FINAL REPORT

ON

OSMOREGULATION IN A HALOPHILIC CYANOBACTERIUM, *APHANOTHECE HALOPHYTICA* : BIOSYNTHESIS OF A COMPATIBLE SOLUTE, GLYCINEBETAINE

BY

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SUBMITTED TO

THE THAILAND RESEARCH FUND

ACKNOWLEDGEMENT

This 3-year project was financially supported by the Thailand Research Fund (RSA 3880022) and the Research Affairs Division of Chulalongkorn University from September 1995 to August 1998.

ABSTRACT

The present investigation was undertaken to unveil the mechanism of osmoregulation in a cyanobacterium. The halophilic cyanobacterium that we used was *Aphanothece halophytica* which possesses advantageous characteristics of being able to grow in a wide range of salinity ranging from 0.25 M to 3 M NaCl. One aspect of the osmoregulation process in a variety of living organisms involves the synthesis and accumulation of a certain class of compound commonly known as a compatible or an osmoprotectant solute.

In order to study the osmoregulation concerning the biosynthesis of a compatible solute, glycinebetaine in a systematic manner, we divided the project into 3 portions :

1. The conditions in which *A. halophytica* could accumulate glycinebetaine. The results indicated that the presence of high external salinity as well as an osmoticum namely sorbitol stimulated glycinebetaine accumulation. Chemical factor such as the presence of $NaNO_3$ in the growth medium or physical factor such as light could facilitate the increase of glycinebetaine only in salt-stressed cells.

2. The biosynthetic pathway of glycinebetaine. The results of radiotracer experiments showed the likely pathway to be choline → betaine aldehyde → glycinebetaine. The operation of this pathway was supported by the finding that the enzyme activities responsible for these 2 steps; choline and betaine aldehyde dehydrogenases were detected in membrane and cytoplasmic fractions respectively. Increased external salinity could enhance the activities of these 2 enzymes. As in the case of glycinebetaine accumulation, increased external salinity also increased the rate of glycinebetaine synthesis of the cells. The evidence was also provided showing that glycinebetaine could be synthesized from either ethanolamine or glycine. These 2 synthetic routes were also stimulated by high external salinity.

3. The purification and characterization of the enzyme responsible for the last step of glycinebetaine synthesis namely betaine aldehyde dehydrogenase. The results showed that the purified enzyme, after ammonium sulfate fractionation, DEAE-cellulose and hydroxyapatite chromatography was a tetramer of 30 kDa subunits. Both NAD^+ and $NADP^+$ could serve as coenzyme. Sulfhydryl reactive agents could modify enzyme activity. The presence of K^+ and Na^+ at or lower than 0.1 M enhanced enzyme activity. At higher than 0.1 M the

magnitude of activation was decreased for K^+ and inhibition was observed for Na^+. Elevation of external salinity caused an increase in enzyme activity.

บทคัดย่อ

งานวิจัยชิ้นนี้มีจุดประสงค์ที่จะเรียนรู้ถึงกลไกในการควบคุมแรงดันออสโมติกในไซยาโนแบคทีเรียมชนิดชอบความเค็ม *อะฟาโนทีซ ฮาโลฟิทิคา* ซึ่งมีคุณลักษณะที่เป็นประโยชน์ในการศึกษาเนื่องจากมันสามารถเจริญได้ในช่วงความเค็มตั้งแต่ 0.25 ถึง 3 โมลาร์ ของเกลือโซเดียมคลอไรด์ กลไกอันหนึ่งที่สิ่งมีชีวิตหลาย ๆ ประเภทใช้ในการปรับแรงดันออสโมติก ได้แก่ การสร้างและสะสมสารประกอบที่ไม่เป็นอันตรายต่อเซลล์ ซึ่งเราเรียกสารประกอบชนิดนี้เป็นคอมแพททิเบิล หรือออสโมโปรเทคแทนท์โซลูท

เพื่อที่จะทำงานวิจัยชิ้นนี้ให้เป็นระบบ จึงได้แบ่งรูปแบบของการศึกษาการสังเคราะห์คอมแพททิเบิลโซลูทที่มีชื่อว่า ไกลซีนบีเทน ออกเป็น 3 ส่วนดังนี้

1. สภาวะที่ทำให้เซลล์สะสมไกลซีนบีเทน ผลการทดลอง พบว่า ความเค็มสูง ๆ หรือใส่สารที่เพิ่มแรงดันภายนอกเซลล์ เช่น ซอร์บิทอล จะช่วยกระตุ้นให้เกิดการสะสมไกลซีนบีเทนขึ้นมากภายในเซลล์ นอกจากนี้การใส่โซเดียมไนเตรทลงในสารอาหาร หรือแม้กระทั่งการให้แสงสว่างขณะกำลังเลี้ยงเซลล์ก็จะทำให้เซลล์ที่เลี้ยงภายใต้สภาวะความเครียดของเกลือเท่านั้นที่จะมีการสะสมไกลซีนบีเทนเพิ่มขึ้น

2. วิถีการสังเคราะห์ไกลซีนบีเทน จากการทดลองโดยวิธี เรดิโอเทรเซอร์ พบว่าวิถีการสังเคราะห์น่าจะเป็นดังนี้; โคลีน → บีเทนอัลดีฮายด์ → ไกลซีนบีเทน ข้อมูลเพิ่มเติมที่สนับสนุนการทำงานของวิถีการสังเคราะห์อันนี้ได้จากผลการทดลองที่แสดงถึงการทำงานของเอนไซม์ที่เกี่ยวข้องในขั้นตอนที่ 1 และที่ 2 กล่าวคือ สามารถตรวจพบ แอคติวิตีของเอนไซม์โคลีนและบีเทนอัลดีฮายด์ ดีไฮโดรจีเนสได้ในส่วนของเม็มเบรนและส่วนของไซโตปลาสมตามลำดับ และยังพบอีกว่า ความเค็มภายนอกที่สูงขึ้นจะทำให้เอนไซม์ทั้ง 2 ตัว ดังกล่าวมีแอคติวิตีเพิ่มขึ้น ซึ่งสอดคล้องกับผลที่แสดงว่าการเพิ่มขึ้นของความเค็มภายนอกเซลล์ช่วยกระตุ้นให้เซลล์สังเคราะห์ไกลซีนบีเทนได้ดีขึ้น นอกจากนี้ข้อมูลเบื้องต้นบ่งชี้ว่าไม่เพียงแต่โคลีนเท่านั้นที่สามารถเปลี่ยนไปเป็นไกลซีนบีเทน เซลล์สามารถที่จะใช้เอธานอลามีนหรือไกลซีนในการสังเคราะห์ไกลซีนบีเทนได้ด้วย และเช่นเดียวกันความเค็มภายนอกที่เพิ่มขึ้นสามารถเพิ่มการสังเคราะห์ไกลซีนบีเทนโดยใช้สาร 2 ตัวนี้ได้

3. การทำให้เอนไซม์บีเทนอัลดีฮายด์ดีไฮโดรจีเนสบริสุทธิ์และการศึกษาคุณสมบัติของเอนไซม์ พบว่า สามารถทำเอนไซม์ให้บริสุทธิ์โดยการตกตะกอนด้วยแอมโมเนียมซัลเฟต ตามด้วยโครมาโทกราฟฟีโดย ดีอีเออีและไฮดรอกซีอะพาไทท์ ตามลำดับ เอนไซม์ที่ได้ประกอบด้วย 4 หน่วยย่อยที่มีขนาดหน่วยย่อยละ 30 กิโลดาลตัน เอนไซม์สามารถใช้ทั้ง เอนเอดี และเอนเอดีพี เป็นโคเอนไซม์ได้ สารประเภทที่ทำปฏิกิริยากับหมู่ซัลฟ์ไฮดริล สามารถ

