CHAPTER IV

PROTEIN METABOLISM AND AMINO ACID STATUS IN P. AMBIGUUM

AS AFFECTED BY SODIUM CHLORIDE SALINITY

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Salinity and other osmotica-induced water stress are known to affect protein-synthesising machinery like ribosomes and nucleic acids of higher plants. According to Gates and Bonner (1959), water stress affected adversely, the RNA metabolism of tomato leaves. West (1962) studied the protein, nucleotide and RNA metabolism in corn during germination under water stress. According to Kessler et al. (1964; c.f. Sheoran and Garg, 1978) salinity treatment strongly suppresses the RNA and DNA content in a plant; the synthesis of these nucleic acids is also reduced (Nieman, 1965; Rauzer and Hanson, 1966). Results of these investigations have led to an idea that an environment may influence plant growth by inter-acting with genetic control at the level of ribonucleic acid (Shah and Loomis, 1965).

Algae have proved beyond biologists' expectations that they can be depended upon as rich sources of proteins and nutritionally important amino acids. It was the work of Bowden (1951) which established that the free amino acid pool in algae is composed of compounds
similar to those found in flowering plants. He observed that nearly all the amino acids normally occurring in proteins are present in free state in cells of *Chlorella vulgaris*, *C. pyrenoidosa* and *Anabaena cylindrica*. Later, Pillai (1957) observed that the composition of protein in both, young and mature algae, was the same and did not undergo any significant change during growth. A more extensive work was carried out by Lewis and Gonzalves (1960, 1962); their work pertained to the amino acids of marine algae.

Reports on amino acid metabolism of algae as affected by an environmental parameter are scanty. Powden (1952, 1954) and Champinghy (1957) reported differences in amino acid composition of proteins of *Chlorella pyrenoidosa*, depending upon whether the cells were grown on nitrate or urea. More recently, Gilles and Pequeux (1977) reported the effects of salinity on the free amino acid pool of a red alga, *Porphyridium purpureum*. However, as far as salinity is concerned, there is a conspicuous lack of detailed work on the amino acids of blue-green algae; this is especially so if one notes the similar work done on another group of procaryotes – the halophilic bacteria (Baxter, 1959; Brown, 1964; Reistad, 1970; Lanyi, 1974). In a marine blue-green alga *Spirulina maxima*, Clement et al
(1957) have observed a qualitative change in the amino acid composition of protein when compared with that of its non-halophilic counterpart. Tindall et al. (1977) have recently reported a comparative study of amino acids of an obligately halophilic blue-green alga, *Aphanotheca halophytica* and a marine and a fresh-water species of blue-green algae.

In sharp contrast to the work done on blue-green algae, salinity (or water stress) effects on higher plants have been widely reported. Saunier et al. (1968) reported increased amounts of glutamic acid and proline under stress. Barnard and Oakes (1970) considered proline as a potential source of energy in the root tips of *Zea mays* grown under saline stress. Hurit and Poljakoff-Mayber (1977) while studying proline content in roots of *Pisum* and *Tamarix* grown under saline conditions also came across increased levels of free proline as well as that in the protein-bound fraction. Huber et al. (1977) reported inhibition of protein synthesis and an increase in the hydrolysis products of proteins as a result of NaCl and abscisic acid while carrying out eco-physiological studies on Indian arid zone plants.
Several workers have reported a high protein content in some species of blue-green algae viz. a fresh-water form, *Anabaena cylindrica* (63%; Cobb and Hyres, 1964); a marine species, *Spirulina platensis* (65%; Hedenskog and Hofsten, 1970) and an obligate halophile, *Aphanotheca halophytica* (Tindall et al. 1977) which has the highest content of total protein (76% on dry weight basis) among the blue-green algae thus far reported.

