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

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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

งานวิจัยชิ้นนี้มีจุดประสงค์ที่จะเรียนรู้ถึงกลไกในการควบคุมแรงดันออสโมติกในไซยาโนแบคทีเรียชนิดชอบความเค็ม *อะฟาโนทีซ สาไลฟิทิกา* ซึ่งมีคุณลักษณะที่เป็นประโยชน์ในการศึกษาเนื่องจากมันสามารถเจริญได้ในช่วงความเค็มตั้งแต่ 0.25 ถึง 3 โมลาร์ ของเกลือโซเดียมคลอไรด์ กลไกอันหนึ่งที่สิ่งมีชีวิตหลาย ๆ ประเภทใช้ในการปรับแรงดันออสโมติก ได้แก่ การสร้างและสะสมสารประกอบที่ไม่เป็นอันตรายต่อเซลล์ ซึ่งเราเรียกสารประกอบชนิดนี้เป็นคอมแพททินิบิล หรือออสโมไปรเทคแทนท์โซลูท

เพื่อที่จะทำงานวิจัยชิ้นนี้ให้เป็นระบบ จึงได้แบ่งรูปแบบของการศึกษาการสังเคราะห์คอมแพททิเบิลโซลูทที่มีชื่อว่า ไกลซินบีเทน ออกเป็น 3 ส่วนดังนี้

1. สภาวะที่ทำให้เซลล์สะสมไกลซินบีเทน ผลการทดลอง พบว่า ความเค็มสูง ๆ หรือใส่สารที่เพิ่มแรงดันภายนอกเซลล์ เช่น ซอร์บิทอล จะช่วยกระตุ้นให้เกิดการสะสมไกลซินบีเทนขึ้นมากภายในเซลล์ นอกจากนี้การใส่โซเดียมในเตรทลงในสารอาหาร หรือแม้กระทั่งการให้แสงสว่างขณะกำลังเลี้ยงเซลล์ก็จะทำให้เซลล์ที่เลี้ยงภายใต้สภาวะความเครียดของเกลือเท่านั้นที่จะมีการสะสมไกลซินบีเทนเพิ่มขึ้น

2. วิถีการสังเคราะห์ไกลซินบีเทน จากการทดลองโดยวิธี เรดิโอเทรเซอร์ พบว่าวิถีการสังเคราะห์น่าจะเป็นดังนี้: โคลีน → บีเทนอัลดีฮายด์ → ไกลซินบีเทน ข้อมูลเพิ่มเติมที่สนับสนุนการทำงานของวิถีการสังเคราะห์อันนี้ได้จากผลการทดลองที่แสดงถึงการทำงานของเอนไซม์ที่เกี่ยวข้องในขั้นตอนที่ 1 และที่ 2 กล่าวคือ สามารถตรวจพบ แอคติวิตีของเอนไซม์โคลีนและบีเทนอัลดีฮายด์ ดีไฮโดรจีเนสได้ในส่วนของเมมเบรนและส่วนของไซโตปลาสมตามลำดับ และยังพบอีกว่า ความเค็มภายนอกที่สูงขึ้นจะทำให้เอนไซม์ทั้ง 2 ตัว ดังกล่าวมีแอคติวิตีเพิ่มขึ้น ซึ่งสอดคล้องกับผลที่แสดงว่าการเพิ่มขึ้นของความเค็มภายนอกเซลล์ช่วยกระตุ้นให้เซลล์สังเคราะห์ไกลซินบีเทนได้ดีขึ้น นอกจากนี้ข้อมูลเบื้องต้นบ่งชี้ว่าไม่เพียงแต่โคลีนเท่านั้นที่สามารถเปลี่ยนไปเป็นไกลซินบีเทน เซลล์สามารถที่จะใช้เอธานอลามีนหรือไกลซีนในการสังเคราะห์ไกลซินบีเทนได้ด้วย และเช่นเดียวกันความเค็มภายนอกที่เพิ่มขึ้นสามารถเพิ่มการสังเคราะห์ไกลซินบีเทนโดยใช้สาร 2 ตัวนี้ได้

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

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

CHAPTER I

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

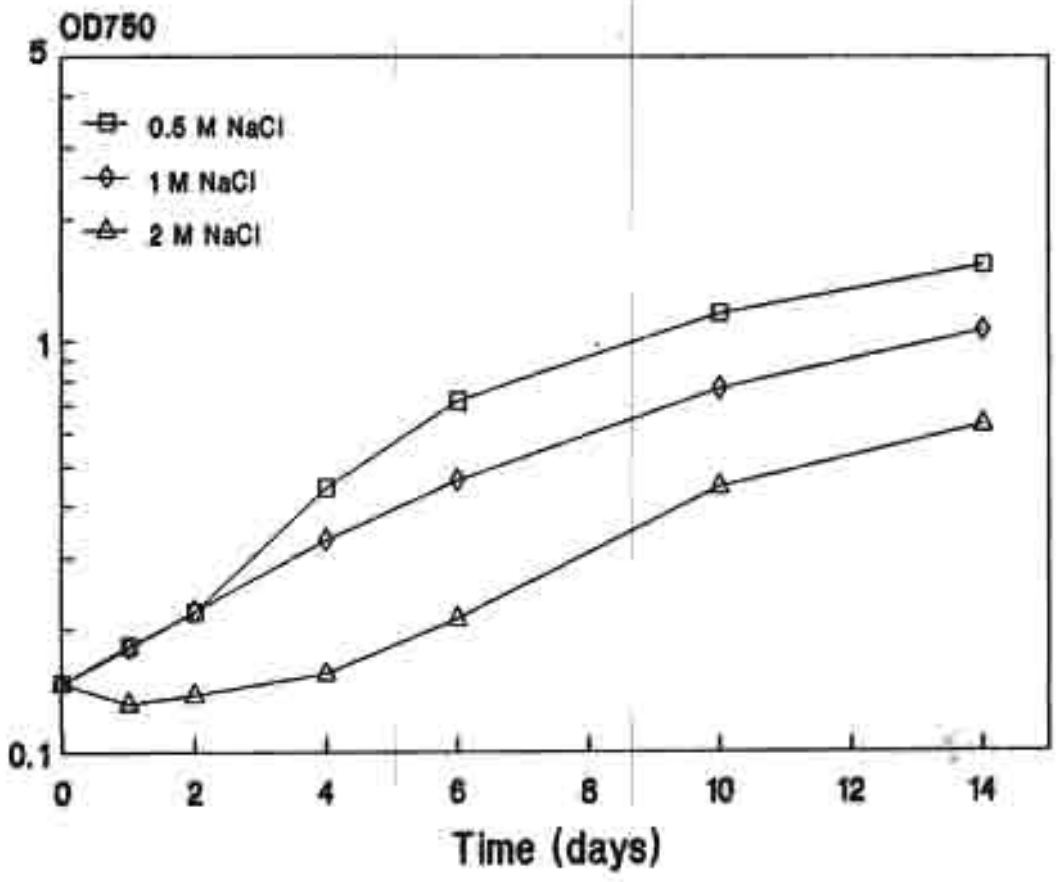

Fig. 1. Effect of external salinity on growth of *A. halophytica*.

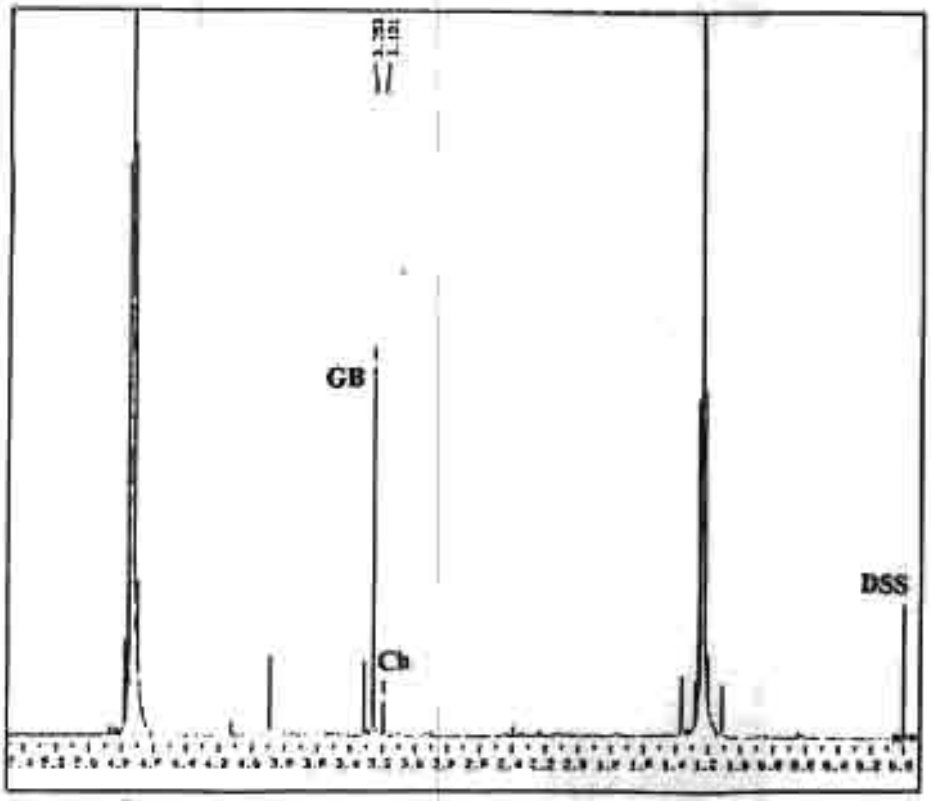

Fig. 2. ^{1}H-NMR spectrum of extract from *A. halophytica* grown in normal culture medium.

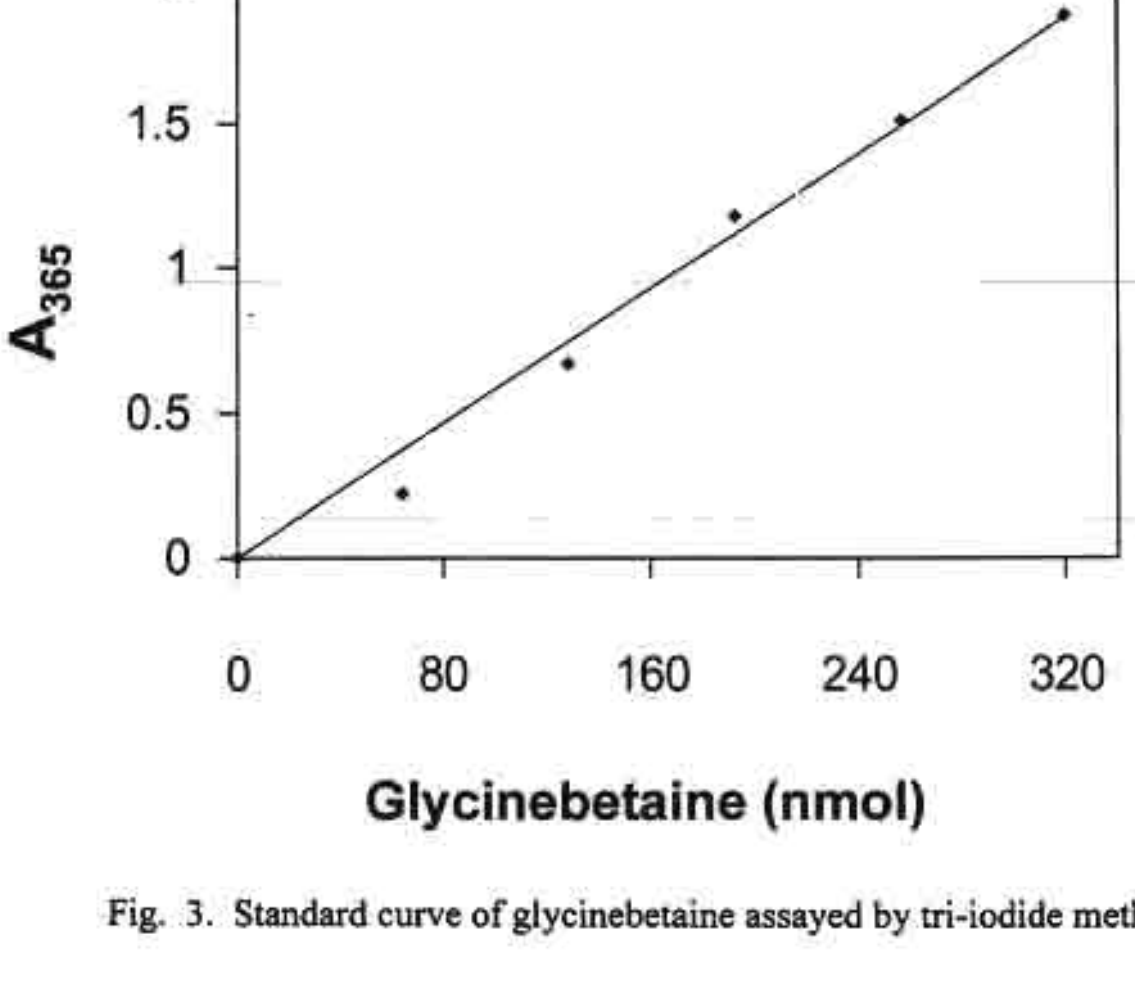

Fig. 3. Standard curve of glycinebetaine assayed by tri-iodide method.