เปลี่ยนแปลงแอคติวิตีของเอนไซม์ โปแทสเซียมและโซเดียมอิออนที่ความเข้มข้นตั้งแต่ 0.1 โมลาร์ลงมาช่วยกระตุ้นแอคติวิตี แต่ถ้าความเข้มข้นสูงกว่า 0.1 โมลาร์ พบว่าขนาดของการกระตุ้นโดย K^+ จะลดลง ในขณะที่จะเกิดการยับยั้งของแอคติวิตีโดย Na^+ การเลี้ยงเซลล์ภายใต้ความเค็มภายนอกสูง ทำให้เซลล์มีแอคติวิตีของเอนไซม์เพิ่มขึ้น

CONTENTS

CHAPTER 1

Introductory Chapter

The osmotic strength in the environment is usually fluctuating and many physical and chemical factors are involved in the changes of the osmotic strength. The survival of an organism therefore depends on its ability to adapt to fluctuations in the external osmolarity. The term 'osmoregulation' has been used to stand for the mechanism of cellular adaptation to osmotic stress. The studies on osmoregulation have been done by many investigators but only relatively recently that the mechanisms responsible for osmotic adaptation have been elucidated. There are remarkable similarities between bacteria and plants in their cellular responses to osmotic stress. For example, when plants or bacteria are subjected to water or salt stress they carry out osmotic adjustment by accumulating inorganic ions and organic solutes. Since many metabolic processes are inhibited by high ionic strength the inorganic ions are mostly sequestered into vacuoles whereas the organic solutes which are not inhibitory to metabolism are accumulated in the cytoplasm. In bacteria including cyanobacteria the exclusion of inorganic ions, especially Na^+, is accomplished by a H^+/Na^+ antiport system (1, 2). From now on for the sake of simplicity the term osmotic stress will be limited to the stress due to salt or salinity.

The process of adaptation to salt stress can be separated into two phases : short-term responses that occur from 1 ms to 1 h after transfer to high salinity and long-term acclimation that occurs over the course of several hours to several days. The uptake and exclusion of inorganic ions are believed to play important roles in short-term responses and the synthesis and accumulation of organic compatible solutes is the major response in long-term acclimation (3). So far various classes of compatible solutes have been found in plants and bacteria (including cyanobacteria); they are 1) amino acids such as glutamate, glutamine, proline, -aminobutyrate, alanine, 2) sugars such as sucrose, trehalose, glucosylglycerol, 3) quaternary ammonium compounds such as glycinebetaine, prolinebetaine. Among these compounds, glycinebetaine has received a lot of attention during the last decade.

Glycinebetaine is effective in the stimulation of growth of a number of bacteria species at high osmolarity (4, 5). In higher plants, a large number of families and genera have been reported to accumulate glycine betaine to levels that can be osmotically significant (6). In contrast to the many studies of glycinebetaine in higher plants and

bacteria, only few studies were reported in cyanobacteria. Reed *et al* (7) reported that glycinebetaine was detected in osmotically significant quantities in 4 strains of cyanobacteria grown in high external salinity. A study by Mackay *et al* (8) also revealed that halotolerant cyanobacteria accumulated glycinebetaine in response to osmotic stress whereas the fresh water cyanobacteria accumulated disaccharides and/or glucosylglycerol.

Despite the widespread occurrence of glycinebetaine, the osmotic regulation of the synthesis and/or accumulation is well understood only in several microbial systems and certain plants. The synthesis of glycinebetaine can be achieved by two systems; 1) by import from the environment such as in *E. coli*, 2) by *de novo* synthesis from choline such as in spinach.

In the attempt to study the osmoregulation in a cyanobacterium, we used *Aphanothece halophytica* as a model organism to investigate 1) the mechanism of the accumulation of glycinebetaine, 2) the pathway for the biosynthesis of glycinebetaine and 3) the purification of the enzyme involved in the biosynthesis of glycinebetaine. The following 3 chapters will be the results of such an investigation.

CHAPTER 2

Factors Affecting the Accumulation of Glycinebetaine in a Halophilic Cyanobacterium, *Aphanothece halophytica*

Summary

The effects of various factors on the accumulation of glycinebetaine have been investigated in a halophilic cyanobacterium *Aphanothece halophytica*. The increase of NaCl in the culture medium from 0.5 to 1.0 and 2.0 M caused an elevation of glycinebetaine content to about 2-and 8-fold respectively. Cell growth was retarded in hypersalinity condition. Determination of glycinebetaine by either ^{1}H-NMR or tri-iodide method gave closely agreeable results although the latter method required preliminary purification of the sample. The presence of 1 M sorbitol in the culture medium could also induce the increase of glycinebetaine by about 5-fold. Sodium nitrate at 20 mM caused about a 5-fold increase of glycinebetaine in salt-stressed cells but no change was observed in non-stressed cells. Similarly the increased glycinebetaine about 3 fold occurred only in salt-stressed cells under the influence of light.

Introduction

Organisms living in hypersaline environments have specific mechanisms that enable them to adjust their internal osmotic status. One such mechanism usually involves the accumulation of inorganic ions and some organic solutes. Since high intracellular concentrations of inorganic ions may be detrimental to cellular functions, many organisms appear to overcome this problem by accumulating one or more organic solutes of low molecular weight such as amino acids, polyols, and quaternary ammonium compounds, that can act as compatible solutes (9). Among the so-called compatible solutes, the quaternary ammonium compound glycinebetaine (N, N, N-trimethylglycine: betaine) has recently become the subject of intensive studies (6). The osmoprotective role of glycinebetaine is evident in a number of diverse microbial systems, including enteric bacteria (10), soil bacteria (11), halophilic bacteria, methanogenic archaebacteria (13) and cyanobacteria (8). In higher plants the accumulation of glycinebetaine has been demonstrated to be an adaptive response to hyperosmotic stress (14). The function of glycinebetaine as an osmolyte has also been reported in mammalian renal cells (15) as well as in invertebrate cardiac cells (16). Besides its physiological role as an osmoprotectant, glycinebetaine also functions as methyl group donor where this methyl group is incorporated into alkaloids (17), methionine (18) and cobalamin (19). Furthermore, glycinebetaine can be utilized as carbon and nitrogen sources by some microorganisms (20).

In cyanobacteria, the highly halotolerant strains accumulate glycinebetaine as a major osmolyte whereas the less tolerant strains accumulate either sucrose or glucosylglycerol (3, 7, 8). Glycinebetaine was shown to prevent the dissociation of cyanobacterial ribulose-1, 5-bisphosphate carboxylase and restored the activity inhibited by 0.25 M KCl (21) and to reverse the inhibition of glutamine synthase by up to 2 M NaCl in two cyanobacteria (22).

Despite the importance of glycinebetaine in many physiological functions, the mechanism governing its accumulation inside the cells has been well studied only in higher plants (23, 24). In the present study we looked at the factors that could affect the accumulation of glycinebetaine in a halophilic cyanobacterium, *Aphanothece halophytica*.

Materials and Methods

Culture conditions

A. halophytica was grown photoautotrophically in a BG 11 medium plus 18 mM $NaNO_3$ and Turks Island Salt Solution (see Appendix 1). Cells were grown in 250-ml flasks containing 100 ml of medium on a rotary shaker with an incident white light illumination of 60 μmol $m^{-2}s^{-1}$ at 30°C without CO_2 supplementation. The concentration of NaCl in the culture medium was adjusted by adding NaCl as required. Growth of the cells was followed by measuring the turbidity of the culture at 750 nm.

Determination of glycinebetaine in *A. halophytica*

Culture of *A. halophytica* was centrifuged at 8,000 g for 20 min to collect the cells. The cell pellet was extracted by incubation in boiling 80% (v/v) ethanol for 5 min as described by Reed (25). To ensure complete extraction the pellet was re-extracted with 80% (v/v) ethanol and incubated for 18 h at 25°C. The combined suspension after removal of cell pellet was pooled and evaporated to dryness before subjecting to analysis by ^{1}H-NMR and UV-spectrophotometry.

