FINAL REPORT

NITRATE TRANSPORT OF THE HALOPHILIC CYANOBACTERIUM, APHANOTHECE HALOPHYTICA GROWN UNDER NORMAL AND SALT STRESS CONDITIONS

BY

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Abstract

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Project Title : Nitrate transport of the halophilic cyanobacterium, *Aphanothece halophytica* grown under normal and salt stress conditions

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The transport of nitrate by *Aphanothece halophytica* grown under normal and salt-stress conditions was investigated. Nitrate transport was monitored by measuring nitrate remaining in the assay medium using an anion-exchange high performance liquid chromatography. Kinetic studies revealed that the affinities of nitrate to normal and salt-stress cells were not significantly different, K_s = 416 and 450 μM respectively. However, the maximum velocity of nitrate uptake for normal cells was about 2 fold of that for salt-stress cells. Both cells required the presence of Na^+ for the uptake of nitrate. Ammonium inhibited nitrate uptake whereas the presence of methionine sulfoximine was unable to release the inhibition by ammonium. Nitrite was found to competitively inhibit nitrate uptake with a K_i value of 84 μM. Both chloride and phosphate anions had no effect on nitrate uptake. DL-glyceraldehyde, an inhibitor of CO_2 fixation, reduced nitrate uptake. Monensin inhibited nitrate uptake in concentration-dependent manner suggesting an involvement of a Na^+-electrochemical gradient. Amiloride, an inhibitor of Na^+/H^+ antiporter, reduced nitrate uptake. Carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) and N, N′-dicyclohexylcarbodiimide (DCCD) each severely inhibited nitrate uptake suggesting that a pH gradient generated by H^+/ATPase played a role in the transport of nitrate. Exogenously added glucose or lactate led to an increase of nitrate uptake rendering a further support for the involvement of ATP and electrochemical potential for the transport of nitrate into *A. halophytica*.

Key Words : Nitrate transport, Cyanobacteria, Salt stress

บทคัดย่อ

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ชื่อโครงการ : การขนส่งไนเตรตของไซยาโนแบคทีเรียมชนิดชอบความเค็ม, *อะฟาโนทีคี ฮาโลฟิทิคา* ที่เจริญภายใต้สภาวะปกติและสภาวะความเครียดของเกลือ

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ได้ทำการศึกษาการขนส่งไนเตรตเข้าสู่เซลล์ของไซยาโนแบคทีเรียมชนิดชอบความเค็ม, *อะฟาโนทีคี ฮาโลฟิทิคา* ซึ่งเจริญภายใต้สภาวะปกติและสภาวะความเครียดของเกลือ โดยทำการตรวจวัดปริมาณไนเตรตที่เหลือในสารละลายด้วยวิธีไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโทกราฟี พบว่า เซลล์ที่เจริญภายใต้ 2 สภาวะดังกล่าวจับตัวกับไนเตรต โดยมีค่า K_s ที่ไม่แตกต่างกันนัก กล่าวคือมีค่า 416 และ 450 ไมโครโมลาร์ตามลำดับ สำหรับเซลล์ที่เจริญภายใต้สภาวะปกติและสภาวะความเครียดของเกลือ อย่างไรก็ตามพบว่าความเร็วสูงสุดของการนำไนเตรตเข้าสู่เซลล์ที่เจริญภายใต้สภาวะปกติมีค่าประมาณ 2 เท่าของเซลล์ที่เจริญภายใต้สภาวะความเครียดของเกลือ การนำไนเตรตเข้าสู่เซลล์ต้องอาศัยการทำงานร่วมกันของ Na^+ ด้วย ทางด้านการศึกษาผลของอิออนต่าง ๆ พบว่าแอมโมเนียมยับยั้งการนำไนเตรตเข้าสู่เซลล์ โดยที่การยับยั้งนี้ยังคงมีอยู่ถึงแม้ว่าจะเติมสารเมไธโอนีนซัลฟอกซิมีนลงไปก็ตาม สำหรับไนไตรต์นั้นพบว่าสามารถยับยั้งการนำไนเตรตเข้าสู่เซลล์แบบแข่งขันโดยมีค่า K_i 84 ไมโครโมลาร์ ในขณะที่คลอไรด์และฟอสเฟตไม่มีผลต่อการนำไนเตรตเข้าสู่เซลล์ กลีเซอราลดีฮัยด์ซึ่งเป็นสารยับยั้งกระบวนการตรึงคาร์บอนไดออกไซด์สามารถลดอัตราการนำไนเตรตเข้าสู่เซลล์ ในด้านการศึกษาแหล่งพลังงานของการนำไนเตรตเข้าสู่เซลล์นั้น ทั้งโมเนนซินและอะมิโลไรด์ ซึ่งยับยั้งกระบวนการก่อให้เกิด Na^+ และ H^+ เกรเดียนท์ตามลำดับ สามารถลดอัตราการนำไนเตรตเข้าสู่เซลล์ ในทำนองเดียวกัน คาร์บอนิลไซยาโนด์คลอโรฟินิลไฮดราโซน (ซีซีซีพี) ซึ่งเป็นสารที่ทำลายแรงผลักดันเนื่องจากโปรตอน สามารถยับยั้งการนำไนเตรตเข้าสู่เซลล์ ในส่วนของไดไซโคลเฮกซิลคาร์โบไดอิไมด์ (ดีซีซีดี) ซึ่งเป็นสารยับยั้งการย่อยสลายเอทีพีก็สามารถยับยั้งการนำไนเตรตเข้าสู่เซลล์ นอกจากนั้นยังพบอีกว่าการเติมแหล่งพลังงานจากภายนอกในรูปของกลูโคสหรือแลคเตทสามารถเพิ่มอัตราการนำไนเตรตเข้าสู่เซลล์ได้ จากข้อมูลทั้งหมดข้างต้นสามารถบ่งชี้ว่าแหล่งพลังงานในรูปของเอทีพีหรือในรูปของความต่างศักย์ของเคมีไฟฟ้ามีบทบาทต่อการนำไนเตรตเข้าสู่เซลล์ *อะฟาโนทีคี ฮาโลฟิทิคา*

คำหลัก : การขนส่งไนเตรต, ไซยาโนแบคทีเรีย, ความเครียดของเกลือ

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Nitrogen Assimilation

After carbon, oxygen and hydrogen, nitrogen is the most abundant element in all living cells. Nitrogen is a constituent of a large number of important compounds found in all living cells. Particular notable examples are amino acids, proteins (enzymes) and nucleic acids (RNA and DNA), while in other examples polyamines and chlorophylls, may play a major role in some organisms. Most animals do not have the capacity to assimilate inorganic nitrogen, nor to synthesize half the amino acids found in protein, unless assisted by bacteria (e.g. in the rumen of sheep and cattle).

Nitrogen is available to all living cells in the three basic forms: free nitrogen gas, and as combined inorganic or organic compounds. The ability to reduce or fix nitrogen gas is found only among the prokaryotes and exclusively in the photosynthetic cyanobacteria. Nitrogen fixation is the conversion of gaseous nitrogen (N_2) to ammonia. Nitrogen fixation requires nitrogenase, an oxygen-sensitive irons-, sulphur-, molybdenum-containing enzyme complex that also brings about the reduction of other substrates containing triple covalent bonds (nitrous oxide, cyanides, isocyanides, cyclopropene, and acetylene).

Nitrate is a major source of nitrogen for photosynthetic organisms including cyanobacteria, algae and plants (Guerrero et al, 1990). Nitrate assimilation takes place by three sequential steps: (1) nitrate transport into the cell by a specific nitrate permease; (2) reduction to nitrite by assimilatory nitrate reductase; and (3) further reduction to ammonium by

assimilatory nitrite reductase. The resulting ammonium is then incorporated into central metabolism through the action of glutamine synthetase and glutamate synthase.

Upon entering the cells, nitrate is converted to nitrite by nitrate reductase. There are two types of nitrate reductase known. The first, and better-known enzyme, is found in eukaryotic algae. This nitrate reductase complex is similar to that found in fungi and higher plants, consisting of haem (cytochrome *b-557*), flavin adenine dinucleotide (FAD), and molybdenum (Mo) as a prosthetic group. The best characterized algae enzyme is from *Chlorella*; this enzyme has a molecular mass of about 350,000 and a complex structure (Solomonson and Barber, 1990). The enzyme catalyzes the reduction of nitrate to nitrite by reduced pyridine nucleotides according to the reaction:

$$NO_3^- + NAD(P)H + H^+ \longrightarrow NO_2^- + NAD(P)^+ + H_2O$$

With the higher plant enzyme there is evidence that Mo is contained in a small complex (molecular mass less than 30,000) that can be fairly easily separated from the bulk of the enzyme. Haem is present as a cytochrome *b-557* which is reduced by NADH and reoxidized by NO_3^-; cyanide stops its reoxidization by NO_3^- but not its reduction by NADH. The pathway of electrons from NAD(P)H to nitrate through nitrate redutase from eukaryotes may be depicted as:

$$NAD(P)H \longrightarrow (FAD \longrightarrow \text{cyt } b\text{-}577 \longrightarrow Mo) \longrightarrow NO_3^-$$

The pyridine nucleotide specificity of the enzyme differs in different algae. Nitrate reductase is always active with NADH as the electron donor and, in many algae, only with this. Some algae, however, are not able to utilize NADPH.

The second type of nitrate reductase is found in prokaryotic cells including cyanobacteria. The prokaryotic nitrate reductase is simpler and smaller (molecular mass of about 75,000); it also contains

molybdenum but not flavin or cytochrome (Manzano et al, 1976). The important difference from the enzyme of eukaryotes is that it does not use pyridine nucleotide as electron donor but reduced ferredoxin. It therefore catalyzes the reaction:

$$NO_3^- + 2Fd + 2H^+ \longrightarrow NO_2^- + 2Fd + H_2O$$

Activation of cytosolic nitrate reductase has been studied in depth over the last 20 years. In higher plants, there are a number of examples of nitrate reductase activation by ferricyanide, light, and higher levels of CO_2, but it seems that redox potential ($NADH/NAD^+$) could be an important regulator of proportion of nitrate reductase found in active form (Solomonson and Barber, 1990). There is a light/dark modulation of nitrate reductase, via nitrate reductase phosphorylation / dephosphorylation reaction, dependent on Mg^+ and ATP and requiring regulator proteins. These proteins are a protein kinase (PK) which phosphorylates nitrate reductase but without inactivating it, an inhibitor protein (IP) which binds to phospho-nitrate reductase and inactivates it, and a protein phosphatase (PP) which reactivates nitrate reductase (Glaab and Kaiser, 1995).