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 ($nmol/10^6$)	
		^{1}H-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.

CHAPTER 3

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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 dramatic 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. Increasing external NaCl concentration in the growth medium 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 the increase in the external NaCl concentration. Choline dehydrogenase activity was approximately 2-fold higher than betaine aldehyde dehydrogenase activity in both control and salt-stressed cells. *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

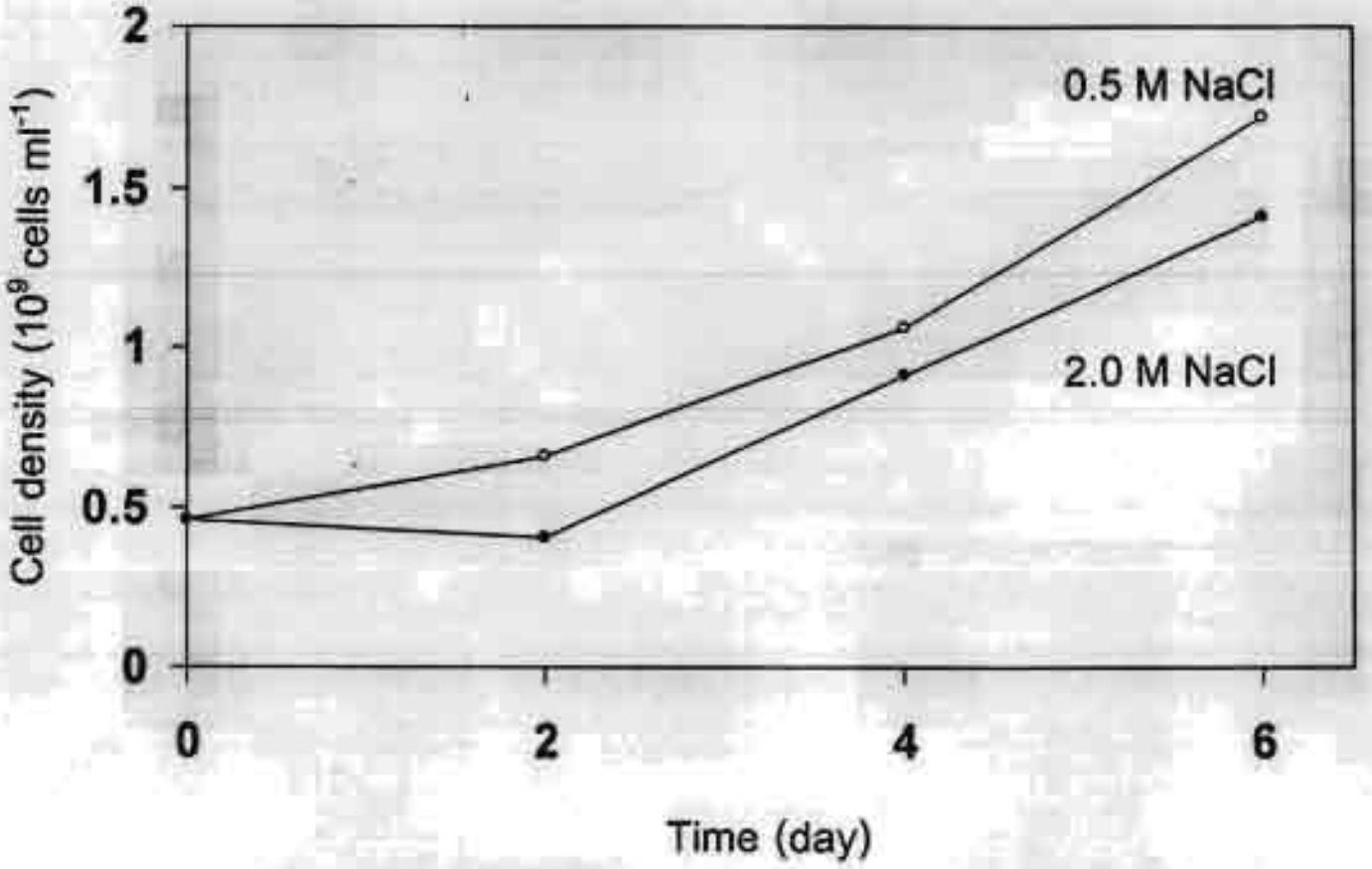


Fig. 1. Growth of *A. halophytica* under salt stress.

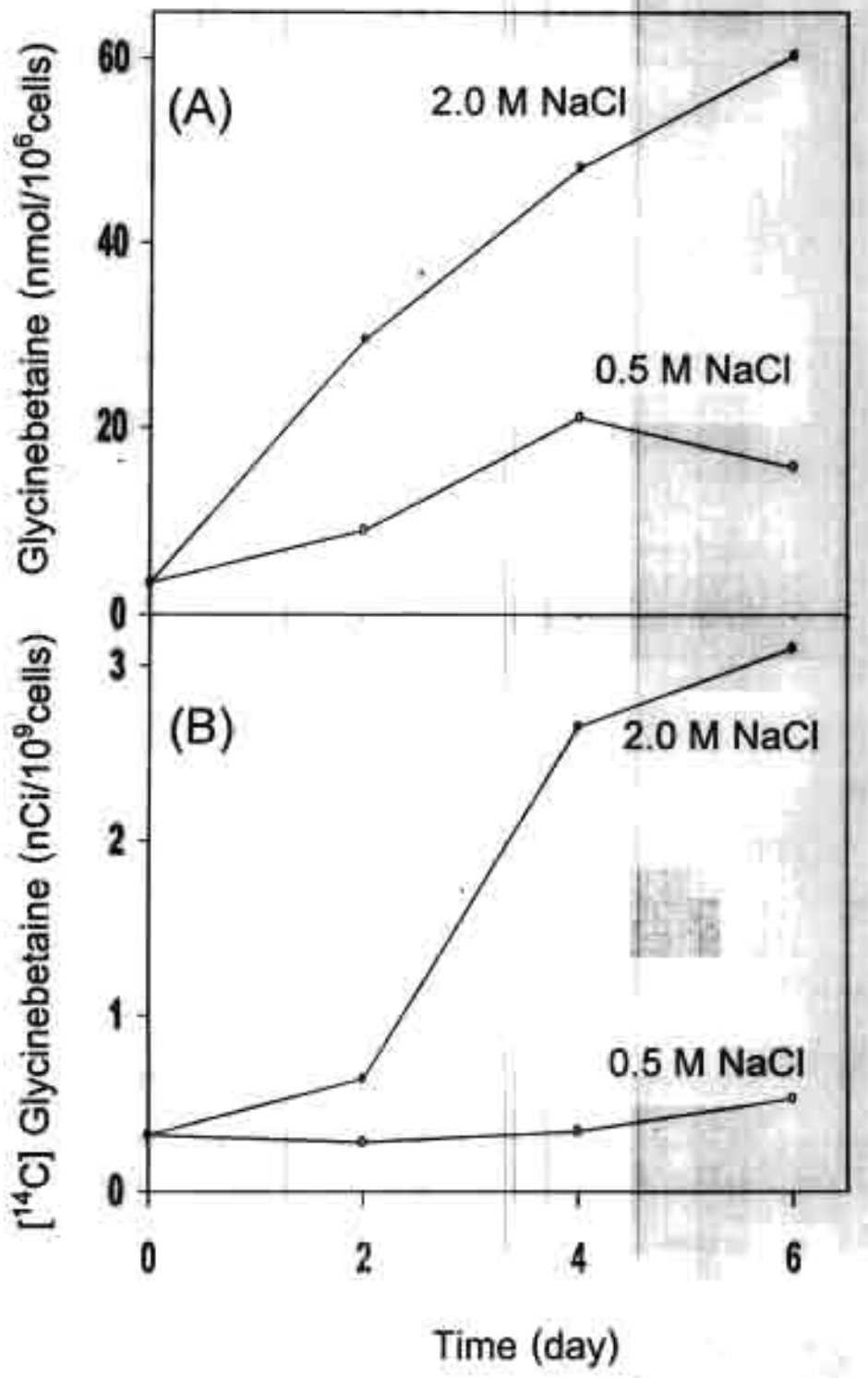


Fig. 2. Effect of various periods of salt stress on glycinebetaine accumulation (A) and [^{14}C] glycinebetaine synthesis (B). The synthesis was determined by labeling cells with [^{14}C] choline for 3 h.