However, the first report of the effects of salinity on the total protein of a blue-green alga, as has been rightly claimed by the authors, was published as recently as 1977 (Tindall et al., on *A. halophytica*). Khatri (1979) has also reported considerable shifts in the protein metabolism of *Chroococcus minutus* grown in varying concentrations of NaCl and KCl. In higher plants, studies involving salinity and protein content are not as meagre as those on cyanophytes. Weimberg (1970) reported reduction in the protein content of pea roots grown on media containing NaCl, KCl, Na₂SO₄ and K₂SO₄. Prisco and O'leary (1970) also came to similar conclusions for bean seedlings. Prisco and Vieira (1976) observed a delay in protein breakdown and turnover in *Vigna sinensis* grown under saline conditions.
For studying protein synthesis, the use of labelled amino acids, mainly leucine, has assumed quite an importance. Some literature, often of a mildly conflicting nature, exists pertaining to this aspect. Steward and Ridwell (1958) studied the incorporation of radioactivity into the proteins of cultured plant tissues; as a result, they proposed an existence of two fractions of proteins—one active and the other, inactive—of which the former showed increased synthesis as evidenced by increased incorporation of radioactivity. Ben-Zioni et al. (1967) observed a reduced uptake and incorporation of labelled leucine into proteins of *Nicotiana rustica* grown under saline stress, suggesting reduced protein synthesis. Khamene and Poljakoff-Mayber (1968) after conducting a similar experiment on the root tips of pea attributed the reduced uptake to changes in the permeability properties of a cell due to salinity. In contrast to these studies, increased incorporation of labelled nitrogen and leucine (and thus improved protein synthesis) in plant tissues under salinisation was reported by Hellel et al. (1975) and Shevyakov and Leonova (1975) respectively.

Against this background, it is clear that reports concerning effects of salt stress (as ionic and/or osmotic stress) on
RNA, amino acid and protein metabolism of blue-green algae are inadequate; a clear picture as to the total protein, protein synthesis and decay and amino acid status together as a single inter-linked physiological entity under salt stress is yet to emerge. This in view, it was thought worthwhile to study the protein and amino acid metabolism of a halotolerant, terrestrial cyanophyte, *Phormidium ambiguum* as affected by increasing concentrations of NaCl in the growth medium.
**MATERIAL AND METHODS**

*P. ambiguus* was grown in control, 0.5%, 1.0% and 2.0% NaCl as detailed in Chapter 1. RNA was extracted according to the method of Smillie and Krotov (1960). Algal cells were harvested on 18th day of growth and subjected to crushing in a mortar in cold methanol with the help of a pestle and acid-washed, sterilised sand. The resultant slurry was centrifuged and supernatant was discarded. The residual pellet was extracted with boiling 95% ethanol : ether, 2:1 for two minutes (over a water bath). The final residual pellet was hydrolysed in 5.0 ml of 0.3 N KOH for about 18 hours at 37°C. The hydrolysate was centrifuged; the supernatant now contained RNA and DNA. To this supernatant was added equal volume of a mixture of chloroform and amyl alcohol in the ratio of 24:1. The contents were well mixed and centrifuged in glass tubes till three distinct layers separated. The uppermost layer was cooled to less than 0°C and later, 5.0 ml of ethanol and a pinch of MgCl₂ were added. At the same temperature, perchloric acid was added to this mixture to adjust its pH to pH 2 and the contents were allowed to
stand at 0°C for 40 minutes. This process removed DNA and proteins. The precipitate was centrifuged-off and the supernatant now contained only RNA. To this was added 0.3 M KOH till the final pH was adjusted to 8.0 the mixture was made to volume.

RNA was estimated colorimetrically employing the orcinol method (Markham, 1955). Orcinol reagent was prepared by

freshly mixing 10 volume of 1.0 %
orcinol with 40 volume of conc. HCl and 1 volume of FeCl₃·6H₂O (10 %). 0.2 ml of the final solution (pH 8.0, mentioned above) containing pentose sugar was mixed with 2.0 ml of orcinol reagent, stoppered and heated for eight minutes on a water bath at 100°C and cooled. The solution was made to volume and was read for measurement of optical density in Systronic photoelectric colorimeter at 660 nm. Total RNA was then calculated using the following regression equation (P.16)

(Table 6, Plate VI, Fig. 3).