The reduction of NO_2^- to NH_4^+ is catalyzed by ferredoxin nitrite reductase, characteristic of photosynthetic organisms (the second type of nitrite reductase, NAD(P)H-nitrite reductase is found in nonphotosynthetic organisms). This enzyme appears to be much the same in algae and in leaves of higher plants. It is a small molecule with a molecular mass of 60,000-70,000. This enzyme contains sirohaem which is an iron tetrahydroporphyrin and where NO_2^- probably attaches, it also contains an iron-sulfur center which participates in electron transport. The reaction catalyzed is:

$$NO_2^- + 6\,Fe + 8\,H^+ \longrightarrow NH_4^+ + 6\,Fe + 2H_2O$$

In the leaves of higher plants, nitrite reductase has been localized definitely in the chloroplast; information about its localization in algae cells is lacking, but some results suggest a close linking between nitrite reduction and the photochemical reaction in the chloroplast.

1.2 Regulation of nitrate assimilation

As the assimilation of nitrogen into protein requires both energy and organic skeletons, it is not surprising that there are major interactions between N-assimilation and photosynthetic metabolism (Bassham et al, 1981). The assimilation of both NH_4^+ and NO_3^- is dependent on photosynthesis, that is assimilation requires light and CO_2; removal of either of these prevents assimilation. *Chlamydomonas reinhardtii* does not assimilate nitrate and ammonium unless a suitable source of carbon is provided. Suitable sources are CO_2 in light or, in darkness, acetate or the carbon reserves accumulated in nitrogen-starved cells. Nitrate metabolism is strongly light dependent in green algae. Nitrate uptake can be stimulated by light, leading to indirect activation of nitrate reductase. The enzyme nitrate reductase can also be directly activated by light; light can furthermore induce nitrate reductase synthesis.

Nitrate uptake is generally believed to be the rate-limiting step in nitrate assimilation, and the overall rate of nitrate assimilation is tightly regulated at the nitrate uptake step by the metabolic carbon/nitrogen status of cells (Flores and Herrero, 1994). It has been known for a long time that ammonium suppresses nitrate uptake in many algae species and also in various higher plants. It has been demonstrated many times that in the presence of both ammonium and nitrate in the medium, the NH_4^+ is assimilated first, and only when it has gone is NO_3^- utilized. Preferential uptake of NH_4^+ has been shown often for both marine and

freshwater algae. However, there have been few reports showing preferential assimilation of nitrate; this phenomenon has been reported e.g. for *Pandorina* and *Haematococus*. There are several reasons for this preferential assimilation of ammonium. Active nitrate reductase is not formed in the presence of NH_4^+ nor is the NO_3^- uptake system. And even if active nitrate reductase and an NO_3^- uptake system are present, the addition of NH_4^+ can lead to a rapid cessation of NO_3^- utilization. There are at least three mechanisms by which nitrate reductase activity can disappear from cells. These include two sorts of reversible inactivation phenomena and an irreversible loss of enzyme due, presumably, to degradation. The effect of addition of NH_4^+ to cells assimilating NO_3^- is complex. The first effect appears to be an inhibition of NO_3^- uptake but this is followed by loss of nitrate reductase (and nitrite reductase) activities. The loss of nitrate reductase activity will be partly due to reversible inactivation, and partly due to irreversible loss of enzyme with the rate of proteolytic breakdown of nitrate reductase possibly being greater in the presence of NH_4^+. At the same time addition of NH_4^+ stops the synthesis of nitrate reductase. The regulation of the formation of nitrite reductase has received much less attention.

1.3 Nitrate uptake in cyanobacteria

Nitrate is a major source of nitrogen for cyanobacteria. Nitrate assimilation in cyanobacteria involves two steps: (i) the uptake process and (ii) enzymic reduction. The enzyme involved in the reduction of nitrate to nitrite in cyanobacteria is ferredoxin-dependent nitrate reductase (E.C. 1.7.7.2). It consists of a single polypeptide of 58-85 kDa, a molybdenum cofactor, four non-heme iron and four labile sulfide (two Fe_2S_2 clusters) per molecule (Mikami and Ida, 1984). The enzyme

nitrite reductase (E.C. 1.7.2.1), which mediates the reduction of nitrite to ammonium, is a polypeptide of 52-68 kDa, having Fe_4S_4 cluster and a siroheme as prosthetic group. Ammonium resulting from nitrate an nitrite is assimilated mostly through the GS/GOGAT pathway rendering glutamate, the principal nitrogen donor in the biosynthesis of other organic nitrogen compounds. The transport of nitrate has been the least understood step of nitrate assimilation, although its importance has been recognized for a long time (Beevers and Hageman, 1983). The first unequivocal demonstration of a gene responsible for nitrate transport and of its essential role in nitrate assimilation was done in the cyanobacterium *Synechococus* sp. PCC7942 (Omata et al, 1989), and the subsequent works have shown the structure of the nitrate transporter of the cyanobacterium (Omata et al, 1993). Clusters of genes involved in cyanobacterium *Synechococus* sp. PCC 7942 nitrate assimilation have been mapped. They are in an operon that is regulated by nitrogen availability and are cotranscribed. The operon consists of two nitrogen regulated genes nirB and ntcB, one structural gene nirA for nitrite reductase, four genes for nitrate transport (nrtA, nrtB, nrtC, and nrtD), and one structural gene NarB for nitrate reductase. Nitrate transport is essential for the growth of the cyanobacterium at physiological concentrations of nitrate and has been shown to be involved in the active transport of nitrite as well.

Comparison of the structures of nitrate transporters from eukaryotic and prokaryotic, photosynthetic and non- photosynthetic organisms indicate that the nrt nitrate/nitrite transporter represents a prokaryotic nitrate transporter distinct from the nitrate transporter of eukaryotes. The activity of the nitrate-assimilation system of cyanobacteria is regulated transcriptionally in response to changes in nitrogen availability ; biosynthesis of the NrtA protein, expression of

nitrate-transporting ability, and expression of NR and NiR activities are all subjected to nutritional repression by ammonium (Herrero et al, 1981). In *Synechococus* sp. PCC 7942, the concerted regulation of the activities related to nitrate assimilation is ascribed to co-transcription of gene nirA-nrtBCD-narB as a single operon. (Suzuki et al, 1992). Ammonium, either added to the medium or generated internally by reduction of nitrate, negatively regulates the transcription of the nirA operon. Since L-methionine sulfoximine (MSX), an inhibitor of ammonium fixation by glutamine synthetase, induces development of NR and NiR activities in *Synechococus* (Herrero et al, 1981), it has been assumed that a nitrogenous compound(s), resulting from the assimilation of ammonium, inhibits the expression of the nitrate assimilation system (Guerrero and Lara, 1987). Nitrate assimilation by cyanobacteria is subjected not only to transcriptional regulation but also to post-translational regulation (Guerrero and Lara, 1987). Addition of ammonium to the medium causes prompt cessation of nitrate uptake in nitrate-utilizing cells of *Synechococcus* sp. PCC 6301, a strain closely related to the strain PCC 7942 (Lara et al, 1987). As in the regulation of the nirA operon, fixation of ammonium to Glu is required for the negative effects of ammonium to prevail on the activity of nitrate transport. It is supposed that a metabolite(s) of Glu acts as a negative effector of nitrate transport.

By monitoring intracellular accumulation of nitrate in *Synechococus* sp. PCC 7942, Rodriguez et al showed a selective and strong dependence of nitrate transport on millimolar concentrations of Na^+ (Rodriguez et al, 1992). Monensin, an ionophore that collapses the eletrochemical gradient of Na^+, was shown to depress nitrate transport. Based on detailed kinetic studies on the sodium-dependent nitrate transport, Rodriguez et al proposed a sodium/nitrate symport driven by

the energy of eletrochemical gradient of Na^+ as the most likely mechanism of nitrate transport.

1.4 Relationship of cyanobacteria salt tolerance and nitrate

Salt overloading in soils is by far the major hindrance for plant growth, and a crucial problem for agriculture. Each organism displays a salinity tolerance range, which includes its optimal growth conditions, though larger ranges of salt concentrations (resistance range) may still be compatible with cell survival. Crop plants are particularly limited in their tolerance range, whereas other photosynthetic organisms, including microalgae and cyanobacteria, may display wider acclimation capacities. Exposure of cells to salt concentrations that are physiologically above those present intracellularly threatens them via two deleterious effects, namely, increase of both the osmotic pressure and the ion concentration. The water potential decreases, leading to loss of water by the cells, and simultaneously to influx of ions into the cytoplasm. Stress from NaCl, in particular, causes a dramatic increase in the concentration of inorganic ions.

Cyanobacteria, the only prokaryotes performing oxygenic photosynthesis and probable ancestors of chloroplasts, constitute valuable models for the study of the molecular mechanisms involved in tolerance to high salinity, or to its corollary, drought, a major agricultural problem. The critical demands of cyanobacteria exposed to high salinity, i.e., accumulation of osmoprotectors and extrusion of sodium ions, are met through immeiate activation and/or long term (protein synthesis-dependent) adaptation of various processes: (i) uptake and endogenous biosynthesis of osmotica, the nature and amount of which are strain and salt concentration dependent (ii) probable modifications of membrane lipid composition (iii) increased energetic

capacity, at the level of cyclic electron flow around photosystem I (through routes induced under these conditions) and cytochrome c oxidase, and (iv) enhancement of H^+-ATPase activity and active extrusion of sodium ions.

Extrusion of sodium ions phenomena have an important influence on the halotolerance of various organisms. Cyanobacteria do not accumulate Na^+ although a transient net Na^+ uptake may occur in response to hypersaline upshock (Reed et al, 1985). It was shown further that the ability to curtail Na^+ influx can also be induced by certain environmental factor like alkaline pH or presence of combined nitrogen in the form of nitrate or ammonium in the growth medium (Reddy et al, 1989). Presence of certain nitrogenous compounds in the growth medium significantly enhanced the salt tolerance of freshwater cyanobacterium *Anabaena* sp. strain L-31 as well as the brackish water cyanobacterium *Anabaena torulosa*. Amoung these, nitrate, ammonium, and glutamine were most effective followed by glutamate and aspartate. These nitrogenous compounds also inhibited Na^+ influx in both *Anabaena* sp. with the same order of effectiveness as that observed for protection against salt stress. The inhibition of Na^+ influx on addition of the nitrogenous substances was rapid; nitrate and ammonium inhibited Na^+ influx competitively. Uptake of nitrate and ammonium increased during salt stress but was not correlated with growth. Intracellular levels of nitrate and ammonium were found to be inadequate to constitute a major component of the internal osmoticum. These suggest that inhibition of Na^+ influx by nitrate and/or amonium is part of a mechanism for protection of cyanobacteria against salt stress.

1.5 Objectives of the project

So far the knowledge on nitrate uptake by cyanobacteria has been very limited. The effect of salinity on the uptake of nitrate is poorly understood. Moreover, the source of the driving force for the transport of nitrate is still controversial. These, coupled to our interest in the mechanism of salt tolerance in cyanobacteria, prompted us to investigate the uptake of nitrate by a unique halotolerant cyanobacterium *Aphanothece halophytica* which is capable of growth in a wide range of salinity. Specifically the objectives of this project are as follows :

1.5.1 Study the kinetics of nitrate uptake by *A. halophytica* grown under non-stress and salt-stress conditions

1.5.2 Study the regulatory aspects of nitrate uptake by non-stressed and salt-stressed *A. halophytica*

1.5.3 Study the energetic aspects of nitrate uptake by non-stressed and salt-stressed *A. halophytica*

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains

Aphanothece halophytica was initially isolated from Solar Lake in Israel. The organism was kindly provided by Dr. T. Takabe of Research Institute of Meijo University, Japan.