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

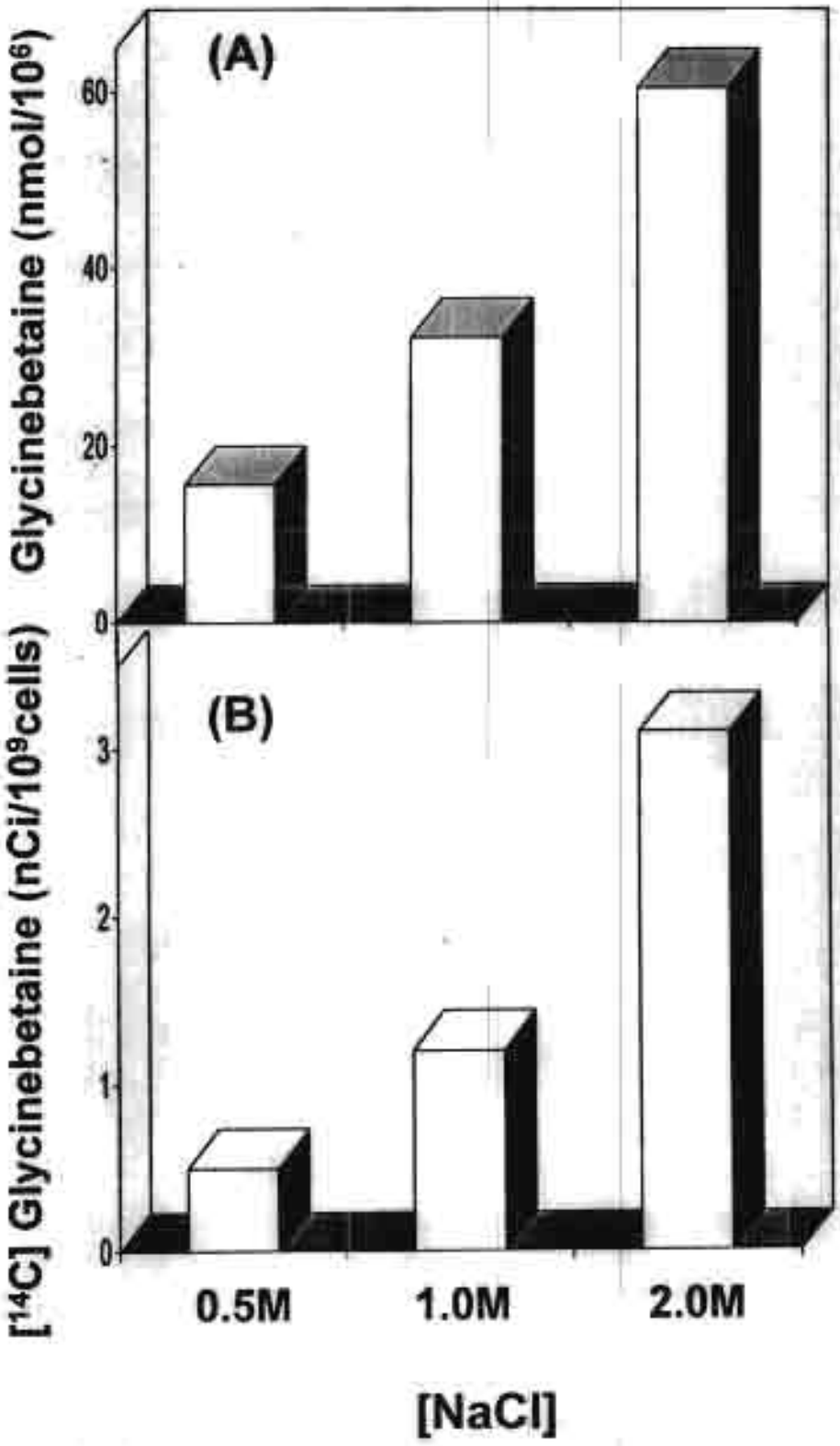


Fig. 3. Effect of external salinity on glycinebetaine accumulation (A) and [^{14}C] glycinebetaine synthesis (B). Cells were grown under various external salinity for 6 d. [^{14}C] glycinebetaine synthesis was determined by labeling cells with [^{14}C] choline for 3 h.

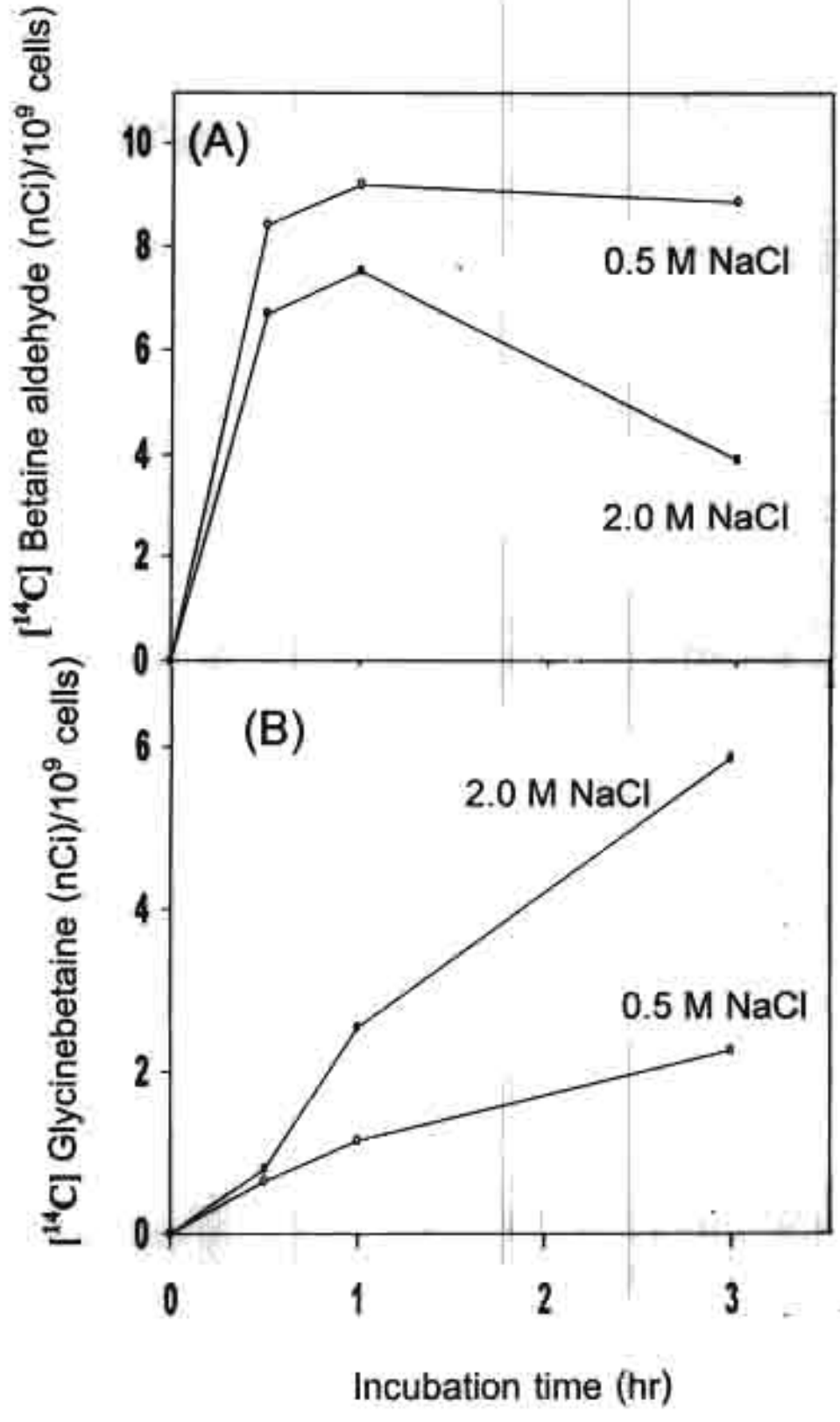


Fig. 4. Time course of the synthesis of [^{14}C] betaine aldehyde (A) and [^{14}C] glycinebetaine from [^{14}C] choline.

first 30 min of labeling and reached maximum at 1 h. After 1 h slight drop of betaine aldehyde occurred in the control cells and much lower betaine aldehyde was detected in salt-stresed cells. If choline-glycinebetaine pathway via betaine aldehyde were to operate in *A. halophytica* the drop in betaine aldehyde would result in the enhanced level of glycinebetaine. This was indeed the case as shown in Fig. 4 B. Slight increase and marked increase of glycinebetaine occurred in control and salt-stressed cells respectively after 3 h. The primary aim of this study was to confirm the existence of the choline → glycinebetaine pathway in *A. halophytica*. The results in Figure 4 clearly established that this pathway was utilized by *A. halophytica*. Furthermore the increased accumulation of glycinebetaine under salt-stressed condition would be due to the increased synthesis by this pathway.

Synthesis of [^{14}C] glycinebetaine from [^{14}C] ethanolamine

When [^{14}C] ethanolamine was incubated with either the control or salt-stressed cells incorporation of radioactivity into [^{14}C] glycinebetaine was readily detectable and seemed to reach maximum after 1 h (Fig. 5). Approximately 4 fold higher level of glycinebetaine synthesis was observed in salt-stressed cells during 1 h incubation. The possible intermediate of ethanolamine → glycinebetaine pathway was shown to be choline in higher plants (50, 51). It was also shown that the potential of both the methylating reaction sequence; i.e., the conversion of ethanolamine to choline and the oxidation sequence; i.e., the conversion of choline to glycinebetaine increased upon salt stress (50, 61). For *A. halophytica*, more refined experiments are needed to determine the effect of salt stress on the methylation of ethanolamine. The experiments must take into account the possibility that the main biosynthetic sequence of the methylation reactions may involve the phosphoryl and the phosphatidyl bases which have been reported in higher plants (51, 62, 63).

Synthesis of [^{14}C] glycinebetaine from [^{14}C] glycine

In the first 30 min following incubation of *A. halophytica* with 40 μM [^{14}C] glycine the salt-stressed cells synthesized about 4-fold more glycinebetaine than the control cells (Fig. 6). Very little synthesis was observed in both control and salt-stressed cells after 30 min. The

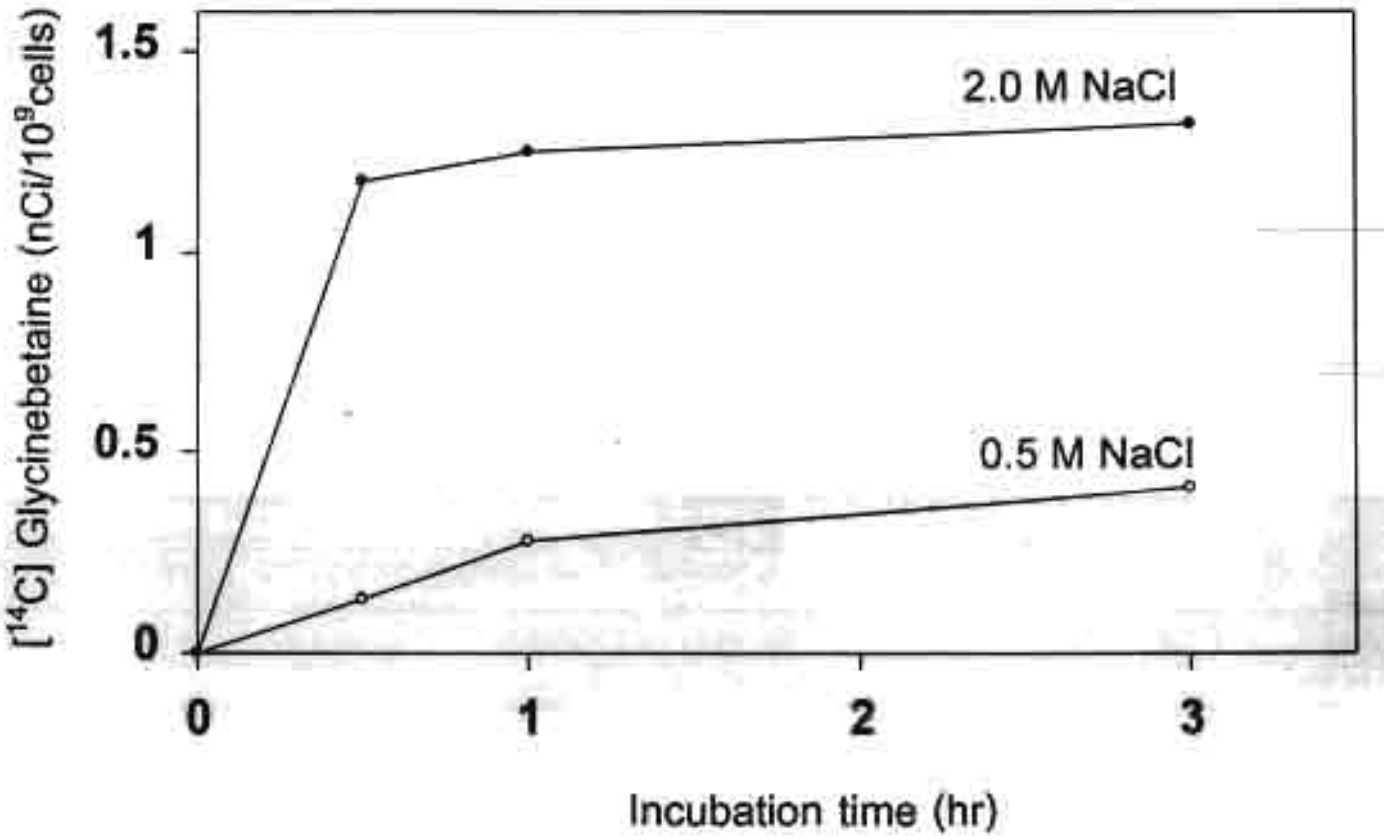


Fig. 5. Time course of the synthesis of [^{14}C] glycinebetaine from [^{14}C] ethanolamine.