Standard curve was obtained using yeast RNA which was dissolved in distilled water adjusted to pH 8.0 with 0.3 M KOH. Known concentrations of RNA (0.01 - 0.15 mg) were
processed as above and the standard curve was obtained using the following regression equation:

\[ y = -0.0182 + (0.4226 \times x) \]

where \( y = \text{Conc. of RNA} \)
\( x = \text{O.D. at 660 nm} \) (Plate VI, Fig. 2)

Amino acids were extracted according to the method described by Lewis (1958).

Known quantity of algal material was harvested of 18th day (log-phase) of growth and homogenised in ground glass tissue homogeniser. 80% ethanol was added to the homogenised mass and the mixture was left overnight at 4°C. It was centrifuged next day and the alcoholic supernatant was collected; the residue was washed twice with cold, 80% ethanol. The supernatant was evaporated to dryness in vacuo. The residue was extracted with 1.0 ml of 10% iso-propanol. This formed the free amino acid pool.
The residue left after removing the alcoholic supernatant was hydrolysed; for the purpose, 15 ml of 6 N HCl was added to the residue and the mixture was transferred to sealing tubes. As a modification, the tubes were vacuum-sealed and the mixture was hydrolysed at 100°-105° C for 24 hours. The sealed tubes were then removed, cooled and the contents were centrifuged; the pellet was twice extracted with 80% ethanol and the supernatant was evaporated in vacuo. The residue was neutralised in a vacuum-dessicator contained NaOH by keeping the same in it for 24 hours; the neutralised residue was then extracted with 1.0 ml of 10% iso-propanol; this formed the protein-bound amino acid fraction.

Standard chromatographic techniques did not give satisfactory results as far as quantification of amino acids is concerned; those of the free pool were observed to be too less in quantity (very light ninhydrin reaction and only glutamic acid spot was strongly visible); and there was streaking of some spots; this was thought to be due to intracellular ions, probably of sodium and chlorine (of the external growth medium). The samples were therefore analysed on a Beckmann amino acid analyser (Courtesy: Uhabba Atomic Research Center, Bombay).
Amino acids were quantified according to the following procedure.

The equation which relates to the peak area ($H \times W$), concentration of amino acids ($n$ moles) and $HW$ constant is

$$C_{HW} = \frac{H \times W}{n \text{ moles}}$$

where, $H =$ Net height of the peak
$W =$ Width of the peak at half its height

and $C_{HW} =$ HW constant.

To determine the HW constants, 4 calibration runs of authentic amino acid mixture of known concentration (0.5 ml containing 0.25 $n$ moles of each amino acid in this experiment) were made. After calculating the net height and width of each amino acid peak, the standard $C_{HW}$ constants were calculated (as an average of such constants for 4 runs). These constants were used for calculating the concentration of the unknown amino acids of the algal samples using the same formula viz:

$$n \text{ moles of unknown amino acid} = \frac{H \times W}{C_{HW}}$$
where \( C_{am} \) is the constant for the authentic amino acid; this was converted to \( \mu \) moles/g dry wt. (Table 5 A, B)

The micro-kjeldahl methods given by Fruton and Simmonds (1960) and Baldwin (1963) for estimating total nitrogen were followed here with slight modifications. In a microkjeldahl flask was taken a pre-weighed algal sample, 1.0 g of the catalyst mixture of copper sulphate, potassium sulphate and selenium dioxide (10:20:1) and 5.0 ml of conc. \( \text{H}_{2}\text{SO}_{4} \). The contents were heated over low flame initially till the frothing stopped; heating was later continued and digestion was prolonged for 4-6 hours till a clear mixture was obtained. It was then cooled and the contents as well as several washings were transferred to the kjeldahl's distillation apparatus. Saturated NaOH solution (40%) was added dropwise until the solution turned black. Steam was then passed through this mixture and the liberated ammonia was absorbed in 100 ml of saturated boric acid containing 2-3 drops of indicator (5 parts of 0.1% (w/v) alcoholic solution of bromocresol green and 2 parts of 0.1% alcoholic solution of methyl red - together diluted to 30 ml with 95% alcohol) until it became green. It was then titrated against 0.02 N
HCl until a pink colour indicated the end-point. Percent nitrogen and then % protein was calculated according to the following formulae:

\[
\% \text{ Total Nitrogen} = \frac{100 \times 0.0014 \times N \times Y}{0.1 \times g}
\]

where, \(N\) = Normality of HCl

\(Y\) = Titration value in ml

\(g\) = dry wt. of the sample in grams

\[
\% \text{ Total Protein} = \% \text{ Total nitrogen} \times \text{factor 6.25}
\]

(Sharmawardene et al, 1972; Table 6, Plate VI, Fig. 4).