2.2 Culture of *A. halophytica*

Ten percent inoculum of *Aphanothece halophytica* was inoculated into a 250 ml flask containing 100 ml of Turk Island salt solution + modified BG_{11} medium (see Appendix I) with 0.5 M NaCl, the pH of the medium was adjusted to 7.6 and grown on a rotary shaker with 160 rpm at 30°C, 60 $\mu Em^{-2}s^{-1}$ of continuous irradiance. After 10 days, 10 ml culture was inoculated into the same medium containing either 0.5 M NaCl or 2.0 M NaCl and shaken on a rotary shaker (160 rpm) at 30°C with 2000 lux of continuous illumination.

2.3 Determination of nitrate uptake

2.3.1 Determination of nitrate content by HPLC

The nitrate content was determined by anion-exchange HPLC method using analytical Hypersil-10 sax column (10 μm porous silica gel packing 250 mm x 4.6 mm i.d.) recommended by Romero et al. (1989). HPLC analyses were performed with HP series 1050, 4 pumps and UV detector, which was set at 210 nm. 30 mM potassium dihydrogen phosphate buffer pH 3 was

used as mobile phase and operated at the flow rate of 1.0 ml/min.

2.3.2 Time courses of nitrate uptake

Ten percent of *Aphanothece halophytica* were inoculated into a 250 ml flask containing 100 ml of Turk Island salt solution + modified BG_{11} medium with 0.5 M NaCl, the pH of the medium was adjusted to 7.6 and grown on a rotary shaker with 160 rpm at 30°C, 60 $\mu Em^{-2}s^{-1}$ of continuous irradiance. After 10 days, 10 ml culture was inoculated into the same medium containing either 0.5 M NaCl (normal) or 2.0 M NaCl (salt stress) and shaken on the rotary shaker (160 rpm) at 30°C with 60 $\mu Em^{-2}s^{-1}$ of continuous irradiance. After 10 days, the culture was centrifuged at 2000 g for 10 min and the cell pellet was suspended in 0.5 M sorbitol, 25 mM Hepes-KOH buffer pH 8.3, 12 mM $NaHCO_3$ containing either 0.5 M NaCl or 2.0 M NaCl followed by the addition of 100 μM $NaNO_3$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by rapid filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1

2.4 Characterization of nitrate uptake

2.4.1 Kinetics of nitrate uptake

A. halophytica was grown in the medium, harvested and osmotic stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll /ml. To

determine the kinetic constants of the nitrate uptake system, initial rates of uptake were determined over a wide range of nitrate concentrations (100 to 1000 μM $NaNO_3$) using cells grown under normal and salt stress conditions (0.5 M and 2.0 M NaCl). The half saturation value (K_s) and maximum velocity (V_{max}) were calculated from x-axis and y-axis intercepts respectively of the double-reciprocal plot, i.e., 1/[s] vs 1/[v].

2.4.2 Na^+ requirement of the uptake system

Cell were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. Initial rates of uptake were determined in the presence of various Na^+ concentrations using NaCl as a source.

2.5 Effect of various ions on nitrate uptake

2.5.1 Effect on NH_4^+ on nitrate uptake

Cells were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes-KOH buffer pH 8.3 and 12 mM $NaHCO_3$. The assay was started by simultaneous addition of nitrate and ammonium with irradiance at 60 $\mu Em^{-2}s^{-1}$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1. To study the effect of ammonium concentrations on nitrate uptake,

initial rates of uptake were determined in the presence of various NH_4^+ concentrations using NH_4Cl as a source. To study the effect of L-methionine D, L-sulfoximine (MSX, an inhibitor of ammonium assimilation) to remove negative effect of NH_4^+, preincubation of the cells with 1 mM MSX for 30 min in the dark was done before assay. The assay was started by simultaneous addition of nitrate and ammonium with irradiance. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1.

2.5.2 Effect of anions on nitrate uptake

Cells were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes-KOH buffer pH 8.3 and 12 mM $NaHCO_3$. The assay was started by simultaneous addition of nitrate and nitrite (or nitrate and chloride, or nitrate and phosphate) with irradiance at 60 $\mu Em^{-2}s^{-1}$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1. To study the effect of nitrite concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various nitrite concentrations.

2.5.3 Effect of DL-glyceraldehyde (DLG) on nitrate uptake

Cells were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes-KOH buffer pH 8.3 and 12 mM $NaHCO_3$. Preincubation of the cells with 30 mM DLG for 30 min in the dark was done before assay. The assay was started by simultaneous addition of 100 μM $NaNO_3$ with irradiance at 60 $\mu Em^{-2}s^{-1}$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1. To study the effect of DLG concentrations on nitrite uptake, initial rates of uptake were determined in the presence of various DLG concentrations.

2.6 Effect of various inhibitors on nitrate uptake

Cells were grown in the medium, harvested and salt stress experiment was done as described in 2.3.2 with an amount of cells equivalent to 25 μg of chlorophyll a/ml. The reaction mixture contained 0.5 M sorbitol in 25 mM Hepes-KOH buffer pH 8.3 an 12 mM $NaHCO_3$. Preincubation of the cells with 20 μM monensin (or 100 μM amilorie or 50 μM DCCD or 10 μM CCCP) for 30 min in the dark was done before assay. The assay was started by simultaneous addition of 100 μM $NaNO_3$ with irradiance at 60 $\mu Em^{-2}s^{-1}$. At indicated time intervals (0 min, 2 min, 4 min, 6 min, 8 min and 10 min) the reaction was stopped by filtration through a 0.45 μm nylon filter membrane. The total supernatant was determined for remaining nitrate content by HPLC as described in section 2.3.1. To study the effect of various inhibitors concentrations on nitrate uptake, initial rates of uptake were determined in the presence of various inhibitors concentration.

CHAPTER 3

RESULTS

3.1 Characterization of nitrate uptake system

3.1.1 Kinetics of nitrate uptake

We first determine the concentration needed to saturate the uptake of nitrate by *A. halophytica* under normal and salt stress conditions (0.5 M and 2.0 M NaCl). Figure 3.1 shows that nitrate uptake rates under normal condition were higher than those under salt stress condition. When the cells were assayed with 100 μM $NaNO_3$ the linear increase of nitrate uptake occurred during the first 2 min and the uptake was rather constant after that. Initial uptake rates were determined over a wide range of nitrate concentration (100 to 1000 μ M). The nitrate uptake system was saturable and displayed typical Michaelis-Menten type kinetics. The Lineweaver-Burk transformation of the data under these conditions was performed (Fig. 3.2) and the line of best fit was obtained by using a least squares linear regression. The apparent K_s values for normal and salt stress conditions were 416 and 450 μM respectively, the maximum velocity values (V_{max}) were 9.1 and 5.3 μmol/min/mgChl respectively.

3.1.2 Effect of Na^+ on nitrate uptake

It has been reported that sodium is required for Nrt-dependent nitrate uptake in *Synechococcus* sp. strain PCC 7942 (Rodriguez et al, 1994). We therefore determined whether nitrate uptake by *A. halophytica* is influenced by external sodium. The rate of nitrate uptake

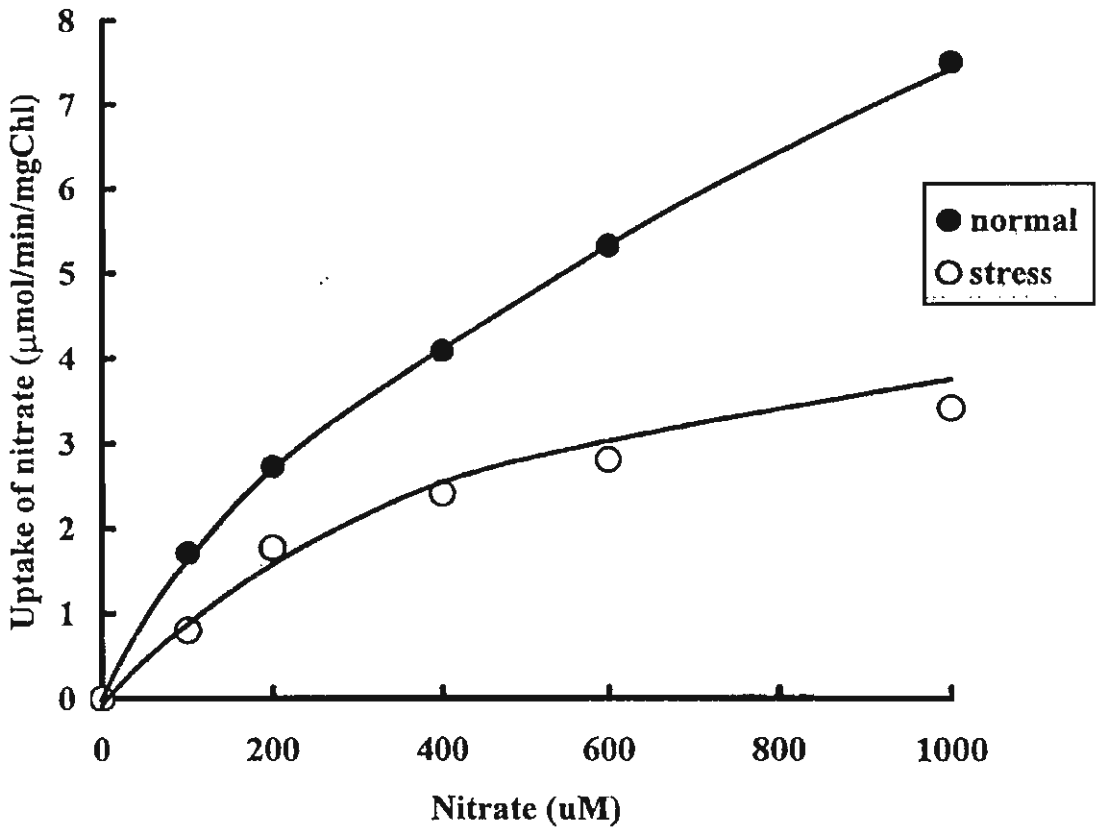

Figure 3.1 Kinetics of nitrate uptake by A.halophytica under normal and salt stress conditions

