GENERAL INTRODUCTION
In biological sense, organisms and their environment are in fact inseparable. As the environment being labile, no organism is ever subjected to constant environmental conditions and are exposed to fluctuations in the environment. This change in environment imposes considerable stress on the inhabiting organisms, which, in turn, have to adjust or adapt to it in order to survive and live effectively in the changed environment and this physiological adaptation to changed environment is one of the fundamental properties of the living organisms.

Temperature is an important labile environmental factor that it exerts a profound influence on enzyme systems, cells, tissues, individual organisms, populations and communities. Further, temperature limits the distribution of animals and controls the rate of their chemical reactions involved in growth and metabolism (Prosser, 1973; Precht et al. 1973).

Based on thermal characteristics, animals are categorised as homeotherms, which can effectively regulate their body temperature and poikilotherms which can't regulate their body temperature.

The internal temperature and rate functions of these poikilotherms should ideally fluctuate in accordance with the
environmental temperature. Such poikilotherms are at the mercy of the nature and will be restricted in their activity and distribution to a narrow thermal zone of habitat (Prosser, 1973; Das, 1984).

Now, it has been well established that some poikilotherms, some what like homeotherms can regulate their metabolism and activity in the adaptive direction against wide range of thermal variations. Such regulation occurs at the whole animal, cellular and subcellular levels (Precht, 1958; Hochachka, 1967, 1969; Fry, 1971; Hazel, 1972; Precht et al. 1973; Hazel and prosser, 1974 ). It is in this sense that studies on metabolic compensation to thermal acclimation in poikilotherms assume significance.

Most of the studies in this area are confined to marine poikilotherms from cold and temperate zones not to the tropical poikilotherms, especially from the freshwater and terrestrial habitats. These were believed earlier not to show any regulation in their metabolism against environmental thermal variations on the assumption that the thermal set-up in the tropics is more or less uniform throughout the year (Vernberg, 1959). But off late it has been well established that even the tropical poikilotherms do regulate their metabolism and activity against environmental
thermal fluctuations just as their counterparts from cold and temperate zones (Rao, 1967; Parvatheswara Rao, 1972a, b; Prosser, 1973; Hazel and Prosser, 1974; Das, 1984). As such a fresh water crab, inhabitant of paddy fields of Rayalaseema, a drought prone area characterised by wide variations in thermal environment was selected for the present study.

In general, adaptation of a poikilotherm to thermal stress is manifested in three different time periods. (1) Metabolic adaptation may take place instantaneously perhaps with in few hours after exposure to thermal stress. (2) Metabolic adaptation may occur after prolonged periods, usually several weeks of exposure to new thermal regime, and (3) Metabolic adaptation may manifest over an evolutionary time span (Somero, 1969; Hochachka and Somero, 1973; Hazel and Prosser, 1974).

Again the responses of animals to thermal stress have been categorized into two types.

1. Immediate or short-term responses observed immediately following transfer of the animal to the stress medium and 2. Stabilized or long-term responses manifested after a prolonged period following transfer of the animal to the stress medium. This period may be hours or days or weeks or even years

As indicated before, poikilotherms react to imposed thermal stress by exhibiting immediate short term responses followed by stabilized long-term responses which indicate the completion of acclimation also referred as adaptation by some other workers.

Metabolic pathways operating upon cold and warm acclimations are different (Hochachka and Hayes, 1962; Rao, 1967; Hochachka, 1967, 1969; Hochachka and Somero, 1973). Further the sequence of events in time-course of acclimation may reflect corresponding acclimatable changes in cellular enzymes (Sidell et al. 1973). The study of time-course of acclimation to cold and warm temperatures not only tells us the time required for completion of acclimation to respective thermal regime but also some qualitative aspects underlying such acclimation.

Measurement of oxygen consumption has been employed more than any other experimental parameter to monitor changes in metabolism associated with thermal acclimation (Hazel and Prosser, 1974) when a poikilotherm is transferred from its
habitat temperature to a new thermal regime there will be a initial shoot-up or initial shoot-down in the metabolic rate. This is followed by a gradual stabilization of the metabolic rate at certain level, which is maintained during its subsequent sojourn in the new thermal regime. The attainment of such stabilized rate indicates the completion of acclimation (Kinne, 1964 a,b). However, the relationship between stabilized rate in the new thermal regime and the original normal rate at the habitat temperature differs between different poikilotherms. Accordingly, Precht (1958) has classified metabolic compensation to thermal stress into 5 different patterns.

(1) Over adaptation: Where the new stabilized rate of metabolism in the adaptation medium goes beyond the original normal rate at the habitat temperature.

(2) Perfect adaptation: Where the stabilized rate of metabolism in the new thermal regime perfectly coincides with the original normal rate at the natural habitat.

