CHAPTER II

MATERIAL AND METHODS
2.1. BIOLOGY OF OZIOTELPHUSA SENEK SENEK

Oziotelphusa senex senex is a freshwater crab occurring abundantly in freshwater streams and paddy fields in and around Bangalore. This crab lives in partially water-filled burrows in the edges of fields and streams. It leads most of its life semiterrestrially rather than submerged under water. It feeds on insects and worms, and often goes cannibalistic on younger crabs. Despite the fact that Oziotelphusa senex senex is a strict inhabitant of freshwaters, the crab has a unique capacity to tolerate a wide range of salinities (Krishnamoorthy et al., 1976; Subrahmanyan, 1979).

2.2. LABORATORY MAINTENANCE OF CRABS

The crabs were caught while they are in burrows by introducing the hand into the burrows. There will be possibilities of experiencing their bites while collecting, but proper handling of their chelate legs would avoid such situations. Only crabs in a weight range of 20-25 gm (carapace length, 3.8 cm) were collected. They were brought to the laboratory, separated by sex and kept in glass troughs containing tap water. They were exposed to natural photoperiod and the medium temperature was 25 ± 1°C. During their sojourn in the laboratory for
adjustments to the laboratory conditions, the crabs received *ad libitum* quantities of frog muscles. The water in the troughs was replaced daily with tap water (unchlorinated borewell water) to obviate the effects of excrements. The crabs were thus maintained for at least one week to obviate the effects of environmental change (i.e., from the field to the laboratory). Transportation resulted in autocauly of certain crabs and such crabs were separated out and were not used for experiments.

2.3. METHODS OF ADAPTATION

Since natural sea water was not available, artificial sea water (Lyman and Flemming, 1940) was used in the investigations as the adaptive medium. The salt composition of the artificial sea water was as follows:

<table>
<thead>
<tr>
<th>Salt</th>
<th>g per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>23.476</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>4.981</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>3.917</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.102</td>
</tr>
<tr>
<td>KCl</td>
<td>0.664</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.192</td>
</tr>
<tr>
<td>KBr</td>
<td>0.096</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>0.026</td>
</tr>
<tr>
<td>SrCl₂</td>
<td>0.024</td>
</tr>
<tr>
<td>NaF</td>
<td>0.003</td>
</tr>
</tbody>
</table>

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34.481
Sea water of different strengths viz., 10, 20, 30, 40, 50, 60, 70, 80 and 90% sea water were prepared by mixing artificial sea water (100% = 34.481% o S) with freshwater (unchlorinated borewell water). The crabs were transferred from one medium to the other through ascending grades of salinities. In each intermediate grade, they were maintained for about 2 days. In the final grades of adaptation, they were maintained for 30-40 days before they were actually sacrificed for experiments. About 2 to 3% mortalities were observed during the course of adaptation.

2.4. ESTIMATION OF STANDARD OXYGEN CONSUMPTION

Oxygen consumption was estimated in both freshwater and salinity-adapted crabs, using the respiratory chambers described by Welsh and Smith (1961). The oxygen content of the medium in the respiration chambers was estimated by Winkler's iodometric method (Welsh and Smith, 1961; Carritt and Carpenter, 1966). Since handling and meal (food) are known to influence the rate of oxygen consumption (Newell, 1970); crabs were fed 12 hours prior to the experiments and were then used. The oxygen consumption was measured when the crabs were at rest in the respiratory chambers.
2.5. ESTIMATION OF CATIONS AND CHLORIDE IN THE BLOOD

Blood was drawn gently into a hypodermal syringe from the ambulatory legs. The needle of the syringe was inserted into the hemolymph cavities through the arthrodisal membrane of the crab's leg while drawing the blood. Blood cationic concentration was estimated by flame photometry (Burriel and Ramirez, 1957) after diluting the blood suitably with glass distilled water. The chloride was determined in one ml samples, by Sendroy's method modified by Robertson and Webb (1939). The silver iodate was prepared in the laboratory according to the procedure suggested by Robertson and Webb (1939).

2.6. ENERGETICS

A known amount of frog thigh muscle was offered to a crab, and it was allowed to feed on it at its own in a separate container, for a day. The next day, the left overs were collected and the water adhering to the tissue was blotted out and dried. The water content of the tissue was calculated. The amount of tissue consumed in terms of dry weight was also calculated. The experiment was repeated for about one week with the same crab to calculate the average daily intake of food. The fecal excrements were gathered everyday, dried at 110°C in an
oven and weighed. The amount of food ingested was calculated by noting the difference between the amount consumed and that is excreted.