Incorporation of \(1^{14}C^{14}\) - L - Leucine into proteins of \(P. \text{ ambiguum}\) was considered as a measurement of the rate of protein synthesis. The alga was grown in varying concentrations of NaCl as detailed in Chapter I. Log-phase cells were harvested (18th day) and washed with sterile growth media under aseptic conditions.

Ambient solution was prepared by adding labelled leucine to
a beaker containing 5.0 ml of distilled water; radioactivity in 0.1 ml of this solution was determined using a thin window Ge counter. Final radioactivity of the ambient solution was calculated as 'counts per minute' per ml (cpm/ml) after correction for background counts; this value was 9.4410 cpm/ml in this experiment.

Bits of thalli of *P. ambiguum* were incubated in control and NaCl media supplemented with known amount of radioactivity from the ambient solution; these were harvested at the time-periods of 10, 30, 60 and 120 minutes, washed thoroughly and quickly with sterile carrier solution. Thallus bits were then homogenised using an ultra-sonic vibrator; after drying the tissue homogenate on pre-weighted chromium planchettes, radioactivity was counted. Total uptake was then calculated for each NaCl concentration for each time period and expressed as cpm/mg dry wt (Table 7, Plate VII).

To study incorporation of labelled leucine, Trichloroacetic acid (TCA) was added to a 1.0 ml aliquote of the algal homogenate so as to obtain a final TCA concentration of 15 % (w/v). The mixture was allowed to stand for 30 minutes and later centrifuged to obtain a clear supernatant; residual pellet was repeatedly washed with 15 % TCA and centrifuged; the supernatent so collected contained
TCA-soluble radioactivity; this fraction was dried on a chromium planchette and its strength was then calculated as cpm.

The residual pellet which represents the TCA-insoluble protein fraction was extracted with absolute alcohol, dried and its strength, calculated as cpm. The ratio of TCA-soluble : TCA-insoluble radioactivity was calculated which gave an idea as to the incorporation of radioactivity from labelled leucine into the proteins of *P. ambiguum* (Table 7; Plate VII).
RESULTS

The total content of RNA in cells of *E. ambiguus* was adversely affected by increasing concentrations of NaCl in the growth medium (Table 6; Plate VI, Fig. 3). It ranged from 1.9 mg/g. dry wt. in the cells grown in control to 1.0 mg in those in 2.0% NaCl, representing an almost 50% decrease; these values were only slightly less in cells of 0.5 and 1.0% NaCl (1.6 and 1.5 mg respectively).

Amino acid composition of *E. ambiguus* as affected by varying concentrations of NaCl has been presented in tables 5 A and 5 B. In general, the number and quantity of amino acids in free pool was much less than those of the protein-bound fraction; cells grown in 2.0% NaCl showed a marked increase in the number of free amino acids, most of which were found to be present generally in traces in cells of control, 0.5 and 1.0% NaCl; while leucine showed a sharp increase, (at 2.0% level) proline and most of the aliphatic amino acids also showed
considerable increases in their content. Glutamic acid was present in a relatively large amount in cells of control but was observed to be nearly half its value in salt-treated cells.

Protein-bound amino acids were conspicuous by the presence of considerably large quantities of as many as 16 of them throughout the salt regime (Table 5A). Overall, protein-bound amino acids of 1.0% - treated cells showed larger amounts of amino acids than the rest; cells grown in 2.0% NaCl showed the least amount of protein-bound amino acids in general.