(3) Partial adaptation: Where the new stabilized rate of metabolism is half way between the initial shoot-up or initial shoot-down as the case may be and the normal rate at the habitat temperature.
(4) No adaptation: Where the metabolic rate is maintained at the initial shoot-down or initial shoot-up level itself as the case may be during its subsequent sojourn in the new thermal regime and

(5) Inverse adaptation: Where the metabolic rate continues to increase or decrease beyond the initial shoot-up or initial shoot-down level as the case may be during subsequent sojourn of the animal in the adaptation medium.

However, the pattern of acclimation may vary in different animals at different periods depending upon differences in thermal history and in the same animal, acclimation to cold and warm temperatures may follow different patterns. Further, not all tissues examined exhibit the acclimation pattern of whole animal nor do all tissues examined exhibit the same degree of acclimation (Hazel and Prosser, 1974).

The fact is that the studies on the time-course of acclimation to thermal stress in tropical poikilotherms especially on fresh water crustaceans are scanty. So this study of time-course of acclimation of oxygen consumption to cold and
warm temperatures at whole animal and tissue level was included in the present study.

Although thermal acclimation in poikilotherms has been extensively studied, very little is known about the mechanisms by which the animals adapt. It has long been speculated that acclimation is hormonally controlled (Rao, 1962; Saroja, 1962; Vijayalakshmi, 1964; Nayeemunnisa, 1966; Nayeemunnisa and Rao, 1975; Silverthorn, 1975b; Ivanovic et al. 1975a, b, 1978, 1980, 1982, 1985; Kale, 1976; Mc Carthy, 1976, 1978; Nagabhushanam and Hanumante, 1977; Pruitt and Dimock, 1979; Kulkarni and Kamath, 1983; Haldani et al. 1984; Charmantier et al. 1984a).

About three decades ago, hormone research in decapod crustacea had been in its infancy and it had been assumed that the factors controlling physiology of crustaceans and their biochemistry was relatively simple. But now it is a well known fact that neuroendocrine control mechanisms in crustaceans are as complex as in vertebrates (Raghaviah, 1977; Reddy, 1981; Lofts and Holmes, 1981; Josefsson, 1983; Ramamurthi and Reddy, 1983; Charmantier et al. 1984a, b; Arechiga et al. 1985; New Comb et al. 1985; Reddy and Ramamurthi, 1987).
Welsh (1930), Hanstorm (1931, 1934, 1939, 1947), and Kleinholz (1936) were first to recognise the presence of neurosecretory cell groups in the eyestalks and their importance as a source of crustacean hormones. These authors named these cell groups present in medulla interna and medulla terminalis as "Sinus gland" and "X" organ. Later Gabe (1953, 1954, 1956, 1966) has demonstrated "Y" organ analogous to insect moulting gland in crustaceans. Significance of "X" organ—sinus gland complex in crustacean metabolism was demonstrated by later workers (Lofts and Holmes, 1981; Charmantier et al. 1984a; Newcomb, 1983; Arechiga et al. 1985; Newcomb et al. 1985).

et al. 1984; Gangotri et al. 1986, 1987) etc., were reported in the eyestalks of crustaceans. These hormones are reported to regulate respiration (Silverthorn 1973, 1975a, b; Vasantha et al. 1979; Reddy and Ramamurthi, 1987), Osmoregulation (Heit and Fingerman, 1973, 1975; Kamemoto, 1976; Davis, 1979; Charmantier et al. 1984a, b) and these hormones are also reported to control carbohydrate (Venkataramaniah and Ramamurthi, 1980; Reddy, 1981; Reddy and Ramamurthi, 1987; Gangotri et al; 1987), Protein (Raghavaiah, 1977; Ramamurthi and Reddy, 1983) and lipid (Chandramouli, 1977: Raghavaiah, 1977) metabolisms in crustaceans.

In crustaceans eyestalk hormones are reported to play an important role not only in controlling the metabolism of animals at habitat temperature but also assigned a role in controlling changes in the metabolism during the process of thermal acclimation (Ivanovic et al. 1975a, b, 1978, 1980, 1982, 1985; Mc Carthy, 1976, 1978; Kulkarni and Kamath, 1983; Haldani et al. 1984; Charmantier et al. 1984a). Further it was also reported that the sequence of changes in the metabolism of crustaceans on acclimation to low the high temperatures are triggered and regulated through one or more humoral agents and thus metabolic compensation to imposed thermal stress is achieved (Silverthorn, 1973, 1975a, b; McCarthy, 1976, 1978; Pruitt and Dimock, 1979; Kulkari and Kamath, 1983; Charmantier et al. 1984a).

Since acclimation to thermal stress involves a number of active processes and hence energy expenditure, the purpose of metabolic regulation during thermal acclimation might be to meet altered energy demands. Hence in this investigation, metabolic rate and some enzyme and biochemical constituents involved in energy metabolism were studied.

Tissue gravimetry plays an important role in assessing physiological state of the animal, especially in stress
conditions (Prosser, 1973) and ionic contents plays an important role not only in maintaining osmotic balance but also in the regulation of metabolism against environmental changes (Sreenivasamoorthy, 1985) and hence body weight, haemolymph volume, hydration levels and some inorganic ions were studied.

