CHAPTER II

EFFECTS OF VARYING, EXTERNAL CONCENTRATIONS OF SODIUM CHLORIDE ON CERTAIN ENZYME ACTIVITIES OF P. AMBIGUUM

INTRODUCTION ... ... ... ... 21

MATERIAL AND METHODS ... ... ... 26

RESULTS ... ... ... ... 32

DISCUSSION ... ... ... ... 33
INTRODUCTION

It is true that much is known about the structure, action and specificity of enzymes in blue-green algae (Lewin, 1962; Carr and Whitton, 1973; Stewart, 1974), although looking at the progress made in this branch for higher eucaryotic plants, a lot remains to be desired. This is especially the case as far as the effects of salinity are concerned. In the present study concerning a terrestrial cyanophyte *P. ambiguum*, two oxidative enzymes—Peroxidase* and Catalase**—and two hydrolysing enzymes—Protease* and RNase**—were studied vis-a-vis NaCl-salinity.

Peroxidase occurs in nearly all plant cells. It is an iron-porphyrin enzyme which by now has been known to be involved in several aspects of plant growth and metabolism. Rubin and Ivanova (1963; c.f. Gagnon, 1968) showed that peroxidase can provide an alternate route for oxidation of reduced nicotinamide adenine dinucleotide. In addition to the classical function of protecting an organism from harmful effects of hydrogen peroxide, peroxidase—H$_2$O$_2$ system has been known to

* E.C. 1.11.1.7
** E.C. 1.11.1.6
* + E.C. 3.4.4.-
** + E.C. 3.1.4.1.

Enzyme numbering system has been taken from Barman (1969).
oxidise a wide variety of biologically occurring compounds in the presence of manganese vis. flavoproteins (Galston et al., 1953), butyric acids (Kentcon, 1955) and pyridine nucleotides (Akazawa and Conn, 1956). Siegel (1955) has pointed out the broad hydrogen-donor specificity of plant peroxidases. Alexander (1964) has put forward peroxidase as a constituent of terminal oxidation. Altman et al. (1966) observed a positive relationship between respiration rate, peroxidase and catalase in citrus roots. Siegel and Galston (1967) have ascribed a significant role for peroxidase in the regulation of cell growth and differentiation.

Catalase was first designated as an enzyme that destroys.

\[ \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

The enzyme is characterised by the fact that it uses \( \text{H}_2\text{O}_2 \) both, as a donor of hydrogen and as a substrate in its catalytic decomposition, to form \( \text{O}_2 \) and \( \text{H}_2\text{O} \). In addition, Bjorn (1967) has observed that catalase can affect the peroxidative oxidation of a variety of organic donors in the same fashion as peroxidases (alcohols, aldehydes, phenols etc). A high level of catalase and a low level of peroxidase activity was related to a high rate of protein synthesis (Verma and Huystee, 1970).

* c.f. Khatri, 1979
Both protease and ribonuclease (RNase) are hydrolytic enzymes effecting breakdown of proteins and ribonucleic acids. The end product of the hydrolysis of proteins is always a mixture of amino acids. Tazawa and Miwa (1953; c.f. Patel, 1974) have shown that gelatine was hydrolysed by the extracts of Porphyra and Eisenia species. Wilson, who has done extensive work on RNase of corn (1963 a, b; 1968) has suggested two types of RNases- RNase A releases oligonucleotides as early products plus 2', 3'-cyclic nucleotides which increase as the digestion time is increased; RNase 'B' release all 4', 5'-nucleotides and other A₂₆₀-absorbing materials.

In higher plants, views differ as to the salinity effects on enzyme activities. Porath and Poljakoff-Mayber (1968) reported increased activity of oxidative enzymes in higher plants and algae vis-a-vis salinity. However, Weimberg (1970) and Greenway and Osmond (1972) found no changes in the levels of enzymes. Peroxidase activity has been observed to be inhibited by NaCl (El Pouly and Jung, 1972)
and stimulated by Strogonov et al. (1956). El Fouly and Jung (1972) also reported a stimulation of catalase activity by NaCl, a view quite opposite to that of Bhardwaj (1964). Algal peroxidases in relation to their anomalous substrate specificities have been studied by Siegel and Siegel (1970). Behaviour of catalase in some members of chlorophyceae and cyanophyceae has been reported by Awasthi (1967) and Chua (1971). Catalase activity in thermal blue-green algae in relation to temperature has been reported by Kubin (1959).

