Biochem. J. (1989) 259, 545–548 (Printed in Great Britain)

Determination of intracellular nitrate

José M. ROMERO, Catalina LARA* and Miguel G. GUERRERO
Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla y C.S.I.C., Apdo. 1113, 41080 Sevilla, Spain

A sensitive procedure has been developed for the determination of intracellular nitrate. The method includes: (i) preparation of cell lysates in 2 M-H_3PO_4 after separation of cells from the outer medium by rapid centrifugation through a layer of silicone oil, and (ii) subsequent nitrate analysis by ion-exchange h.p.l.c. with, as mobile phase, a solution containing 50 mM-H_3PO_4 and 2% (v/v) tetrahydrofuran, adjusted to pH 1.9 with NaOH. The determination of nitrate is subjected to interference by chloride and sulphate when present in the samples at high concentrations. Nitrite also interferes, but it is easily eliminated by treatment of the samples with sulphamic acid. The method has been successfully applied to the study of nitrate transport in the unicellular cyanobacterium *Anacystis nidulans*.

INTRODUCTION

The study of nitrate transport across the plasma membrane has lagged well behind that of other steps in nitrate assimilation, mainly because of the difficulties found in estimating intracellular nitrate (see [1]). The use of $^{13}NO_3^-$, which has allowed the kinetic evaluation of nitrate transport in *Klebsiella* [2], has the drawback of the extremely short half-life (10 min) of this radionuclide. Conventional methods for nitrate determination have only succeeded when applied to vacuolated cells, such as diatoms [3,4], which can build up high intracellular nitrate pools. Most microalgae and bacteria are devoid of vacuoles, and their intracellular nitrate pools are below the detection limit of most conventional techniques, including the use of a nitrate electrode [5], u.v. determination [6] and biological conversion of nitrate into nitrite followed by colorimetric detection of the latter [7].

During the last few years, several analytical procedures for the separation of anions by ion-exchange h.p.l.c. have been developed. They include anion detection by conductimetry [8,9], indirect fluorimetry [10] or u.v. absorption [11–13]. On the basis of the latter we have developed a simple and sensitive method to determine low intracellular pools of nitrate in unicellular cyanobacteria (blue–green algae). This has enabled us to measure nitrate accumulation in *Anacystis nidulans* and to provide evidence of the existence of a regulated nitrate-transport system in this organism [14]. The procedure involves the rapid lysis of cells by centrifugation through a layer of silicone oil into a denaturing agent [15], followed by analysis of nitrate by ion-exchange h.p.l.c. The method, its advantages and possible sources of interference are described and discussed here.

EXPERIMENTAL

Ion-exchange h.p.l.c.

Ion-exchange h.p.l.c. analysis was performed with Waters equipment consisting of a WISP 710 B automatic injector, a 6000A pump, a 720 system controller, a Lambda Max 481 variable-wavelength LC-spectrophotometer and a M730 data module (printer/plotter/integrator). The analytical column (4.6 mm × 25 cm) was a strong-anion-exchange one containing Whatman Partisil-10 SAX (10 μm particle size). Between the pump and the injector a guard precolumn (4.6 mm × 25 cm) packed with silica gel (37–53 μm particle size) was placed in order to saturate the mobile phase with stationary phase, thus avoiding loss of resolution in the analytical column [16]. Both the analytical and the guard column were placed in a temperature-controlled chamber at 25 °C. Under our working conditions, column performance was adequate for about 1500 analyses.

The mobile phases were prepared with h.p.l.c.-grade water obtained either by filtering deionized bidistilled water through Millipore Norganic Cartridges or directly by a Milli-Q (Millipore) system. The quality of water is of crucial importance in the analysis. Before use, eluants were filtered through Millipore HA membranes (0.45 μm pore size) and degassed in an ultrasonic bath for 30 min. Two mobile phases were used: a 50 mM-sodium phosphate buffer, pH 3.0 [13], henceforth referred to as 'eluant A'; and a solution containing 50 mM-H_3PO_4 and 2% (v/v) tetrahydrofuran (adjusted to pH 1.9 with NaOH solution at 25 °C), henceforth referred to as 'eluant B'.