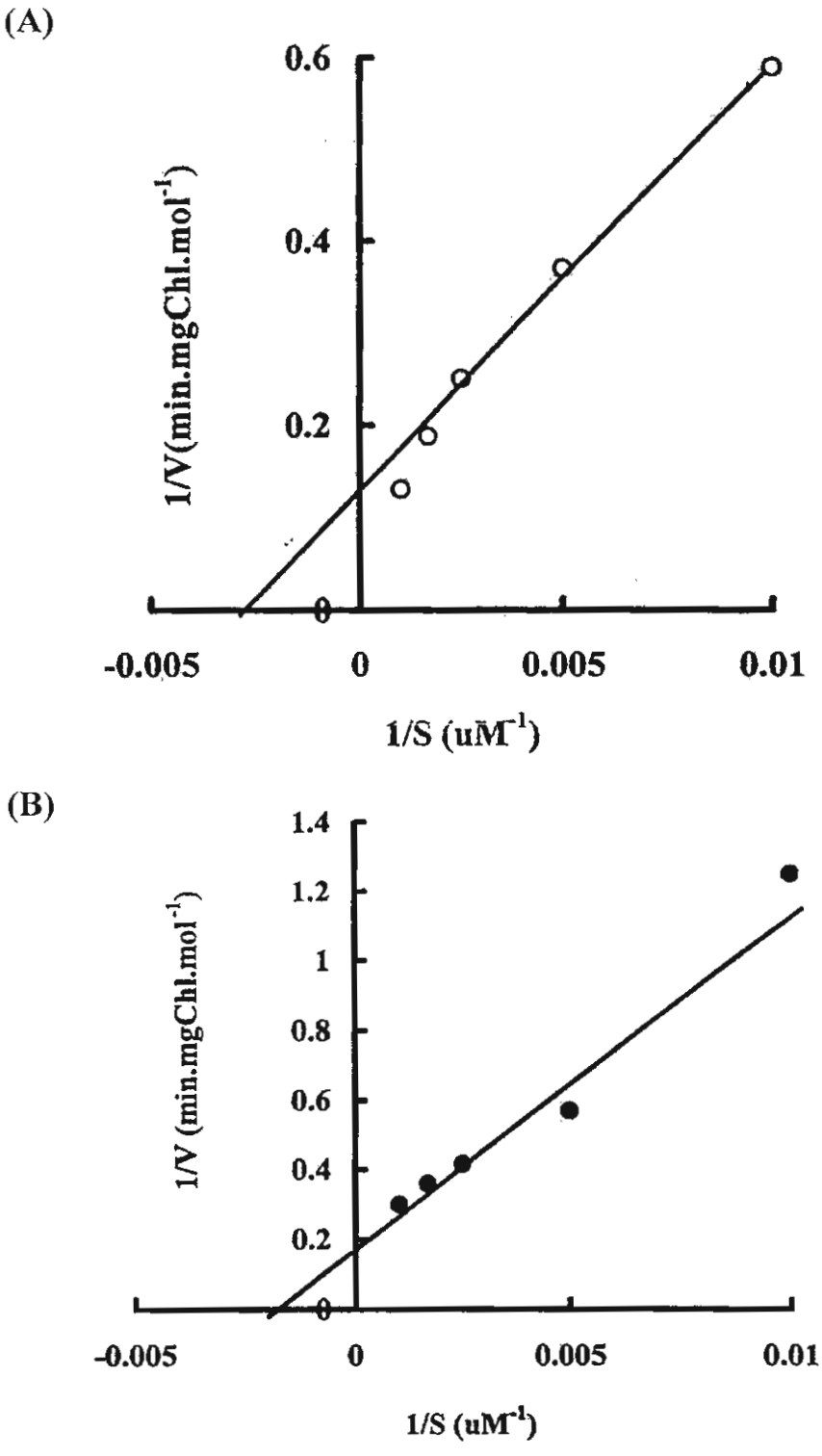

Figure 3.2 Lineweaver-Burk transformation of the data from cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M (B)

was determined in reaction mixtures containing 0 to 10 mM NaCl. Figure 3.3 show that Na^+ could activate nitrate uptake in both normal and salt stress conditions. At a fixed concentration of nitrate used, the uptake of nitrate appeared to reach saturation at 1 mM Na^+ for both conditions. In addition, nitrate uptake was not inhibited by Na^+ as high as 10 mM.

3.2 Effect of various ions on nitrate uptake

3.2.1 Effect of NH_4^+ on nitrate uptake

Figure 3.4 shows the effects of 100 μM ammonium chloride on nitrate uptake in the presence and absence of mM L-methionine sulfoximine (MSX). At 100 μM ammonium chloride, the inhibition of nitrate uptake was hardly observed in the first 2 minutes for both normal and salt stress conditions. However, slightly lower uptake occurred after 2 minutes. MSX did not appear to release the inhibition by ammonium for both normal and salt stress conditions. When higher than 100 μM ammonium chloride was tested, i.e. at 200 μM ammonium, nitrate uptake rate was reduced for both normal and salt stress conditions (Fig. 3.5). Again, MSX had no effect on the release of inhibition by ammonium.

3.2.2 Effect of NO_2^- on nitrate uptake

Nitrite, an alternative nitrogen source, behaves as an effective competitive inhibitor of nitrate uptake. Figure 3.6 shows the effect of 100 μM $NaNO_2$ on nitrate uptake. In both cases (normal and salt stress conditions) the addition of nitrite to the uptake media inhibited the net uptake of nitrate. Figure 3.7 illustrates the response of nitrate uptake rate to four concentrations of nitrite (50, 100, 200, and 400 μM). In

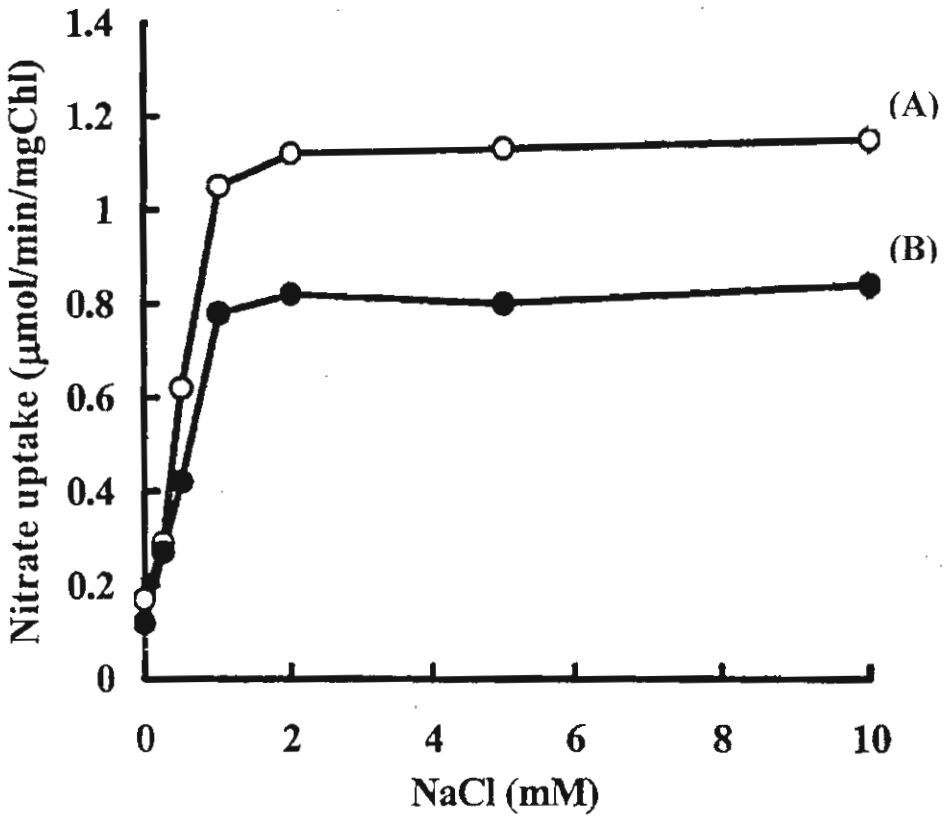

Figure 3.3 Effect of Na^+ concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

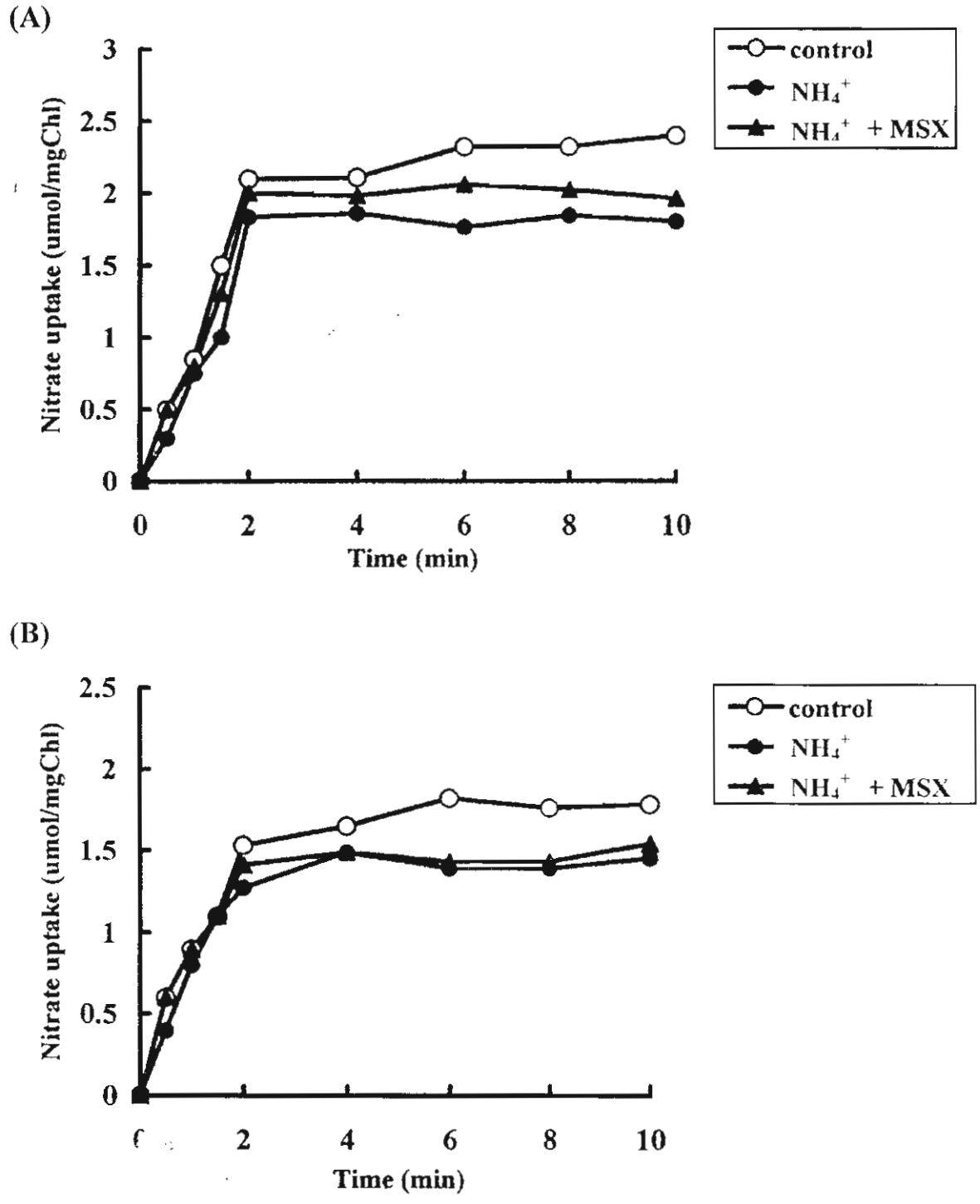

Figure 3.4 Effect of ammonium and MSX on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