Aliphatic and acidic amino acids (Table 5B) formed the bulk of the protein-bound fraction in general, particularly glutamic acid which showed highest content in cells of 1.0% NaCl (366.9 μmols/g. dry wt.). The ratio of acidic : basic amino acids was appx. 3:1, in cells of control rising slightly to appx. 4:1 for cells under salt stress. Glycine, alanine and leucine comprising the aliphatic group, were also present in large quantities under all growth conditions. While proline content increased in cells grown in 1.0% and 2.0% NaCl, leucine showed uniform increase under increasing salt concentrations. Individually, glutamic acid and proline formed the bulk of protein-bound amino acids (Table 5A).
The total protein content of *P. ambiguum* as affected by varying concentrations has been included in Table 6; Plate VI, Fig. 4. From nearly 40% (dry wt. basis) in the cells of control, total cellular protein showed a decreasing tendency, going down by nearly 65% in the cells grown in 2.0% NaCl (27% approx.).

In general, uptake of $^{14}$C-L-leucine by the cells of *P. ambiguum* increased as a function of time (Table 7; Plate VII). It was also found to be higher in cells of control when compared with any salt concentration. In cells grown in 0.5% NaCl, initial uptake (10 mins.) was less when compared with those of control; thereafter, the rate of uptake was faster than in control as incubation period increased (Plate VII).

Incorporation of radioactivity from labelled leucine into the proteins of *P. ambiguum* more or less increased under all salt concentrations, with increase in the incubation period. However, such incorporation was significantly higher in cells of 0.5% NaCl (as denoted by a low TCA-soluble : insoluble ratio) when compared to those of control and 1.0%
NaCl; this signified faster rate of protein synthesis at 0.5% NaCl level as compared to almost parallel rates in cells of control and 1.0% NaCl (Plate VII). Cells grown in 2.0% NaCl showed the least incorporation of radioactivity signifying a slow rate of protein synthesis therein.

All chemicals used here were of pure, analytical grade. Authentic amino acids and yeast RNA were obtained from Sigma (USA). Labelled leucine was supplied by B.A.R.C.; Bombay; the rest of the chemicals were obtained either from SDH (India) or Sarabhai Chemicals, India.
DISCUSSION

The total RNA content of *E. ambiguus* was adversely affected by increasing concentrations of NaCl in its growth medium. Since RNase activity has not been observed in this alga (Chapter II), it can be said that it is the synthesis of RNA that is adversely affected by salinity thereby affecting the total RNA content. This is in accordance with the views expressed by Nieman (1965) and Rausser and Hanson (1966) for higher plants. Gates and Bonner (1959) however reported that a similar loss (in tomato leaves) in RNA is due mainly to the activity of RNase rather than impaired synthesis. Genckel et al (1967) found that drought caused disappearance of polysomes in corn and bean leaves and appearance of free ribosomes and dimers; they attributed this to the increased activity of RNase. This suggestion has however been questioned by Dhindsa and Sewley (1976) who proposed that the ribosome runoff from mRNA coupled with a failure to re-form an initiation complex rather than RNase activity is the primary cause of polysome loss during dessication. In any case, the loss in RNA content under saline stress is bound to affect the quantity and quality of aminoacids and proteins of *E. ambiguus*; earlier, qualitative changes or proteins as
evidenced by changing electrophoretic patterns have already been discussed (Chapter III). As far as amino acids, total proteins and protein synthesis is concerned, the results were quite intriguing.

As noted earlier, a protein content of 76% (dry wt.) in an obligate halophile, *A. halophytica* has been the highest reported so far for any cyanophyte (Tindall et al., 1977). They also observed a gradual loss in this protein content as salinity increased in what they termed as the first study of its kind.

In the present study, a fairly good amount of total cellular protein was observed in cells of *E. ambiguum* grown in control (appx. 40% ; Table 6; Plate VI, Fig. 4). In this case too, the protein content was adversely affected by increasing salinity. Weimberg (1970) also reported a reduction in total protein of pea seedlings grown in saline media; Prisco and O'leary (1970) reported similar results for bean seedlings.