The dried residue was dissolved in 1.5 ml of D_2O and 0.6 ml of solution was transferred to a 5 mm NMR tube to which was added 5 μl of 1% sodium 2, 2-dimethyl-2-silapentate-5-sulfonic acid (DSS). The ^{1}H-NMR spectrum was run on a JEOL JMN-A 500 Fourier transform NMR spectrometer operating at a frequency of 500 MHz at a probe temperature of 29°C (26). The quantitation of glycinebetaine was obtained by comparing integrated peak intensity against a standard curve.

The analysis for glycinebetaine content by UV-spectrophotometry was done by dissolving the dried residue in 1.5 ml of distilled water and loading onto a Dowex-50W column (1 x 3 cm, H^+ form) to remove other quaternary ammonium compounds. The column was washed with 10 ml of distilled water followed by 20 ml of 2 M NH_3 to elute glycinebetaine. The eluate obtained was dried by lyophilization. The dried pellet was analyzed for glycinebetaine by tri-iodide assay according to Storey and Wyn Jones (27). The dried pellet dissolved in 0.25-0.6 ml of distilled water was added with 0.2 ml of acid potassium tri-iodide solution. The mixture was shaken for at least 90 min in an ice

bath, 2 ml of ice-cold water was then added rapidly to the mixture to reduce the absorbance of the blank. This was quickly followed by 5 ml of 1, 2-dichloroethane and the two layers were mixed by stirring. The absorbance of the lower organic layer was measured spectrophotometrically at 365 nm. The quantitation of glycinebetaine was obtained by comparing the absorbance against a standard curve. The values shown in the figures and tables represent the mean of 2 independent experiments.

Other methods

The cell growth was monitored by measuring the turbidity of the culture at 750 nm. Cell number was estimated by a haemocytometer.

Results and Discussion

Effect of salinity on growth of *A. halophytica*

The growth of *A. halophytica*, previously maintained in 0.5 M NaCl, in response to increased salinity is shown in Figure 1. Although *A. halophytica* requires NaCl for its normal growth , the increased concentration of NaCl to 1 M and 2 M retarded the growth rate during the first 6 days of cultivation. However, after 6 days the growth rate did not appear to be affected by NaCl. High salinity can cause the cessation of growth and eventually lead to cell death if the organism does not have the mechanism to withstand salinity stress. *A. halophytica* responded to high salinity in a manner that its growth was delayed during the initial period of salinity stress. The analogous phenomenon was also observed in a fresh water cyanobacterium *Synechococcus* 6311 (28). After transferring *Synechococcus* 6311 to salinity in the range 0.2 to 0.4 M NaCl, the photosynthetic activity was decreased initially. However, the cells later increased the photosynthetic activity by 2-fold. The capacity of *A. halophytica* to sustain growth at high salinity has been attributed to the increased photosynthetic activity and the increase in the ribulose-1, 5-bisphophate carboxylase activity and content of the cells (29). Since the growth rate of the salinity-stressed *A. halophytica* was not increased, it was likely that the products resulting from increased photosynthetic activity were diverted to the process of osmoregulation. To test this possibility we attempted to find out how the increased salinity could alter the content of glycinebetaine inside *A. halophytica*.

Effect of salinity on glycinebetaine content of *A. halophytica*

The quantitation of glycinebetaine was done by 2 methods, namely the ^{1}H-NMR spectrometry and the tri-iodide assay by UV-spectrophotometry. Figure 2 represents the ^{1}H-NMR spectrum of quaternary ammonium compounds extracted from *A. halophytica* grown in 0.5 M NaCl-containing medium. Glycinebetaine was clearly separated from choline with the peak at 3.25 ppm and 3.19 ppm respectively. The tri-iodide assay for glycinebetaine was based on the precipitation of quaternary ammonium compounds by iodine to form periodides (30). The periodides were subsequently extracted by 1, 2-dichloroethane and subjected to ultraviolet absorption at 365 nm. Figure 3 shows the linear