Miller (1959) has found that filtrates of alga Monodina subterranea filtrates has a fairly good activity of protease. O'Heocha and Curley (1961) showed that proteolytic enzyme was found to be most active in extracts of cells of a marine alga. Another hydrolytic enzyme, RNase has been found at very low levels in blue-green algae when compared to sea weeds (Norton and Roth, 1967 a); they have (1967 b) partially purified RNase from Anacystis nidulans. However, salinity effects on these hydrolytic enzymes have not been widely reported. Prisco and Vieira (1976) did not observe any change in proteolytic activity of bean seedlings grown in NaCl media. Chino et al. (1964-69) observed increased protease activity in barley plants under continuous water stress. Lazar et al. (1973) observed an increase in RNase activity in protoplasts of tobacco leaves. Sheoran and Garg (1978) observed inhibition of RNase in cotyledons and roots of mung bean seedlings.

It becomes apparent from the foregoing account, that while there are scanty and conflicting reports for enzymes of higher plants as affected by NaCl, similar studies for blue-green algae are still rarer. Moreover, a study of some enzymes (within the limits imposed by the availability of facilities and chemicals) was considered interesting in view of the intriguing results of a study on protein metabolism (Chapter IV). This in view of an attempt has been made to understand certain enzyme activities in the cell-extracts of *E. aabigiuuta* grown in media with varying concentrations of sodium chloride.
P. ambiguum was grown under varying concentrations of NaCl as detailed in Chapter I and harvested on 18th day. Extraction of enzymes and their assays were carried out at 4°C unless otherwise mentioned.

Peroxidase activity was determined by the methods of Chance and Maehly (1955). Algal sample was homogenised with the help of mortar, pestle and acid-washed, sterilised sand, using phosphate buffer 0.15 M, pH 7.0 as extraction medium.

The supernatant after centrifugation served as a source of enzyme. The reaction mixture was prepared by adding 2.0 ml phosphate buffer, 1.0 ml of 20 mM aqueous pyrogallol (substituting for guaiacol which gave negative reaction) and 1.0 ml of enzyme extract. Blank was run by preparing the reaction mixture in the above manner except that the enzyme was excluded and replaced by 1.0 ml of buffer. Later, 0.02 ml of 10 vol. H₂O₂ was added to the reaction mixture and blank and colour was allowed to develop at room temperature. At the end of 10 min.-period, optical densities of both, reaction mixture and blank were read at 470 nm on a Systronic photoelectric colorimeter. The difference in O.D. readings
between the two gave the actual peroxidase activity; the results were calculated further and the enzyme activity was expressed as O.D./min./mg. protein (Table 4; Plate III, Fig. 2). Enzyme protein in the same extract was quantified following the method of Lowry et al. (1951).

Catalase activity was determined by the manometric method of Chance and Maehly (1955). Algal sample was homogenised with 1.0 gm of CaCO₃ using phosphate buffer, 0.1 M, pH 7.0 and the supernatant served as a source of enzyme. Known volume of the supernatant was placed in a bottle. A small tube, containing 5.0 ml of 10 volume H₂O₂ was carefully placed in the same bottle avoiding any mixing of the two solutions. The bottle was corked and connected to a manometer; later, it was placed on an automatic shaker to allow the two solutions to mix completely. The resultant evolution of oxygen was reflected on the water level in the manometer. The time taken for the evolution of 2.0 ml of oxygen was recorded. Enzyme protein in the same extract was quantified following the method of Lowry et al. (1951). Enzyme activity was expressed as ml of O₂ evolved/min./mg protein (Table 4; Plate III, Fig. 2).
Protease activity was assayed employing a combination of methodologies of Penner and Ashton (1967) and Cruz et al. (1970). Algal sample was homogenised using 0.01 M phosphate buffer, pH 7.0 as extraction medium. The supernatant after centrifugation served as a source of enzyme.

