Planta (1991) 184:47–52

Planta

Measurement of intracellular nitrate concentrations in *Chara* using nitrate-selective microelectrodes

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Received 23 August; accepted 15 November 1990

Abstract. Nitrate-selective microelectrodes have been made using a quaternary ammonium sensor, methyltridodecylammonium nitrate, in a polyvinylchloride matrix. These electrodes showed a log-linear response from 0.1 to 100 mol · m^{-3} nitrate with a typical slope of 55.6 mV per decade change in nitrate concentration. The only physiologically significant interfering anion was chloride but the lower limit of nitrate detection was 0.5 mol · m^{-3} in the presence of 100 mol · m^{-3} chloride which means this interference will not be important in most physiological situations. These microelectrodes were used to measure nitrate concentrations in internodal cells of *Chara corallina* cultured under low nitrate and nitrate-replete conditions for 6 to 30 weeks. Cells maintained in low nitrate only showed measurements which were less than the detection limit of the electrodes, while cells grown under nitrate-replete conditions showed two populations of measurements having means of 1.6 and 6.2 mol · m^{-3}. Chemical analysis of the high-nitrate cells indicated that they contained a mean nitrate concentration of 5.9 mol · m^{-3}. As vacuolar nitrate concentration would dominate this whole-cell measurement, it is concluded that the higher concentration measured with the electrodes represents vacuolar nitrate concentration and the lower value represents the cytoplasmic concentration. This intracellular distribution of nitrate could only be achieved passively if the electrical potential difference across the tonoplast is between +25 and +35 mV.

Key words: *Chara* (nitrate compartmentation) – Compartmentation (nitrate) – Cytosol (nitrate) – Nitrate compartmentation – Nitrate-selective microelectrodes – Vacuole (nitrate)

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Abbreviations: APW = artificial pond water; Hepes = 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid; MTDDA.NO_3 = methyltridodecylammonium nitrate

Introduction

The measurement of nitrate concentrations in the vacuole and cytosol is a necessary prerequisite to characterising nitrate transport at both the plasma membrane and tonoplast. Vacuolar nitrate concentration can be estimated by chemical analysis of fully vacuolated tissues because the vacuole dominates such measurements, but cytosolic nitrate concentration is more difficult to obtain. Estimates have been made using the technique of compartmental tracer-flux analysis with ^{13}N- or ^{15}N-labelled nitrate (Lee and Clarkson 1986; Macklon et al. 1990) or with the nitrate analogue $^{36}ClO_3^-$ (Deane-Drummond and Glass 1982). However, values of cytosolic nitrate concentration obtained using this approach range from as low as 1 to 10 mmol · m^{-3} in cells of the giant alga, *Chara corallina* (Deane-Drummond 1985) to as high as 40–50 mol · m^{-3} in onion roots (Macklon et al. 1990). This value for *Chara* is different from a value of 2.2 mol · m^{-3} measured in cytoplasm isolated directly from cells of *Chara australis* (Okihara and Kiyosawa 1988). It is not known whether the differences are the consequence of errors in the tracer-flux method or result from experimental treatments imposed on the tissues.

Other approaches to estimating cytosolic nitrate concentration include tissue-fractionation procedures (Martinoia et al. 1986) and anaerobic nitrite analysis for spinach (Steingröver et al. 1986). These yielded cytosolic nitrate concentrations of around 4 mol · m^{-3} for barley mesophyll cells and 7 mol · m^{-3} for spinach leaves. These estimates are, however, very dependent on the assumed values of cytosolic volume.

A more direct approach would be to use nitrate-selective microelectrodes. Ion-selective microelectrodes have been used to measure a number of ions in plant cells including H^+ (Felle 1987), K^+ (Penny and Bowling 1974), Ca^{2+} (Miller and Sanders; 1987, Felle 1988), and Cl^- (Penny et al. 1976). Nitrate-selective macroelectrodes are commercially available and recently a minia-

turized version has been used to measure nitrate fluxes at the external surface of barley roots (Henriksen et al. 1990). In this paper, we describe further reduction in the dimensions of such electrodes to a size suitable for intracellular impalement. We have fabricated double-barrelled nitrate-selective microelectrodes and used these to make the first direct measurements of intracellular nitrate concentrations in *Chara corallina*.