The dried muscle and excreta were analysed for total soluble sugars, protein and carbohydrate following standard biochemical methods. Total sugars were estimated by anthrone method (Carrol et al., 1956). This method would also estimate the glycogen present in the sample as the acid anthrone hydrolyzes all the glycogen present in the sample. The total protein was estimated by microkjeldahl method, and the total lipid gravimetrically after extracting it with chloroform: methanol (3:1 v/v) mixture in soxlet apparatus (Folch et al., 1957). Calorific conversions of the basic food stuffs ingested were made according to Brody (1945).

2.7. SEPARATION OF MITOCHONDRIA

All the nine gills from each branchial chamber were excised, rinsed in homogenizing solution containing 0.25M sucrose, 6 mM EDTA, 20 mM imidazole-HCl buffer, pH 6.8, briefly blotted, and placed in ten volumes of fresh homogenizing solution. Appreciable quantities of sodium deoxychlorate was added to the ice-cold homogenizing solution to make a final concentration of about 0.1% (Hendler et al., 1972). The tissue was homogenized in a
glass Potter-Elvehjem apparatus using 20 complete strokes at 600 revolutions per minute. The debris was removed through a cheese cloth. Insoluble suspended matter was removed by centrifugation at 600 x g maximum for 20 minutes. Finally the ice-cold homogenate was centrifuged at 11,000 x g maximum for 40 minutes to sediment the mitochondria in an International refrigerated centrifuge (IEC). The sediment was separated by decanting the supernatant, resuspended in 5 ml of the medium and centrifuged at 11,000 x g maximum for 20 minutes. The pellet obtained was separated, suspended in 5 ml fresh imidazole-HCl buffer (pH 6.8) and used for enzyme assays. The protein of the suspension was measured colorimetrically (Lowry et al., 1951). The clear supernatant obtained at 11,000xg was collected for enzyme assays. This contained most of the cytoplasmic proteins and the protein was estimated by colorimetric method of Lowry et al. (1951). Bovine serum albumin was the colorimetric standard.

2.8. ASSAY OF MEMBRANE-BOUND ENZYMES

1) (Na$^+$ + K$^+$)-ATPases: (Na$^+$ + K$^+$)-ATPase activities were assayed according to Towle et al. (1976). Triplicate samples of dialyzed mitochondrial suspensions and the cytoplasmic homogenate (each containing about 20 µg protein) were preincubated for 10 minutes at 30°C with
the assay medium. Dialysis was carried out at 4°C for 3 hours. The assay medium contained 4 mM ATP, 20 mM imidazole-HCl buffer (pH 7.8), 100 mM NaCl, 30 mM KCl and 5 mM MgCl₂ (all in final concentration). Triplicates under identical conditions preincubated with denatured suspension served as controls. Denaturation was done by keeping the suspension in boiling water for 10 minutes. Inhibitors like 0.1 mM Ouabain, 0.1 M sodium azide, 0.2% actinomycin-D, 0.1M Diamox (Acetazolamide) and 0.1 M Amiloride were used. These chemicals were obtained from Sigma. The reaction was started by the addition of 0.2 ml imidazole neutralized ATP, resulting in a final volume of the assay medium. Following incubation at 30°C for 30 minutes, the reaction was stopped by adding 2 ml of ice-cold 10% trichloroacetic acid (w/v). After centrifugation at 10,800 x g maximum for 10 minutes, the inorganic phosphate in the supernatant was determined according to Fiske and Subbarow (1925). The difference of ATPase activity found between a sample with Na⁺ and K⁺ and that without these ions was calculated and was expressed as μmoles Pi released per minute per milligram protein.

