair on nitrate reductase and, as a control, on glutamine synthetase activity. Table 2 shows that ascorbate or PMS alone had essentially no effect, that mannitol halved, and ascorbate and PMS together nearly abolished glutamine synthetase activity. Ascorbate, PMS or the ascorbate/PMS pair had no effect on cellular nitrate reductase activity (Table 2), in agreement with previous results obtained with reduced viologen dyes as source of the oxygen free radical species (Paneque *et al.*, 1982; Llobell *et al.*, 1984).

Table 2. *Effect of the ascorbate/PMS pair on nitrate uptake and activities of glutamine synthetase and nitrate reductase in A. chroococcum*

Enzyme activities were determined *in situ* in samples of cell suspensions that had been assayed for nitrate uptake for 15 min in the presence, as indicated, of mannitol or ascorbate/PMS, and are expressed as mU (mg protein)⁻¹. Other experimental conditions as for Fig. 2 and in Methods.

Energy source	Glutamine synthetase	Nitrate reductase	Nitrate uptake [nmol (mg protein) ⁻¹ min ⁻¹]
None	838	29	0
Mannitol	432	30	33
PMS	805	29	0
Ascorbate	718	32	0
Ascorbate/PMS	22	28	18

In summary, our results support the participation of a nitrate-active transport system acting before the nitrate reduction step and closely linked to the cell energy metabolism. This conclusion agrees well with data obtained from kinetic analysis of nitrate transport in *K. pneumoniae* using the radioisotope ¹³N (Thayer & Huffaker, 1982), which have provided evidence to support the proposal that in bacteria nitrate transport is necessary for nitrate assimilation.

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REFERENCES

- BETLACH, M. R., TIEDJE, J. M. & FIRESTONE, R. B. (1981). Assimilatory nitrate uptake in *Pseudomonas fluorescens* studied using nitrogen-13. *Archives of Microbiology* **129**, 135–140.
- CAWSE, P. A. (1967). The determination of nitrate in soil solution by ultraviolet spectrophotometry. *Analyst* **91**, 311.
- CEJUDO, F. J., DE LA TORRE, A. & PANEQUE, A. (1984). Short-term ammonium inhibition of nitrogen fixation in *Azotobacter*. *Biochemical and Biophysical Research Communications* **123**, 431–437.
- CRESSWELL, R. C. & SYRETT, P. J. (1981). Uptake of nitrate by the diatom *Phaeodactylum tricornutum*. *Journal of Experimental Botany* **32**, 19–25.
- FALKOWSKI, P. G. (1975). Nitrate uptake in marine phytoplankton: (nitrate-chloride)-activated ATPase from *Skeletonema costatum* (Bacillariophyceae). *Journal of Phycology* **11**, 323–326.
- FLORES, E., GUERRERO, M. G. & LOSADA, M. (1983). Photosynthetic nature of nitrate uptake and reduction in the cyanobacterium *Anacystis nidulans*. *Biochimica et biophysica acta* **722**, 408–416.
- GUERRERO, M. G., VEGA, J. M., LEADBETTER, E. & LOSADA, M. (1973). Preparation and characterization of a soluble nitrate reductase from *Azotobacter chroococcum*. *Archiv für Mikrobiologie* **91**, 287–304.
- HAROLD, F. M., BAARDA, J., BARON, C. & ABRAMS, A. (1969). Inhibition of membrane-bound adenosine triphosphatase and of cation transport in *Streptococcus faecalis* by *N,N'*-dicyclohexylcarbodiimide. *Journal of Biological Chemistry* **244**, 2261–2268.
- HAYASHI, S. & LIN, E. C. C. (1965). Capture of glycerol by cells of *Escherichia coli*. *Biochimica et biophysica acta* **94**, 479–487.
- HOFMAN, A. (1972). Über die energetische Kopplung der Nitrataassimilation von Grünalgen an Atmung und Photosynthese. *Planta* **102**, 72–84.
- JACKSON, M. A., JACKSON, J. B. & FERGUSON, S. J. (1981). Direct observation with an electrode of uncoupler sensitive assimilatory nitrate uptake by *Rhodospseudomonas capsulata*. *FEBS Letters* **135**, 275–278.
- KATOH, T. (1963). Nitrate reductase in the photosynthetic bacterium, *Rhodospirillum rubrum*. Adaptive formation of nitrate reductase. *Plant and Cell Physiology* **4**, 199–215.
- LARSSON, C.-M. & ANDERSSON, M. (1982). Uptake and photoreduction of NO₃⁻ and NO₂⁻ in *Scenedesmus*: interaction with CO₂ fixation. In *Photosynthesis*, vol. IV, pp. 741–750. Edited by G. Akoyunogly. Philadelphia: Balaban International Science Services.
- LEVINE, R., OLIVER, C. N., FULKS, R. M. & STADTMAN, E. R. (1981). Turnover of bacterial glutamine synthetase oxidative inactivation precedes proteolysis. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 2120–2124.
- LLOBELL, A., BARCENA, J. A., REVILLA, E. & PANEQUE, A. (1984). Inactivating effect of hydrogen peroxide on nitrate reductase from *Azotobacter chroococcum*. *Biochemistry International* **9**, 273–280.
- MORPETH, F. F. & BOXER, D. H. (1985). Kinetic analysis of respiratory nitrate reductase from *Escherichia coli* K12. *Biochemistry* **24**, 40–46.
- NISHIKIMI, M., RAO, N. A. & YAGI, K. (1972). The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. *Biochemical and Biophysical Research Communications* **86**, 849–854.