Material and methods

Chara corallina was cultured in the laboratory in river mud overlaid with a simple salt solution. Internodal cells were cut from the culture and 20–30 cells were placed in each of three beakers containing 150 ml of either artificial pond water (APW: 0.2 mol · m^{-3} K_2SO_4, 1.0 mol · m^{-3} NaCl, 1.0 mol · m^{-3} $CaSO_4$, 5.0 mol · m^{-3} 2-(N-morpholino)ethanesulfonic acid (Mes)-NaOH pH 5.8) or APW in which the NaCl was replaced by either 1.0 mol · m^{-3} $NaNO_3$ or 1.0 mol · m^{-3} $NaNO_3$ plus 4.5 mol · m^{-3} $Ca(NO_3)_2$. A chloride concentration of 20 mmol · m^{-3} was measured in these nitrate-supplemented APW solutions using ion chromatography; the nitrate level in unsupplemented APW was 2 mmol · m^{-3}. The internodal cells were maintained in these solutions at 15–20° C on a laboratory bench with a photon fluence rate of 8 μmol E · m^{-2} · s^{-1}. The solutions were replaced weekly and under these conditions the cells continued to expand and produce new growth. Only growing internodal cells, 20–30 mm in length were used and at the time of measurement these cells had been maintained in each type of APW for 6–30 weeks. For the microelectrode measurements the cells were clamped into a Perspex perfusion chamber mounted on a microscope stage.

The nitrate-sensor cocktail consisted of the following reagents mixed by weight (w/w), 23% polyvinylchloride (PVC, high-molecular-weight polymer); 1% methyltriphenyl phosphonium bromide (a lipophilic cation); 65% 2-nitrophenyl octyl ether; 5% nitrocellulose; 6% methyltridodecylammonium nitrate (MTDDA.NO_3). The cocktail was dissolved in approx. 4 volumes of tetrahydrofuran (THF), sufficient to give a backfilling liquid which could be drawn into a 30 G needle and 1-cm^3 glass syringe. The nitrate-selective component of the cocktail was the quaternary ammonium compound, MTDDA.NO_3, which was synthesized by the method of Wegmann et al. (1984). All chemicals were analytical grade and were obtained from Fluka Chemicals, Glossop, Derbyshire, UK.

Double-barrelled microelectrodes were prepared by twisting together two single pieces of filamented borosilicate glass each having an outer diameter of 1.0 mm and an inner diameter of 0.58 mm (Hilgenberg Glass, Malsfeld, FRG). Twisting was done during the heating and pulling steps of micropipette preparation using a Narishige (Tokyo, Japan) PE–2 vertical micropipette puller. Both barrels of such electrodes had resistances of 5 to 10 MΩ when filled with 100 mol · m^{-3} KCl solution. The barrel designated to be the nitrate-selective barrel was silanized by placing a few drops of a solution of 1% (w/v) tributylchlorosilane in chloroform on its blunt end. Care was taken to ensure that the reagent did not enter the other (membrane potential measuring) barrel. The microelectrode was then placed under a heating lamp giving a temperature of 140° C at the micropipette surface. The silanizing solution quickly vapourised giving the ion-selective barrel a hydrophobic coating. This barrel was then backfilled with the nitrate-sensor cocktail dissolved in THF. After backfilling, the double-barrelled pipettes were placed tip down for 48 h in silica gel-dried air at room temperature. After this time, the excess THF had evaporated leaving a plug of nitrate sensor in PVC in the tip of the nitrate-sensing barrel. An hour before use, both barrels were backfilled, 100 mol · m^{-3} KCl in the membrane-potential-measuring barrel and 100 mol · m^{-3} $NaNO_3$ + 100 mol · m^{-3} KCl in the nitrate-sensing barrel. Intracellular nitrate measurements were made using a high-input impedance differential electrometer (model FD 223; World Precision Instruments, New Haven, Conn., USA). The electrometer output passed via an A/D converter (Labmaster DMA/PGH; Scientific Solutions, Solon, Ohio, USA) at a sampling frequency of 10 Hz to an Opus PC V microcomputer.