1.0 ml. of enzyme aliquot was added to 1.0 ml of 0.5% casein dissolved in the phosphate buffer, 0.01 M, pH 7.0 (w/v). The mixture was left to incubate for 2 hours at 40°C. The same mixture with buffer substituting for enzyme extract served as control. At the end of the incubation period, 1.0 ml of 15% Trichloroacetic acid (w/v) was added to the mixture to stop enzyme reaction and precipitate out excess casein. After 20 minutes, the contents were centrifuged and clear supernatant obtained. A portion was diluted four-fold and read at 280 nm in a Beckman Du-2 spectrophotometer. Enzyme protein in the same extract was quantified following the method of Lowry et al. (1951). Protease activity was expressed as O.D. (280 nm)/min/mg/protein. (Table 4; Plate VI, Fig. 1).
Two methodologies were employed to detect RNase activity in the extracts of *P. ambiguum*. The method of Tanaka (1961) which he followed for a fungus includes the use of 0.5 ml of 6 mg/ml (w/v) yeast RNA solution as substrate, 0.5 ml of 0.2 M phosphate buffer, pH 1.0 as extraction medium and 0.2 ml of 0.75 % (w/v) uranyl acetate in 25 % (v/v) perchloric acid as an inhibitor of RNase activity; optical density readings were taken at 260 nm, in a Beckman DU-2 Spectrophotometer. Another method utilised was that of Brown and Marshall (1977) followed while studying properties of partially purified RNase activity in *Chlorella*. Sodium citrate buffer, 0.1 M, pH 5.2 was used as an extraction medium and 1.0 % yeast RNA solution (w/v) as the substrate. After incubation, 0.2 ml of 2.5 M cold perchloric acid served as an inhibitor of enzyme activity. However, both these methods and their modifications failed to detect any RNase activity in the extracts of *P. ambiguum*. The method of Tanaka (1961) has been successfully employed for *Anabaena* sp. by Wahal et al. (1974). Attempts to achieve results by modifying molarities and hydrogen ion concentrations of buffers and incubation temperatures and periods proved futile. This was the case even when an excess quantity of algal material was taken for extraction. It was therefore concluded
that RNase activity in this case was undetectable and possibly, absent.

The experiments for assaying enzyme activities in extracts of *P. ambiguus* were repeated several times to study trends. It was only after confirming a particular trend, that an ideal case was selected for discussion in the current study.

Algal proteins were measured by the method of Lowry et al. (1951). The final colour is a result of biuret reaction of proteins with copper ion in alkali and reduction of phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophane present in the treated protein. This colour is photocolorimetrically measured to determine the protein content.

Reagents:

A. 2.0 % Na$_2$CO$_3$ in 0.1 N NaOH (w/v)
B. 0.5 gm CuSO$_4$ $\cdot$ 5H$_2$O in 1.0 % Na or K-tartarate (w/v)
C. Alkaline CuSO$_4$ solution, i.e. 50 ml of reagent (A) + 1.0 ml of reagent (B)
D. Folin-reagent

$3.0$ N CENTRON (INDIA) MAKE DILUTED TO 1.0 N

Protein standard:

10.0 mg of casein was dissolved in 100 ml of 0.1 N NaOH. From this different concentrations ranging from 0.01 mg/ml - 0.09 mg/ml were prepared (10 ug/ml-90 ug/ml).
Procedure:

Different aliquotes of standard protein solution (Casein—containing 0.01 mg-0.09 mg/ml) and 5.0 ml of the reagent 'C' were added in the test tube, mixed and allowed to stand for 10 min at room temperature. 0.5 ml of 1.0 N of Folin reagent D was added rapidly. After 30 minutes the optical density was read on a Systronic Photoelectric Colorimeter at 660 nm. The procedure was repeated in triplicate for each casein concentration. Standard curve of casein was plotted based on the following regression formula (Plate III, Fig. 1).

\[ y = 0.00242 + (0.3189 \times x) \]

where, \( y \) = concentration of protein
\( x = \text{O.D. (660 nm)}. \)  