STATEMENT OF THE PROBLEM:

A scan through the available literature on thermal acclimation of poikilotherms clearly indicates that studies on the role of eyestalk hormones in the regulation of physiological and biochemical processes during thermal acclimation of crustaceans, especially of freshwater crabs are scarce. In view of this, the present study was undertaken to study the role of eyestalk hormones in the acclimation of freshwater field crab, Oziotelphusa senex senex.
MATERIALS & METHODS
EXPERIMENTAL ANIMAL:

The fresh water field crab, Oziotelphusa senex sene (Fabricius) was used as an experimental animal in the present

CLASSIFICATION:

Phylum : Arthropoda
Subphylum : Mandibulatae
Class : Crustacea
Subclass : Malacostraca
Series : Eumalacostraca
Super Order : Eucarida
Order : Decapoda
Sub order : Reptantia
Section : Brachyura
Genus : Oziotelphusa
Species : Senex
Variety : Senex
Procurement and maintenance of test species:

Oziotelphusa senex senex is an edible fresh water field crab, which normally inhabits the paddy fields and irrigation canals of south India. They make burrows in the bunds and canals in the paddy fields and lives in partially water filled burrows. They are eurythermal and can survive longer periods on land, but they do not inhabit brackish or saline waters, they are carnivorous, feeding on worms, insects etc., and cannibalistic feeding on younger crabs.

The crabs collected from the paddy fields and irrigation canals around Tirupati were brought to the laboratory and maintained in large glass troughs containing tap water (temperature 28 ± 1°C) and were exposed to natural photoperiod. During laboratory sojourn, the crabs received ad libitum quantities of minced meat every alternate day and the water in the troughs was changed once or twice in a day, depending on the necessity. The crabs were maintained thus for at least one week in the laboratory to obviate the effect of environmental changes.

Only intermolt (stage $C_4$) male crabs of identical size, weighing 25 ± 2 grams were selected for conducting further
investigations, injured animals were not used for experimentation.

THERMAL ACCLIMATION:

To study the effect of thermal acclimation, crabs were divided into 3 groups. The first group of animals were maintained at room temperature (28°C). The second and third groups of crabs were acclimated to cold (18°C) and warm (38°C) temperatures in a thermostatically controlled water bath. For acclimation a 23 day sojourn for cold and 21 day sojourn for warm temperatures was allowed. It was experimentally verified and confirmed that these periods are sufficient for the completion of acclimation to cold and warm temperatures. After the completion of acclimation, the second and third groups are designed as cold-acclimated and warm-acclimated crabs. The first group of crabs kept at laboratory temperature served as controls. To ensure that equal thermal stress was imposed on the crabs during acclimation to cold and warm temperatures, care was taken to see that the thermal gradient is equal on both the sides of acclimation relative to the normal room temperature.

During the course of acclimation crabs were fed normally. Care was taken to control, as for as possible, every
other factor, as physiological effects of changes in ambient temperature is known to modify other variables. Feeding was stopped one day before the commencement of the experiment to avoid variations in the metabolism, if any, due to differential feeding. All estimations were made at the same time on each day to eliminate the effect of circadian rhythms (Chandrasekhara Reddy et al.1978).

**TIME COURSE OF ACCLIMATION:**

Whole animal: The time course of acclimation in the rate of oxygen consumption to cold (18°C) and warm (38°C) temperature was studied separately in six individuals of O, senex senex to assess the degree of acclimation and also the time (days) taken to acclimate to the imposed thermal stress, cold or warm as the case may be. This period was calculated by daily measurement of oxygen consumption, till a stable level of oxygen consumption is obtained.

For measuring whole animal oxygen consumption, Winkler's method as given by Welsh and Simth (1953) was adapted. The set-up of apparatus used in this experiment was similar to that of described by Saroja (1959). The apparatus mainly consisted of a reservoir (R) and a respiratory chamber (RC). A 500ml wide
mouthed bottle was used as a respiratory chamber. The size of the bottle was such that it was not too big for the enclosed crab to give a considerable difference in oxygen content between initial and final samples. The chamber was coated with black paint to avoid any activity due to light. The mouth of the respiratory chamber was filled with a four holed rubber stopper (S). Through one of the holes a centigrade thermometer (T) is fitted to find the fluctuations in temperature, if any. Through the second, third and fourth holes an inlet tube (T₁) an outlet tube (T₃) and a small straight tube (T₂) were fitted to find whether the chamber is airtight. T₁, T₂ and T₃ were fitted with small bits of rubber tubing to which pinch cocks were fixed which designated as P₁, P₂ and P₃. The inner ends of T₁ and T₂ are stopped at the bottom of the rubber stopper, while T₃ extended almost up to the bottom of the respiratory chamber. The inlet tube was connected to the reservoir by a glass tube, through which water is drawn into the respiratory chamber. The chamber, with the crab (C) inside, made air free by opening the pinch cocks P₁ and P₂. P₂ was closed and the initial sample was drawn into the sample collecting bottle (SB) through T₃. Now P₃ and P₁ were closed. At the time of drawing the final sample, after 1 hour interval, T₂ was opened by removing P₂ in order to let the water out through T₃, which was opened by removing P₃. The respiratory chamber was kept in a water bath (WB) to maintain constant temperature.
SET UP OF APPARATUS USED FOR MEASURING THE WHOLE ANIMAL OXYGEN CONSUMPTION
Before beginning the experiment crabs were left in running tap water for about 10 min to facilitate them to come to a state of normality from a state of excitement, in any. After the equilibration period was over, one crab was kept in each respiratory chamber without causing any damage to the animal and the initial sample was collected immediately as described above. Then the crab was allowed to respire for one hour. Immediately after one hour the final sample was collected. The oxygen consumed by the animal was calculated by using the difference between the readings of initial and final samples. The values are represented as ml O$_2$/h.

