CHAPTER II

MATERIALS AND METHODS

MATERIALS

1. Equipment

Equipment/Model	Company/Country
pH meter PHM 83	Radio, Copenhagen; Denmark
Autoclave HA-3D	Hirayama Manufacturing Cooperation, Japan
Laminar Flow BVT-124	International Scientific Supply, Co. Ltd. Thailand
Microscope	Olympus, U.S.A.
Digital Lux Meter FT 710	Taiwan
Spectrophotometer DU 650	Beckman, U.S.A.
Illuminated/Refrigerated Orbital Incubator 101400:XX2C	Sanyo, England.
Water bath Buchi 461	Switzerland.
Centrifuge H-103 N Series	Kokusan.
Siliga Gel Plates	LK5D silica gel 150 A°, Whatman; U.S.A.

Equipment/Model	Company/Country
Rotary Shaker	New Brunswick Scientific Edison, N.J. : U.S.A.
Fraction Collector Model 2211	Pharmacia LKB, Sweden.
Micropipette	Gilson, France
Scintillation Counter	Pharmacia LKB Wallac, Rackbeta 1218; England
Ultracentrifuge	Beckman, U.S.A.

2. Chemicals

Chemicals	Company/Country
Calcium chloride	Merck Ag Darmstadt, Germany.
Magnesium sulfate	
Magnesium chloride	
Potassium chloride	
Acrylamide	
Ferric sulfate	Mallinckrodt Chemical, U.S.A.
Tritron x-100	Packard, U.S.A.
Chloroform	England
Glycine betaine	Sigma, U.S.A.
Betaine aldehyde chloride	
Choline chloride	
Dowex 50W (50x4-200, H^+ form)	
(50-100 mesh, 2% cross-linkage)	
DTT (Dithiothreitol)	
EDTA (Ethylenediaminetetraacetic acid)	
Ethylene Dichloride (1,2-dichloroethane)	
Lysozyme	
Nitro Blue Tetrazolium	
Phenazine Methosulfate	
Coomassie Brilliant Blue	BDH Laboratory Chemical Ltd.

3. Specimen

Aphanothece halophytica was isolated from marine lake in Israel. The organism was kindly provided by Dr. T. Takabe of Nagaya University, Japan.

METHODS

1. Separation of quaternary ammonium compounds.

The quaternary ammonium compounds namely choline, betaine aldehyde, and glycine betaine were separated by ion-exchange chromatography. The mixture of 50 mM each of these quaternary ammonium compounds in 0.5 ml H_2O containing 0.3M sodium bisulfite was supplied to a column (0.9 by 1.3 cm) packed with Dowex 50W (50x4-200, H^+ form, Sigma). The glycine betaine aldehyde-bisulfite addition product was eluted with 15 ml of 0.2 M sodium bisulfite, glycine betaine was eluted with 15 ml of 2 M NH_3 (Lanfald and Strom 1986), and then choline was eluted by 5 ml of 2 M HCl. The total eluate was allowed to dry by lyophilization, and then the dry pellet was suspended in 0.5 ml of H_2O. The eluted quaternary ammonium compounds were checked by spotting them a thin- layer 20x20 cm silica- gel plate (Whatman company). The sample and authentic commercial compounds [50mM each of choline, betaine aldehyde, and glycine betaine from Sigma as standards were dropped onto the layer a little at a time and dried between each application. The spot must be completely dry before the plate is put into the solvent. The plate was later developed in a mixture containing chloroform / methanol / 0.1 M HCl (65 : 30 : 4, v/v) for 1.5 h. Markers of choline, betaine aldehyde, and glycine betaine were identified by visualization with Dragendorff' s reagent (Appendix 2). This method also provided a means of assessing the efficiency of separation for choline, betaine aldehyde, and glycine betaine with ion- exchange chromatography.