- PADAN, E., ZILBERSTEIN, D. & ROTTENBERG, H. (1976). The proton electrochemical gradient in *Escherichia coli* cells. *European Journal of Biochemistry* **63**, 533–541.
- PANEQUE, A., DEL CAMPO, F. F., RAMIREZ, J. M. & LOSADA, M. (1965). Flavin nucleotide nitrate reductase from spinach. *Biochimica et biophysica acta* **109**, 79–85.
- PANEQUE, A., BARCENA, J. A., CORDERO, N., REVILLA, E. & LLOBELL, A. (1982). Benzyl viologen-mediated *in vivo* and *in vitro* inactivation of glutamine synthetase in *Azotobacter chroococcum*. *Molecular and Cellular Biochemistry* **92**, 33–41.
- REVILLA, E., LLOBELL, A. & PANEQUE, A. (1985). The assimilatory nitrate uptake in *Azotobacter chroococcum*. Induction by nitrate and by cyanate. *Journal of Plant Physiology* **118**, 165–176.
- SCHLOEMER, R. H. & GARRETT, R. H. (1974). Nitrate transport system in *Neurospora crassa*. *Journal of Bacteriology* **118**, 259–269.
- SERRA, J. L., LLAMA, M. J. & CADENAS, E. (1978). Nitrate utilization by the diatom *Skeletonema costatum*. I. Kinetics and nitrate uptake. *Plant Physiology* **62**, 987–990.
- SHAPIRO, B. & STADTMAN, E. R. (1970). The regulation of glutamine synthesis in microorganisms. *Annual Review of Microbiology* **24**, 501–524.
- SNELL, F. D. & SNELL, C. T. (1949). *Colorimetric Methods of Analysis*, vol. 3, pp. 804–805. New York: Van Nostrand.
- SYRETT, P. J. (1981). Nitrogen metabolism of microalgae. *Canadian Bulletin of Fisheries and Aquatic Sciences* **210**, 182–210.
- THAYER, J. R. & HUFFAKER, R. C. (1982). Kinetic evaluation, using ^{13}N , reveals two assimilatory nitrate transport systems in *Klebsiella pneumoniae*. *Journal of Bacteriology* **149**, 198–202.
- ULLRICH, W. R. (1974). Der nitrat- und nitritabhängige photosynthetische O_2 -Entwicklung in N_2 bei *Ankistrodesmus braunii*. *Planta* **116**, 143–152.
- ULLRICH, W. R. (1983). Uptake and reduction of nitrate: algae and fungi. In *Encyclopedia of Plant Physiology*, vol. 15, pp. 276–298. Edited by A. Läuchli & R. L. Bielecki. Berlin, Heidelberg & New York: Springer Verlag.
- WILSON, D. B. (1974). Source of energy for the *Escherichia coli* galactose transport systems induced by galactose. *Journal of Bacteriology* **120**, 866–871.