To start with, the rate of oxygen consumption of each of the twelve crabs was measured at normal temperature (28°C) over a period of five days at 24h intervals. On the fifth day six crabs each were transferred from the normal temperature to the cold and warm temperatures. There after they were maintained in the same acclimation temperatures and their oxygen consumption was measured continuously at 24h interval till the attainment of "Stabilized rate" of oxygen consumption in the new thermal regime. The attainment of stabilized rate indicate that the crabs were acclimated to the respecting temperature. Each measurement of oxygen consumption was made in six crabs and the mean value
Considered for all practical purposes.

Excised Tissues: The time course of acclimation in the rate of oxygen consumption of excised tissues namely the hepatopancreas (HP), Claw muscle (CLM) and gill (GL) of O. senex senex to cold (18°C) and warm (38°C) temperatures was determined to ascertain whether or not the acclimation pattern of these three tissues is comparable with that of the whole animal.

The rate of oxygen consumption of the HP, CLM and GL throughout acclimation to cold and warm temperatures was measured in a Warburg constant volume respirometer (Gallenkamp, England) as per the procedure described by Umbriet et al. (1972). The manometers were calibrated with mercury. Brodie's fluid was used as the manometric fluid. The Warburg flasks were cleaned before use. Crustacean ringer prepared as per the procedure of Van Harreveld (1936) was used as the suspension medium and it is given below.

Composition of the ringer:

- Sodium chloride (NaCl) ... 12.00g
- Potassium chloride (KCl) ... 0.40g
- Calcium chloride (CaCl₂) ... 1.50g
- Magnesium chloride (MgCl₂) ... 0.25g
- Glucose (C₆H₁₂O₆) ... 1.00g

Dissolved in water and made up to 1 litre. The pH of the ringer
was adjusted to 7.4 by the addition of 0.1N NaOH.

The ringer was prepared afresh every week and stored in
the refrigerator. Whenever required, crabs were sacrificed and
the tissues HP, CLM and GL were dissected out into individual
microbeakers containing ice cold crustacean ringer. Then they
were blotted and weighed in a sartorius electrical balance and
teased into thin slices. The tissue slices were then transferred
into individual sterilized warburg flasks. A drop of penicillin
was added to each preparation to check bacterial growth. Into
central well of each warburg flask 0.3 ml of 10% potassium
hydroxide solution was added carefully and a filter paper wick
was placed inside the central well for absorbing carbon dioxide
released during respiration. Finally the flasks were mounted on
the manometers, filled with brodies fluid and their initial
levels were preadjusted using a knob. Thus in each manometer the
gas phase was air and the liquid phase was ringer solution. The
entire process of tissue preparation was carried out in a
sterilised cold room where the temperature was maintained at
15±1°C. After checking the air tightness of the manometers,
oxygen consumption of the HP, CLM and GL was measured at required
temperature over a continuous period of 30 min at 10 min
intervals. The tissue preparation was given about 10 min.
equilibration period prior to the commencement of the
measurements. The rate of oxygen consumption of individual
tissues was expressed as μl o2/g/h.
To begin with the rate of oxygen consumption of the HP, CLM and GL of normal crabs was measured over a period of five days at 24h intervals. On the fifth day, two batches of crabs were subjected to cold and warm acclimations as described earlier. After one hour of exposure the rate of oxygen consumption of the HP, CLM and GL was measured at cold and warm temperatures. Further measurements were made in the HP, CLM and GL of crabs maintained in the same acclimation temperatures at 24h intervals till the rate of oxygen consumption stabilized at a new level in the new thermal regime. Each measurement of tissue oxygen consumption was made in six crabs and their mean value is taken into consideration.

To study the effect of eyestalk extracts both the eyestalks of cold-acclimated and warm-acclimated crabs were removed at the proximal end with indectomy scissors and the stubs were cauterized. The eyestalks were held on ice until a sufficient number were obtained and their extracts were prepared separately.