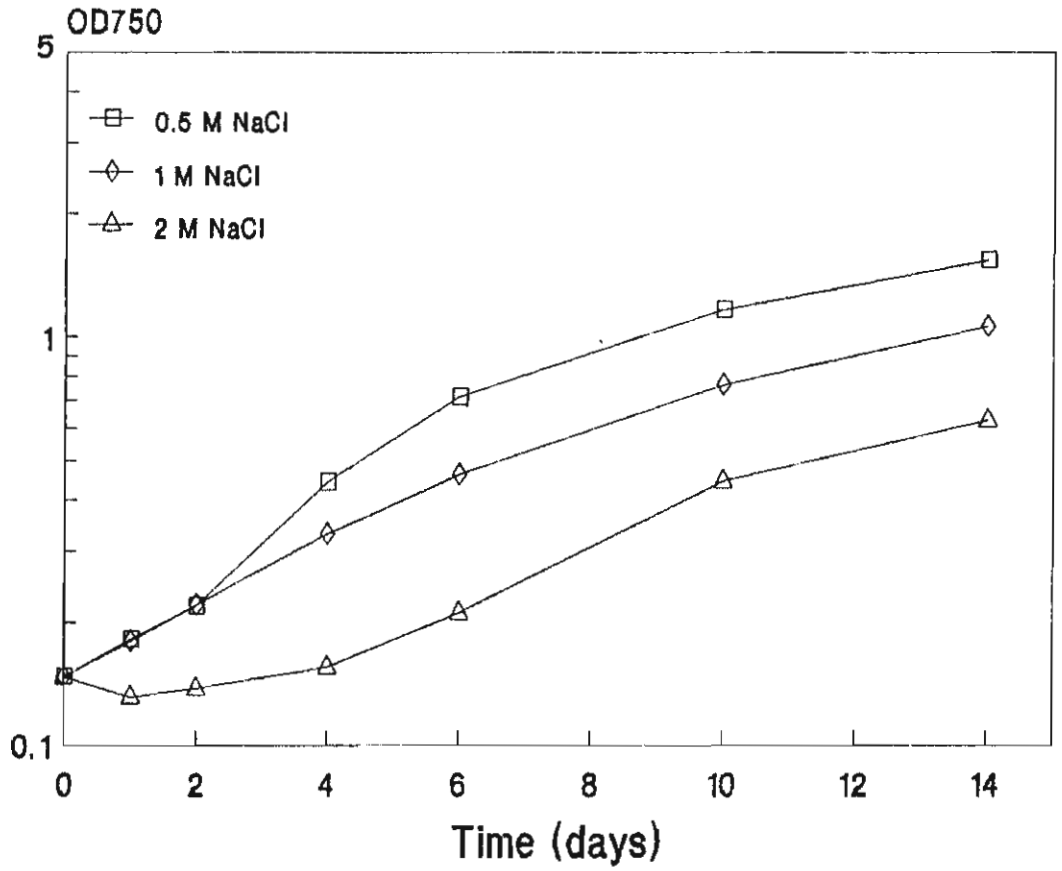

Fig. 1. Effect of external salinity on growth of *A. halophytica.*

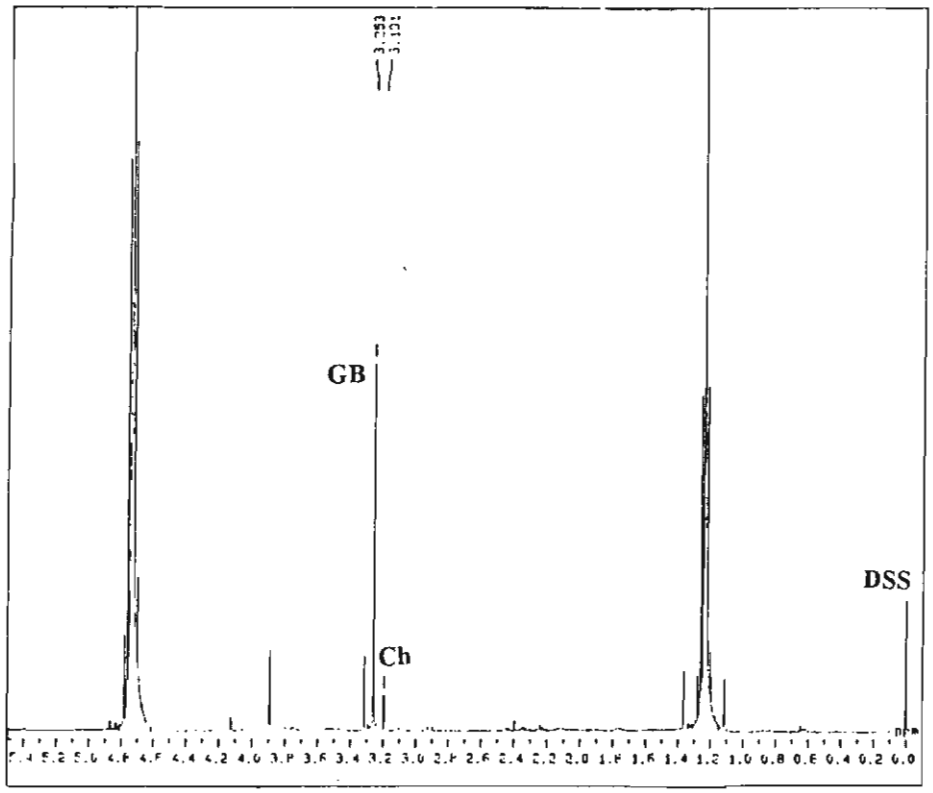

Fig. 2. ^{1}H-NMR spectrum of extract from *A. halophytica* grown in normal culture medium.

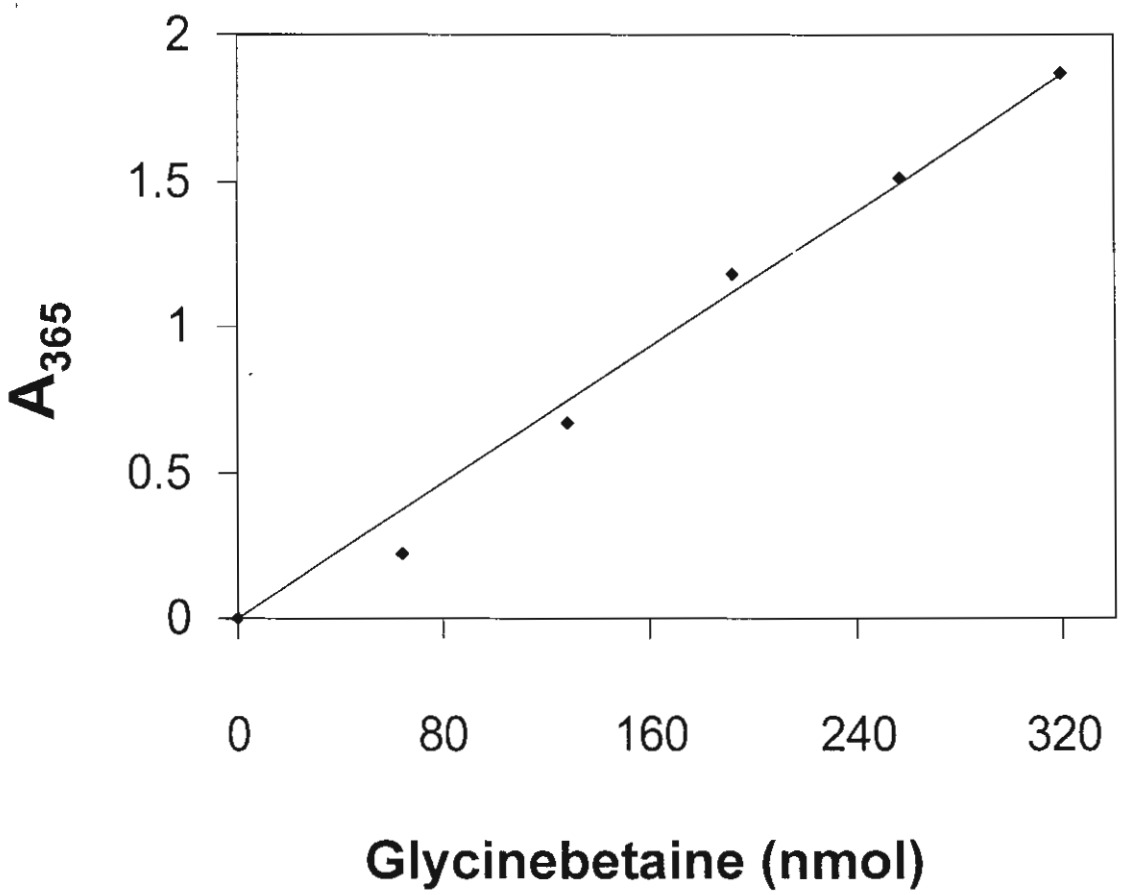

Fig. 3. Standard curve of glycinebetaine assayed by tri-iodide method.

standard curve of glycinebetaine. The linear regression analysis gave the equation y = 5.752 x. This method was able to detect nanomol level of glycinebetaine. Nevertheless, before using this method it is essential that other co-existing quaternary ammonium compounds must be removed by ion-exchange column. We used Dowex-50 W column to purify glycinebetaine and the purity (checked by thin layer chromatography) as well as the yield were very satisfactory, only one spot of glycinebetaine was detected and the column gave 100% recovery of glycinebetaine.

The accumulation of glycinebetaine induced by salinity in *A. halophytica* was determined by transferring the cells normally grown in 0.5 M NaCl-containing medium to the medium containing 1.0 and 2.0 M NaCl. Table 1 shows that higher salinity could induce the increased content of glycinebetaine inside the cells. The increase of glycinebetaine appeared to be proportional to the increase of NaCl. It is therefore likely that glycinebetaine serves as a major osmolyte in *A. halophytica*. The accumulation of glycinebetaine not only plays a role in osmoregulation but also contributes to the beneficial effect on the metabolic activities of the cells. In *Synechocystis* DUN 52 glycinebetaine exhibited protective effects on glutamine synthase activity (22). Also in *Spirulina subsalsa*, glycinebetaine was able to protect glucose-6-phosphate dehydrogenase against inhibition by NaCl (31). Moreover, previous studies in *A. halophytica* have shown that the inhibition of ribulose-1, 5-bisphosphate carboxylase by Cl^- can be relieved in the presence of glycinebetaine (21, 32). It is worth mentioning here that the analysis of glycinebetaine as determined by ^{1}H-NMR and tri-iodide methods gave comparable results (Table 1). In the later determination of glycinebetaine only the tri-iodide method was used.

Effect of sorbitol on glycinebetaine content of *A. halophytica*

The increase of salt in the culture medium can increase the level of glycinebetaine in *A. halophytica*. This increase can be attributed to either the direct influence of increased salinity or to the indirect influence of the decrease in the external water potential. To test this hypothesis, an organic solute namely sorbitol was used instead of NaCl as an external osmoticum. Glycinebetaine content in *A. halophytica*

Table 1. Effect of external salinity on intracellular glycinebetaine and growth of *A. halophytica*

NaCl (M)	Cell density ($x10^6$ /ml)	Glycinebetaine ($nmol/10^6$)	
		1H-NMR	Tri-iodide
0.5	23.4	9.7	9.1
1.0	16.3	26.9	26.4
2.0	4.3	76.0	78.7

grown in the presence of 1 M sorbitol was about 5-fold higher than that in the absence of sorbitol (Table 2). The result suggested that the increased content of glycinebetaine was induced by an osmotic effect rather than by a salinity-specific effect. The osmotic effect due to sorbitol also resulted in a retardation of cell growth. Cell density was increased by about 1.5-fold in sorbitol-grown cells as compared to about 10-fold in control cells. Although *A. halophytica* was able to grow in the presence of 1 M sorbitol its growth rate was much lower than that in NaCl at the same osmolarity (data not shown). Sorbitol is a non-ionizable carbohydrate compound and cannot penetrate into the cells whereas Na^+ and Cl^- can be taken up by the cells. This suggests that the uptake of ions by the cell is an important first step to trigger the cell mechanism for readjustment of the cell volume at the initial stage of osmotic stress. The osmotic stress by sorbitol could likely be sensed by the cells later rather than sooner as compared to that by NaCl.