The Ca⁺⁺ ATPase activity in the suspensions was estimated according to MacLennon (1970). The incubating mixture consisted of 1 ml suspension (0.1 mg protein, denatured in controls) in 1% albumin (Bovine serum albumin,
Biochemicals Unit, V.P. Chest Institute, Delhi) with a mixture of solution containing 2 ml of 0.1 M tris-maleate buffer (pH 7.3), 0.5 ml of 0.005 M CaCl₂ and 0.005 M ATP.

ii) Carbonic anhydrase. Carbonic anhydrase activity was assayed in both mitochondrial and cytoplasmic fractions. The method adopted was similar to that of Roughton and Booth (1946). The detailed procedure followed to estimate the specific activity of the enzyme is as follows. 3 ml veronal buffer (0.02 M veronal in 0.022 M sodium chloride; pH 7.95) with three drops of bromothymol blue indicator and 2.3 ml of distilled water were mixed in a 15 ml stoppered vial and cooled over ice cubes. 5 ml of ice-cold carbonated water (saturated with CO₂) were added to the mixture. The time for non-enzymic drop in pH in the contents was measured with the help of a stop-watch. Then to a similarly mixed sample the enzyme solution was added and the time for pH drop was noted. The rate of enzymic hydration of CO₂ in moles per liter per second was calculated according to Roughton and Booth (1946).

2.9. COLLECTION AND ESTIMATION OF EXCRETORY PRODUCTS

A group of 3 or 4 salinity adapted crabs were transferred into a measured quantity of the respective medium, at 25°C (in BOD incubator, Mosser's, Toshniwal, India) and
maintained for 3 hours. The medium was later used for the analysis of excretory products.

Urea was estimated enzymatically in the following way: 50 mg urease powder was added to 50 ml of medium which was earlier buffered with phosphate buffer (pH 6.8) (Folin and Swedberg, 1930). After incubating the medium at 30°C for 30 minutes, it was transferred to the distillation flask. 25 ml of 40% KOH was added to the incubated medium and distilled to collect the expelled ammonia into 2% boric acid. The boric acid captured the ammonia and it was estimated colorimetrically by Nesslerization (Oser, 1965, p.1218). The free ammonia and ammonium salts in the medium were estimated following distillation and Nesslerization (Oser, 1965). Trimethylamine (TMA) was estimated by the method of Shewan et al. (1969).

The non-protein nitrogen (NPN) of the medium was determined by the microkjeldahl method. The amino acids were detected by the method of Danielson (Oser, 1965, p.1048) and the proteins by biuret colour reaction (Layne, 1957).

The uric acid in the medium was estimated by the arsenophosphotungstic acid colorimetric method (Oser, 1965, p.1239). To homogenize the uric acid, the medium
was treated with equal volumes of 20% NaHCO₃ solution. This dissolved the crystals of uric acid in the medium. The standards of the above excretory products were purchased from the British Drug House (Glaxo Labs., India Ltd., Bombay).

2.10. PAPER CHROMATOGRAPHY OF AMINO ACIDS

Blood samples deproteinized with absolute alcohol were concentrated in a boiling water bath for two-dimensional paper chromatographic analysis. The solvent systems used consisted of (1) 4:1:1 butanols:acetic acid:water, and (2) 5:3 hexane:benzene. Spots were identified after spraying 0.1% ninhydrin in acetone, over the chromatogram. After identification, the coloured spots of the chromatogram were cut and eluted into 0.1% CuSO₄ in absolute alcohol to estimate the amino acid content colorimetrically at 480 nm.

2.11. TOTAL NINHYDRIN POSITIVE SUBSTANCES (TNPS)

Weighed tissues were ground in 50% TCA and centrifuged at 600 x g. The TNPS were estimated colorimetrically in the supernatant according to the method of Danielson (Oser, 1965). Chromatographically pure glycine (BDH chemical) was used as standard.
2.12. **CALCULATION OF INTRACELLULAR CONCENTRATION OF IONS**

The exact volume of the extracellular fluid of the tissues should be known in order to calculate the intracellular concentration of any ion correctly. Particularly in the invertebrates this is extremely difficult (Martin et al., 1958). However, it is known that in *Callinectes sapidus*, the muscle extracellular space occupies 22% of the entire muscle space (Lange, 1968). For the nervous tissue of the same crab, it is 25% (Keynes and Lewis, 1956). The level of tissue hydration (i.e., the water content of tissue) was made use of in calculating the concentration of ions per kg tissue water.

\[
\frac{C_{\text{wt}}}{\frac{\text{wt}}{\text{kg}}} \times 1000 = C_{\text{TH}} 
\] .. (1)

where,

- \( C_{\text{wt}} \) = concentration of ions in the wet tissue \( (\text{mM/kg wet tissue}) \);
- \( \text{TH} \) = level of tissue hydration;
- \( C_{\text{TH}} \) = concentration of ion in the tissue water \( (\text{mM/kg tissue water}) \).