2. Efficiency of separation of quaternary ammonium compounds by ion-exchange column

A 200 ml sample of exponentially grown culture of *A. halophytica* (Figure 6) was centrifuged at 2,000 g for 10 min. The cell pellet was suspended in 0.5 ml of 50 mM Hepes- NaOH, pH 7.5 containing 0.5 M NaCl followed by the addition of 1 μCi [methyl-^{14}C] choline (55mCi/mmol). The suspension was incubated at room temperature, 60 min with continuous shaking. The reaction was stopped by addition of 1 ml methanol and then centrifuged at 2,000 g for 5 min. The pellet was washed twice with 0.5 M NaCl solution before extraction with 5 ml boiling 80% ethanol for 5 min and re-extracted with 5 ml 80% ethanol at room temperature for 18 h. The suspension was centrifuged at 2,000 g for 5 min. The total supernatant was dried by oven at 65°C. The radioactive quaternary ammonium compounds present in the pellet were isolated by ion-exchange column. The dry pellet was suspended in 0.5 ml of H_2O containing 0.3M sodium bisulfite, and 1 mM each of unlabed choline, betaine aldehyde and glycine betaine. The reaction mixture was applied to a column (0.9 by 1.3 cm) packed with Dowex 50W (50x4-200, H^+ form, Sigma.). The labeled betaine aldehyde-bisulfite, labeled glycine betaine, and labeled choline was sequentially eluted as described in 1. The total radioactivity of quaternary ammonium compounds was determined by liquid scintillation counting.

3. Time courses of [methyl-^{14}C] choline oxidation

Ten percents inoculum of *Aphanothece halophytica* was inoculated into a 250 ml flask containing 100 ml of Turk Island Salt Solution + modified BG_{11} medium (see Appendix 1, Thongekkaw 1995) with 0.5 M NaCl, the pH of the medium was adjusted to 7.6 and grown on a rotary shaker with 160 rpm at 30°C, 2,000 lux of continuous illumination. After 10 days, 10 ml culture was inoculated into the same

Figure 6 Culture of *Aphanothece halophytica* grown in Turk Island Salt Solution + modified BG_{11} medium at day 14 in a 250 ml flask

medium containing either 0.5 M NaCl or 2.0 M NaCl and shaken on a rotary shaker (160 rpm) at 30°C with 2,000 lux of continuous illumination. After 3 days, the culture was centrifuged at 2,000 g for 10 min and the cell pellet was suspended in 0.5 ml of 50 mM Hepes- NaOH, pH 7.5 containing either 0.5 M NaCl or 2.0 M NaCl followed by the addition of 1 μCi [methyl-^{14}C] choline (55mCi/ mmol). At indicated time intervals (0min, 30 min, 1 h, and 3 h) the reaction was stopped by the addition of 1 ml of methanol and then centrifuged at 2,000 g for 5 min. The pellet was washed twice with 0.5 or 2.0 M NaCl solution. The pellet was extracted with 5 ml boiling 80% ethanol for 5 min and re-extracted with 5 ml 80% ethanol at room temperature for 18 h before centrifugation at 2,000 g for 5 min. The total supernatant was dried by oven at 65°C. The radioactive quaternary ammonium compounds present in the pellet was isolated by ion-exchange chromatography. The dry pellet was suspended in 0.5 ml of H_2O containing 0.3M sodium bisulfite, and 1 mM each of unlabeled choline, betaine aldehyde and glycine betaine. The reaction mixture was applied to a column (0.9 by 1.3 cm) packed with Dowex 50W (50x4-200, H^+ form, Sigma.). The radioactive betaine aldehyde-bisulfite, labeled glycine betaine, and labeled choline were eluted as described in 1. The radioactivity of quaternary ammonium compounds was determined by liquid scintillation counting.

4. Formation of ^{14}C-glycine betaine from various precursors.

A. halophytica was grown in Turk Island Salt Solution + modified BG_{11} medium containing 0.5 M NaCl, the pH of the medium was adjusted to 7.6. At 10 days, 10 ml culture was inoculated into fresh medium containing either 0.5 M NaCl or 2.0 M NaCl and shaken on a rotary shaker (160 rpm) at 30°C with 2,000 lux of continuous illumination. After 3 days, the culture was centrifuged at 2,000 g for 10 min and the cell pellet was suspended in 0.5 ml of 50 mM Hepes- NaOH, pH 7.5 containing either 0.5 M