Before making intracellular measurements the nitrate-selective microelectrodes must be calibrated in nitrate solutions which have a constant background ionic strength. For calcium-selective microelectrodes this is achieved by ensuring the calibration buffers have a K^+ concentration of 125 mol · m^{-3} and an ionic strength of about 140 mol · m^{-3} (Tsien and Rink 1981), and the same values were adopted for the nitrate-selective electrodes. The anion chosen for inclusion in the nitrate calibration solutions should give minimal interference. Hydrogenphosphate (HPO_4^{2-}) gave the smallest selectivity coefficient of the ions tested (see *Results*) so K_2HPO_4 was used to give a constant background ionic strength in the calibration solutions. The solutions were buffered at pH 7.9 with 5 mol · m^{-3} 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid (Hepes) to ensure that phosphate was present as the divalent anion. Table 1 lists the compositions of calibration solutions used. When the ionic strength of the calibration solution was doubled by altering the K_2HPO_4 concentration the output voltage from nitrate-selective microelectrodes in a 1 mol · m^{-3} nitrate solution changed by only 4 mV (data not shown). This indicates that the differences between vacuolar and cytosolic composition are unlikely to affect the results significantly unless one compartment contains a large concentration of an interfering anion. Total nitrate was extracted from cells with boiling water and was measured by the method of West and Ramachandran (1967).

Table 1. Composition of the solutions used to calibrate the nitrate-selective microelectrodes for intracellular measurements. All solutions were at pH 7.9 to ensure the major anion determining ionic strength, other than nitrate, was HPO_4^{2-} · pNO_3 is the negative $\log_{10}$ [NO_3^- activity]. (Nitrate activity was calculated using the SOLCON programme written by Dr. D. C. S. White, Biology Department, University of York, UK)

Nitrate activity (mol · m^{-3})	pNO_3	Hepes (mol · m^{-3})	KNO_3 (mol · m^{-3})	K_2HPO_4 (mol · m^{-3})	Ionic strength (mol · m^{-3})
0.01	5.0	5	0.0121	50.0	140
0.1	4.0	5	0.122	50.0	140
0.5	3.3	5	0.63	50.0	141
1.0	3.0	5	1.22	50.0	141
5.0	2.3	5	6.30	48.0	140
10.0	2.0	5	12.20	46.5	140
20.0	1.7	5	25.00	43.0	141
100.0	1.0	5	130.00	15.0	142

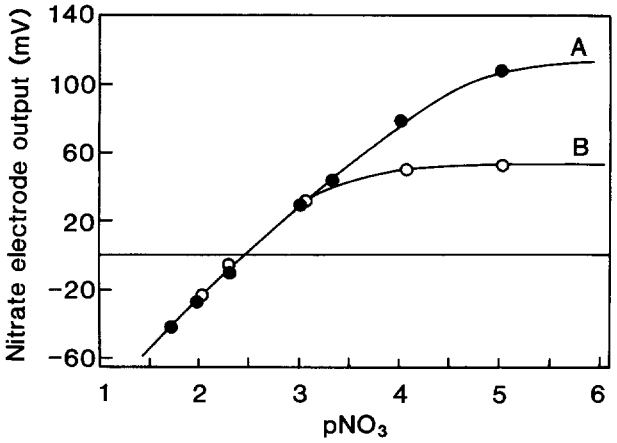

Fig. 1. Calibration of a typical nitrate-selective microelectrode in the absence (*A*) and presence (*B*) of 100 mol · m^{-3} KCl, $pNO_3 = -\log_{10}[NO_3^-$ activity]

Table 2. Interference of other anions with the nitrate-selective microelectrodes. Selectivity coefficients were determined by the fixed-interference method (Ammann 1986) with the interfering anion at the concentration indicated. A selectivity coefficient greater than 1 indicates the electrodes are more selective for that anion than for nitrate and a value less than 1 that they are less selective