(Plate III)

To calculate enzyme protein, 0.9 ml of enzyme source (algal homogenate) was mixed with 0.1 ml of 1.0 N NaOH. 5.0 ml of reagent 'C' were added to the mixture, mixed and allowed to stand for 10 mins. 0.5 ml of 1.0 N Folin reagent 'D' was added rapidly. After 30 minutes, the optical density was read at 660 nm. Enzyme protein (soluble) in 1.0 ml of algal homogenate was the calculated using the above regression formula. The experiment was repeated in triplicate and the final protein value was used to obtain the units of enzyme activity (e.g. O.D./min./mg protein).
RESULTS

Table 4 presents the data of the enzyme activities in *P. ambiguum* as affected by NaCl concentrations. These values have been represented on Plate III and Plate VI, Fig. 1.

Peroxidase activity increased from 0.05 units in cells of control to 0.12 units in those grown in 0.5% NaCl. The activity was only slightly less in cells grown in 1.0% NaCl but was considerably reduced in those of 2.0% NaCl (0.013 units), (Plate III).

Catalase activity showed increasing tendency throughout. At 2.0% NaCl level, the activity was nearly three times (0.144 units) that of control (0.153 units), (Plate III).

Proteolytic activity doubled in cells grown in saline media (when compared to those in control), (Plate VI).

RNase activity could not be detected. The possibility of it being absent in this particular organism is not ruled out.
As far as the oxidising enzymes and salinity effects are concerned, there is not much to draw upon from the past work carried out on algae in general. Siegel and Siegel (1970) reported their evaluation of substrate specificity among algal peroxidases. Murphy and O'Hoobha (1973) have also given some details of peroxidase in a green and a red alga. Wahal et al. (1973) noted the presence of peroxidase activity in the heterocysts of *Anabaena cylindrica*. Much of the work done on *Chlorella* has been carried out by Trubachev (1968) and Gurevich and Elina (1972) for peroxidase and special peroxidase. Patel (1974) noted peroxidase activity in several green and blue-green algae. Effect of varying salinity on peroxidase of *Chroococcus minutus* was reported by Khatri (1979) who observed a depression in its activity with an increase in salinity as opposed to an increase reported here. A reference to the work on algal catalase has been made in the relevant paragraph.

A positive correlation has been known to exist between respiration rate and activities of oxidative enzymes peroxidase and catalase (Altman et al., 1966). While studying peroxidase and catalase activities in wheat seedlings grown in NaCl media, El Fouly and Jung (1972) stated that high activities of these terminal oxidases is in agreement with
the earlier report of increased respiration rates in some crop plants grown in NaCl media (Nieman, 1962). In the present study, peroxidase and catalase generally increased under salt stress. This is indicative of increased respiration under abnormal environmental conditions which the alga survives; a similar can be drawn here with the situation in diseased plants - that infections on plants by fungi and other pathogens alter the metabolism of host plants, especially the oxidising enzymes (Agarios, 1969).

To a plant peroxidase can be attributed many functions as discussed earlier. In the present study, one correlation is very superficially seen; it is between increased peroxidase activity and faster relative growth rates (RGR) of cells of *P. ambiguum* under salt stress in general (Chapter I). In higher plants, peroxidase activity arises when active cell differentiation is going on (Galston, 1951). However, when examined closely, peroxidase activity in the present study during harvest-time, 18th day) is highest in the cells grown in 0.5 % NaCl (Table 4) whose RGR is actually slower than cells in 1.0 % NaCl (Plate 1, Fig. 2); again, in cells grown in 2.0 % NaCl, the RGR value is more than both, cells in control as well as 0.5 % NaCl whereas peroxidase activity is at its lowest (Table 4; Plate III). It therefore seems that the statement of Galston can hold true for the results of the present study only if a very generalised view of
non-saline and saline conditions is taken vis-a-vis growth rates and enzyme activity. Otherwise, initial stimulation of peroxidase in the present investigation and later suppression confirms the observation of Todd (1972) that milder stress stimulates some enzyme activities but a severe one suppresses the same. Weinberg (1970) found increases in peroxidase activity in pea seedlings affected with NaCl; however, El-Fouly and Jung (1972) observed decreased activity in wheat seedlings grown in saline media.