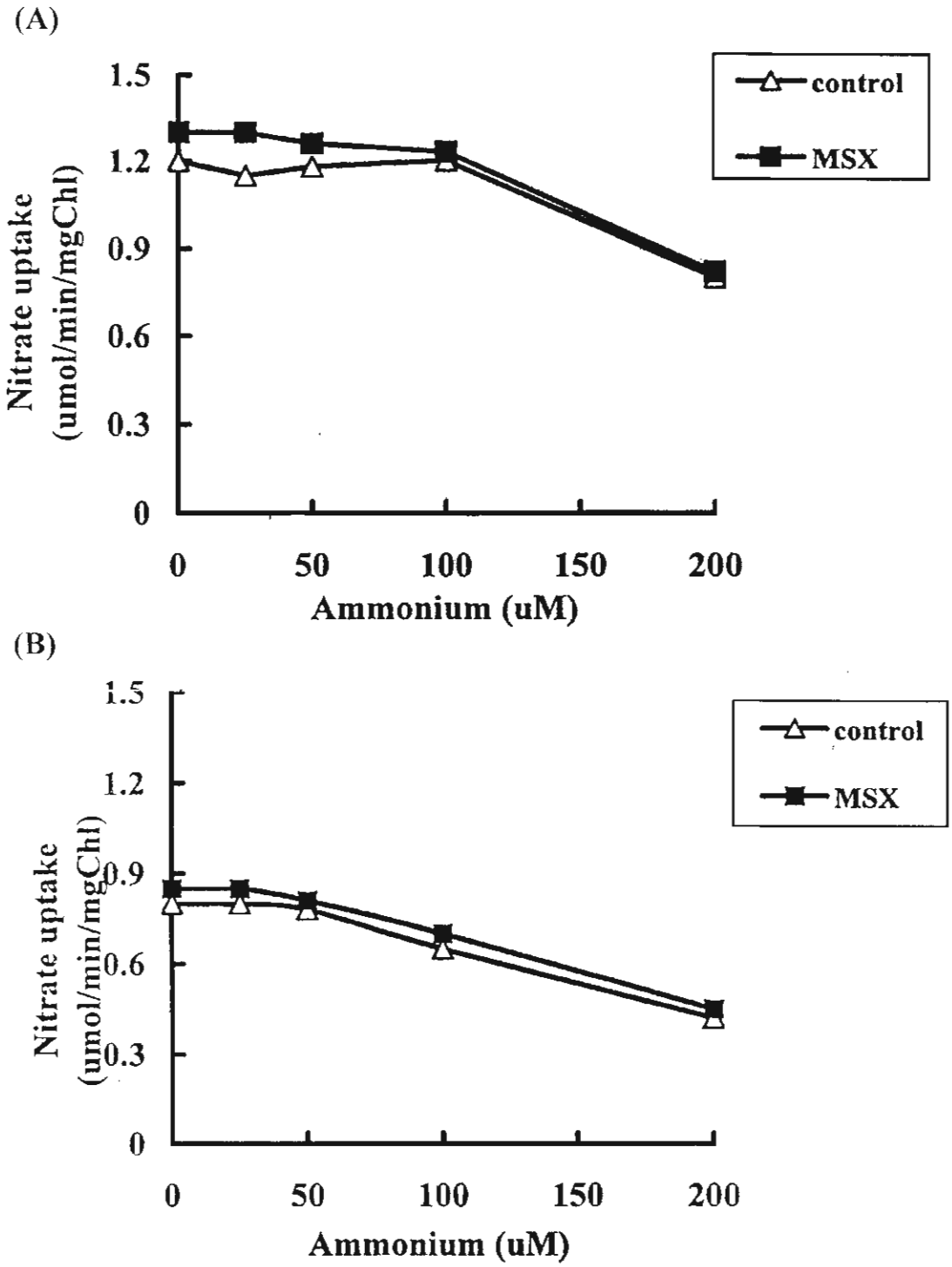

Figure 3.5 Effect of ammonium concentration with or without MSX on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

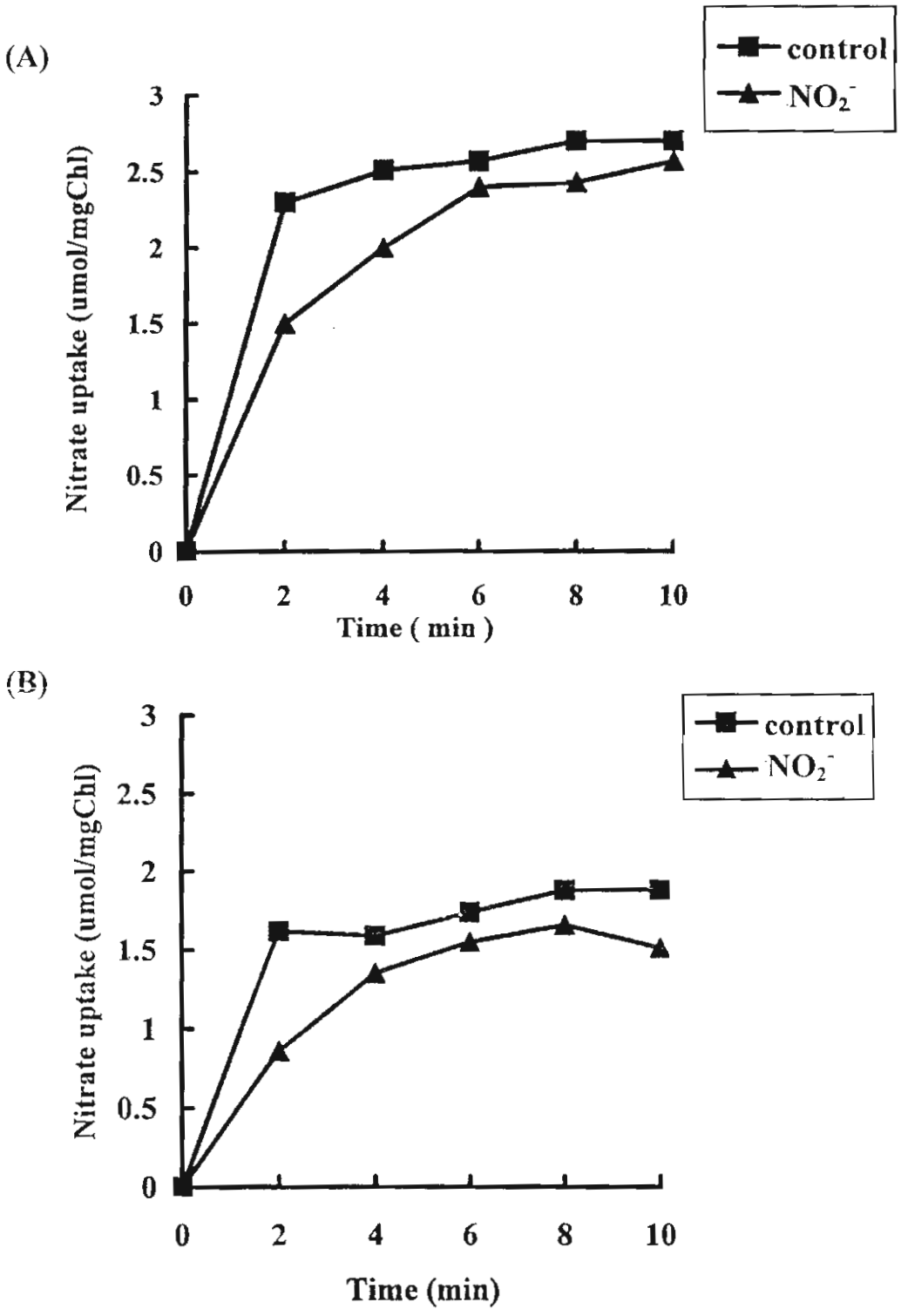

Figure 3.6 Effect of nitrite on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

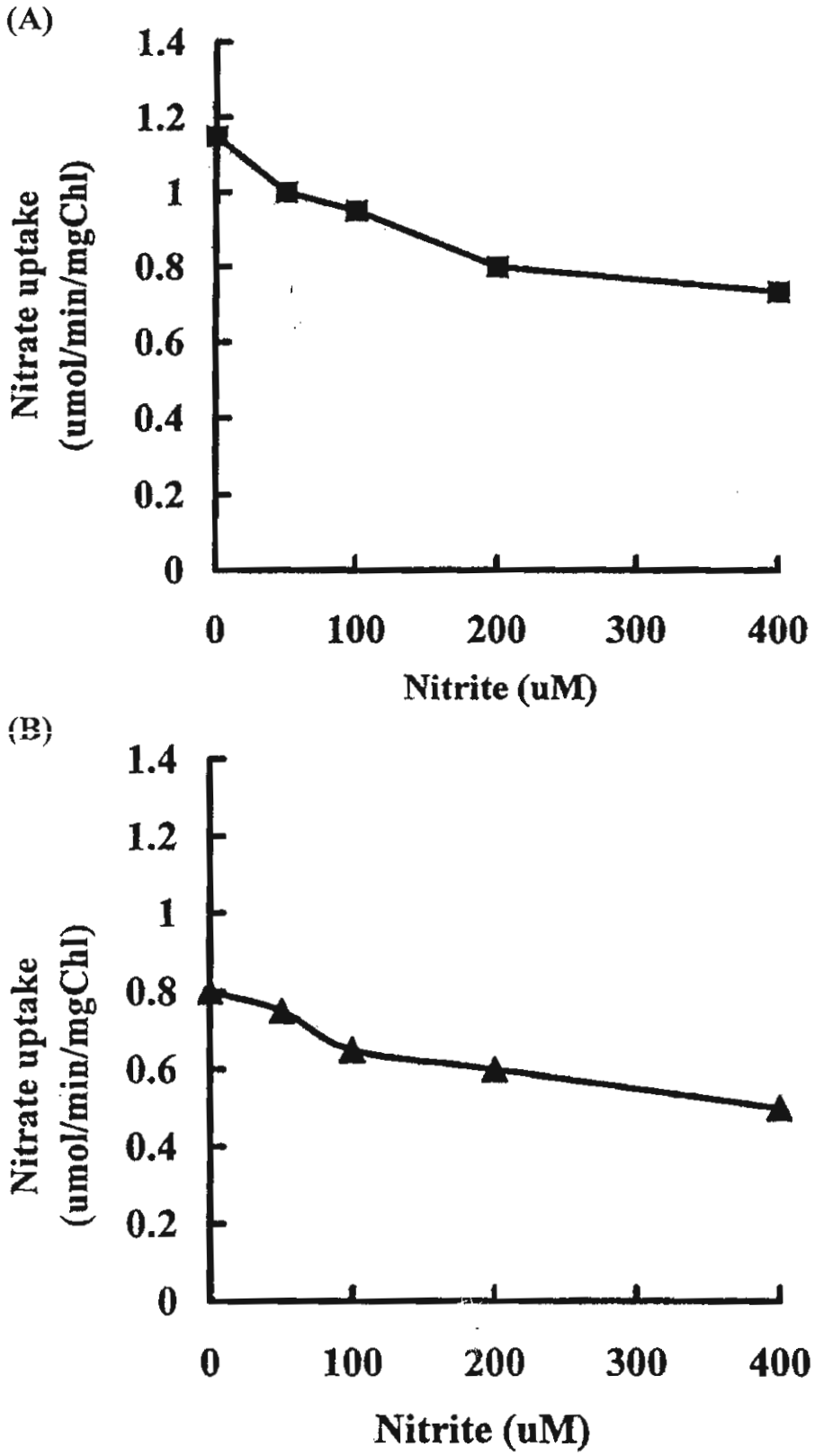

Figure 3.7 Effect of nitrite concentration on nitrate uptake with cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

comparison to the maximum rate (no nitrite), increasing the concentration of NO_2^- in the uptake solution gradually inhibited the uptake of NO_3^-. The inhibition by nitrite appeared to be competitive because the regression lines of the double reciprocal plots at different concentrations of nitrite approached a common intercept (Fig. 3.9, inset). The value of inhibition constant, K_i, obtained by plotting the concentration of nitrite versus slope, was 84 μM (Figs. 3.8 and 3.9). This indicates that nitrite binds to the nitrate transporter with very high affinity.