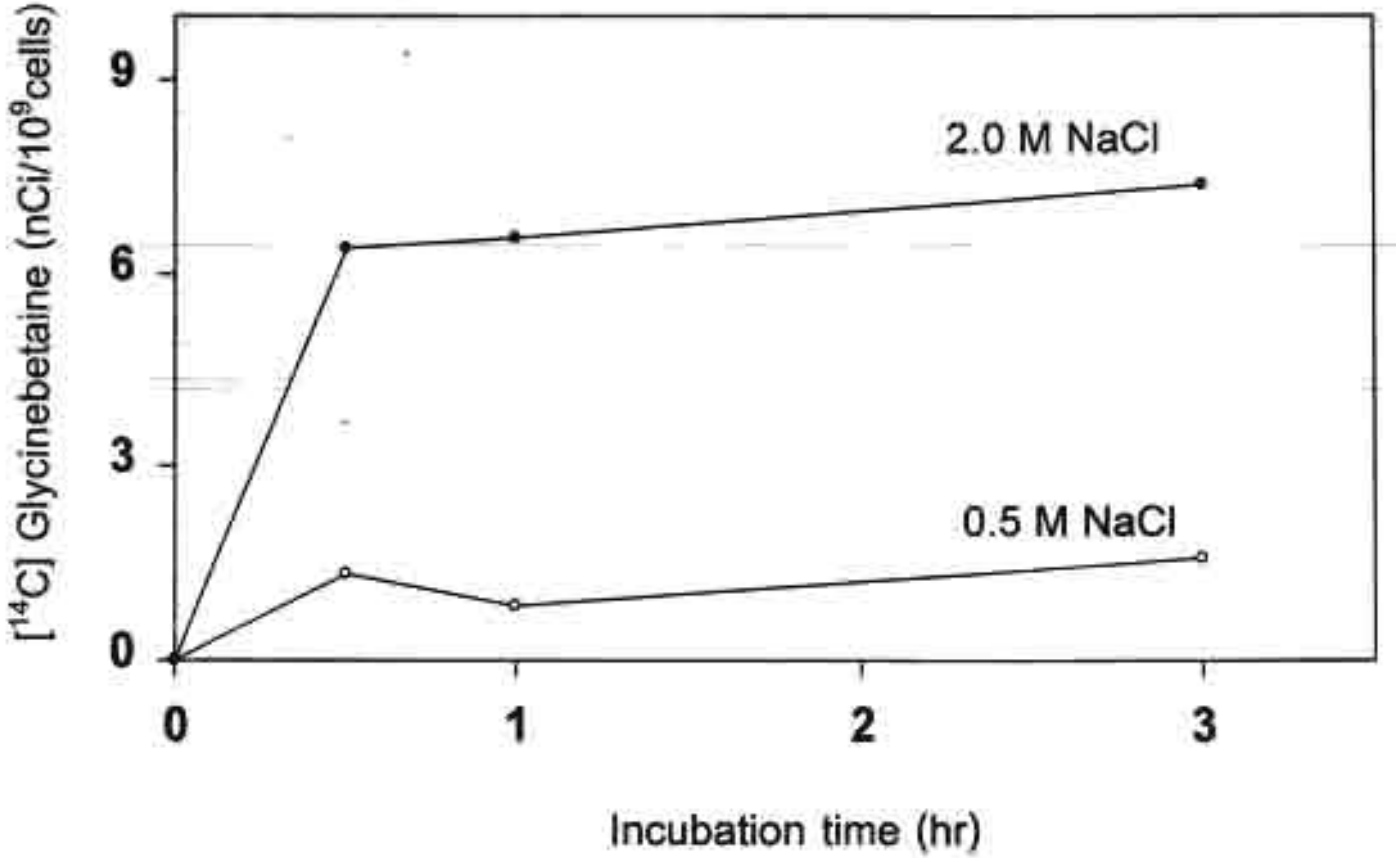


Fig. 6. Time course of the synthesis of [^{14}C] glycinebetaine from [^{14}C] glycine.

halt in glycinebetaine synthesis after 30 min was mainly due to the possibility that [^{14}C] glycine was extensively metabolized to products unrelated to glycinebetaine pathway. There are at least 2 mechanisms by which the 2-carbon moiety of glycinebetaine is derived from glycine. Glycine decarboxylase, one of the enzymes of C-2 glycolate pathway in higher plants, can decarboxylate glycine to form serine which can generate ethanolamine after decarboxylation again. Ethanolamine can then undergo a series of methylation to form choline and then glycinebetaine is formed as a result of the oxidation of choline. In the second mechanism glycinebetaine may be formed by the methylation of glycine. ^{13}C-NMR labeling study has shown that *Methanohalophilus* strain FDF 1 synthesized glycinebetaine by this mechanism (53). Glycine methyltransferase has also been reported in rat liver tissues (52). It should be mentioned here that the operable and functional biosynthetic pathway shoud be supported and substantiated by the presence or the participation of enzymes involved.

Effect of external NaCl concentrations on choline and betaine aldehyde dehydrogenase activities

At the onset of the experiment, the localization of choline and betaine aldehyde dehydrogenase was determined. It was found that the majority (about 80%) of the former localized in the membrane fraction whereas the latter (about 75%) was found in the cytoplasmic fraction. This finding was contradictory to the report by Landfald and Strom (47) where choline and betaine aldehyde dehydrogenase were exclusively localized in membrane and cytoplasmic fraction respectively in *E. coli* cells grown in the presence of choline.

Both choline and betaine aldehyde dehydrogenase activities increased with increasing NaCl concentration (Fig. 7). Detailed analysis showed that salt stress induced about 64% increase in choline dehydrogenase activity and about 125% increase in betaine aldehyde dehydrogenase activity suggesting the more sensitive response towards salt stress for the latter enzyme.

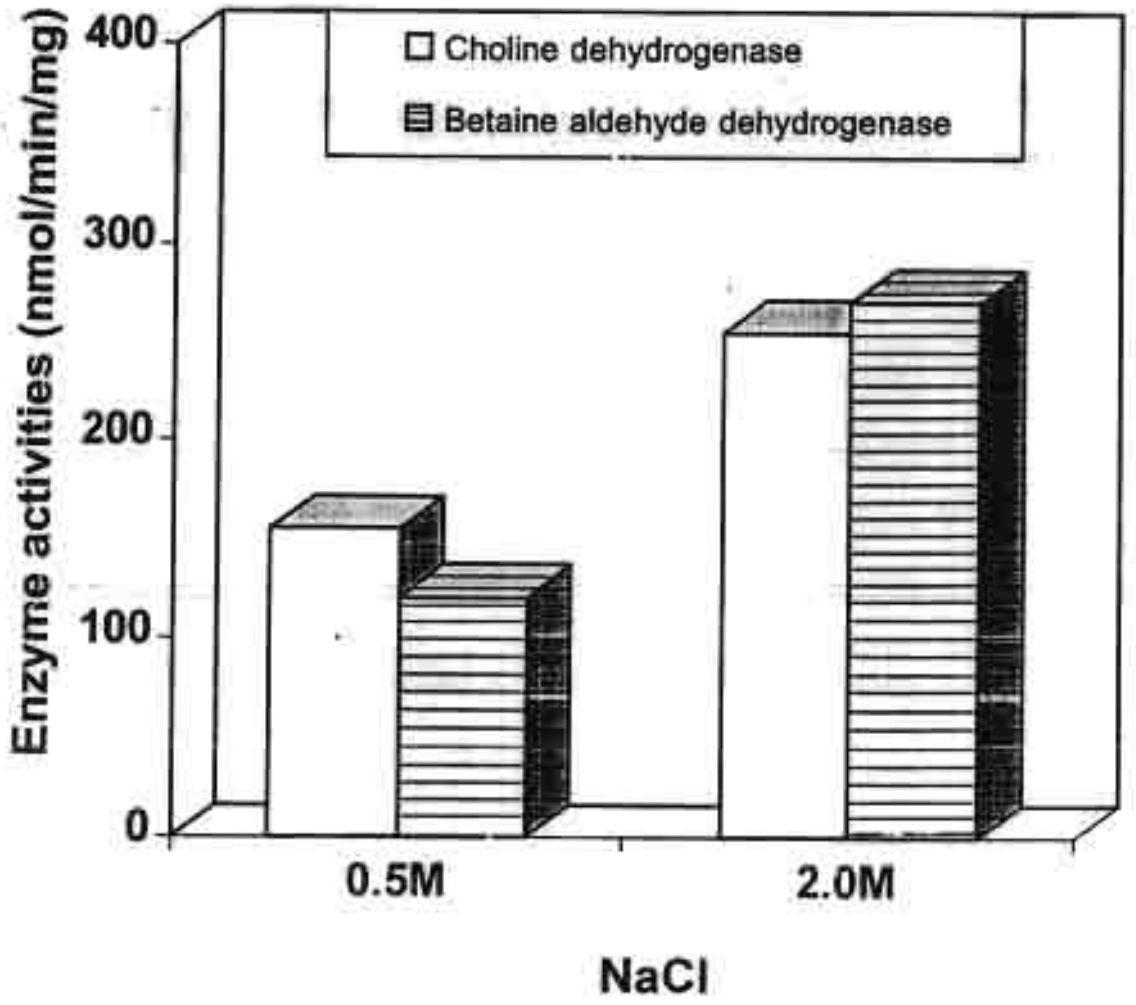


Fig. 7. Effect of external salinity on choline and betaine aldehyde dehydrogenase activities.

CHAPTER 4

Betaine Aldehyde Dehydrogenase From a Halotolerant Cyanobacterium, *Aphanothece halophytica* : Purification, Properties, and Regulation by Salinity

Summary

Betaine aldehyde dehydrogenase (EC 1.2.1.8) was purified from a halotolerant cyanobacterium *Aphanothece halophytica*. Purification was achieved by ammonium sulfate fractionation of lysozyme-disrupted cells, followed by DEAE-cellulose chromatography and hydroxyapatite chromatography. The enzyme was purified about 18-fold with a final specific activity of 298.6 μmol min^{-1} mg^{-1} protein. The enzyme was found to be a tetramer of identical 30 kDa subunits. The optima pH and temperature for the enzyme were 7.5 and 25°C respectively. Both NAD^+ and $NADP^+$ could be used as coenzyme with K_m values of 71.4 μM and 100 μM respectively. The enzyme activity was strongly inhibited by acetaldehyde. N-methylated substrate analogs could also inhibit the enzyme activity and only slight inhibition was observed for glycine betaine. Dithiothreitol enhanced enzyme activity whereas *p*-chloromercuriphenylsulfonate completely abolished the activity. The enzyme was activated by KCl and NaCl at low concentrations up to 0.1 M above which the magnitude of activation was decreased for KCl and the inhibition occurred for NaCl. The elevation of external salinity resulted in the increase of the specific activity of the enzyme.

Introduction

Aphanothece halophytica is a halotolerant cyanobacterium capable of growing at external concentration of up to 3M NaCl (7, 64). The adjustments by *A. halophytica* to counter-balance the high external salinity included the accumulation of inorganic K^+ (65) and an organic quaternary ammonium compound, namely glycine betaine (7). The accumulation of glycine betaine as an osmoregulatory compound was also reported in a marine cyanobacterium, *Spirulina subsalsa* (31). In many bacteria (11, 66, 67), plants (6) and marine animals (9), glycine betaine was also found to accumulate in response to hyperosmotic conditions.