Preparation of various types of eyestalk extracts :

Aqueous extract : Aqueous extract was prepared as per the procedure given by Ranga Rao (1965). Aqueous extract was prepared
by crushing the eyestalks in precooled mortor with a pestle in a small amount of distilled water. The resulting suspension was decanted into a glass tube immerced in ice jacket. This procedure was repeated to get the particles into a suspension. The suspension was centrifuged at 2500 rpm for 20 min at 0°C. The resulting supernatant was diluted with cold distilled water to give a concentration of two eyestalk equivalents per 50 μl.

Acetone extract: Acetone was prepared according to procedure given by Bartell et al. (1971). Eyestalks were homogenized in acetone. The suspension was centrifuged at 1500 rpm for 10 min and washed 3 times, recentrifuged and the resulting supernatant was allowed to evaporate overnight. The residue obtained was washed with chloroform: methanol (1: 2) and centrifuged. The supernatant was decanted and the final residue was resuspended in crustacean saline (Van Harreveld, 1936) to give a dosage of two eyestalk equivalents per 50 μl.

Boiled ethanol extract: Boiled ethanolic extract was prepared according to Silverthorn (1975a). The eyestalks were homogenized in a small amount of 80% ethanol. The solution was collected and the procedure was repeated twice. The solution obtained was heated in boiling water for 10 min in a test tube. The extract
was centrifuged at 2500 rpm for 20 min and the clear supernatant was allowed to dry at room temperature overnight. The residue was resuspended in crustacean saline to give a dosage of two eyestalk equivalents per 50 μl.

**Saline extract**: The Saline extract was prepared according to Bartell et al. (1971). The saline extract was prepared by crushing the eyestalks in a small amount of ice cold crustacean saline (Van Harreveld, 1936). The resulting suspension was centrifuged at 2500 rpm for 20 min at 0°C. The resulting supernatant was diluted with crustacean saline to give a dosage of two eyestalk equivalents per 50 μl.

**Ether extract**: Ether extract was prepared according to procedure given by Vijayalakshmi (1964). Eyestalks were homogenized in a small amount of 80% ethanol. The solution was collected and the procedure was repeated twice. The extract was centrifuged at 2500 rpm for 20 min. The resulting supernatant was taken in a test tube and equal amount of ether was added to it and shaken well. The mixture was again centrifuged and the upper ether layer removed and discarded. The remaining extract was allowed to evaporate at room temperature overnight. The residue was resuspended in crustacean saline to give a dosage of two eyestalk equivalents per 50 μl.
Injection of eyestalk extract: Crabs were injected with a dose of 50 µl of extract equivalent to two eyestalks, through the articulating membrane at the base of the coxa of the fourth pair of walking legs.

The following types of animals were used:

1. Eyestalk extract from cold-acclimated crabs was injected to normal crabs, for which normal crabs injected with 50 µl of saline served as controls.

2. Eyestalk extract from cold-acclimated crabs was injected to warm-acclimated crabs, for which warm-acclimated crabs injected with 50 µl of saline served as controls.

3. Eyestalk extract from warm-acclimated crabs was injected to normal crabs, for which normal crabs, injected with 50 µl of saline served as controls.

4. Eyestalk extract from warm-acclimated crabs was injected to cold-acclimated crabs, for which cold acclimated crabs injected with 50 µl of saline served as controls.

Tissue processing:

All investigations were carried out simultaneously in control and experimental crabs. Prior to the estimations, the
crabs were dissected out and the hepatopancreas, claw-muscle and gill were carefully isolated. The tissues were blotted and weighed in the electrical balance to the nearest milligram. Separate homogenates were prepared for all estimations.

Determinations/Measurements:
Whole-animal oxygen consumption

Whole animal oxygen consumption of control and experimental crabs was measured by Winkler's iodometric method as described by Welsh and Smith (1953) as described earlier.

Tissue oxygen consumption:

The rate of oxygen consumption of the hepatopancreas, claw-muscle and gill of control and experimental crabs was measured using constant volume respirometer (Gallenkamp, England) as per the procedure given by Umbriet et al. (1972) as described earlier.

Aldolase: (Fructose 1,6-diphosphate, D-glyceraldehyde-3-phosphate lyase, EC.1.2.1)

Aldolase activity was assayed by the method of Bergmeyer (1965).
10% (W/V) homogenates of the hepatopancreas and claw-muscle and 5% homogenate of the gill were prepared in 0.25 M sucrose solution and centrifuged at 2500 rpm for 15 min. The clear supernatant was used for enzyme assay. The reaction mixture in a final volume of 3 ml contained: 1.75 ml of collidine-hydrazine buffer (pH 7.4), 0.25 ml of fructose 1,6 diphosphate (0.1 M) and 1 ml of enzyme solution. The reaction mixture was incubated at 37°C for 30 min in a thermostatic water bath. The reaction was stopped by the addition of 3.0 ml of 10% (W/V) trichloroacetic acid. The solution was centrifuged at 3000 rpm for 10 min. and to 1 ml supernatant taken in a test tube, 0.75 ml sodium hydroxide (0.75 N) was added. The colour developed was read with in 10 min. in a spectrophotometer (Elico-C1-24) at 540 nm against zero time control. Enzyme activity was calculated according to Bruns (1954) and expressed as μ moles FDP/mg protein/h.