NaCl or 2.0 M NaCl followed by the addition of 1 μCi {[methyl-^{14}C] choline (55mCi/ mmol), [2-^{14}C] ethanolamine (57mCi/ mmol), or [1-^{14}C] glycine (55mCi/ mmol). At indicated time intervals (0min, 30 min, 1 h, and 3 h) the reaction was stopped by the addition of 1 ml of methanol and then centrifuged at 2,000 g for 5 min. The pellet was washed twice with 0.5 or 2.0 M NaCl solution. The pellet was extracted by 80% ethanol, as described in 2. And after the total supernatant was dried by oven at 65°C, the radioactive glycine betaine was isolated from other quaternary ammonium compounds by ion-exchange chromatography. The dry pellet was suspended in 0.5 ml of H_2O containing 0.3M sodium bisulfite, and 1 mM unlabeled glycine betaine. The reaction mixture was applied to a column (0.9 by 1.3 cm) packed with Dowex 50W (50x4-200, H^+ form, Sigma.). The radioactive glycine betaine was eluted by 15 ml of 2M NH_3. The radioactivity of glycine betaine was determined by liquid scintillation counting.

5. ^{14}C-Glycine betaine biosynthesis and glycine betaine accumulation after various periods of stress

5.1 Determination of glycine betaine accumulation

A. halophytica was grown in the medium as described in 3. The concentration of NaCl in Turk Island Salt Solution + modified BG_{11} medium was either 0.5 M NaCl or 2.0 M NaCl. At time intervals (0,2,4,6 days), the culture was harvested at 2,000 g for 10 min. The pellet was extracted by 5 ml of 80 % ethanol at 100°C for 5 min. The suspension was then centrifuged at 2,000g for 5min and extracted again at room temperature for 18 h before centrifugation at the same rate. The total supernatant was allowed to dry by oven at 65°C before dissolving in 1 ml of H_2O. Glycine betaine was separated from other quaternary ammonium compounds by ion exchange chromatography Dowex 50W (50x4-200, H^+ form, Sigma). After washing the column with H_2O, glycine betaine was eluted by 15 ml of 2 M NH_3. The eluate was dried by

lyophilization before dissolving in 1 ml of H_2O. Glycine betaine was determined by tri-iodide assay (Storey and Wyn Jones, 1977). The solution was added with 0.2 ml of the acid potassium triiodide solution (dissolving 7.5 g I_2 and 10 g KI in 1M HCl and filtering). The mixture was shaken and left for at least 90 min in an ice bath with intermittent shaking. Two ml of ice- cooled H_2O was added rapidly to the mixture to reduce the absorbance of the blank and improve replication. This was quickly followed by 5 ml of cold 1,2 - dichloroethane, and the 2 layers mixed by vortex. The absorbance of the lower organic layer was measured at 365 nm by spectrophotometer. The quantitation of glycine betaine was obtained by comparing OD at 365 nm against standard curve (using commercial glycine betaine hydrochloride (Sigma) as a standard, Appendix 5).

5.2 Determination of ^{14}C-glycine betaine biosynthesis

The cells of *A. halophytica* as described in 5.1 were harvested at 2,000 g for 10 min. The pellet was suspended in 0.5 ml of Hepes- NaOH, pH 7.5 containing either 0.5 M NaCl or 2.0 M NaCl followed by the addition of 1 μCi of [methyl-^{14}C] choline (55 mCi/ mmol) and allowed to incubated at room temperature. After 3 h, the reaction was stopped by 1 ml of methanol and then centrifuged at 2,000g for 5 min. The pellet was washed, and extracted as described in 3. The pooled supernatant was dried by oven at 65°C. The [^{14}C] glycine betaine was separated from other radioactive quaternary ammonium compounds by Dowex 50W (50x4-200, H^+ form) as described in 4.1. Radioactivity of [^{14}C] glycine betaine was determined by liquid scintillation counting.

6. Disruption and centrifugal fractionation of cells.

A. halophytica was grown in Turk Island Salt Solution + modified BG_{11} medium containing either 0.5 or 2.5 M NaCl , the pH of the medium was adjusted to 7.6. A 100 ml culture in 250 ml flask was shaken on a rotary shaker (160 rpm) at 30°C with

2,000 lux of continuous white light illumination. At 10 days,cells (3.6 g and 3.8 g wet weight for 0.5 M and 2.0 M NaCl, respectively) were suspended into 10.8 ml and 11.4 ml (in 0.5 M and 2.0 M NaCl samples, respectively) of grinding buffer (50 mM Hepes-NaOH, pH 7.5 containing 10% glycerol, 10 mM EDTA and 5 mM DTT) containing 2mg/ml lysozyme and stirred at 4°C for 90 min. After lysozyme treatment, the mixture was centrifuged at 33,000 g, 20 min to remove whole cells and large fragments. The supernatant was then centrifuged at 180,000 g for 60 min. The high-speed supernatant was collected and the pellet (membrane fraction) was washed once by suspending it in the grinding buffer and centrifuging at 180,000 g for 60 min. The final pellet was resuspended in the same buffer.