The extracellular volume for the muscle of *Oziotelphusa senex senex* is not known. Assuming it to be equivalent to that in *Callinectes sapidus* (Lange, 1968), a value of 22%
was arbitrarily chosen for calculation of intracellular levels of osmotic constituents.

The intracellular concentration of osmotic constituents is given by the following equation:

\[ \text{TTH} - \text{ECH} = \text{ICH} \quad \text{.. (2)} \]

where,

\( \text{TTH} \) = tissue total hydration (g of water per kg wet tissue);

\( \text{ECH} \) = extracellular hydration (volume); and

\( \text{ICH} \) = intracellular hydration.

Since, \( \text{ECH} \) is assumed to be 22%,

\[ \text{TTH} - 220 = \text{ICH} \quad \text{.. (3)} \]

\( (\text{ECH} = 22\% = 220 \text{ ml/1000 g}). \)

The tissue extracellular spaces are filled with blood or lymph. Therefore, the extracellular fluid is presumably similar to blood in its osmotic composition.

Hence,

\[ \frac{C_b}{1000} \cdot 220 = L_{ecf} \quad \text{.. (4)} \]

where,

\( C_b \) is the concentration of the ion in blood;
220 is the extracellular volume;
\( L_{ecf} \) is the level of the ion in the extracellular fluid of the tissue.

When \( L_{ecf} \) is subtracted from the concentration of ions in the wet tissue \( (C_{wt}) \), the intracellular level of the ion would be obtained.

\[
C_{wt} - L_{ecf} = L_{ic} \quad \ldots \quad (5)
\]

Then,

\[
\frac{L_{ic} \times 1000}{ICH} = C_{ic} \quad \ldots \quad (6)
\]

where,

\( C_{ic} \) is the concentration of the intracellular ion or osmotic effector.

2.13. **ASSAY OF GLUTAMINASE ACTIVITY**

Assays were performed on whole homogenates from the gill, hepatopancreas and blood. A 20% homogenate was realized in an iced 0.1 M tris-HCl buffer at pH 7.5 with a Potter-Elvehjem apparatus fitted with a glass pestle turning 450 turns per minute. Enzymic activity (L-glutamine amidohydrolase E.C. 3.5.1.2) was evaluated from the amount of ammonia formed from 5 mM L-glutamine at pH 8.0 and
at 30° C. A 20 mM tris-HCl buffer solution (3.9 ml) at pH 8.0 containing 5 mM L-glutamine and 60 mM Na₂HPO₄ was preincubated in 15 ml centrifuge tubes in a water bath at 30° C. The contents were shaken well during the incubation. After 10 minutes, into each tube 0.1 ml of distilled water or 0.1 ml of 20% homogenate was added. After 60 minutes of incubation the reaction was stopped by the addition of 100 ml 30% TCA solution. The proteins were separated by centrifugation at 1500 x g. The ammonia content was evaluated per ml or g of tissue per hour and the specific activity of the enzyme was expressed as μmoles NH₃ formed per mg protein per hour. The ammonia content was determined colorimetrically using Nessler's reagent.

The same procedure was adopted for the determination of asparaginase activity using L-asparagine as substrate in place of L-glutamine.

2.14. ASSAY OF ARGINASE ACTIVITY

Fresh 10% homogenates of hepatopancreas were prepared in 0.02 M tris-HCl buffer (pH 7.5) by homogenizing the tissue for 5 min in a tissue grinder. The homogenate was centrifuged at 1500 x g for 20 minutes and the clear supernatant was obtained for assays. The protein in the homogenate was measured according to Lowry et al. (1951). The arginase activity (E.C. 3.5.3.1) was measured by the
rate of urea formation. The reaction mixture contained
0.5 ml of 0.1 M tris-HCl buffer (pH 9.5), 1.0 ml of 0.5 M
arginine (pH 9.5), 0.1 ml of 0.15 M NaCl and 0.05 ml of
0.1 M MnCl₂ and the enzyme solution to make a final volume
of 2.0 ml. The reaction mixture was preincubated for 10
minutes prior to addition of the enzyme at 30°C. The
reaction was started by adding the enzyme solution (0.35 ml)
to the mixture. The incubation was carried out for 1 hr.
The reaction was stopped with 0.5 ml of 25% TCA. The
mixture was coloured with the pigments present in the
hepatopancreas. These were removed by filtering the
mixture after treating it with activated charcoal. The
urea content in the filtrate was estimated by the colori-
metric procedure of Archibald (1945) as modified by Ratner
(1955). One unit of the arginase activity is defined as
μmole of urea formed per hour per mg of protein at 30°C.