Lactate dehydrogenase (LDH) : (L-Lactate : NAD Oxidoreductace, EC. 1.1.1.27).

LDH activity was assayed by the method of Srikanthan and Krishnamurthy (1955).

10% (W/V) homogenates of the hepatopancreas and claw-muscle and 5% (W/V) homogenate of the gill were prepared in 0.25
M ice cold sucrose solution and centrifuged at 2500 rpm for 15 min. The supernatant was used for enzyme assay. The reaction mixture in a final volume of 2.0 ml contained: 100 μ moles sodium phosphate buffer (pH 7.4); 40 μ moles lithium lactate; 0.1 μ mole NAD and 4 μ moles INT. The reaction was initiated by the addition of 0.5 ml supernatant and incubated at 37°C for 30 min. in a thermostatic water bath. The reaction was stopped by the addition of 5 ml of glacial acetic acid. The colour developed was extracted into 5 ml toluene and was read at 495 nm in a spectrophotometer against a reagent blank.

Enzyme activity was expressed as μ moles of formazan formed/mg protein/h.

Succinate dehydrogenase (SDH) : (Succinate : acceptor oxidoreductase. EC. 1.3.99.1).

SDH activity was assayed by the method of Nachlas et al. 1960.

10% (W/V) homogenates of the hepatopancreas and claw muscle and 5% (W/V) homogenate of the gill were prepared in 0.25 M ice cold sucrose solution and centrifuged at 2500 rpm for 15 min. The supernatant was used for enzyme assay. The reaction
mixture in a final volume of 2.0 ml contained: 100 μ moles sodium phosphate buffer (pH 7.4), 40 μ moles sodium succinate, 4 μ moles INT. The reaction was initiated by the addition if 0.5 ml of supernatant and the reaction mixture was incubated at 37°C for 30 min. in a thermostatic water bath. After incubation, the reaction was stopped by the addition of 5 ml of glacial acetic acid. The formazan formed was extracted overnight at 5°C into 5ml toluene. The colour developed was read at 495 nm in a spectrophotometer against a reagent blank.

SDH activity was expressed as μ moles of formazan formed/mg protein/h.

Isocitrate dehydrogenase (ICDH) : (Isocitrate : acceptor oxidoreductase. EC.1.1.1.4).

ICDH activity was assayed by the method of Korenberg and Pricer (1951).

10% (W/V) homogenates of the hepatopancreas and claw-muscle and 5% (W/V) homogenate of the gill were prepared in 0.25 M ice cold sucrose solution and centrifuged at 2500 rpm for 15 min. The clear supernatant was used for enzyme assay. The reaction mixture in a final volume of 2.0 ml contained: 20 μ
moles DL-isocitrate, 100 μ moles of sodium phosphate buffer (pH 7.4), 4 μ moles INT, 0.2 moles of NAD. The reaction was initiated by the addition of 0.5 ml of supernatant and the contents were incubated at 37°C for 30 min. in a thermostatic water bath. After incubation the reaction was stopped by the addition of 5 ml of glacial acetic acid and the formazan formed was extracted overnight at 5°C into 5 ml toluene. The colour was measured in a spectrophotometer at 495 nm against a reagent blank.

ICDH activity was expressed as μ moles of formazan formed/mg protein/h.

Aspartate amino transferase (AAT) : (L-aspartate: 2-oxoglutarate aminotransferase. EC. 2.6.1.1).

AAT activity was assayed by the method of Reitman and Frankel (1957) as described by Bergmeyer (1965).

10% (W/V) homogenates of the hepatopancreas, claw-muscle and gill were prepared in 0.25 M ice cold sucrose solution and centrifuged at 2500 rpm for 20 min. The supernatant was used for enzyme assay. The reaction mixture in a final volume of 1.0 ml contained: 100 μ moles sodium phosphate buffer (pH 7.4), 40 μ
moles L-aspartate, 2 μ moles α-Ketoglutarate and 0.2 ml supernatant. The reaction mixture was incubated at 37°C for 60 min. in a thermostatic water bath. After incubation the reaction was stopped by the addition of 1 ml of 2,4-dinitrophenyl hydrazine solution (ketone reagent) and the mixture was allowed to stand at room-temperature for 20 min. Zero time controls were maintained for all the samples by the addition of 1 ml ketone reagent to the mixture prior to the addition of enzyme source. To all the tubes 10 ml of 0.4 N sodium hydroxide was added and the colour developed was read in a spectrophotometer at 545nm against a reagent blank.

AAT activity was expressed as μ moles of pyruvate formed/mg protein/h.

Alanine aminotransferase (A1AT) : (DL-alanine : 2-oxoglutarate aminotransferase. EC. 2.6.1.2).

A1AT activity was assayed by the method of Reitman and Frankel (1957) as described by Bergmeyer (1965).