The observed loss in proteins of *E. ambiguum* grown under varying concentrations of NaCl did not however reflect on its amino acid status. Protein-bound amino acids of cells grown in 1.0% NaCl showed relatively larger quantities than those in 0.5% and control (Table 5); otherwise, even at
2.0% level, wherein the cells showed nearly 65% decrease in total protein, the amino acids and their quantity was not affected to that extent. In case of the obligate halophile, *A. haloparvica*, a striking consistency in its protein-bound amino acids was observed (Tindall et al, 1977) despite a loss in total protein under an increasing molarity of sodium chloride; however, they did not ascribe any reason to this observation. Steward and Sidwell (1958), while studying the nitrogen metabolism of cultured plant tissue, observed only slight differences in the amino acid composition of protein in fast-growing and slow-growing tissues indicating that some factor other than the amino acid composition is responsible for the enhanced loss of proteins in the fast-growing tissue. These aspects have been discussed later on after taking into consideration protein synthesis and proteolysis and the rest of the amino acid situation that is, after taking a concise view of the protein metabolism of *P. ambiguum* as constituted of several physiologically inter-dependent components.

Protein-bound amino acids of *P. ambiguum* were observed to be in large amounts when compared with those of the free pool; they generally had an excess of acidic amino acids over basic, the ratio being only slightly higher under the entire salt regime when compared with that of control
It has been suggested that halophilic bacteria contain relatively large amounts of protein in their cell envelopes (Brown and Shorey, 1963); later, Brown (1964) established that increasing the number of \(-\text{COOH}\) groups in a bacterial membrane can increase its halophilic character. Thus, as he further stated, the bacterial salt requirement is always accompanied by a high proportion of acidic amino acids in membrane proteins and that, salt may be required to neutralise the excess negative charges of proteins thus allowing a proper conformation of their molecules. The observed halotolerance of *E. ambiguus* can be partly attributed to this aspect; in another blue-green alga, *A. halophytica*, Tindall et al (1977) also observed a preponderance of acidic amino acids over the basic, attributing the fact to the obligately halophilic nature of the organism.

It would not perhaps be out of place to make a brief mention here on the amino acids of phycocyanin-protein vis-a-vis the halotolerance of a cyanophyte. An increase in the ratio of acidic to basic amino acids of the phycocyanin of the halophilic alga was noted by Tindall et al (1977); they also observed its amino acid composition to be nearly identical to that of bulk protein and bulk protein minus phycocyanin; c-Phycocyanin content of *P. australis* (Plate II) increased with an increase in NaCl concentration and was highest at 0.5%.
and 1.0 % level; in the light of Tindall et al's finding cited above, it is possible that the bulk of the increase in the amounts of amino acids in the protein-bound fraction of *P. ambiguum*, especially at 1.0 % NaCl level could be a result of increased phycocyanin content. Although Kao et al (1973) observed differences in the characteristics of phycocyanin from non-halophilic and halophilic species of cyanophytes, Tindall et al (1977) noted that phycocyanin from all sources show certain halophilic characteristics (e.g. high ratio of acidic to basic amino acids) regardless of external environmental extremes.

As far as the free pool is concerned, cells of *P. ambiguum* generally showed only traces of amino acids when compared to their massive presence in the protein-bound fraction (Table 5 A). Variations in the amino acid content of the free pool can be due to variations in their synthesis and/or proteolytic activity. Protease activity (Chapter II; Plate VI) was much higher in cells grown in varying salt concentrations than those of control; protein synthesis (labelled leucine incorporation; Table 7, Plate VII) was observed to be faster in cells of 1.0 % and 0.5 % NaCl than those of control. Thus, in general, except at 2.0 % NaCl level, protein synthesis and break down i.e. protein turnover is quicker leading to only traces of amino acids being
present in the free pools. Steward et al. (1956) attributed this aspect to the utilisation of free amino acids as to their carbon in respiration and nitrogen for the resynthesis of proteins. Joshi (1976) suggested the possibility of initially produced amino acids being utilised for PGA, sugar phosphates and starch in some marine algae.