Chromatography was performed in the isocratic mode at a flow rate of 1 ml/min. Anions were detected by their absorbance at 210 nm.

Preparation of acid cell lysates and nitrate-transport assay

Acid extracts were prepared from cells rapidly separated from the reaction mixture by centrifugation through a layer of silicone oil [15]. Polyethylene centrifuge tubes (0.4 ml) containing three phases were used. The lower phase (20 μl) was 2 M-H_3PO_4. The middle silicone layer (80 μl) was a 2: 1 mixture of Versilube F-50 (Serva) and Silicone 14615-3 (Janssen Chimica). The upper layer (300 μl) contained the assay mixture, consisting of 25 mM-Tricine/NaOH/KOH buffer, pH 8.3, 10 mM-$NaHCO_3$, 50 μM-KNO_3, and an amount of *Anacystis nidulans* cells equivalent to 10 μg of chlorophyll *a*.

Nitrate-transport assays were initiated by simultaneous nitrate addition and illumination, and stopped by rapid centrifugation (10000 *g*, 1.5 min) in a Beckman

* To whom correspondence and reprint requests should be sent.

Microfuge II. By this procedure, the cells, rapidly filtered through the silicone layer, are lysed by the H_3PO_4 solution releasing acid-soluble compounds, all insoluble material being pelleted at the bottom of the tube. Nitrate was analysed in aliquots of the acid cell lysate by ion-exchange h.p.l.c. The values obtained were corrected for extracellular nitrate carried by the cells through the silicone barrier in samples centrifuged immediately after nitrate addition.

Organism and culture conditions

Anacystis nidulans (*Synechococcus leopoliensis* 1402-1 from the Göttingen University Culture Collection, Göttingen, Germany) was grown photoautotrophically with nitrate as the sole nitrogen source as previously described [17]. Cells were harvested by centrifugation after 24 h growth (15–20 μg of chlorophyll $a \cdot ml^{-1}$), washed with 25 mM-Tricine/NaOH/KOH buffer, pH 8.3, and resuspended in the same buffer. Chlorophyll *a* was estimated after extraction with methanol as in [18]. Cells were treated with tungstate [19] in order to obtain cells with low nitrate reductase activity [14].

RESULTS AND DISCUSSION

Selection of lysis solution and mobile phase

Critical factors in the estimation of intracellular nutrient pools are the achievement of rapid separation of cells from the assay medium and the avoidance of dilution during the preparation of cell extracts. These requirements are fulfilled by the silicone-oil-centrifugation technique [15], in which the cells pass rapidly from a relatively large volume of assay medium (0.3 ml) into a small volume of lysis solution (20 μl) by filtration through a layer of silicone oil.

As the Partisil columns used in ion-exchange h.p.l.c. are damaged at neutral or alkaline pH values, being otherwise stable at low pH, the use of an acid for the lysis of cells appeared appropriate and simple. In order to select an acid whose anionic form does not interfere with nitrate in the chromatographic analysis, the elution properties of different anions were determined (Table 1). With the mobile phase described in [13] consisting of 50 mM-phosphate buffer, pH 3 (eluant A), the retention times for phosphate, perchlorate and chloride were equal, or very similar, to that of nitrate, although nitrite and sulphate were well separated. This pointed to H_2SO_4 as a candidate, but *Anacystis* cell lysates in 0.25 M-H_2SO_4 did not show, under these conditions, good resolution in the region of the nitrate peak (results not shown). Several other mobile phases were assayed, the main changes in the elution pattern of anions being observed by changing the pH of the eluant. Table 1 also shows the retention times of anions using eluant B [a solution containing 50 mM-H_3PO_4 and 2% (v/v) tetrahydrofuran adjusted at pH 1.9 with NaOH solution] as mobile phase. In this case, strong anions such as nitrate, sulphate, perchlorate or chloride were retained by the column and were eluted with similar retention times, but phosphate was only weakly bound to the column and was eluted soon after the chromatographic front.