Effect of nitrate on glycinebetaine content of *A. halophytica*

As glycinebetaine is a nitrogen-containing compound, it is therefore relevant to study whether $NaNO_3$ which is one major component in the culture medium of *A. halophytica* can affect the content of glycinebetaine. The level of glycinebetaine in cells grown in a non-stressed condition was hardly affected by 20 mM Na NO_3 whereas in salt-stressed cells the presence of $NaNO_3$ induced the increase of glycinebetaine by about 6-fold affer 7 days growth (Table 3). It should be noted here that the condition of salt stress by itself without $NaNO_3$ already contributed to about 2-fold increase of glycinebetaine. Since nitrogen for this increased glycinebetaine was not provided in the culture medium the sources of available nitrogen was likely to derive from the metabolic degradation of some nitrogen containing biomolecules like proteins or nucleic acids. The reduced level of many proteins has been reported in tobacco cells adapted to salt or water stress (33). This could be a result of either reduced gene expression of the affected proteins or the increased degradation of the proteins. It remains to be clarified whether salt stress in *A. halophytica* can cause an alteration in gene expression of some proteins especially those responsible for the synthesis of glycinebetaine. However, overall results in Table 3 suggested that the supply of $NaNO_3$ could alleviate the salt stress in *A.*

Table 2. Effect of sorbitol on intracellular glycinebetaine and growth of *A. halophytica*

Days	Without Sorbitol		With 1 M Sorbitol	
	Glycinebetaine (nmol/10^6)	Cell density (10^6/ml)	Glycinebetaine (nmol/10^6)	Cell density (10^6/ml)
0	2.9	1.4	2.9	1.4
7	3.1	14.2	15.1	2.2

Table 3. Effect of nitrate on intracellular glycinebetaine of *A. halophytica.*

Days	Glycinebetaine (nmol/10^6)	
	0.5 M NaCl	1.5 M NaCl
0	2.5	2.4
7	2.8	15.3

halophytica through the availability of nitrogen for the synthesis of glycinebetaine.

Effect of light on glycinebetaine content of *A. halophytica*

Photosynthetic organisms require light for their initial energy transduction process. *A. halophytica* is a unicellular cyanobacterium capable of O_2-evolving photosynthesis characteristic of plant cells. The relation of light to salt stress can be investigated with respect to the accumulation of glycinebetaine. As shown in Table 4 light did not change the level of glycinebetaine in non-stressed cells. When stressed with 1.5 M NaCl, a 3-fold increase in glycinebetaine was observed. In the dark the cells did not change their glycinebetaine regardless of the salt stress condition. The natural occurrence of *A. halophytica* is in the hypersaline lake with abundant sunshine. The organism is able to use the light to create the proton gradient which finally results in the generation of ATP. This ATP would spare the use of organic nutrients for energy generation, and there by release a higher proportion of those nutrients for biosynthesis of required compounds of which glycinebetaine would be among them. In this respect it would be worth investigating how the inhibitor of ATP synthesis could affect the process of glycinebetaine accumulation and synthesis.

Table 4. Effect of light on intracellular glycinebetaine of *A. halophytica.*

Days	Glycinebetaine (nmol/10^6)			
	0.5 M NaCl		1.5 M NaCl	
	Dark	Light	Dark	Light
0	2.2	2.2	2.4	2.4
7	2.0	2.4	2.2	7.5

CHAPTER 3

Accumulation of Glycinebetaine and Its Synthesis from Radioactive Precursors in Salt-Stressed Cyanobacterium, *Aphanathece halophytica*

Summary

Salt-stressed (2.0 M NaCl) *A. halophytica* exhibited initially delayed growth during the first two days and eventually attained the same growth rate as the control (0.5 M NaCl) cells. Glycinebetaine accumulation was increased slightly in control cells but drastic increase of glycinebetaine occurred in salt-stressed cells during 6 days growth. There was no apparent increase in the synthesis of [^{14}C] glycinebetaine in the control cells as contrast to the marked increase in its synthesis in salt-stressed cells. Salt stress induced both the accumulation and the synthesis of glycinebetaine. Time course experiments provided evidence that [^{14}C] choline was oxidized first to [^{14}C] betaine aldehyde and finally to [^{14}C] glycinebetaine in *A. halophytica.* The supporting data for such pathway was obtained from the results showing the localization of choline and betaine aldehyde dehydrogenase activities in the membrane and cytoplasmic fractions respectively. The activities of these 2 enzymes were also enhanced by salt stress. Betaine aldehyde dehydrogenase seemed to be more responsive towards salt stress than choline dehydrogenase. *A. halophytica* was able to utilize [^{14}C] ethanolamine and [^{14}C] glycine to synthesize [^{14}C] glycinebetaine. In both cases higher rate of glycinebetaine synthesis was found for salt-stressed than control cells.

Introduction

Glycinebetaine (N, N, N-trimethylglycine) is a compatible solute which accumulates to a level considered to be osmotically significant in a large number of plant and bacteria species (6, 34). The osmoprotective effects of glycinebetaine have been well described for bacteria (34, 35) and plants (36, 37). Glycinebetaine is less inhibitory to enzyme activity than equivalent concentration of inorganic ions (38). Partial protection against NaCl or KCl inhibition by glycinebetaine has been reported for several higher plants (39-41) and cyanobacteria enzymes (21, 31, 32).

The synthesis of glycinebetaine in several plant families is by a two-step oxidation of choline, via the intermediate betaine aldehyde (42, 43); choline → betaine aldehyde → glycinebetaine. The first step is catalyzed by a ferredoxin-dependent choline monooxygenase which is a soluble enzyme located in the chloroplast stroma (44). The second step is catalyzed by a pyridine nucleotide-dependent betaine aldehyde dehydrogenase. The choline-glycinebetaine pathway in animals and microorganisms is different from plants in the first step. Choline dehydrogenase which is a membrane-bound enzyme has been found to catalyze choline to betaine aldehyde in mammals (45, 46) and in bacteria (47-49).

The biosynthetic route of glycinebetaine beyond choline proposed to occur in higher plants (50, 51) is as follows : serine → ethanolamine → methylethanolamine → dimethylethanolamine → choline. The direct methylation of glycine to form choline is likely to occur in animals (52) and probably in bacteria (53) but so far not in plant tissues.

The accumulation of glycinebetaine in a variety of organisms including cyanobacteria has been well documented (6). Nevertheless the synthesis of glycinebetaine has never been reported for cyanobacteria. The work presented here showed that the halophilic cyanobacterium *A. halophytica* could synthesize glycinebetaine from radioactive precursors such as glycine, ethanolamine and choline and that the salt stress condition could enhance the level of glycinebetaine synthesis.

Materials and Methods

Organism and growth condition

A. halophytica was grown photoautotrophically as described in chapter 2.

Radiotracer experiments

The culture of exponentially grown *A. halophytica* was centrifuged at 2,000 g for 10 min. The cell pellet was suspended in 0.5 ml of 50 mM HEPES-NaOH buffer pH 7.5 containing either 0.5 M or 2.0 M NaCl. The suspension was incubated with 40 μM [methyl-^{14}C] choline (55 mC_i/mmol) [or [2-^{14}C] ethanolamine (57 mC_i/mmol) or [1-^{14}C] glycine (55 mC_i/mmol)] at 30°C. At indicated time intervals the reaction was stopped by 1 ml of methanol followed by centrifugation at 2,000 g for 5 min. The pellet was washed twice with either 0.5 M or 2.0 M NaCl before subjecting to extraction.