The biosynthesis of glycine betaine in leaves of higher plant has been well studied. Glycine betaine is synthesized by a two step oxidation of choline via the intermediate betaine aldehyde with the aid of choline monooxygenase and betaine aldehyde dehydrogenase (BADH) in spinach chloroplasts (23, 24, 68). In bacteria the transformation of choline into glycine betaine is catalyzed by the membrane-bound enzyme choline dehydrogenase and the cytoplasmic enzyme BADH (47, 49).

Despite the widespread occurrence of glycine betaine its synthesis and osmotic regulation are well understood only in some bacteria (11, 66, 67), certain plant (6) and animal (16, 69) cells. To our knowledge, so far the biosynthesis of glycine betaine has never been reported in cyanobacteria. In the present study, we attempted to partially purify *A. halophytica* BADH and determine its properties as well as its response to hyperosmotic condition.

Materials and Methods

Materials

Aphanothece halophytica was originally isolated from Solar lake in Israel. The organism was kindly provided by Dr. T. Takabe, Nagoya University, Japan. Acrylamide was purchased from Merck. Coomassie brilliant blue was from BDH Laboratory Chemical. Other chemicals were of reagent grade and were from Sigma Chemical.

Organism and growth conditions

Aphanothece halophytica was grown photoautotrophically at 30°C in BG11 medium plus 18 mM $NaNO_3$ and Turk Island salt solution as previously described (29, 64). Cotton-plugged 250 ml conical flasks containing 100 ml of medium each were used and shaken on a rotatory shaker without supplementation of CO_2 gas. The culture flasks were incubated at 30°C with illumination of about 3,000 lux provided by cool white fluorescent lamps.

Purification of betaine aldehyde dehydrogenase

All steps were carried out at 4°C. The enzyme was purified by the following procedure :

i) *Ammonium sulfate precipitation.* The cells at the late log phase (14 days) were first broken by suspending the cell pellet in 50 mM HEPES-KOH pH 7.5 containing 2 mg/ml lysozyme. The suspension was incubated at 37°C for 1 h before centrifugation at 14,000 xg for 20 min. The supernatant was fractionated with ammonium sulfate and the precipitate obtained at 35-70% saturation was dissolved in a small volume of 10 mM Tris-HCl pH 7.5 containing 1 mM DTT and 10% (v/v) glycerol before dialysis against the same buffer.

ii) *DEAE-cellulose column chromatography.* The dialysate from 1) was loaded onto DEAE-cellulose column (2.5 x 17 cm) pre-equilibrated with 10 mM Tris-HCl buffer pH 7.5 containing 1 mM DTT and 10% (v/v) glycerol. The proteins were eluted with a linear gradient of 0-1.0 M NaCl prepared in the buffer at a flow rate of 0.5 ml/min. Peak enzyme activity eluted at about 0.25 M NaCl. The fractions (5 ml)

containing enzyme activity were pooled and subjected to further purification by hydroxyapatite column.

iii) *Hydroxyapatite column chromatography*. The pooled fractions from ii) were applied to a hydroxyapatite column (2.0 x 6.5 cm) pre-equilibrated with 10 mM potassium phosphate buffer pH 7.5 containing 1 mM DTT and 10% (v/v) glycerol. The proteins were eluted with a linear gradient of 10 to 200 mM potassium phosphate buffer containing 1 mM DTT and 10% (v/v) glycerol. The fractions of peak enzyme activity were pooled.

Enzyme assay

BADH activity was assayed spectrophotometrically as described by Pan *et al* (56) with slight modification. The final 1 ml reaction mixture contained 50 mM HEPES-KOH buffer pH 7.5, 10 mM DTT, 1 mM EDTA, 0.5 mM NAD^+ , 0.5 mM betaine aldehyde and an enzyme fraction. In case where the effect of various compounds were tested, they were also included in the reaction mixture. The reaction was initiated with the addition of betaine aldehyde. The slope of the increased A_{340} against time during incubation at 25°C was the measured enzyme activity.

Estimation of enzyme molecular weight

The molecular weight of native BADH was estimated by gel filtration on a Sephadex G-200 column (1.8 x 105 cm) in 10 mM Tris-HCl pH 7.5 and 1 mM DTT using thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), acid phosphatase (95 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), chymotrypsinogen A (23.2 kDa) and cytochrome c (12.3 kDa) as standards.

The molecular weight of BADH in the denatured state was determined by SDS-polyacrylamide gel electrophoresis on a 10% slab gel according to Laemmli (19). The standard proteins used were phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14.4 kDa).

Other methods

The protein content was determined by a sensitive dye-binding method according to Bradford (57). The content of protein eluted from the column was monitored by measuring A_{280}. The values in the figures and tables represent the mean of 2 independent experiments.

Table 1. Purification of betaine aldehyde dehydrogenase from *Aphanothece halophytica*

Purification step	Total protein (mg)	Total activity ($\mu mol\ min^{-1}$)	Specific activity ($\mu mol\ min^{-1}\ mg^{-1}$)	Yield (%)	Purification (fold)
Crude extract	420.3	6,923	16.5	100	1.0
35-70% $(NH_4)_2SO_4$	157.9	4,848	30.7	70	1.8
DEAE-cellulose	2.1	596	290.8	8.6	17.6
Hydroxyapatite	0.6	192	298.6	2.8	18.1

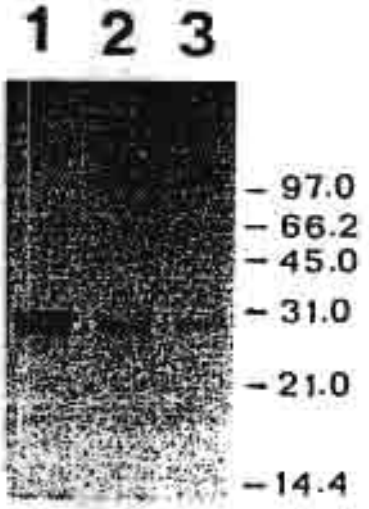


Fig. 1. SDS-polyacrylamide gel electrophoresis of purified *Aphanothece halophytica* betaine aldehyde dehydrogenase on a 10% slab gel. Lanes 1, 2 and 3 were 5, 2.5 and 1.25 μg of purified enzyme after DEAE-cellulose step. Numbers on the right indicate standard molecular weight markers in kilodalton.

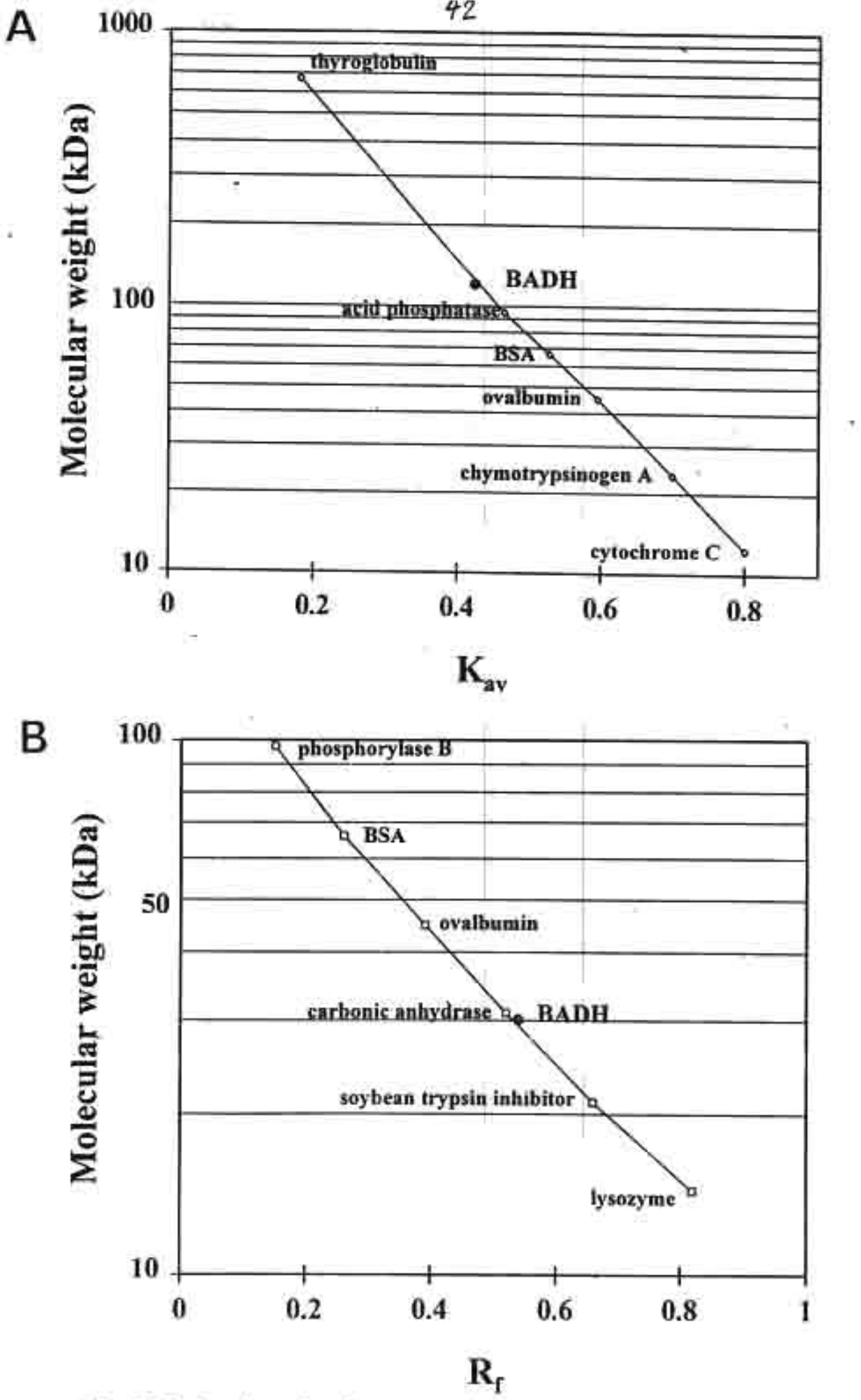


Fig. 2. Estimation of native (A) and subunit (B) molecular weight of betaine aldehyde dehydrogenase from *Aphanothece halophytica* by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively.