Enzyme catalase occurs in peroxisomes of leaves which account for the oxidation of glycollate, the associated O₂ requirement for photorespiration and the consequent production of hydrogen peroxide. In some green algae, production of H₂O₂ by glycollate dehydrogenase did not occur (Nelson and Tolbert, 1970) and catalase was about 10% of that in higher plants; this relatively low algal catalase was still considered an active system by Stewart (1974). In blue-green algae however, H₂O₂ production is widespread as reported by Stevens et al. (1973) who surveyed 36 axenic isolates of this group. In *D. ambiguum*, presence of hydrogen peroxide has already been indicated by the presence of peroxidase activity. While catalase activity in the present study was observed to be fairly good, the same was not observed in another species of *Phormidium* (Harvey, 1924; c.f. Awasthi, 1967); Kubin (1959) noted its presence in some
thermophilic blue-green algae. Awasthi (1967) hinted at a close catalase-chlorophyll relationship while studying the behaviour of catalase in some members of Chlorophyceae and Cyanophyceae. Catalase in *P. ambiguum* was not only an active system but was also affected markedly by shifts in the external levels of salinity. A sharp increase in catalase activity (Table 4; Plate III) in cells throughout the NaCl regime seems to indicate a necessity to destroy increased \( \text{H}_2\text{O}_2 \) levels and thus, increased toxicity, due to salt stress. Welsberg (1970) also observed an increase of up to 350% in catalase activity of salinity-affected wheat seedlings; a similar trend was observed by El-Fouly and Jung (1972). There is however no definite information as to the site of \( \text{H}_2\text{O}_2 \) production in blue-green algae. Patterson and Myers (1973) concluded that it was due to photosynthetic generation of excess reductant, the production site being probably on the reducing side of photosystem I. However, the dark production of \( \text{H}_2\text{O}_2 \) by some blue-green algae observed by Stevens et al. (1973) indicates an origin of \( \text{H}_2\text{O}_2 \), not directly linked with photosynthesis.

In view of the deleterious effects of NaCl on the ribonucleic acids of *P. ambiguum* observed in Chapter IV (Plate VI), it was thought interesting to have a check on enzyme R\( \text{N} \)ase; its activity however was not detected. Earlier, Horton and Roth (1967 a) have observed very low levels of R\( \text{N} \)ase activity in
some non-marine algae when compared to sea weeds; they later purified an RNase from *Anacystis nidulans* (1967 b). Kessler and Tishel (1962) found increased levels of ribonuclease in some drought resistant plants grown under water stress. Dove (1967) also got similar results. Sheoran and Garg (1978) and Patel (1979) however reported depressed RNase activity in plant parts grown in saline media and under water stress (osmotic) respectively.

Proteolytic activity in cells of *E. ambiguum* more than doubled under NaCl stress (Table 4; Plate VI, Fig. 1). In cells of marine algae, protease activity was found to be most active (O’Hwhelock and Curley, 1961 c.f. Lewin, 1962). Chinoy et al. (1964-69) observed increased protease activity in barley plants under water stress. Prisco and Vieira (1976) did not observe any effects of NaCl on proteolytic activity of bean seedlings.

Increased protease activity under salt stress in the present context has its own significance. More and more protein breakdown occurs under stress making more and more amino acids available to remain in free pool, to be incorporated into proteins or to be made available for other physiological processes e.g. respiration; this should result in reduction in the content of total protein which in fact has been observed (Chapter IV). However, it’s best not to view this increased proteolytic activity in isolation; an attempt has
been made later to study this vis-a-vis protein synthesis and protein turnover.

As for the enzymes in general and salinity effects, most reports, including the present one, seem to be at variance with each other - at least to a certain extent. In the present investigation, only a preliminary screening of some enzymes was carried out; the kinetics of their activities could not be studied. It is apparent that more sophistication is required to further study some more enzymes, specially those of metabolic pathways, to add to our meagre knowledge of salinity effects on enzymes of blue-green algae.