Anion	Concentration of anion (mol · m^{-3})	Selectivity coefficient
SCN^-	100	9.16
NO_2^-	100	0.106
Cl^-	100	0.035
$H_2PO_4^-$	100	0.0074
HCO_3^-	40	0.0067
$Acetate^-$	100	0.0044
$Oxalate^{2-}$	25	0.0057
$Malate^{2-}$	100	0.0042
SO_4^{2-}	50	0.0018
HPO_4^{2-}	100	0.0001

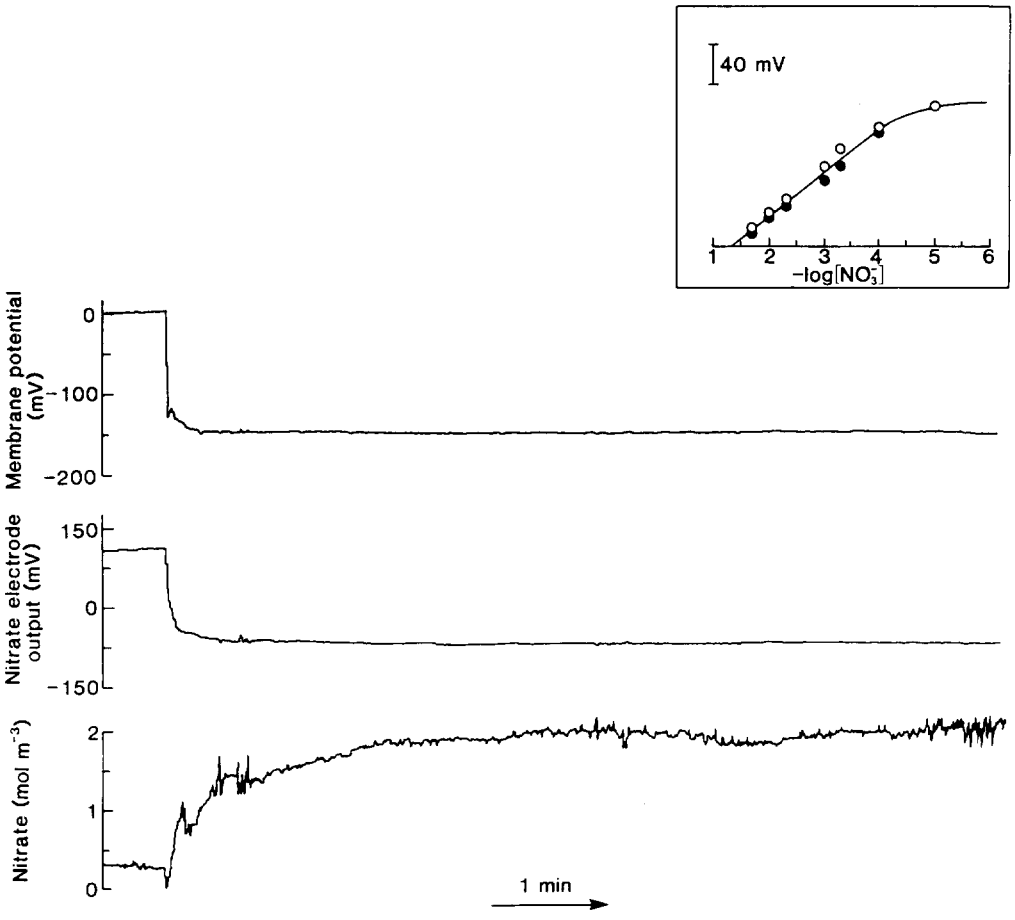

Fig. 2. Intracellular microelectrode recordings for *Chara corallina* showing a typical impalement sequence. The *upper trace* shows output from the membrane-potential-sensing barrel (mV), the *middle trace* shows, output from the nitrate-sensing barrel (mV) and the *lower trace* shows nitrate concentration (mol · m^{-3}). This cell had been grown in a solution containing APW supplemented with 1 mol · m^{-3} nitrate. The *inset* shows the calibration data before (○) and after (●) impalement. The curve was fitted to the combined data with a simplified Nicolsky-Eisenman equation of the form $E = E_0 - S\log\{[NO_3^-] + K\}$, in which E is the measured output of the nitrate-selective electrode, E_0 is the constant electrode reference potential, S is the slope and K is a term which subsumes the concentrations of the interfering ions and the selectivity coefficients of the electrode for those ions. Fitted parameter values: $E_0 = -54.3$ mV; $S = 51.8$ mV ; $K = 74$ mmol · m^{-3}