Table 1. Retention times of anions eluted with different mobile phases

Solutions containing 20 μM-nitrate alone or mixtures of 20 μM-nitrate with either 20 μM-nitrite, 2 M-H_3PO_4, 0.5 M-H_2SO_4, 0.5 M-$HClO_4$ or 0.25 M-HCl were analysed in a 10 μl injection volume. Anions were detected by absorbance at 210 nm. Unretained solutes were eluted with a retention time of 2.2 min.

Anion	Retention time (min)	
	Eluant A	Eluant B
Chloride	8.07	10.80
Nitrate	8.15	10.85
Nitrite	6.15	4.35
Perchlorate	8.30	10.90
Phosphate	8.15	4.47
Sulphate	9.90	10.70

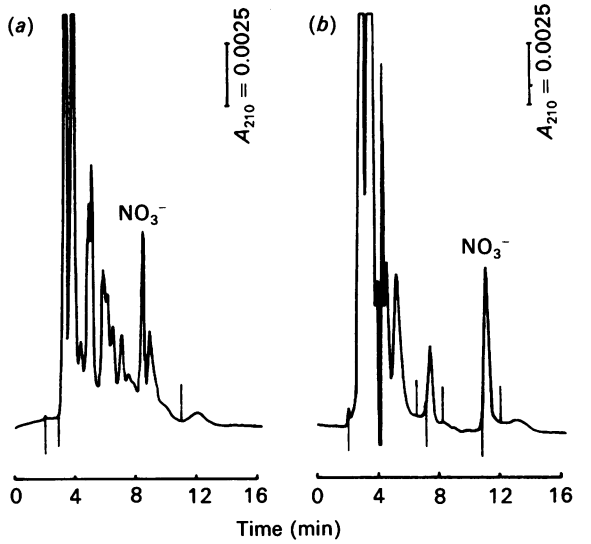

Fig. 1. H.p.l.c. chromatograms of an *A. nidulans* acid extract with two different mobile phases

Cell lysates were obtained by silicone-oil centrifugation into 2 M-H_3PO_4. Ion-exchange h.p.l.c. was performed, as described in the Experimental section, on a 10 μl injection volume. Eluant A (*a*) or eluant B (*b*) were used as mobile phases.

Fig. 1 shows chromatograms of an *A. nidulans* extract obtained by silicone-oil centrifugation of the cells into 2 M-H_3PO_4, using eluants A and B as mobile phases. At pH 3, u.v.-absorbing material of the extracts as well as the phosphate ions from the lysis solution interfered with the nitrate peak. At pH 1.9, most of the anions are in their protonated form and pass through the column, only anions with very low pK_a, such as nitrate, being retained. Also, tetrahydrofuran increased slightly the retention time of nitrate. Under these conditions, the nitrate peak appeared clearly isolated from other anions, being easily quantified by integration. Consequently, H_3PO_4 and eluant B were selected as lysis solution and mobile phase respectively.

Interference with nitrate determination

As indicated above, sulphate and chloride, if present in the biological material analysed, can interfere with nitrate determination by the present procedure. The three anions are eluted with analogous retention times (Table 1),

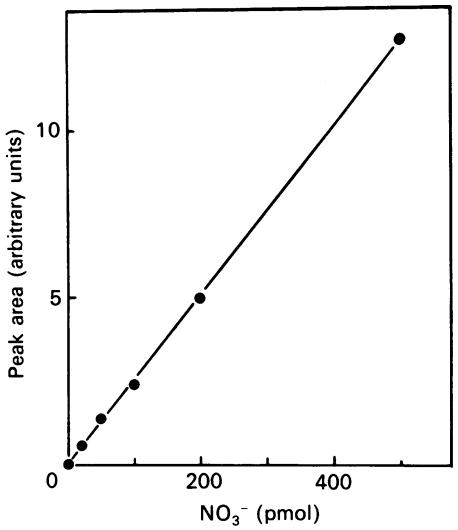

Fig. 2. Correlation between the amount of nitrate applied to the h.p.l.c. system and the nitrate peak area

Standard solutions of KNO_3 in 2 M-H_3PO_4 were analysed as described in the Experimental section, eluant B being used as the mobile phase.

although the absorption coefficients at 210 nm of sulphate and chloride are about 10^5 and 10^3 times lower respectively than that of nitrate. Appropriate controls in the absence of nitrate must be analysed to confirm the absence of u.v.-absorbing material in the 10–11 min-retention-time region.