3.2.3 Effect of other anions on nitrate uptake

Previous studies on several genotypes of the genus *Hordeum* compared net uptake of nitrate from the solution containing equimolar concentration of chloride. The kinetics of net nitrate uptake suggested that the presence of chloride might inhibit nitrate uptake (Smith 1973). We tested the hypothesis by adding nitrate with various anions to the assay medium. Figure 3.10 shows the effects of chloride and phosphate on nitrate uptake. Both 100 μM chloride and 100 μM phosphate did not inhibit nitrate uptake compared with the control treatment in both normal and salt stress conditions. The results suggest that chloride and phosphate have no effect on nitrate uptake in *A. halophytica*.

3.3 Effect of various inhibitors on nitrate uptake

3.3.1 Effect of DL-glyceraldehyde (DLG)

As previously reported in *Synechococcus* sp. PCC 7942 (Rodriguez et al, 1998), nitrate uptake was severely inhibited by preincubation of the cells with DLG (a selective inhibitor of CO_2 fixation). We have tested this hypothesis in *A. halophytica* cells by

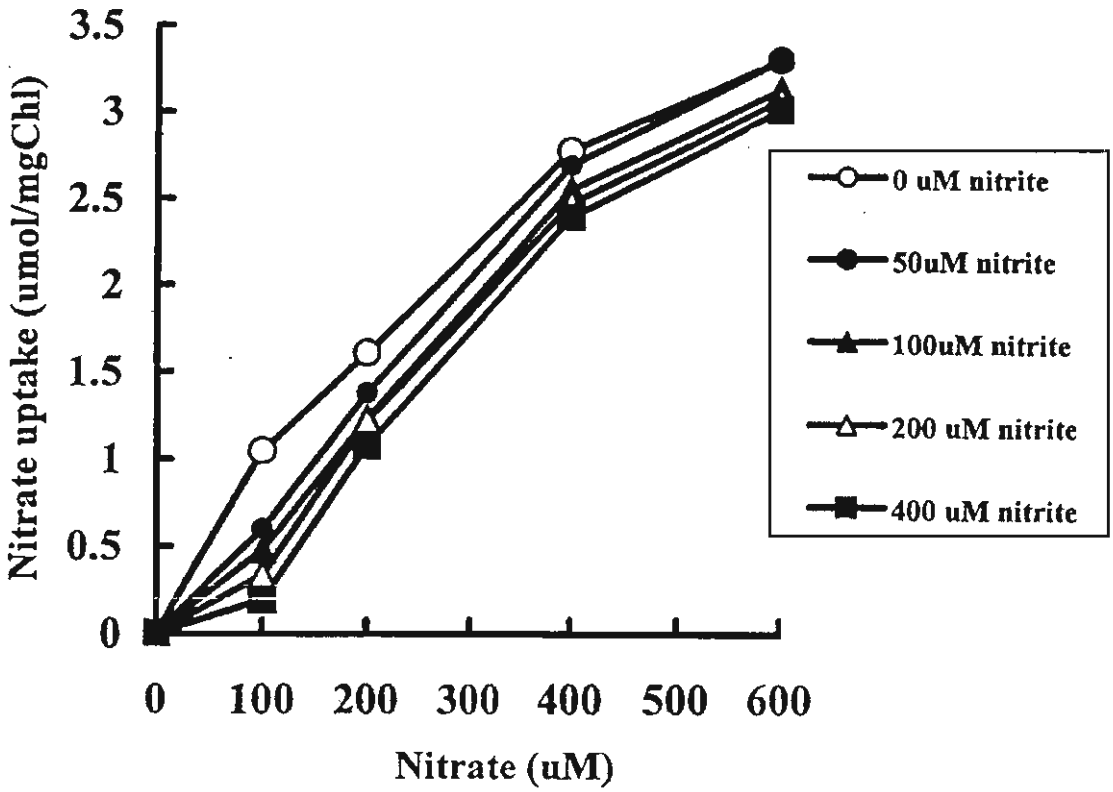

Figure 3.8 Kinetics of nitrate uptake as influenced by various concentrations of nitrite

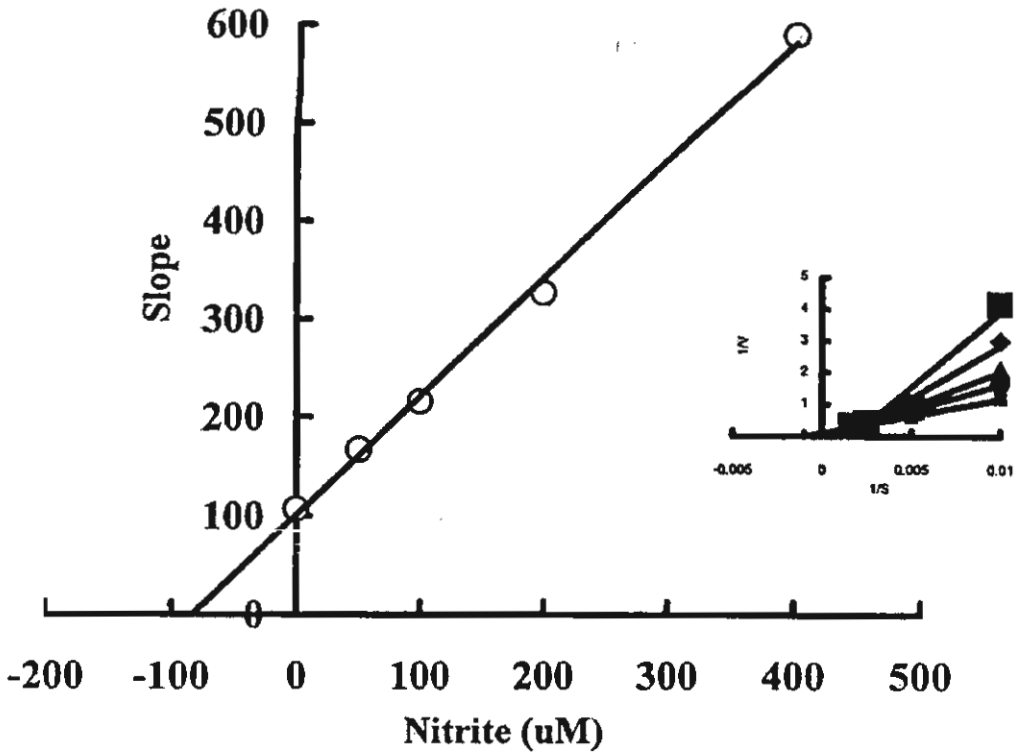

Figure 3.9 Relationship between various nitrite concentrations and slopes obtained from double reciprocol plots

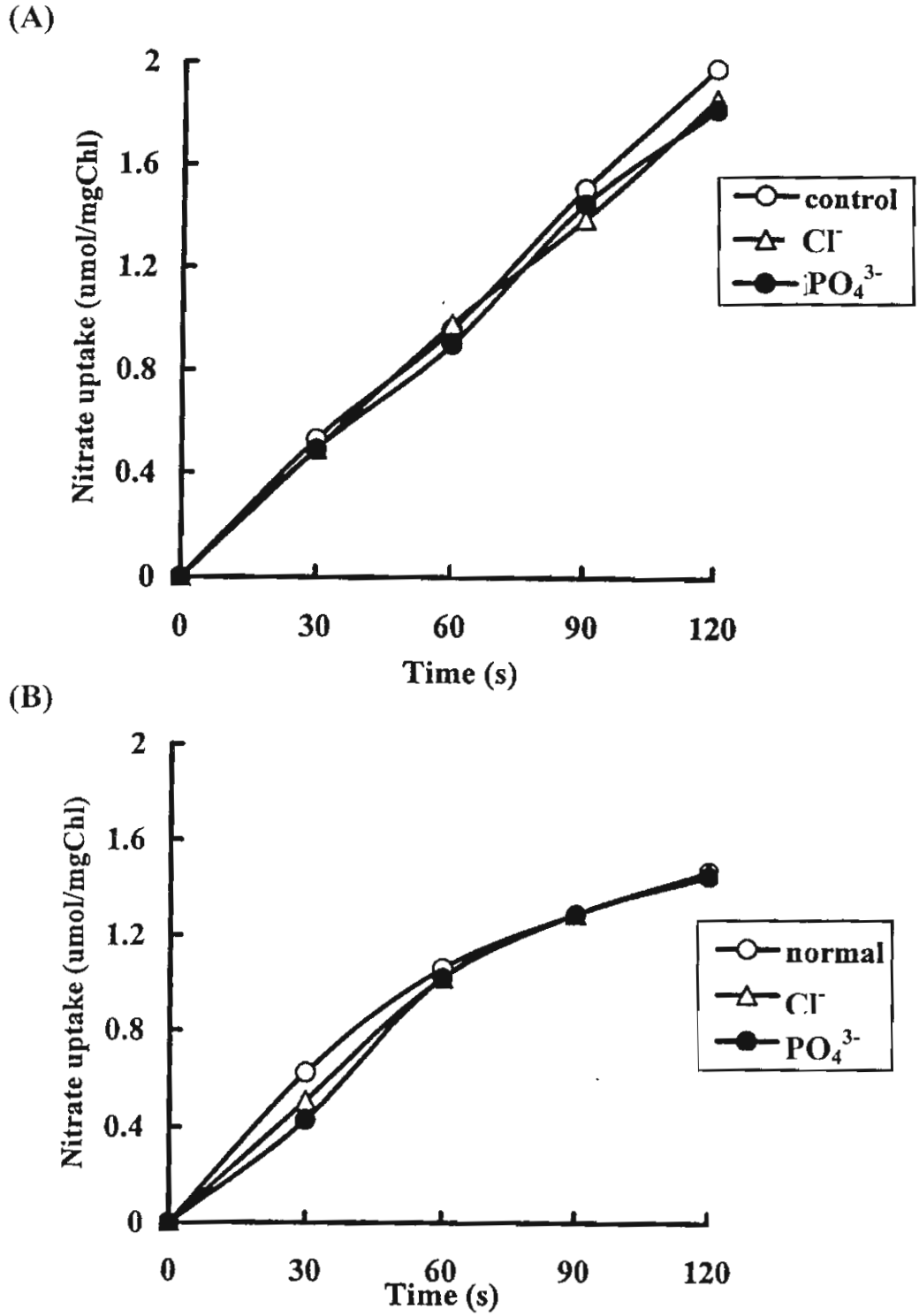

Figure 3.10 Effect of various anions on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

preincubation of the cells with 30 mM DLG for 30 min in the dark prior to the nitrate uptake assay. In both normal and salt stress cells the data show that when DLG, a selective inhibitor of CO_2 fixation, was present in the assay medium, cells of *A. halophytica* consumed nitrate from the medium, but the rate of nitrate uptake was slower than that in the absence of DLG (Fig. 3.11). The nitrate uptake rates declined with increasing concentration of DLG (Fig. 3.12). These results strongly indicated the dependence of nitrate uptake upon active carbon assimilation. The results presented here confirm that nitrate uptake in *A. halophytica* is partly dependent on active CO_2 fixation.