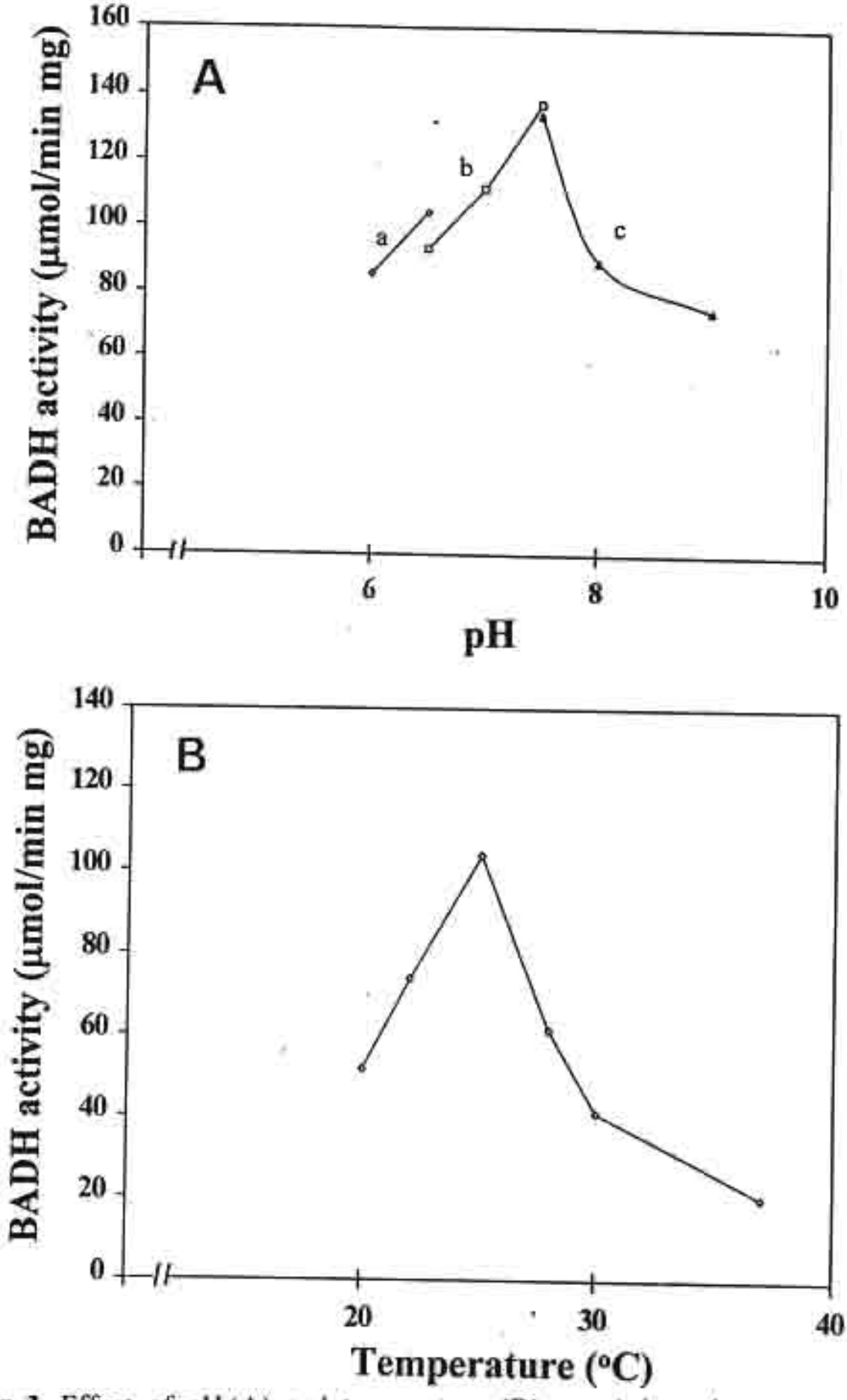
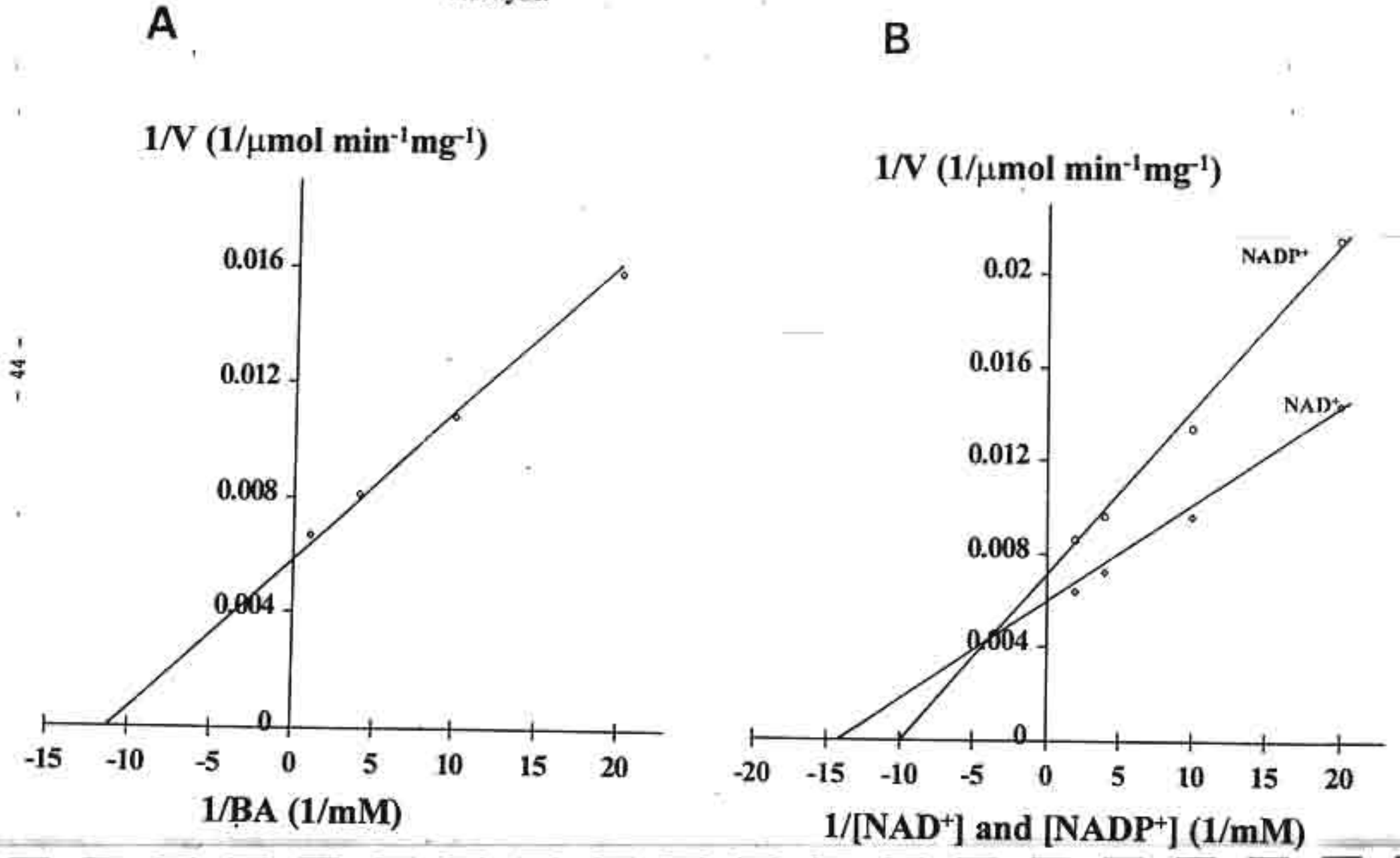


Fig. 3. Effect of pH (A) and temperature (B) on *Aphanothece halophytica* betaine aldehyde dehydrogenase activity. The buffer systems used in (A) were a) 50 mM potassium phosphate buffer, b) 50 mM HEPES-KOH buffer and c) 50 mM Tris-HCl buffer.

Fig. 4. Double reciprocal plots of activity of betaine aldehyde dehydrogenase against substrate concentration. (A) Betaine aldehyde (BA) as variable substrate at fixed 0.5 mM NAD^+. (B) NAD^+ or $NADP^+$ as variable substrate at fixed 0.5 mM betaine aldehyde.



presented in the form of Lineweaver Burk plots as shown in Fig. 4. The apparent Michaelis constants (K_m) were estimated to be 91 μM for betaine aldehyde (Fig. 4A) and 71.4 μM and 100 μM for NAD^+ and $NADP^+$ respectively (Fig. 4B). The maximal reaction velocities (Vmax) were found to be 175.4 and 138.8 μmol min^{-1} mg^{-1} when NAD^+ and $NADP^+$ were used as coenzyme respectively.

Effect of substrate-analogs on BADH activity

Four compounds bearing resemblance to betaine aldehyde substrate were tested for inhibitory effect on BADH activity. Table 2 shows that acetaldehyde was a very potent inhibitor, the BADH almost completely lost its activity in the presence of acetaldehyde. The other three compounds which were N-methylated substrate analogs showed different degree of inhibition towards BADH activity. Ethanolamine was the most effective inhibitor whereas glycine betaine, the product of the BADH-catalyzed reaction, was the least effective inhibitor.

Effect of sulfhydryl-reactive reagents on BADH activity

Sulfhydryl-reactive reagents that were used in this study were DTT and PCMS. BADH activity was strongly enhanced by DTT and severely inhibited by PCMS (Table 3). The inhibitory effect of PCMS was mostly relieved when the enzyme was preincubated with DTT. The inhibition by PCMS could be reversed about one half by the subsequent addition of DTT. Taken together, the overall results appeared to indicate that one or more sulfhydryl group at the enzyme catalytic site is essential for enzyme activity.

Effect of salts on BADH activity

Four different types of salts with respect to cations were tested for their effects on BADH activity. The control (no salt) contained 50 mM Tris-HCl pH 7.5 instead of 50 mM HEPES-KOH for buffering capacity. Salts with monovalent cations, i.e., Na^+ and K^+ at low concentrations gave an increase in enzyme activity (Fig. 5). K^+ concentration higher than 0.1 M reduced the magnitude of enzyme activation and the enzyme showed no response to K^+ at or higher than 1 M K^+. Elevated Na^+ at or higher than 0.25 M inhibited enzyme activity. Divalent cations were very inhibitory even at low concentrations. A 50%

Table 2. Inhibition of betaine aldehyde dehydrogenase by analogs of betaine aldehyde

Substrate analog at 100 mM	Remaining activity (%)
Glycine betaine	88.5
Choline	77.1
Ethanolamine	27.0
Acetaldehyde	0.9

The activity of the enzyme without substrate analog was 96.7 μmol min^{-1} mg^{-1} and was taken as 100%.

Table 3. Effect of dithiothreitol (DTT) and *p*-chloromercuriphenylsulfonate (PCMS) on the activity of betaine aldehyde dehydrogenase

Treatment	Relative activity (%)
Control	100
5 mM DTT (30 min)	274.2
0.1 mM PCMS (10 min)	0
5 mM DTT (30 min), then 0.1 mM PCMS (30 min)	90.3
0.1 mM PCMS (10 min), then 5 mM DTT (30 min)	51.6

The specific activity of the enzyme without DTT (control) was 57.8 μmol min^{-1} mg^{-1}.

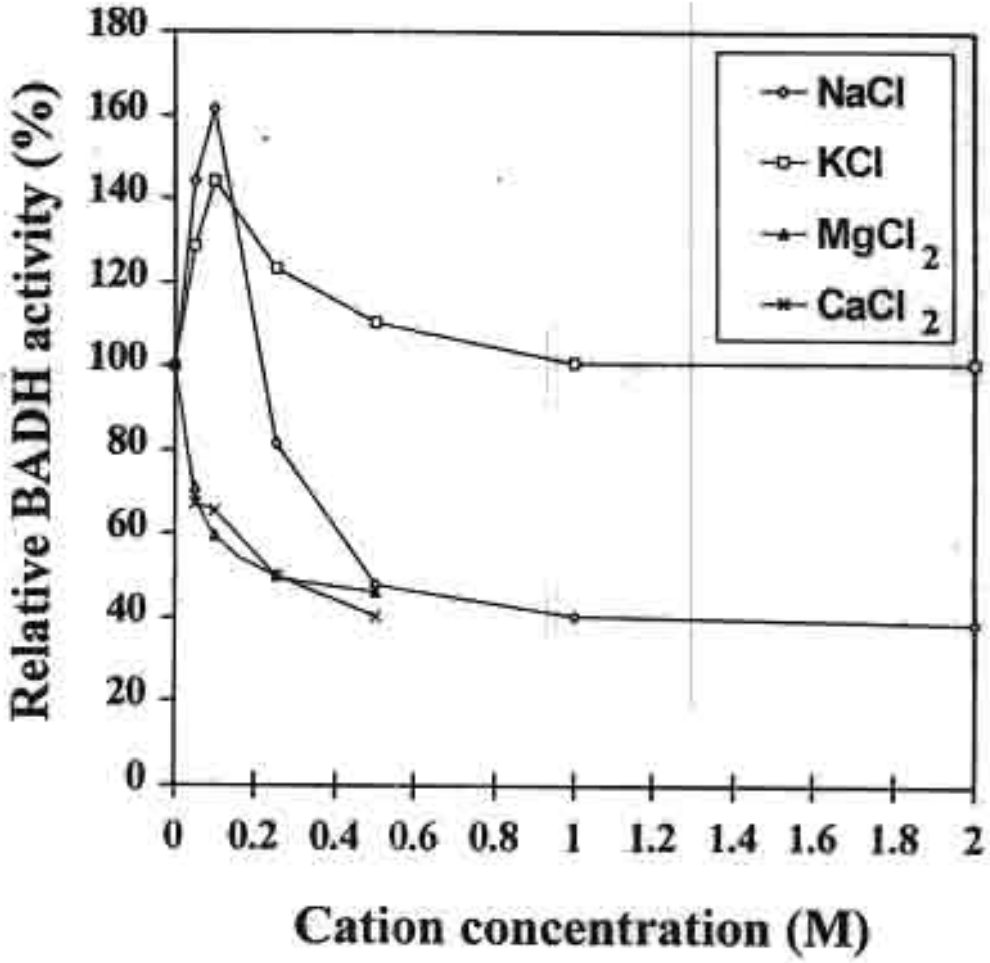


Fig. 5. Effect of salts on the relative activity of betaine aldehyde dehydrogenase from *Aphanothece halophytica.*

reduction in enzyme activity occurred in the presence of 0.25 M Mg^{2+} or Ca^{2+}.