Special attention must be paid to the possible interference of nitrite and the means to eliminate it. At low pH, spontaneous oxidation of nitrite to nitrate occurs [13,20]. At pH 1.9 even on-column oxidation could be observed in standard solutions. Sulphamic acid has been used for the removal of nitrate from aqueous solutions without influencing nitrate recovery [6,21]. Under our chromatographic conditions, addition of 20 mM-sulphamic acid to standards containing up to 200 μM-nitrite completely eliminated nitrite without otherwise affecting the h.p.l.c. analysis of nitrate in either nitrate standards or in nitrate-plus-nitrite mixtures. If the biological samples to be analysed contain nitrite, sulphamic acid must be included in the lysis solution in order to avoid nitrite oxidation to nitrate during the assay. In our case, no nitrite was present in either normal NO_3^--grown or tungstate-treated *Anacystis* cells, as indicated by the similar nitrate recovery in extracts prepared in H_3PO_4 or in H_3PO_4 supplemented with 20 mM-sulphamic acid.

Sensitivity of nitrate determination

Fig. 2 shows the correlation found between the amount of nitrate applied to the chromatographic system and the area of the corresponding peak. The response was linear, at least in the range between 20 and 5000 pmol of nitrate in a 10 μl injection volume. The values obtained were highly reproducible, varying by a mean of 1.8% in 24 h. For nitrate amounts lower than 20 pmol, variability increased, the resulting peak being very much dependent on the integration parameters chosen.

As shown in Fig. 1(*b*), nitrate from cell extracts was eluted as a distinct peak on a stable baseline and, consequently, amounts of nitrate as low as 20 pmol in a 10 μl injection volume could be accurately quantified. Table 2 illustrates the analysis of nitrate in *A. nidulans* extracts obtained by silicone-oil centrifugation, at the indicated times, of the nitrate-transport assay mixtures. Nitrate accumulation was observed in NO_3^--grown tungstate-treated cells, unable to reduce nitrate, whereas addition of NH_4^+, which induces a rapid inhibition of nitrate utilization [1], suppressed the nitrate-transporting capacity [14].

Table 2. Nitrate accumulation and its prevention by NH_4^+ in *A. nidulans* cells

Assays of nitrate transport were performed, as described in the Experimental section, in NO_3^--grown tungstate-treated *A. nidulans* cells. Data are those of a representative experiment. The values have been corrected for extracellular nitrate carried by the cells through the silicone barrier, which accounted for 32 pmol in a 10 μl injection volume. In general conditions were as in Fig. 1(*b*).

	Nitrate (pmol)	
Assay time (s)	No addition	$+NH_4^+$ (0.5 mM)
0	0	0
30	19	0
50	65	0
80	130	0

Concluding remarks

The method presented here for the determination of intracellular nitrate has proven adequate for the determination of nitrate transport in cyanobacteria [14]. It is sensitive and simple, although some precautions have to be taken in case of the presence of other inorganic anions such as nitrite, which can be effectively removed by sulphamic acid treatment of the extracts, or sulphate and chloride, whose presence in interfering amounts has to be previously excluded. The procedure seems also potentially adequate for the study of nitrate transport in other microalgae and in higher-plant protoplasts and organelles.

We are grateful to Professor M. Losada for generous encouragement and help, to Mr. T. Coronil and Ms. R. Rodríguez for kindly solving troubles with the h.p.l.c. equipment and to Ms. J. Pérez de León and Ms. A. Friend for skilful secretarial assistance. This work was supported by grant no. BT 85/0045 from Comisión Asesora de Investigación (Spain).

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Received 21 September 1988; accepted 3 November 1988