Extraction and separation of metabolites

The pellet was extracted with boiling 80% (v/v) ethanol for 5 min as described by Reed (25). The re-extraction with 80% ethanol was carried out at room temperature for 18 h. The combined extract was centrifuged at 2,000 g for 5 min and the supernatant was dried at 65°C. The dried residue was suspended in 0.5 ml distilled water containing 0.3 M sodium bisulfite and 1 mM each of betaine aldehyde and glycinebetaine. This suspension was loaded onto a Dowex-50 x 4-200, H^+ form (Sigma), column (0.9 x 1.3 cm) and the elution of [^{14}C] betaine aldehyde-bisulfite addition product by 15 ml of 0.2 M sodium bisulfite, of [^{14}C] glycinebetaine by 15 ml of 2 M NH_3 and of [^{14}C] choline by 15 ml of 2 M HCl was done as described by Landfald and Strom (47). The radioactivity in the eluates was determined by liquid scintillation counting. To check for the possibility of the chemical degradation of radioactive precursors or products during extraction, the cell pellet without prior radioactive labeling was added with the same quantity of radioactive precursor and subjected to extraction as described

above. The results indicated very little degradation of radioactive precursors.

Determination of glycinebetaine

Glycinebetaine was determined by tri-iodide method as described in chapter 2.

Disruption and centrifugal fractionation of cells

Approximately 4 g (wet wt) of *A. halophytica* grown in the medium containing either 0.5 M or 2.0 M NaCl for 10 days was suspended into 10 ml of grinding buffer (50 mM HEPES-NaOH pH 7.5 containing 10% glycerol (v/v), 10 mM EDTA, 5 mM DTT and 2 mg/ml lysozyme) and stirred at 4°C for 1.5 h. After lysozyme treatment, the mixture was centrifuged at 33,000 g for 20 min to remove whole cells and large fragments. The supernatant was then centrifuged at 180,000 g for 1 h. The high-speed supernatant was collected and the pellet (membrane fraction) was washed once by recentrifugation. The final pellet was resuspended in a small volume of the grinding buffer devoid of lysozyme. The membrane was solubilized by the addition of Triton X-100 to a final concentration of 0.3% (v/v) and incubated with stirring at 4°C for 1 h. The undissolved material was removed by centrifugation at 33,000 g for 20 min.

Choline dehydrogenase and betaine aldehyde dehydrogenase assays

Choline dehydrogenase was assayed spectrophotometrically by a phenazine-linked reduction of cytochrome c according to Russell and Scopes (48). The reaction mixture (1 ml) contained 50 mM HEPES-NaOH pH 7.4, 20 mM choline chloride, 0.33 mM phenazine methosulfate and 50 μM cytochrome c. The reaction was started by the addition of enzyme sample and the increase in absorbance at 550 nm was recorded against time. The slope of the increased A550 at 25°C was taken as initial velocity of the enzyme and the activity was expressed in μmol choline oxidized min^{-1} mg^{-1} protein using an extinction coefficient of 20.5 cm^{-1} mM^{-1} (54) for the difference between reduced and oxidized cytochrome c, and dividing by 2 to allow for the single electron reduction of the cytochrome.

For betaine aldehyde dehydrogenase assay, the spectrophotometric method for the determination of NADH at 340 nm with extinction coefficient of 6.22 cm^{-1} mM^{-1} was used (55, 56). The reaction mixture (1 ml) contained 50 mM HEPES-KOH pH 7.5, 10 mM DTT, 1 mM EDTA, 0.5 mM NAD^+, 0.5 mM betaine aldehyde and appropriate amount of enzyme sample. The reaction was started by the addition of betaine aldehyde and allowed to incubate at 25°C for 3 min before stopping by the addition of 50 µl of 1 M HCl.

Other methods

Protein was determined by a sensitive dye-binding method of Bradford (57) using bovine serum albumin as a standard. Cell number was estimated by a haemocytometer. All the values shown in the figures represent the mean of 2 independent experiments.

Results and Discussion

Effect of salt stress on growth of A. halophytica

A. halophytica culture routinely maintained at 0.5 M NaCl-containing medium was transferred to the medium that contained 2.0 M NaCl and the cell number was monitored over a period of 6 days. The cell density was apparently lower in salt-stressed condition over the tested period (Fig. 1). It is noteworthy that the rate of increase of cell density starting from day 2 was not changed between the control and salt-stressed cells. The possibility existed that during the first 2 days of salt stress no cell growth occurred as a result of the metabolic activities and the required energy being diverted to the initial adjustment of cellular volume by regulating the influx and efflux of certain ions and compounds. The uptake and extrusion of Na^+ in *Synechocystis* PCC 6714 (58) and of choline and glycinebetaine in *E. coli* (59) have been shown to occur during the initial period of hypersaline treatment. It remains to be clarified how the movement of various solutes in and out of *A. halophytica* affect the osmoregulation of the cells. Previously *A. halophytica* was shown to have increased photosynthetic CO_2 fixation upon salt stress (29). Taken together with the results of Figure 1 in the present study showing no difference in the growth rate at a later stage of salt stress it can be envisaged that salt stress induces the increase in photosynthetic CO_2 fixation which in turn plays an important role in the later stage of osmoregulation involving the synthesis and accumulation of a specific compatible solute.

Accumulation and synthesis of glycinebetaine under salt stress

To investigate the effect of salt stress on the accumulation and the synthesis of glycinebetaine, *A. halophytica* culture was grown in 0.5 M (control) and 2.0 M (salt stress) NaCl for various times. The samples were withdrawn and determined for glycinebetaine content and [^{14}C] glycinebetaine synthesis using [^{14}C] choline as a precursor. Accumulation of glycinebetaine increased nearly 20-fold during the 6 days growth of salt-stressed cells whereas only about 5-fold increase was observed in control cells during the same period of growth (Fig. 2A). A slight drop in the content of glycinebetaine at day 6 was probably due to its being metabolized to other products. Although glycinebetaine was