Results

Microelectrodes made using MTDDA.NO_3 showed a linear response from 0.1 mol · m^{-3} to 100 mol · m^{-3} nitrate with a typical slope of 55.6 mV per decade change in nitrate concentration (e.g. curve A in Fig. 1). The selectivity of the microelectrodes to a range of anions was determined by the fixed-interference method (Ammann 1986). The electrodes were almost tenfold more selective for SCN^- than for nitrate (Table 2). However, SCN^- does not accumulate in most plant cells and thus is unlikely to interfere with the physiological application of the electrodes as a nitrate-measuring system. However, care might be needed in some plants which can produce

this anion (Underhill 1980). Of all the other anions tested only chloride and nitrite had selectivity coefficients which indicated that they might interfere if they accumulated to high concentrations relative to nitrate. Interference by nitrite is unlikely to be a problem because this anion did not accumulate in normal barely roots under aerobic conditions (Lee 1979) and when it did accumulate in mutant barley plants it was toxic (Wray 1989). In contrast, chloride can accumulate to high concentrations in the vacuole and is therefore likely to be the most serious interfering ion for intracellular measurements. However, at 100 mol · m^{-3} it had appreciable effects only at 0.5 mol · m^{-3} nitrate (pNO_3 3.3) or less (Fig. 1); this nitrate concentration is the lower limit for nitrate detection in the presence of a high concentration of chloride. Therefore, the electrodes may not be able to detect very low nitrate concentration in compartments containing a high chloride concentration, e.g. the vacuoles of halophytes or some giant algae (see Flowers et al. 1977; Sanders 1981; Okihara and Kiyosawa 1988). The nitrate-selective microelectrodes were insensitive to cations (data not shown) including H^+ at pHs between 3 and 10.

Figure 2 shows a typical intracellular recording for a *Chara corallina* internodal cell impaled with a double-barreled nitrate-selective microelectrode. Measurements were only accepted if the cells had a membrane potential more negative than −120 mV, if cytosolic streaming could be observed throughout the measurement, and if the microelectrodes recalibrated after insertion into the cell (e.g. inset to Fig. 2). The intracellular nitrate concentration was calculated from the line fitted through the combined "before" and "after" calibration data points using a simplified Nicolsky-Eisenman equation (Miller and Sanders 1987).

The use of double-barrelled microelectrodes ensures that both tips are located in the same compartment. However, determining the compartmental location of the microelectrode tip can be a problem in plant cells. For calcium-selective electrodes the compartmental location was determined by the magnitude of the measured free-calcium concentration. All intracellular calcium measurements grouped into two populations: in the mol · m^{-3} range in the vacuole and in the μmol · m^{-3} range in the cytosol (Miller and Sanders 1987). Nitrate concentrations measured in internodal cells grown in APW (2 mmol · m^3 nitrate) all fell into one population in the range 0.5 mol · m^{-3} or less (data not shown), a value at the detection limit of the electrodes. However, the measurements for cells which had been incubated in nitrate-supplemented (both 1 and 10 mol · m^{-3} nitrate) APW yielded two populations (Fig. 3). One group ranged between 0.5 and 2.5 mol · m^{-3} nitrate with a mean of 1.6 mol · m^{-3} and the other ranged from 3.5 to 11 mol · m^{-3} and had a mean of 6.2 mol · m^{-3}. The mean membrane potentials (± SE) of these two populations were 152 ± 6 mV for the lower nitrate concentrations and 144 ± 12 mV for the higher concentrations. Both populations were found in cells grown in APW containing either 1 or 10 mol · m^{-3} nitrate. The chemical analysis of whole *Chara* cells incubated in nitrate-supplemented APW gave total cellular nitrate concentrations in the range 3.5 to 11 mol · m^{-3} with a mean of 5.9 mol · m^{-3} (Fig. 3).