2.15. EXTRACTION AND ESTIMATION OF PHOSPHOLIPIDS

The gill filaments were excised, briefly bottled and
weighed. They were homogenized in 25 ml 1:2 chloroform:
methanol mixture to extract the total lipids and they were
separated from aqueous phase according to Bligh and Dyer
(1959). The solvent mixture containing the lipids was
reduced to 5 ml under vacuum and fractionated into lipid
classes by adsorbant column chromatography (Carrol, 1963).
The total lipid fraction was loaded on a silicic acid column (20 g, Baker Chemical Co.) with chloroform. The neutral lipids and phospholipids were eluted with 700 ml chloroform, and 300 ml methanol respectively. The phospholipid fraction was dried under reduced pressure and stored in methanol.

Thin layer chromatography of phospholipids was performed on glass plates (20 cm x 20 cm) coated with 0.25 mm layers of silica gel G (E. Merck, AG, Darmstadt, Germany). The plates were activated previously at 110°C for 1 hour. 50 μl of lipid extract was applied to the plates. Ascending chromatography was carried out in unlinked tanks. The developer used was a mixture of chloroform:methanol:30% methylamine (65:25:4, v/v/v) (Kuhn and Lynen, 1965). The standard lipids, obtained from Applied Science Laboratories, U.S.A., were spotted along with the samples on the same plate. The lipids separated on the chromatogram were visualized under UV light after spraying 2,7-dichlorofluorescin. The definable lipid spots were identified by comparison with standard lipids. The quantities of the isolated lipid fractions were estimated according to Levine and Chargaff (1951). The following procedure was adopted. The identified or unidentified spot was scraped and pooled from plates and the phospholipids were eluted through a glass chromatographic column (0.8 cm x 1.3 cm)
with chloroform:methanol (1:1, v/v) containing 10% water (Rouser et al., 1967). The elutants were evaporated and the phosphorus content of the residue was estimated according to Barlett (1959). Phospholipid content was expressed in μg atoms of P per g protein of the tissue.

2.16. **CHOLESTEROL EXTRACTION AND ESTIMATION**

The tissue was weighed, homogenized in 10 ml of acetone:alcohol (3:1) mixture, and centrifuged at 600 x g for 20 minutes. The clear supernatant was separated, and to a 2 ml portion of it, 1 ml of 1% digitonin in 80% alcohol was added to precipitate the 3-OH sterols. The precipitation was allowed to take place overnight at 4°C. The sterol digitonides were separated by centrifugation and estimated according to Sperry and Webb (1950) using Liberman-Curchard reagent.

2.17. **BLOOD UREA**

Blood was drawn into a hypodermic syringe as described in Section 2.5. Immediately the blood was neutralized with 10% perchloric acid and centrifuged. 20% KOH was added drop by drop to neutralize the acid, and complete neutralization was checked by pH paper. The resultant perchlorate was removed by centrifugation. The urea in this neutral extract was determined enzymatically following the method of Polin and Svedberg (1930).
2.18. IN VITRO UREA SYNTHESIS

The hepatopancreatic tissue was cut into approximately 1 mm thick slices, weighed and incubated in a mixture of 5 ml of 0.1 M \( \text{PO}_4 \) buffer at 7.6 pH, containing 1 mg carbomyl phosphate, 10 mM arginine, 10 mM citrulline, 10 mM ornithine and 10 mM \( \text{NH}_4 \text{Cl} \). \( \text{CO}_2 \) was bubbled through the mixture for about 15 minutes. After incubating the slices for 3 hours, at 30°C, 1 ml of 50% TCA was added and homogenized. The homogenate was then neutralized by the addition of KOH. The urea content was estimated colorimetrically after subjecting the sample to urease hydrolysis as described earlier (see Section 2.9).