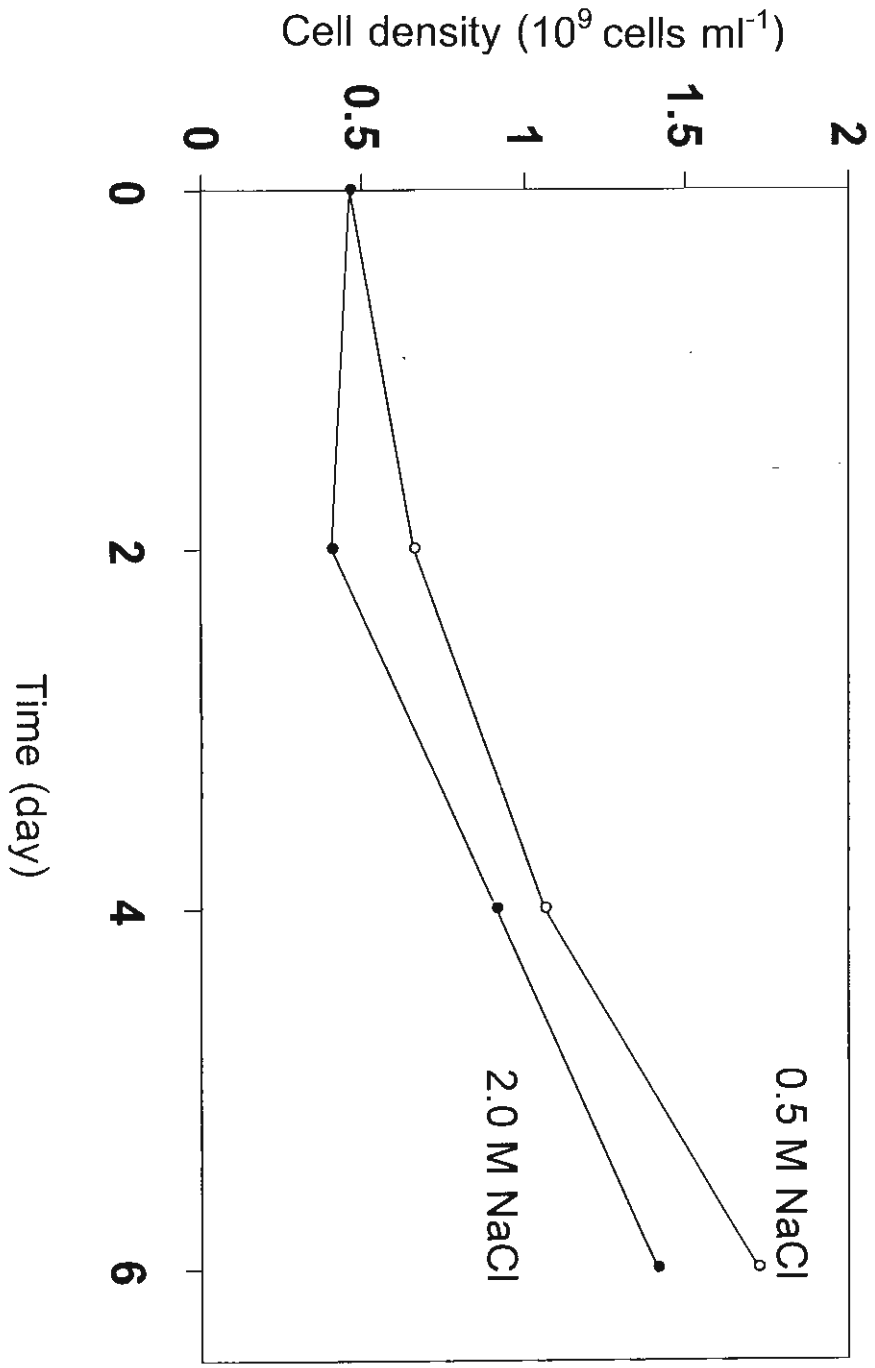

Fig. 1. Growth of *A. halophytica* under salt stress.

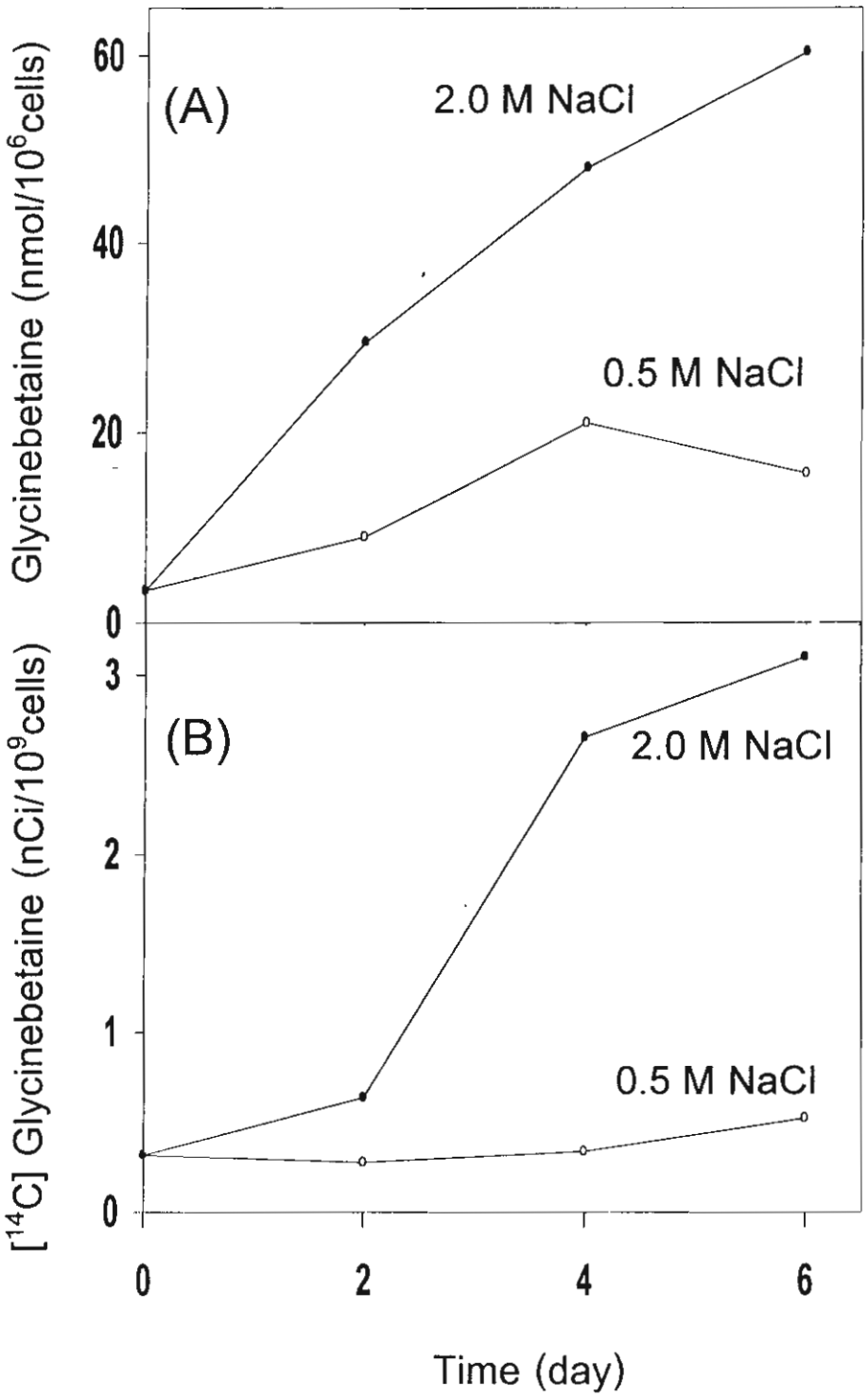

Fig. 2. Effect of various periods of salt stress on glycinebetaine accumulation (A) and [^{14}C] glycinebetaine synthesis (B). The synthesis was determined by labeling cells with [^{14}C] choline for 3 h

considered an end product in many organisms including higher plants (60), the degradation of glycinebetaine through demethylation has been reported in *Rhizobium meliloti* (11).

The synthesis of glycinebetaine in the form of [^{14}C] glycinebetaine also increased in the salt-stressed cells with approximately 10-fold increase when stressed for 6 days (Fig. 2B). The synthesis of glycinebetaine in the control cells was relatively unchanged during 6 days growth. The overall results indicated that salt stress induced the accumulation of glycinebetaine in *A. halophytica*. The increased accumulation of glycinebetaine was accompanied by its increased synthesis. During the first 2 days of salt stress the synthesis of glycinebetaine from choline increased at a slow rate, approximately 2-fold increase. Nevertheless, the accumulation of glycinebetaine during the 2 days stress had become already high. Also in the control cells the accumulation of glycinebetaine increased during 6 days growth but this increased glycinebetaine accumulation was not accompanied by an increase in its synthesis during 6 days growth. It was likely that the conversion of choline to glycinebetaine would not solely be responsible for the synthesis of glycinebetaine in *A. halophytica*. If this were the case it would be of great significance towards the understanding of metabolism of cyanobacteria provided that the other route (s) could be identified. In this connection it should be pointed out that cyanobacteria do have genetic, metabolic and morphological diversities that rival those seen among the totality of other eubacteria.

To investigate how salinity affected the accumulation and synthesis of glycinebetaine we grew *A. halophytica* under various concentrations of NaCl for 6 days and analyzed the content and the synthesis of glycinebetaine. The increase of NaCl caused the increase in both the content and the synthesis of glycinebetaine (Fig. 3). The increase of both the content and the synthesis appeared to be proportional to the increase in external salinity.