10% (W/V) homogenates of the hepatopancreas and claw-muscle and 5% homogenate of the gill were prepared in 0.25 M ice cold sucrose solution and centrifuged at 2500 rpm for 15 min. The
supernatant was used as enzyme source. The reaction mixture in a final volume of 2.0 ml contained: 100 μ moles of sodium phosphate buffer (P<sub>H</sub> 7.4), 100 μ moles DL alanine (P<sub>H</sub> 7.4), 2 μ moles Oxoglutaric acid and 0.5 ml supernatant. The reaction was initiated by the addition of 0.5 ml of enzyme source. The mixture was incubated at 37°C for 30 min. in a thermostatic water bath and the reaction was stopped by the addition of 1 ml of ketone reagent and the contents were allowed to stand for 20 min. at room temperature. To all the tubes 100 ml of 0.4 N sodium hydroxide was added and the colour developed was read in a spectrophotometer at 545 nm against a blank.

The enzyme activity was expressed as μ moles of pyruvate formed/mg protein/h.

Glutamate dehydrogenase (GDH) : (L-Glutamate : NAD oxidoreductase. EC 1.4.1.3):

GDH activity was assayed by the method of Lee and Lardy (1965).

10% (W/V) homogenates of the hepatopancreas and claw-muscle and 5% homogenate of the gill were prepared in 0.25 M ice cold sucrose solution and centrifuged at 2500 rpm for 15 min. The
supernatant was used as enzyme source. The reaction mixture in a final volume of 2.0 ml contained: 40 \( \mu \) moles of sodium glutamate, 100 \( \mu \) mole of NAD and 4 \( \mu \) moles of INT. The reaction was initiated by the addition of 0.5 ml of enzyme source. The mixture was incubated at 37°C for 30 min in a thermostatic water bath and the reaction was stopped by the addition of 5 ml of glacial acetic acid. The formazan formed was extracted overnight at 5°C into 5 ml toluene. The colour was read in a spectrophotometer at 495 nm against a reagent blank.

GDH activity was expressed as \( \mu \) moles of formazan formed/mg protein/h.

**Total Protein:**

The total protein content was determined by the method of Lowry et al. (1950).

1% (W/V) tissue homogenates were prepared in 10% (W/V) trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 min. The residue was dissolved in a known volume of 1 N sodium hydroxide. 0.05 ml of this solution was taken in a test tube and 4ml of alkaline copper solution was added. After 10 min. 0.4 ml of folin-phenol reagent was added and the contents were made up
to 5 ml with glass distilled water. The resultant blue colour was read with in 5 min at 600 nm in a spectrophotometer against a reagent blank. The amount of protein content was calculated from the standard graph prepared with Bovine serum albumin. The total protein content was expressed as mg/g wet weight of tissue.

**Total free aminoacids:**

The total free amino acids were estimated according to the method of Moore and Stein (1948).

The tissues were weighed; homogenized in 10% TCA and centrifuged at 3000 rpm for 10 min. 0.1 ml of supernatant was taken in a test tube and 2 ml of Ninhydrin reagent was added and kept in a boiling water bath. The contents were immediately cooled and the volume was made upto 10ml with glass distilled water. The violet colour of the samples was read at 570 nm against a reagent blank in a spectrophotometer. The amount of free amino acids in the samples was calculated from the standard graph prepared with tyrosine and the values are expressed as mg/g wet weight of tissue.
Ribonucleic acid (RNA):

RNA content was estimated by the method of Schmit and Thannhauser (1948) as described by Smille and Krotkow (1960).

The tissues were weighed; homogenized in 95% (V/V) methanol and centrifuged at 2000 rpm for 5 min and the supernatant was discarded. 10ml of 5% (W/V) TCA was added to the residue, stirred well and centrifuged. To the residue 2:1 ethanol:ether mixture was added and centrifuged. The residue was suspended in 95% ethanol and centrifuged. 5 ml of 5% perchloric acid was added and the contents were kept in hot water bath for 13 min. The contents were cooled and centrifuged. To 1 ml supernatant 1 ml orcinol reagent was added. The contents were boiled in a water bath for 10 min and the volume was made upto 10ml with distilled water. The colour developed was read at 660 nm against a reagent blank.

The RNA content was expressed as mg/g wet weight of tissue.
Total lipids:

The total lipid content was estimated gravimetrically by the method of Folch et al. (1957) as described by Natelson (1975).

The tissue was dried in hot air oven at 70°C. A known amount of dry tissue was homogenized in 1:1 (W/V) chloroform : methanol mixture. The homogenate was slowly heated in a water bath set at 60°C till boiling starts, then cooled to room-temperature. The volume was made up with chloroform and the contents were filtered into a 60 ml separating funnel through a filter paper moistened with chloroform. To this 10 ml normal saline was added and the contents were vigorously shaken. The lower chloroform layer containing lipids was collected. 3 ml aliquots of the chloroform extract were taken into pre weighed beakers of 5 ml capacity and the contents evaporated to dryness at 70°C. The beakers were cooled to room temperature and accurately weighed. The weight of the residue in the beakers corresponds to total lipids and expressed as mg/g dry weight of the tissue.
Free fatty acid content was determined titrimetrically by the method of Natelson (1975).