2.19. AMYLASE ACTIVITY (E.C. 3.2.1.1)

Hepatopancreatic tissue from 5-6 crabs were excised and pooled together. The tissue was homogenized in ice-cold acetone in a Waring Blender, and filtered through Whatman No.1 filter paper. The precipitate was thoroughly washed with fresh quantities of acetone to remove pigments. Finally the powder was washed with ethyl-ether and dried at room temperature. This acetone powder was found to contain stable amylase enzyme of the crab (Krishnamoorthy and Venkatareddy, 1968). To extract the enzyme 1 g acetone powder was suspended in 100 ml 80% glycerol according to
Rorrer and van Weel (1958). The extraction continued overnight at 4°C. The undissolved powder was removed after centrifugation and the enzyme present in the clear supernatant was assayed for its specific activity in the following way.

The specific activity was determined according to the procedure described by Bergmeyer (1974, p.432). α-Amylase catalyzes the hydrolysis of internal 1,4-glucan bonds in polysaccharides resulting in the formation of dextrins and a mixture of reducing sugars. The enzyme is activated by Cl⁻ and hence they are added to the assay mixture. The mixture contained 0.5 ml starch in 20 mM phosphate buffer (pH 7.0) with 6 mM NaCl and 0.48 ml distilled water, pre-incubated at 30°C. The reaction was started with 0.02 ml of enzyme solution (the glycerol extract) and incubated for 1 hr. One ml of 3,4-dinitrosalicylic acid reagent (1 gm in 20 ml of 2 N NaOH, 30 g potassium-sodium tartrate and 100 ml distilled water) was added, heated in a boiling water bath and cooled at room temperature. The optical density of the resultant was measured at 540 nm. To obtain the maltose content as reducing equivalents from a standard curve. The protein of the extract was estimated colorimetrically (Lowry et al., 1951).
2.20. LIPOSE ACTIVITY

The method used consists in the titrimetric estimation of the fatty acids liberated from the catalytic activity of the hepatopancreatic extract on tributyrin (Sigma Co.). The general schema of incubation of the enzyme with the substrate and the titration were according to Bier (1955). 10% homogenate of hepatopancreas in neutral phosphate 1/15 buffer (w/v) was prepared in a tissue grinder. The extract was centrifuged at 600 x g and the clear supernatant was used for enzyme assays.

The substrate was prepared by mixing 100 ml buffer with 1 g tributyrin. The pH of the mixture was adjusted with dilute acetic acid or dilute sodium hydroxide to 7.2. The substrate was preincubated at 30°C for half an hour and 10 ml of the hepatopancreatic extract was added. The incubation was carried for 1 hr. At the end of the incubation period, 1 g of Norit was added to the contents and filtered through Whatman No.1 paper so that all the interfering pigments were removed. 55 ml of the clear filtrate was titrated against standard NaOH (0.02 N) using 0.02% aqueous phenyl red as indicator. The protein of the Norit treated homogenate was determined according to Lowry et al. (1951). Heat denatured homogenates were used as controls.
2.21: PROTEASE ACTIVITY

Ten per cent homogenates of hepatopancreas (pooled from 8-10 crabs) were prepared in neutral phosphate buffer as described in Section 2.20. Solid ammonium sulfate was then added (30.4 g per 100 ml homogenate); the precipitate which formed was collected on a hardened paper and the filtrate was rejected. This precipitate was dissolved in 30 ml distilled water and was treated with 20 ml saturated ammonium sulfate. The mixture was filtered with suction through a Buchner funnel. Washings were done with 0.4 ml saturated ammonium sulfate and the precipitate in the funnel was discarded (Laskowski, 1955). To the filtrate equal volume of saturated ammonium sulfate was added and filtered through hardened paper in a Buchner funnel. A saturated solution of magnesium sulfate in 0.02 N $\text{H}_2\text{SO}_4$ was then poured over the precipitate and filtered. The precipitate contained the trypsinogen. The activation of the enzyme was done by dissolving it in 30 ml of 0.4 M borate buffer (pH 9.0) at 2 to $5^\circ$C. The protein of this extract was measured colorimetrically (Lowry et al., 1951).

The trypsic activity was measured by haemoglobin digestion method of Anson (Laskowski, 1955). The amount of split products in the assay mixture was determined colorimetrically after removing the hemoglobin by filtration.
The specific activity was expressed as mg amino acids obtained due to digestion by mg protein per hour.

2.22. **STATISTICAL ANALYSES**

Statistical analyses of the data were done according to the method suggested by Croxton (1953).