Synthesis of [^{14}C] glycinebetaine from [^{14}C] choline via [^{14}C] betaine aldehyde

When *A. halophytica* was incubated with 40 μM of [^{14}C] choline the 2 products formed were betaine aldehyde (Fig. 4 A) and glycinebetaine (Fig. 4 B). Betaine aldehyde rose markedly within the

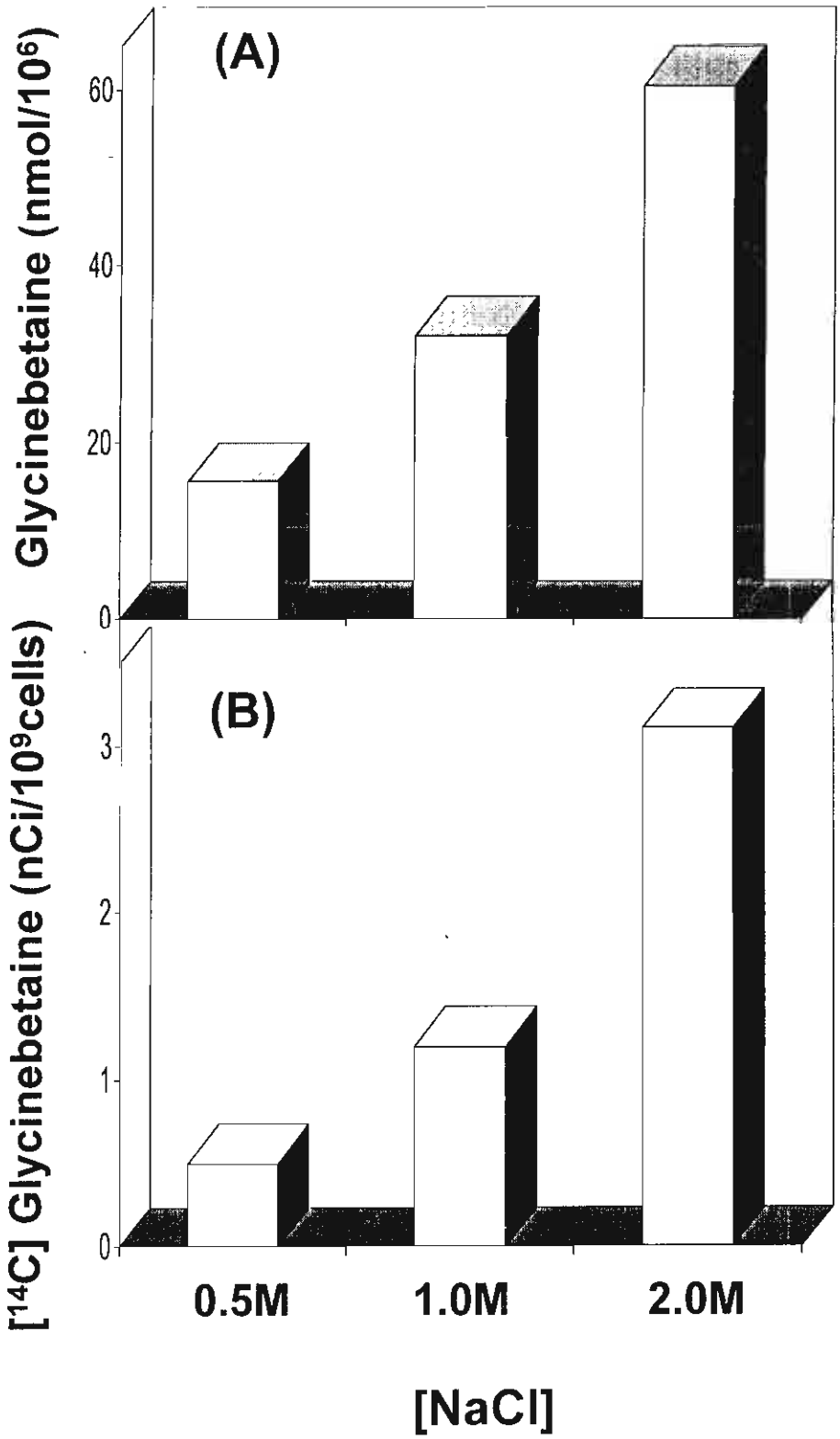

Fig. 3. Effect of external salinity on glycinebetaine accumulation (A) and [^{14}C] glycinebetaine synthesis (B). Cells were grown under various external salinity for 6 d. [^{14}C] glycinebetaine synthesis was determined by labeling cells with [^{14}C] choline for 3 h.

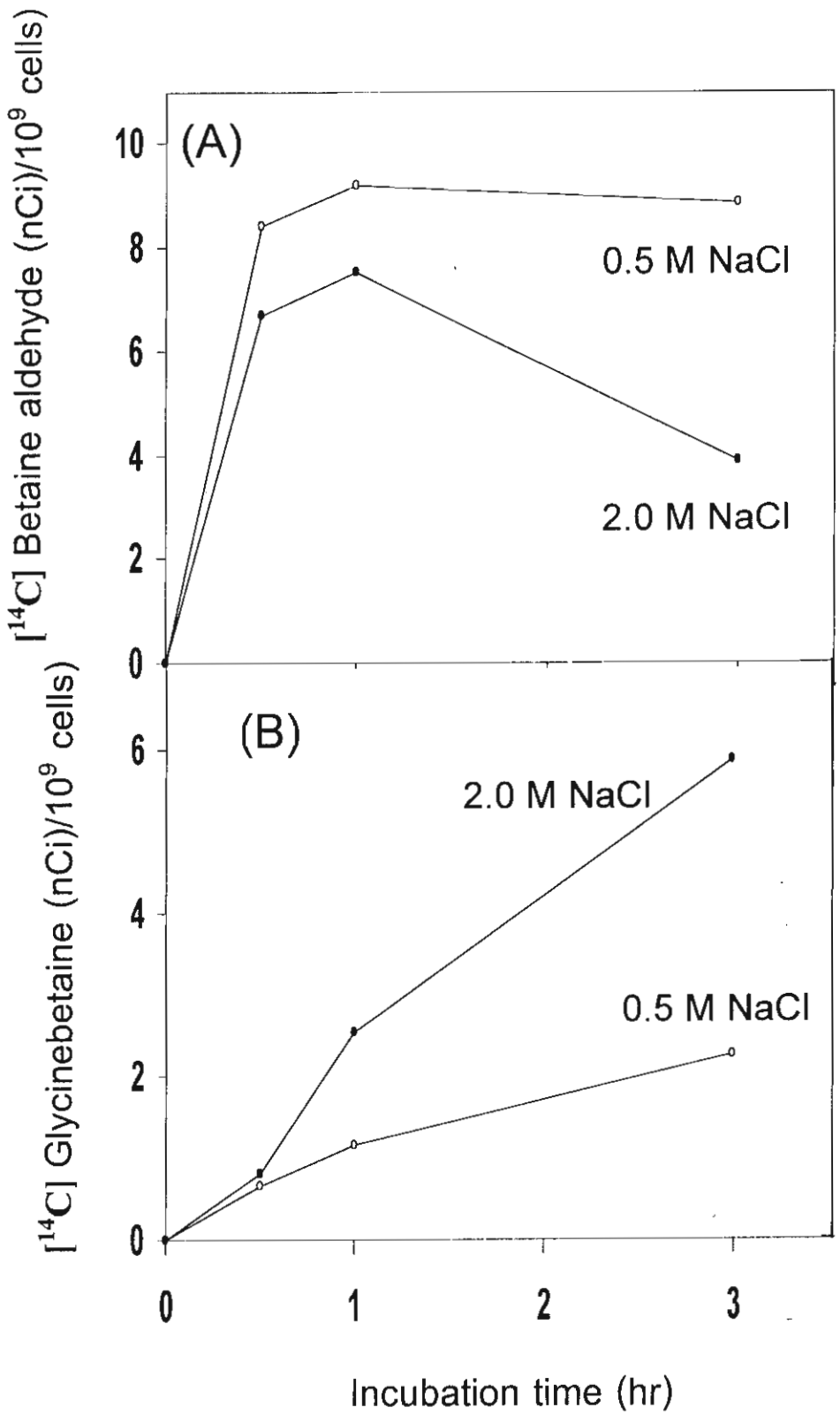

Fig. 4. Time course of the synthesis of [^{14}C] betaine aldehyde (A) and [^{14}C] glycinebetaine from [^{14}C] choline.