3.3.2 Effect of metabolic inhibitors on nitrate uptake

The effects of various inhibitors that interfere with ion gradients, i.e. disrupting Na^+ electrochemical gradients (monensin), inhibiting Na^+/H^+ antiportor and/or Na^+ channel (amiloride), blocking proton channel (DCCD) and protonophore (CCCP) on the initial rate of nitrate uptake were examined. Inhibitors were preincubated with the cells for 30 min prior to the nitrate uptake assay. The results are shown in Figs. 3.13, 3.15, 3.17, 3.19. All inhibitors inhibited nitrate uptake in both normal and salt stress conditions. In comparison to the maximum rate obtained without inhibitors, the nitrate uptake rates declined with increasing concentration of inhibitors (Figs. 3.14. 3.16, 3.18, 3.20).

The concentrations of monensin, amiloride, DCCD and producing 50% inhibition of nitrate uptake in normal condition were 23, 245, 32, and 22 μM, respectively. The concentrations of monensin, amiloride, DCCD and CCCP producing 50% inhibition of nitrate uptake in salt stress condition were 25, 160, 75, and 18 μM, respectively. CCCP and DCCD appeared to be a more potent inhibitor of nitrate uptake in *A. halophytica*. Inhibition of nitrate uptake was almost

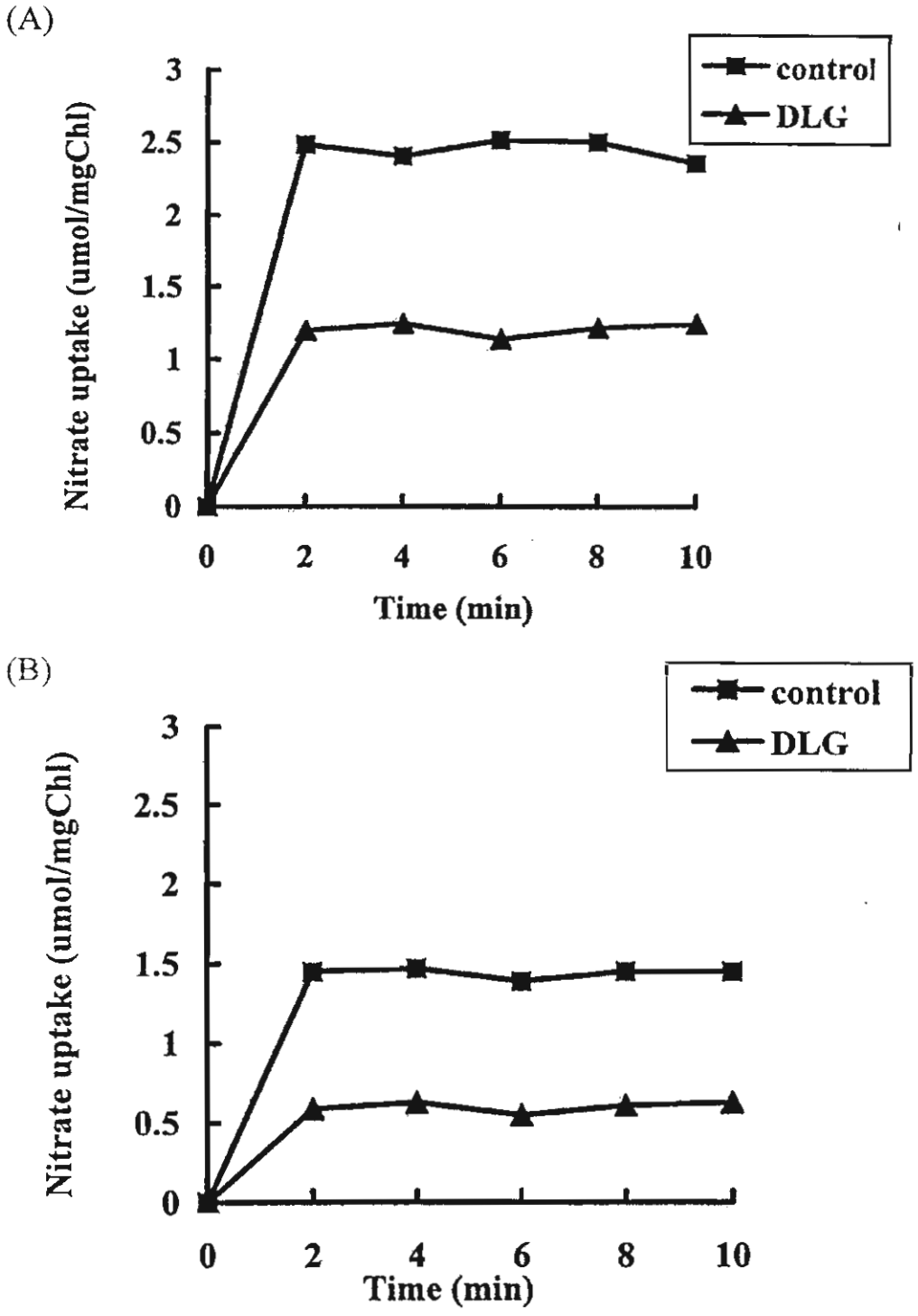

Figure 3.11 Effect of DLG on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

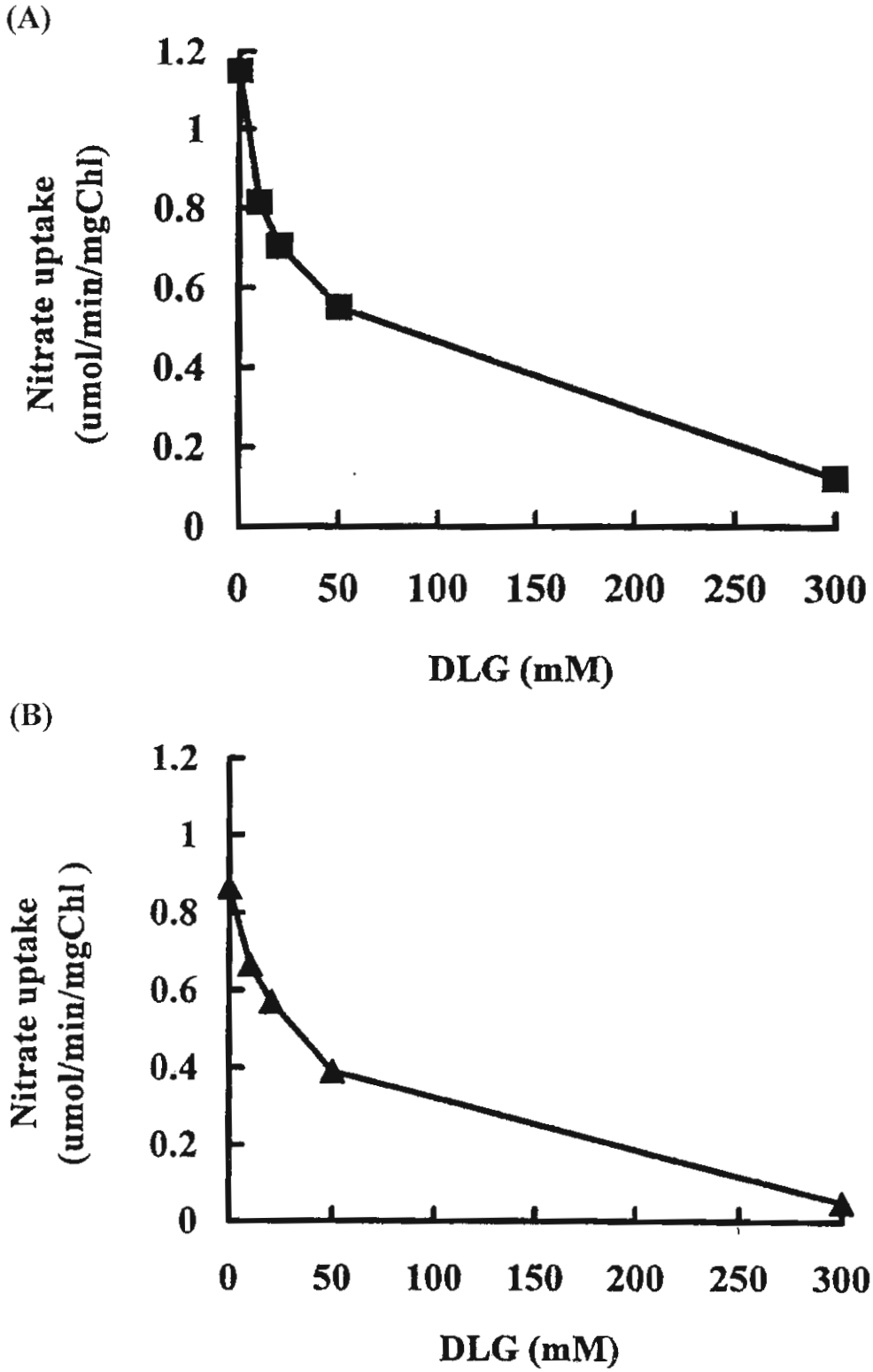

Figure 3.12 Effect of DLG concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