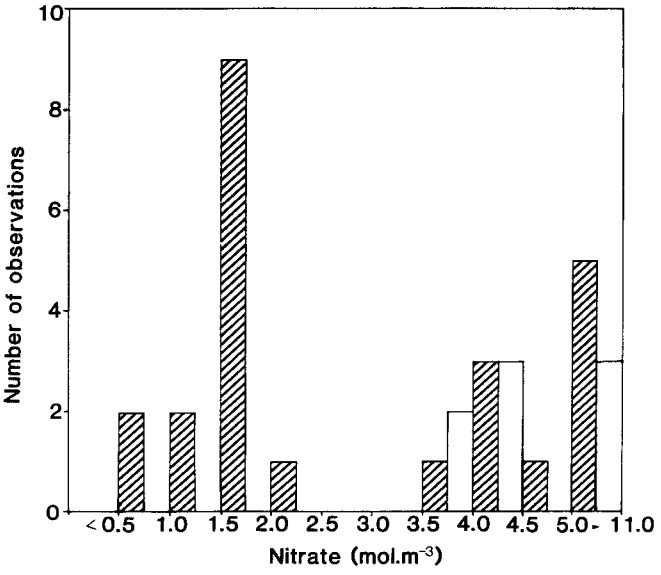

Fig. 3. The distribution of intracellular nitrate concentrations measured in *Chara* internodal cells by chemical analysis of whole cells *(open columns)* or by insertion of nitrate-selective microelectrodes *(shaded columns)*. The histogram shows pooled results for both 1 and 10 mol · m^{-3} nitrate-supplemented APW. The two populations of electrode measurements are believed to represent the cytoplasmic and the vacuolar compartments. Mean values ± SE; for chemical analysis 5.94 ± 1.02 mol · m^{-3} and for the microelectrode measurements 1.57 ± 0.13 and 6.23 ± 0.87 mol · m^{-3}

Discussion

Nitrate-selective microelectrodes suitable for intracellular measurement of nitrate have been successfully developed. Application of these microelectrodes to plant cells should allow the in vivo measurement of both cytosolic and vacuolar nitrate.

Use of the microelectrodes on cells of *Chara* incubated in nitrate-supplemented APW gave two populations of nitrate concentrations with means of 1.6 and 6.2 mol · m^{-3}. It is probable that these represent the cytoplasmic and vacuolar nitrate concentrations, respectively. Both cytoplasm and vacuole must contribute to the whole-cell nitrate concentration of about 6 mol · m^{-3}. In *Chara* the vacuole occupies 90–96% of the intracellular volume, and the cytoplasm 4–10% (Deane-Drummond 1985, Walker and Pitman 1976). If the nitrate concentration in the cytoplasm were to dominate the whole-cell measurement, then this compartment would have to contain nitrate at a concentration between about 60 and 150 mol · m^{-3}. Although 60% of impalements of *Chara* result in insertion of the electrode into the cytoplasm (Miller and Sanders 1987), concentrations of this magnitude were never observed. This means that the whole-cell measurement is probably dominated by the vacuolar nitrate concentration. As the vacuole represents such a large proportion of the intracellular volume, concentrations in the vacuole should be similar to those

in the whole-cell. On this basis, the coincidence between the whole-cell measurement and those for the higher range of values measured with the microelectrodes indicates that the latter represent the vacuolar nitrate concentration and the lower range therefore represents the cytoplasmic nitrate concentration. Of the total measurements made, 54% fell into the lower range, close to the proportion (0.6) of cytoplasmic impalements recorded with calcium-selective microelectrodes (Miller and Sanders 1987) further supporting the suggestion that measurements in this lower range are cytoplasmic. In addition the cytoplasmic and vacuolar nitrate concentrations measured with the electrodes are similar to the values obtained by direct chemical analysis of sap samples isolated from these compartments in *Chara australis* (Okihara and Kiyosawa 1988). Identification and final proof of the compartmental location of the microelectrode tip could be provided by using triple-barrelled microelectrodes which included a pH or calcium-selective third barrel. The large differences in concentration of both H^+ and Ca^{2+} across the tonoplast serving to identify the location of the microelectrode tip.