The number and amounts of amino acids were relatively higher in the free pool of *E. ambiguum* grown in 2.0 % NaCl (Table 5 A); at the same NaCl concentration, protein synthesis is at its lowest rate when compared to that in cells of control (Plate VII) and, protease activity is relatively high (Plate VI, Fig. 1). Thus, faster hydrolytic activity and a slow rate of protein synthesis probably results in the accumulation of amino acids under an extreme NaCl stress as noted above. Saunier et al. (1968) while studying aspects of drought tolerance in Creosote bush also observed a doubling of free amino acids under moisture stress. Genckel et al. (1964) has earlier suggested that such an increase may be a mechanism for preventing a build up of toxic ammonia under extreme stress conditions, a factor which can also be applicable to *E. ambiguum* surviving a salt concentration of 2.0 percent.

Individually speaking, significant increases were noted in the content of free proline and leucine under salt stress in
general (Table 5 A). While proline showed a concomitant increase with increasing salinity, leucine appeared only at 2.0 % NaCl concentration; both these amino acids also showed fair increments in the protein-bound fraction; this possibly suggests that their accumulation in the free pool is due mainly to their fresh synthesis and less due to the hydrolysis of proteins. Both, proline and leucine (in their free state), have been known to play an important osmoregulatory role in plants under physiological drought. Liu and Hellebust (1976 b) while studying the effects of salinity on a marine diatom *Cyclotella cryptica* observed that under stress, proline is readily synthesised from glutamate, arginine and ornithine and its accumulation is mainly in response to abrupt changes in intracellular ionic strength during adaptation to increased salinity. Nurit and Poljakoff-Mayber (1977) also observed increased proline content in the roots of *Pisum sativum* and *Tamarix tetragyna* grown on saline media. Jaeger and Meyer (1977) reported a stimulation in the activities of proline dehydrogenase and glutamate dehydrogenase suggesting fresh synthesis of proline under stress; they further added that the ability of a plant to accumulate proline might be of ecological importance and might be an adaptation mechanism for the plant to overcome periods of drought. As far as free leucine is concerned, it may also play a role similar to that of free proline.
Tindall et al (1977) reported a similar increase in free leucine in *A. halophytica* and attributed to it, a possible osmoregulatory role.

Uptake of $^{14}$C-$\text{L}$-leucine by *P. ambiguum* was observed to go down with an increase in NaCl concentration (Table 7; Plate VII). While discussing respiratory responses of a red alga to cations and their relationship to (Na$^+$-K$^+$) ion transport across a cell membrane, Eppley (1966) suggested that high energy phosphate compounds are involved in ion transport and that utilisation of ATP at the cell surface is quite possible. Raven (1971) showed that active K$^+$-influx in the cells of *Hydrodictyon* is supported by cyclic phosphorylation; he further showed that a considerable portion of ATP supplied by non-cyclic phosphorylation is used by K$^+$-pump, the inevitability of such a transport mechanism for the survival of an organism in saline conditions has been discussed earlier (Chapter I). What can therefore be suggested here is that a similar ATP-utilisation in saline conditions may result in the reduction in the availability of energy required for the uptake of labelled leucine observed in the present study; another possibility could be changes in the permeability properties of a cell due to presence of salinity. Such a possibility has been suggested by Kahane and Poljakoff-Mayber (1968) as a reason for the
reduced uptake of labelled amino acids by pea plants grown in saline media; such changes in the permeability properties could be similar to a decrease in diaminopimelic acid and mucopeptide content of a cell wall as reported by Brown (1964) for halophilic bacteria growing in increasing salinities.

As far as incorporation of labelled leucine into the TCA-insoluble fraction is concerned, it was more or less of the same amount for cells in Control and 1.0 % NaCl suggesting parallel rates of protein synthesis (Table 7; Plate VII). In cells growing in 0.5 % NaCl, the synthesis was at a faster rate; at the extreme salt stress of 2.0 %, protein synthesis was at a much slower rate possibly due to damage to the protein synthesising machinery e.g. RNA, as noted earlier. Ben-Zioni et al (1967), while studying the effects of salt stress on protein synthesis in Nicotiana rustica, observed that stressed tissue has reduced capacity to incorporate labelled leucine; they however found evidence that limitations on uptake of leucine could not explain the observed reduction in its incorporation into proteins. In the present study also, while the uptake went down with an increase in salinity, the same did not match with the increased incorporation, at least upto 1.0 % level. (Table 7). Kellal et al (1975) observed that in barley plants, while salinisation impaired growth and uptake of labelled N, the
incorporation was however improved. This has also been observed in the present study; in general, neither bio-mass nor the growth rates (Plate I) had any correlation with the rates of protein synthesis (Plate VII); if anything, it tended to be of an inverse nature. Shevyakov and Leonova (1975) came to similar conclusions after studying the effects of salts on incorporation of labelled leucine into protein in a system in vitro. According to them, it would appear that metabolic readiness of cells for mitosis is lacking during accumulation of salts in the plants despite intensively proceeding protein synthesis.