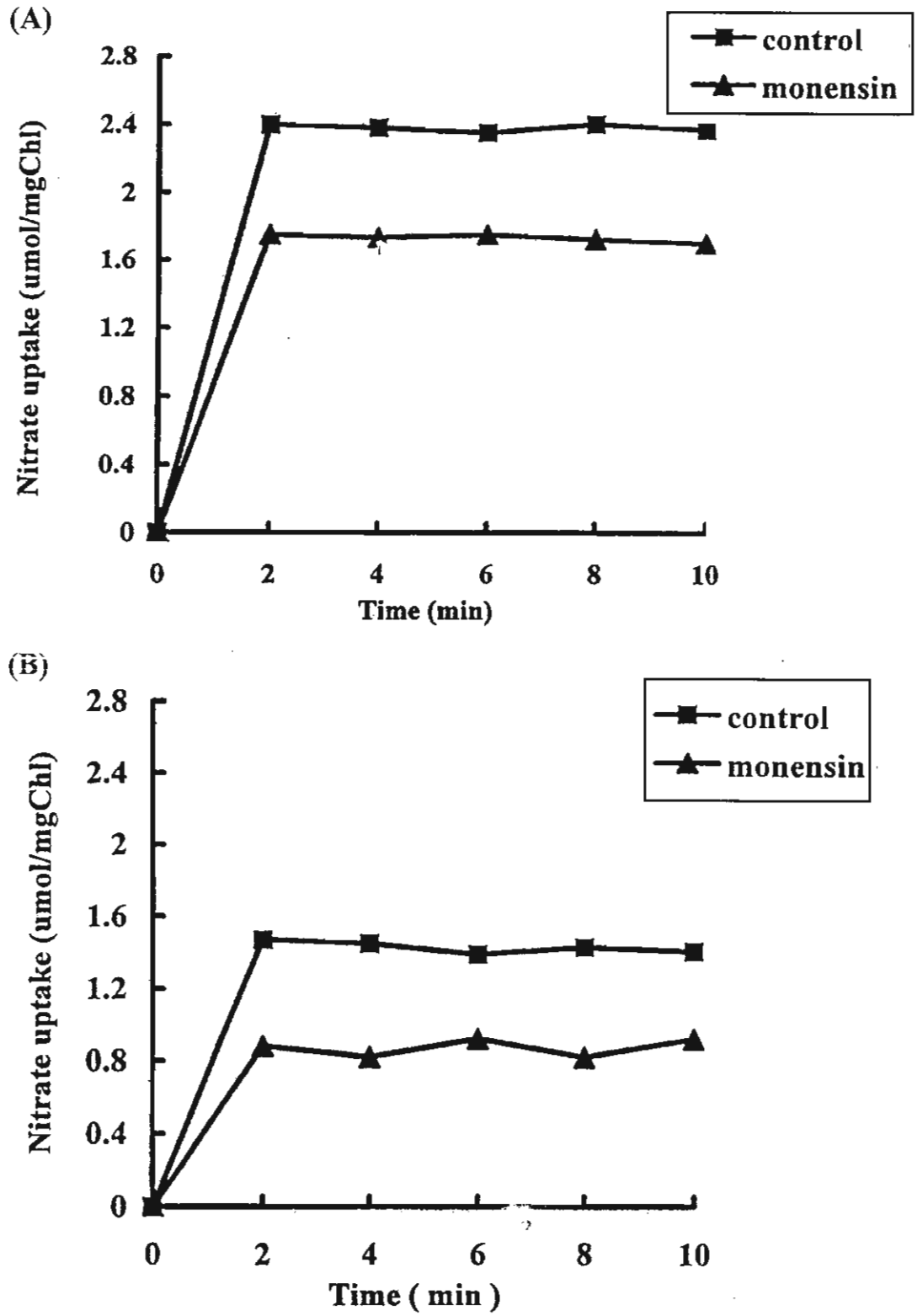

Figure 3.13 Effect of monensin on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

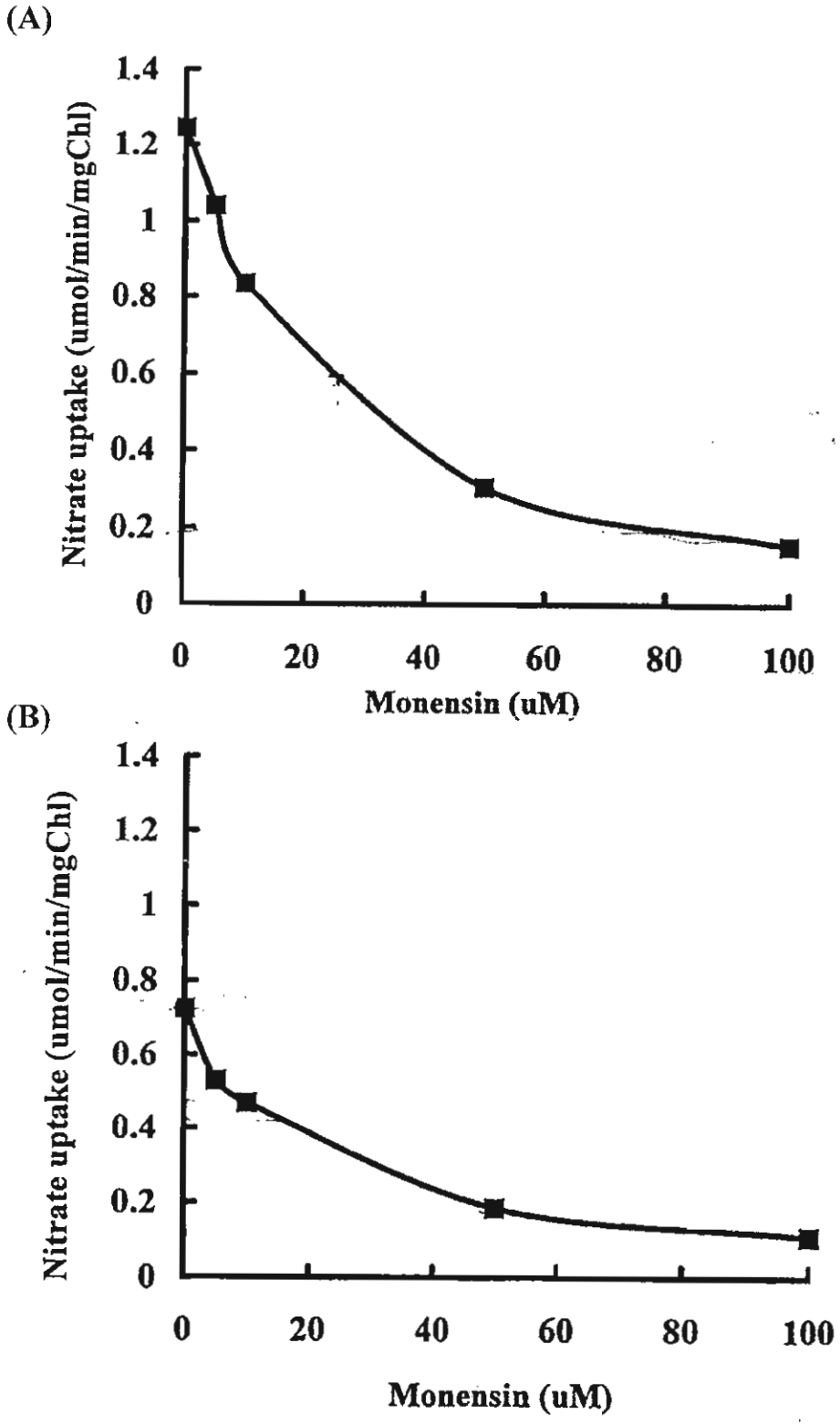

Figure 3.14 Effect of monensin concentration on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)

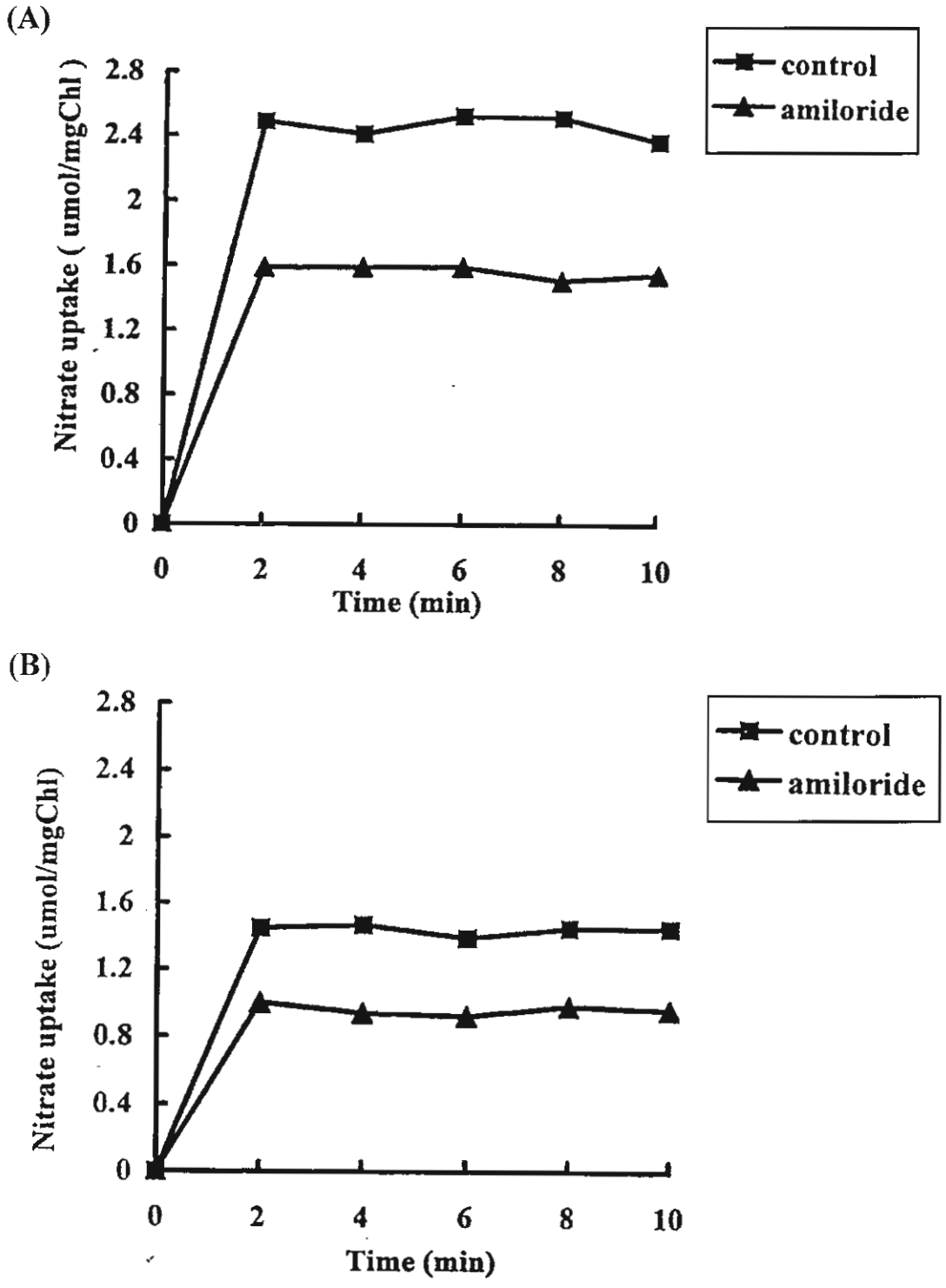

Figure 3.15 Effect of amiloride on nitrate uptake by cells grown under normal 0.5 M NaCl (A) and salt stress with 2 M NaCl (B)