The nitrate-selective microelectrode measurements of cytosolic nitrate concentration in *Chara corallina* lie in the mol · m^{-3} range and are similar to values obtained by compartmental flux-tracer measurements on higher plants (see *Introduction*) but very different from previous measurements for *Chara* (Deane-Drummond 1985). However, the estimates of cytosolic nitrate concentration made by Deane-Drummond (1985) were made in cells pre-treated for 72 h with 2 mmol · m^{-3} KNO_3 and then placed for 2 h in 0.2 mol · m^{-3} KNO_3. Under these conditions the cytosolic compartment was possibily still filling with nitrate. Our measurements were made after the cells had been in nitrate-supplemented APW for several weeks and the cytosolic nitrate concentration seems to have reached a steady state. This is supported by the observed single population of cytosolic nitrate concentrations for cells growing in 1 and 10 mol · m^{-3} nitrate APW (Fig. 3). Thus differences in pre-treatment probably account for the apparent discrepancy between these two cytosolic nitrate concentrations for *Chara* obtained by these different techniques. The measurements with the microelectrodes on *Chara* cells grown with or without nitrate suggest cytosolic nitrate concentration is sensitive to the pretreatment the cells receive. Cells grown in APW without nitrate had only a single population of measurements all below 0.5 mol · m^{-3} nitrate and so presumably cytosolic nitrate is less than or equal to this value. When nitrate was supplied to the cells the concentration of nitrate increased in both vacuole and cytoplasm until a mean cytosolic nitrate concentration of around 1.6 mol · m^{-3} was attained.

Internodal cells of *Chara corallina* accumulated nitrate in the vacuole to only relatively low concentrations when compared with most higher plants (Leigh and Wyn Jones 1986). In nitrate-supplemented APW solutions the external chloride concentration was 20 mmol · m^{-3} (present only as a contaminant in the other salts) while nitrate concentration was 1 or 10 mol · m^{-3}. However, Sanders (1981) found that *Chara* did not replace chloride in the vacuole with nitrate when the external chloride and nitrate concentrations were 9 mmol · m^{-3} and 0.5 mol · m^{-3}, respectively. In the experiments reported in the present paper, the cells were subjected to only limited chloride deficiency because the nitrate-supplemented APW solution was replaced each week restoring the low background levels of chloride (20 mmol · m^{-3}). Therefore, despite the low chloride concentrations, this anion was probably still preferentially accumulated over nitrate.

The mechanism of nitrate transport across the tonoplast remains to be elucidated. However, the compartmental distribution of nitrate in *Chara* could result from passive distribution if the trans-tonoplast potential difference is between +25 and +35 mV. Such a tonoplast resting potential difference is more positive than most estimates for *Chara* but does lie within the range of reported values for giant celled algae (Tester et al. 1987). Tyerman and Findlay (1989) have described an anion channel which could mediate such a passive flux. However, the two populations of nitrate-electrode measurements did not have significantly different membrane potentials suggesting that the trans-tonoplast potential may be close to zero. If the potential difference is close to 0 mV then active transport of nitrate into the vacuole would be necessary. Active transport of chloride is believed to occur at the tonoplast of *Chara* (see MacRobbie 1970).

R.G. Zhen was awarded a Sino-British Friendship Scholarship, sponsored by the British Council. We are grateful to Dr. G. Dawson (Insecticides and Fungicides Department) for his advice on the synthesis of MTDDA.NO_3, Mr. I. Jennings (Department of Biology, University of York) for developing the software used in this work and Dr. R.A. Leigh for critically reading the manuscript.

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