At this stage, it becomes pertinent to take an overall view of protein and amino acid metabolism in *P. ambiguum* as affected by NaCl-salinity. It is by now understood that variations in a single physiological or metabolic process resulting due to an external factor cannot be explained by itself; this is especially so in case of amino acid and protein situation which is never static and therefore needs to be viewed from several angles. In the present investigation, it has been suggested earlier that reduction in RNA may result in simultaneous effects on the size and composition of amino acid pools and the quantity and kinds of proteins in plants undergoing a physiological drought; in general, protein synthesis increased under salt stress or remained at par with that in cells of *P. ambiguum* grown in control; however, total protein as observed at a
particular growth phase showed a decline in its content. In view of this intriguing data, it can be suggested that increased incorporation of labelled leucine does not necessarily mean that synthesis of all protein fractions is increased. Possibly, certain fractions (e.g. enzymes) are adversely affected and more so in comparison with some other fractions, thereby resulting in reduction of total protein in the alga under saline stress. In the present investigation, c-phycoerythrin (biliprotein) content as well as Peroxidase Catalase and Protease activities showed fairly good increments under saline stress in general. However, some other enzymes can be affected otherwise; Huffaker et al (1970) showed a greater decrease in the level of nitrate reductase than of ribulose-1,5-diP 'carboxylase under water stress.

Dhindsa and Cleland (1975) stated that water stress causes both, qualitative change in the types of proteins produced in Avena coleoptile cells (as demonstrated by double labelling ratio technique) and a quantitative reduction in the rate of incorporation of leucine into proteins; they concluded that stress causes differential inhibition of protein synthesis with synthesis of some proteins being affected to a greater extent than synthesis of others. It is important here to note that the number of soluble protein bands did not vary much under increasing salt concentrations (Chapter III).
although there were qualitative changes - all this despite the fact that total protein showed simultaneous loss in its content. Ruesink (1978) while studying leucine uptake and incorporation by Convolvulus culture cells under severe osmotic stress noted an inhibition of protein synthesis by 37% but cautioned that this must not be interpreted to mean that stress is affecting synthesis of each protein to that extent.

Lastly, an important aspect remains to be considered and that is the theory of 'two moieties of proteins' in plant tissues, which seems to have a relevance to certain findings of the present study. It was observed that the results of experiments concerning total protein and protein synthesis in P. ambisum did not reflect on the amino acid status in general. Steward et al (1956), while studying the effects of coconut milk on carrot tissue explants, found that although the quantity of protein was greatly affected, amino acids were not; they visualised the total bulk of protein to be essentially consisting of two moieties; one is metabolically active which, through alternating breakdown and synthesis, can contribute to the total respiration and energy exchanges for the physiological use of the cell; the other moiety is a stable, inactive one but may have more than mere structural properties (eq. enzymes). Steward and Bidwell (1958) indicated
that some factor other than the amino acid complement is responsible for the great increase in the loss of proteins in the fast-growing plants tissues; after studying the incorporation of radioactivity into the protein-bound amino acids, they further proposed two distinct types of proteins - 1) those in which incorporation is increased, much more than total protein and 2) those in which the increased incorporation is much smaller, slightly less than the total protein; this, according to them again suggested two metabolically different moieties of protein. It will perhaps be plausible to adopt this 'two moiety' theory for the overall protein and amino acid metabolism of P. ambiguum vis-à-vis its observed halotolerance.