Effect of external salinity on BADH activity

A. halophytica cells were either grown in the medium containing 0.5 M or 2.0 M NaCl. The cell extracts were subjected to 35-70% ammonium sulfate precipitation before being used for BADH assay. The activity of BADH obtained from cells grown in 2 M NaCl was about 4-fold of that from cells grown in 0.5 M NaCl (Table 4). The result appeared to be in line with the previous finding that showed the increase of glycine betaine level about 4 fold when *A. halophytica* cells were grown in 2 M NaCl as compared to those grown in 0.5 M NaCl (75).

Table 4. Betaine aldehyde dehydrogenase activity from *Aphanothece halophytica* grown in different salinities

NaCl in growth medium (M)	Enzyme activity (μmol min^{-1} mg^{-1})
0.5	1.03
2.0	4.43

Discussion

In the present study we attempted to purify the enzyme which is responsible for the synthesis of glycine betaine. Preliminary experiment by which the cells were broken by sonication failed to retrieve the activity of BADH. However, when we used lysozyme to break the cells we were able to detect the presence of BADH. An essentially pure enzyme preparation was obtained after DEAE-cellulose chromatography. The specific activity of BADH in the present study was somewhat similar to that reported for BADH of horseshoe crab (69) and about one and two orders of magnitude higher than BADH from spinach and *E. coli* respectively (56, 71).

The gel filtration and SDS-PAGE experiments indicated that BADH of *A. halophytica* appeared to be a tetramer with identical 30 kDa subunits. Previous reports on BADH molecular weight and subunit from different organisms were quite variable. BADH of spinach is a dimer with subunits of 60 (72) and 63 (55) kDa. The tetramers of subunits of 55 (71) and 58 (73) kDA were reported for BADH of *E. coli* and *C. didymum* respectively. Taken together it appears that no typical prokaryote or eukaryote BADH exists with respect to native molecular weight and subunit composition.

The BADH from *A. halophytica* preferred NAD^+ (K_m value of 71.4 μM) as a coenzyme, but could also use $NADP^+$ (K_m value of 100 μM). The specificity of BADH with regard to coenzyme is similarly observed for BADH from other sources (55, 56, 69, 71). So far only BADH from *P. aeruginosa* A-16 has been reported to prefer $NADP^+$ to NAD^+ as a coenzyme (74). *A. halophytica* BADH showed a relatively narrow pH optimum of 7.5 similar to that of BADH from horseshoe crab (69). For *E. coli* and spinach the BADH had broader pH optima spanning 2 pH units (55, 71). The V_{max} of *A. halophytica* BADH (175.4 μmol min^{-1} mg^{-1}) in the present study was highest when compared to other species, i.e., more than 100 -fold higher than that for spinach (55, 56) and about 3-times higher than the V_{max} for *E. coli* BADH (71). This suggests that *A. halophytica* is very efficient in converting betaine aldehyde to glycine betaine especially when the organism is under hyperosmotic environments.

The BADH from *A. halophytica* had a relatively high affinity for betaine aldehyde. The K_m value of 91 μM was the lowest

among other species studied so far (55, 56, 69, 71, 74). The analogs of betaine aldehyde could inhibit BADH activity with varying degree of inhibition (Table 2). Aldehyde compound such as acetaldehyde was a strong inhibitor whereas N-methylated compounds such as glycine betaine, choline and ethanolamine showed lesser inhibition than the aldehyde compound. This suggested that the aldehyde functional group of the substrate might play an important role in binding to the catalytic site of BADH. The fact that glycine betaine which is the product of BADH reaction only slightly inhibits BADH may have physiological relevance since this metabolite accumulates in *A. halophytica* at a very high level in response to salt stress (75).

One purpose of the present investigation is to study the response of BADH towards various salts with respect to the cations. It is clear that *A. halophytica* BADH is a salt tolerant enzyme (Fig. 5). Both K^+ and Na^+ activated the enzyme at low concentrations. Even at 1.0 or 2.0 M Na^+ about 40% of the original activity was retained. As for K^+, no inhibition of BADH activity was observed. This phenomenon could well explain why the accumulation of glycine betaine still occurred despite the initial massive accumulation of K^+ inside *A. halophytica* under hyperosmotic condition (7). Although both Mg^{2+} and Ca^{2+} could inhibit BADH activity in *A. halophytica*, the effect of these 2 cations on the synthesis of glycine betaine would be minimal due to their low concentrations inside the cells. It is noted that BADH from horseshoe crab could be activated by 2 fold in the presence of as low as 0.75 μM Ca^{2+} (69).

The observed salt-induced rise in *A. halophytica* BADH specific activity (Table 4) is a good circumstantial evidence in support of the existence of the choline —> betaine aldehyde —> glycine betaine pathway. The extent of the increase of BADH activity was in good agreement with the increase in the content of glycine betaine (75). Previous report by Ishitani *et al* (76) has shown that BADH mRNA of barley leaves increased 8-fold under salt stress and the level decreased upon release of the stress. Future experiments are needed to examine whether the increase of BADH activity in *A. halophytica* under salt stress is caused by an increase of BADH transcripts.

CHAPTER 5

General Discussion and Conclusion

Osmoregulation is the term used to refer to active processes carried out by living organisms during adaptation to the osmotic strength of the environment. Generally osmoregulatory phenomena are categorized into 2 types : 1) !ong-term or steady-state responses that are manifested during the growth of organisms at a constant osmolarity and 2) short-term or transient responses that occur soon after changes in the external osmolarity. Most studies, including the present investigation, have involved sudden shifts in osmolarity; i.e., the short-term responses. The knowledge of the osmoregulatory process occurring in different types of organisms can be beneficial for molecular geneticists as well as plant breeders to construct plant cells, especially those with economic importance, that have traits conferring salinity or drought tolerance. To reach this end basic studies on the molecular mechanism of osmoregulation at the levels of metabolism, enzymes and genes involved, are essential.

The use of cyanobacteria as a model organism is advantageous in that they are prokaryotes evolving themselves between prokaryotic bacteria and eukaryotic higher plants. The information obtained using cyanobacteria should well serve as a link between bacteria and higher plants which may be useful for comparative studies especially those related to evolution process. One such example is the endosymbiont hypothesis which states that cyanobacteria are the most likely ancestor of chloroplasts (77).

The key word for the present investigation is a compatible solute glycinebetaine which was firmly established as a major osmoticum inside *A. halophytica*. It is important that a suitable assay method must first be established for the determination of glycinebetaine. Both the ^{1}H-NMR spectrometry and the tri-iodide method were proved equally satisfactory for the determination of glycinebetaine. However each of these 2 methods has advantages and disadvantages. The former is sensitive but rather costly, not to mention the equipment cost. The tri-iodide method is much less expensive and requires only a simple spectrophotometer. Extra work is needed, however, to separate glycinebetaine from other quaternary ammonium compounds. This can

be accomplished by a small cation exchange column, i.e., a Dowex-50 from Sigma.

The finding that intracellular glycinebetaine was increased in only salt-stressed cells by $NaNO_3$ and light was interesting. The explanation for the observed phenomena may not be obvious at present. However, it remains an intriguing question as to the effect of light on an internal level of N-containing compounds especially $NaNO_3$ for both control and salt-stressed *A. halophytica*.

The major emphasis of the present investigation lies on the elucidation of the metabolic pathway of glycinebetaine synthesis. A number of problems have been encountered during the course of investigation. Initially the technique of paper chromatography was employed to separate the precursor from the intermediates and products. However, later we found that the descending paper chromatographic technique using n-butanol :ethanol : acetic acid : H_2O = 8 : 2 : 1 : 3 (v/v) was not reproducible and that the radioactivity measurement by the radioscanner machine was not sensitive enough. The problems were rectified by using the cation exchange column (Dowex-50 from Sigma) which gave clear separation and complete recovery of precursor, intermediates and products. The separated compounds containing incorporated radioactivity could be conveniently determined by liquid scintillation spectrometry.

Another point worth mentioning here is that in the preliminary radiotracer experiments we labeled the cells with appropriate precursors, i.e., [^{14}C] choline, [^{14}C] ethanolamine and [^{14}C] glycine using growth medium as incubation medium during labeling. We found that the uptake of radioactive precursor by the cells was variable and quite low for [^{14}C] ethanolamine and [^{14}C] glycine. After slight modification by replacing the growth medium as incubation medium by the aqueous buffer pH 7.5, the uptake problem was finally solved.

At this stage it appears certain that the synthesis of glycinebetaine in both control and salt-stressed *A. halophytica* is as follows : choline → betaine aldehyde → glycinebetaine. However, more work is needed in order to ascertain whether the increased synthetic rate can account for the observed increase of glycinebetaine content due to salt stress. Such work will involve the estimation of the internal pool of various metabolites and possibly the fate of glycinebetaine, i.e., can it be degraded? Equally interesting is the question whether the increased synthesis of glycinebetaine is due to salinity or osmotic effect. The

increase of glycinebetaine content has already been shown to be due to the osmotic effect.

The possibility that ethanolamine and glycine can serve as precursors for glycinebetaine synthesis is briefly presented in this study. There are more rooms to further explore this ethanolamine or glycine-derived pathway for glycinebetaine. Some examples are the series of methylation reactions from ethanolamine to choline, the involvement of free or phosphoryl or phosphatidyl form of ethanolamine and choline. Most importantly is the study on the route by which 2-carbon moiety of glycinebetaine can derive from glycine. Direct methylation of glycine to glycinebetaine has never experimentally been reported.

The investigation on the betaine aldehyde dehydrogenase was also not without problems. The main and serious problem was the unstable nature of the enzyme. It was crucial that the extraction buffer contained high content of glycerol (10%, v/v) as well as 1 mM DTT. Furthermore we had to use a very mild and gentle method of breaking the cells by the use of lysozyme. The purified enzyme from *A. halophytica* possessed properties both similar to and different from enzymes from other sources. One distinct property is its molecular structure of a tetramer of 30 kDa subunits. Also the enzyme from *A. halophytica* had higher V_{max} than those from higher plant and bacteria which may suggest that cyanobacteria are more efficient organisms to accumulate glycinebetaine and can better withstand high salinity stress.

In conclusion the investigation on the osmoregulation with respect to the synthesis of glycinebetaine has provided some important information. Nevertheless there are some points that require further experimentation so that a full description of the biosynthesis of glycinebetaine in *A. halophytica* can emerge.

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Proceedings

1. Incharoensakdi, A. and Kum-arb, U. (1996) Osmoregulation in a halophilic cyanobacterium, *Aphanothece halophytica*. In "Proceedings of the 2nd Thai-French Symposium on Plant Molecular Biology", Bordeaux, France, October 6-10, 1996, (Bove, J.M., ed.) pp. 283-292.