Solubilized membranes were obtained by dissolving the final membrane pellet in grinding buffer that also contained 0.3 % Triton x-100 (Lanfald and Strom 1986). The solution was stirred for 60 min at 4°C and the undissolved material removed by centrifugation (33,000 g for 20 min). The solution (cytoplasmic and membrane fraction) was assayed for the content of protein and choline dehydrogenase activity.

7. Determination of Protein

Protein content was determined by Bradford' s method (Bradford, 1976). The protein sample (maximum 100 µl) was aliquoted into a tube and distilled H_2O was added to make a total volume of 100 µl. This was followed by 1 ml of Bradford working solution. The mixture was mixed well by vortex. Absorbance at 595 nm was read after 2 min but before 1 h. The standard curve was constructed using 1mg/ml bovine serum albumin as protein sample.

The compositions of Bradford stock solution and Bradford working solution are as follows:

Bradford stock solution : 100 ml 95% ethanol

200 ml 85% phosphoric acid

350 mg Serva Blue G

Stable indefinitely at room temperature.

Bradford working solution : 425 ml distilled water

15 ml 95% ethanol

30 ml 85% phosphoric acid

30 ml Bradford Stock Solution

Filter through Whatman No. 1 paper, store at room temperature in brown glass bottle. Usable for several weeks, but may need to be refiltered.

8. Choline dehydrogenase assay

The activity of choline dehydrogenase was assayed by the spectrophotometric determination of phenazine-linked reduction of cytochrome *c* at 550 nm. The reaction mixture (1 ml) contained : 20 mM Hepes-NaOH buffer, pH 7.4, 20 mM choline chloride, 0.33 mM phenazine methosulfate and 50 µM cytochrome *c*. The enzyme sample was added, and the increase in absorption at 550 nm was recorded. Activity is expressed in units of µmol choline oxidized per min at 25°C, using an extinction coefficient of 20.5 cm^{-1} mM^{-1} (Fan and Master, 1974) for the difference between reduced and oxidized cytochrome *c*, and dividing by 2 to allow for the single electron reduction of the cytochrome.

9. Polyacrylamide gel electrophoresis (PAGE)

9.1 Non-denaturing PAGE

Discontinuous PAGE was performed on slab gels (10x8x0.75 cm), consisting of 7.5%(w/v) separating gel, and 5%(w/v) stacking gel. Tris-glycine buffer pH

8.3 was used as electrode buffer (see Appendix 4). The sample was treated with sample buffer (see Appendix 4) at 4:1 (sample: sample buffer) ratio. The electrophoresis was run from cathode toward the anode at constant current of 20 mA per slab in a Midget LKB 2001 electrophoresis. The temperature was controlled at 4°C by LKB 2209 Multi-temperature thermostat water bath. The protein bands on the gel were stained with staining solution and destained with destaining solution.

9.2 Detection of proteins in slab gel by coomassie blue staining

After electrophoresis, proteins in the gel were stained by coomassie blue. The gel was immersed in 0.2%(w/v) of coomassie brilliant blue R-250 containing 45% (v/v) methanol and 10%(v/v) acetic acid for at least 20 min on slow shaker. It was then destained with a solution of 10% methanol and 10%(v/v) acetic acid for 1-2 hr, followed by several changes of destaining solution until background of the gel was clear.

9.3 Choline dehydrogenase activity staining

After non-denaturing polyacrylamide gel electrophoresis, the gel was stained for activity by incubating the gel in a solution containing 50 mM choline chloride/ 1 mM phenazine methosulfate/ 1mg/ml nitroblue tetrazolium/ 50 mM Hepes-NaOH, pH 7.5 at 37°C for 30 min in the dark. The presence of choline dehydrogenase activity was confirmed by the appearance of brown colour band.