2. Incharoensakdi, A. and Kum-arb, U. and Wutipraditkul, N. (1997) Biosynthesis and accumulation of glycinebetaine confer salt tolerance in the cyanobacterium, , *Aphanothece halophytica*. In "Proceedings of Chulalongkorn University 80th Anniversary Research Conference", Chulalongkorn University, October 15-17, 1997, pp. 677-684.

Publications

1. Kum-arb, U. and Incharoensakdi, A. "Betaine aldehyde dehydrogenase from a halotolerant cyanobacterium *Aphanothece halophytica* : Purification, properties and regulation by salinity". (Submitted to J. Sci. Soc. Thailand)

2. Incharoensakdi, A. and Wutipraditkul, N. "Accumulation of glycinebetaine and its synthesis from radioactive precursors in salt-stressed cyanobacterium *Aphanothece halophytica*". (Submitted to J. Appl. Phycology)

3. Wutipraditkul, N., Kum-arb, U. and Incharoensakdi, A. "Factors affecting the accumulation of glycinebetaine in a halophilic cyanobacterium, *Aphanothece halophytica*". [Submitted to J. Sci. Res. (Chula. Univ.)]

Publication (Not related to the project)

1. Incharoensakdi, A. and Kitjaharn, P. (1998) Removal of lead from aqueous solution by filamentous cyanobacterium, *Spirulina platensis*. J. Sci. Res. (Chula. Univ.) 23, 37-44.

Appendix 1

Growth Medium for *A halophytica*

1). Turks Island Salt Solution

A solution of 5 l (made with distilled water) of Turks Island Salt Solution consists of the following components :

A). KCl	3.33 g
B). $MgCl_2 . 6H_2O$	2.75 g
C). $CaCl_2 . 2H_2O$	7.33 g
D). $MgSO_4 . 7H_2O$	34.70 g
E). NaCl	140.80 g

2). BG 11 plus NO_3 solution

The components of BG 11 plus NO_3 solution are as follows :

A). $NaNO_3$	150.0 g/l
B). KH_2PO_4	4.0 g/l
C). $MgSO_4 . 7H_2O$	75.0 g/l
D). $CaCl_2 . 2H_2O$	36.0 g/l
E). Na_2CO_3	20.0 g/l
F). Citric acid	6.0 g/l
G). EDTA . Na_2	1.0 g/l
H). $FeSO_4 . 7H_2O$	6.0 g/l

I). Trace element A_5 solution consisting of the following components in 1 l solution

H_3BO_3	2.68 g
$MnCl_2 . 4H_2O$	1.81 g
$ZnSO_4 . 7H_2O$	0.22 g
$Na_2MoO_4 . 2H_2O$	0.39 g
$CuSO_4 . 5H_2O$	0.079 g
$Co(NO_3)_2 . 6H_2O$	0.049 g

The growth medium is prepared by adding 50 ml of item 2.A and 5 ml each of items 2.B. to 2.I. to 5 l of Turks Island Salt Solution.

สัญญาเลขที่ RSA/22/2538

รายงานการเงินในรอบ 1 ปี

ชื่อโครงการ การควบคุมแรงดันออสโมติกในไซยาโนแบคทีเรียชนิดชอบความเค็ม, อะฟาโนทีซ สาโลฟิทิดา : การสังเคราะห์สารประกอบไกลซีนบีเทน

ชื่อหัวหน้าโครงการ รองศาสตราจารย์ ดร.อรัญ อินเจริญศักดิ์

รายงานในช่วงวันที่ 1 กันยายน 2540 ถึงวันที่ 31 สิงหาคม 2541

รายจ่ายประจำงวดปัจจุบัน

หมวด (ตามเอกสารโครงการ)	รายจ่าย จากรายงานครั้งก่อน	รายจ่าย ตรางนี้	รวมสะสม
1. ค่าจ้าง	36,000	36,000	72,000
2. ค่าตอบแทนเบธีวิจัย	90,000	90,000	180,000
3. ค่าตอบแทน (อื่น ๆ)	21,000	21,000	42,000
4. ค่าใช้สอย	4,440.90	4,063.50	8,504.40
5. ค่าวัสดุ	49,316.05	99,341.80	148,637.85
6. ค่าครุภัณฑ์	-	-	-
7.	-	-	-
8.	-	-	-
รวม	200,756.95	250,405.30	451,162.25

จำนวนเงินที่ได้รับและเงินคงเหลือ

งวดที่ 1	ได้รับจาก สกว.	360,000	บาท
	ได้รับจากมหาวิทยาลัย	120,000	บาท
	อื่น ๆ (เช่น ดอกเบี้ย)	9,885.67	บาท
	รวม	489,885.67	บาท
	รายจ่าย	399,981.70	บาท
	เหลือ	89,903.97	บาท
งวดที่ 2	ได้รับจาก สกว.	360,000	บาท
	ได้รับจากมหาวิทยาลัย	120,000	บาท
	อื่น ๆ (เช่น ยกมาจากงวดก่อนหรือดอกเบี้ย)	102,916.63	บาท
	รวม	582,916.63	บาท
	รายจ่าย	393,555.43	บาท
	เหลือ	189,361.20	บาท
งวดที่ 3	ได้รับจาก สกว.	270,000	บาท
	ได้รับจากมหาวิทยาลัย	120,000	บาท
	อื่น ๆ (เช่น ยกมาจากงวดก่อนหรือดอกเบี้ย)	207,662.73	บาท
	รวม	597,662.73	บาท
	รายจ่าย	451,162.25	บาท
	เหลือ	146,500.48	บาท

ชื่อบัญชี
NAME

48292-1 เมธีวิจัย สกว. - อรัญ อินเจริญศักดิ์

ธนาคารไทยพาณิชย์ จำกัด (มหาชน)
THE SIAM COMMERCIAL BANK PUBLIC COMPANY LIMITED
สาขาสภากาชาดไทย

บัญชีเงินฝากออมทรัพย์
SAVINGS ACCOUNT

เลขที่บัญชี
ACCOUNT NO.
045-2 48292-1

ผู้มีอำนาจลงนาม
Authorized Signature

วันที่ DATE	รหัสรายการ TRANS. CODE	ถอน WITHDRAWAL	ฝาก DEPOSIT	คงเหลือ BALANCE	สาขา	ลายมือชื่อ
21/09/95CDD			********100.00	********100.00	0828	
13/10/95QDN			****360,000.00	****360,100.00	0323	
06/12/95CWD		*****22,000.00		****338,100.00	0411	
31/12/95T/I		**********0.00	*******3,868.23	****341,968.23	0000	
05/01/96CWD		*****22,000.00		****319,968.23	0410	
05/02/96CWD		*****22,000.00		****297,968.23	0413	
05/03/96CWD		*****66,000.00		****231,968.23	0828	
14/03/96CWD		*****56,685.00		****175,283.23	0828	
11/04/96CWD		*****22,000.00		****153,283.23	0828	
08/05/96CWD		*****22,000.00		****131,283.23	0828	
09/05/96QDN			****120,000.00	****251,283.23	0414	
[illegible]		*****23,350.00		****227,933.23	0414	
30/06/96T/I		**********0.00	*******6,017.44	*****233,950.67	0000	
05/07/96CWD		*****22,000.00		****211,950.67	0412	
16/07/96CWD		*****23,436.00		****188,514.67	0417	
07/08/96CWD		*****53,042.00		****135,472.67	0409	
05/09/96CWD		*****55,568.70		*****79,903.97	0828	
04/10/96CWD		*****22,000.00		*****57,903.97	0417	
[illegible]		*****23,680.00		*****34,223.97	0828	
21/11/96QDN			****360,000.00	****394,223.97	0417	
18/12/96CWD		*****22,244.00		****371,979.97	0412	
31/12/96T/I		**********0.00	*******4,374.77	****376,354.74	0000	

รหัสรายการ

CDP.CDN ฝากเงินสด		XWD XWN ถอนเป็นเงินโอน	
ODP.ODN ฝากด้วยเช็ค		COR	รายการแก้ไข
XDP. XDN ฝากเป็นเงินโอน		RTC	เช็คคืน
ODP.ODN ฝากด้วยเช็คต่างสาขา		INT	ดอกเบี้ย
CWD.CWN ถอนเงินสด		TAX	ภาษี

1

วันที่ DATE		ถอน WITHDRAWAL	ฝาก DEPOSIT	ยอดคงเหลือ BALANCE		
06/01/97	CWD	*****22,000.00		****354,354.74	0411C	
06/02/97	CWD	*****50,143.00		****304,211.74	0828C	
04/03/97	CWD	*****32,592.60		****271,619.14	0414C	
06/03/97	QDP		****120,000.00	****391,619.14	0410C	
10/04/97	CWD	*****22,202.00		****369,417.14	0417C	
02/05/97	CWD	*****22,175.48		****347,241.66	0418C	
04/06/97	CWD	*****34,481.50		****312,760.16	0414C	
	INT	******8,637.89				
30/06/97	TAX	**********0.00	****321,398.05	0000A		
04/07/97	CWD	*****25,564.00	****295,834.05	0409C		
06/08/97	CWD	*****22,000.00	****273,834.05	0409C		
29/08/97	CWD	*****84,472.85	****189,361.20	0828D		
29/09/97	QDN	****270,000.00	****459,361.20	0828D		
07/10/97	CWD	*****33,573.10	****425,788.10	0417C		
07/11/97	CWD	*****31,049.55	****394,738.55	0415C		
24/11/97	XDN	****120,000.00	****514,738.55	0046C		
03/12/97	CWD	*****22,147.00	****492,591.55	0411C		
21/12/97	XWD	*****38,776.20	****453,815.25	0414C		
31/12/97	CDR	*****38,776.30	****492,591.55	0414L		
31/12/97	CWD	*****38,776.30	****453,815.25	0414C		
	INT	******8,953.20				
31/12/97	TAX	**********0.00	****462,768.45	0000A		

2

วันที่ DATE	TRANS CODE	ถอน WITHDRAWAL	ฝาก DEPOSIT	คงเหลือ BALANCE		
04/02/98	CWD	*****34,397.00	****428,371.45	0411C		1
02/03/98	CWD	*****40,814.00	****387,557.45	0046C		2
03/04/98	CWD	*****33,626.50	****353,930.95	0409C		3
12/05/98	CWD	*****39,867.00	****314,063.95	0828D		4
08/06/98	CWD	*****23,350.00	****290,713.95	0412C		5
	INT	*******9,348.33				6
30/06/98	TAX	**********0.00	****300,062.28	0000A		7
15/07/98	CWD	*****30,650.00	****269,412.28	0409C		8
10/08/98	CWD	~~*****54,167.80~~	****215,244.78	0829C		9
10/08/98	CDR	*****54,167.80	****269,412.28	0828L		10
10/08/98	CWD	*****54,107.50	****215,304.78	0828C		11
						12
11/09/98	CWD	*****69,804.30	****145,500.48	0417C		13
						14
						15
						16
						17
						18
						19
						20
						21
						22

บริการฝาก-ถอนต่างสำนักงาน และบริการอื่นผ่านเครื่องอัตโนมัติ

ธนาคารไทยพาณิชย์ จำกัด (มหาชน) เป็นผู้นำในด้านบริการอิเล็กทรอนิกส์ ช่วยให้ท่านฝากถอน โอนเงิน สอบถามยอดคงเหลือในบัญชี ชำระค่าสินค้าและบริการ และธุรกิจทั่วไป ได้ทั้งจากสาขาที่ ON LINE และตู้อัตโนมัติของบริการอิเล็กทรอนิกส์ในรูปแบบต่างๆ ได้แก่ บริการเงินด่วน ๒๔ ชม. บริการธนาคารทางโทรศัพท์ (TELE-BANKING) บริการโทรเงินสด (TELECASH)

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