After determining the total lipid, the residue in the beakers was dissolved in 2 ml 95% ethanol. To each of the beakers, a drop of phenol red was added as an indicator. The contents were titrated against N/50 potassium hydroxide solution taken in a microburette. The appearance of red tinge indicated the end point. Titration of 2ml 96% ethanol plus indicator served as blank. The free fatty acid content was calculated from the titure value (sample value less the blank value) and expressed as mg/g dry weight of tissue.

Total body weight:

Crabs were taken out of water and numbered on their carapace with a glass marking pen. Their weights were recorded to the nearest milligram using a sartorius electrical balance. 24 hours after injection of Eyestalk extract, they were again weighed. The animals, normal as well as experimental, before being weighed were blotted gently with filter paper to remove the adhered water if any. The difference in weights of the normal and
experimental crabs gives the change in weight due to injection of eyestalk extract and expressed in grams.

Haemolymph volume:

The haemolymph volume of the crabs was determined by the procedure described by Lee (1961). The dye used was 0.25% congo red in 0.9% sodium chloride solution (Padmanabha Naidu, 1966). 100 μl of the dye solution was injected into the crab through the arthroidal membrane at the base of the fourth walking leg using a microsyringe. Vaseline was applied after taking the syringe needle out to prevent the loss of dye as well as the haemolymph. After 10 min the haemolymph was drawn from the crab, and the absorption of the dye in the haemolymph was measured at 480 nm in a spectrophotometer. A calibration curve was prepared using different quantities of the dye. The following equation was employed for the calculation of haemolymph volume.

\[ V = \frac{dg1}{dg2} - a \]

Where 'V' is the haemolymph volume; 'g1' and 'g2' are the amount of the dye injected and the amount of dye in the sample respectively; 'd' is the volume of the sample and 'a' is the volume of the saline injected with the dye.
Hydration levels (water content):

Total body water content (hydration level) was determined in the following manner. Crabs were taken out of the troughs; adhered water, if any, was wiped off gently with a filter paper and their weights were recorded (Wet wt.) Immediately, they were placed in prenumbered corning beakers; allowed to dry at 100°C for 48 h and quickly transferred to a desiccator. After 30 min. they were taken out of the desiccator and weighed again (dry weight). The difference between the dry weight and wet weight gives water content. The same procedure was followed for determining water content of the hepatopancreas, claw-muscle and the gill.

Sodium (Na⁺), Potassium (K⁺) Calcium (Ca++) and magnesium (Mg++):

Sodium, Potassium Calcium and magnesium contents were estimated by flame photometric method as described by Oser (1965).

The hepatopancreas, Claw-muscle and gill of crabs were isolated and weighed. The weighed tissues were wet ashed in 3:2 (V/V) concentrated nitric acid : concentrated sulphuric acid (Dall, 1967). After the tissues were completely dissolved, the
solution was evaporated at 70°C. The residues were dissolved in glass distilled water and made upto 5ml and filtered through whatman No.1 filter paper. Further, appropriate dilutions were made prior to the estimation of ions. The ions were quantitatively estimated using flame photometer (model Cl-24; Elico Hyderabad) and their concentrations were calculated from the calibration curves prepared with different standard solutions of respective ions. The values were expressed as millimoles/g wet weight of the tissue.

Chlorides (Cl⁻):

The chloride content was estimated by the method of Sendroy as modified by Robertson and Webb (1939).

2% (W/V) tissue homogenates were prepared in 10% (W/V) TCA: centrifuged at 5000 rpm for 15 min. 1 ml of supernatant was taken and diluted to 60 ml with glass distilled water in a 100ml standard flask. To this 5 ml of 23% phosphoric acid, 0.2g of silver iodate and a few drops of caprilic alcohol were added. The contents were made upto 100ml with glass distilled water and were filtered through whatman No.1 filter paper. To 20 ml of the clean filterate 2 ml of saturated iodine solution was added in a conical flask and liberated iodine was titrated against 0.1 N
sodium thiosulphate solution using starch as an indicator till the appearance of blue colour. The values are expressed as millimoles/g wet weight of the tissue.

Bicarbonates ($\text{HCO}_3^-$):

The bicarbonate content was estimated by the method of Segal (1965) as given by Henry (1966).

2% (W/V) tissue homogenates were prepared in 10% (W/V) TCA: Centrifuged at 5000 rpm for 10 min. 1 ml of supernatant was taken and diluted to 10 ml with glass distilled water and the $p^H$ was determined. To this when a known volume of 0.05 N hydrochloric acid was added carbon dioxide was released from bicarbonates and an equal amount of hydrogen was also being removed for the formation of water. The excess of acid was then back titrated to original $p^H$ with 0.01 N sodium hyroxide. The bicarbonate content of the tissues was calculated according to the following equation.

$$\text{HCO}_3^- = (\text{ml of acid} \times \text{normality of acid}) - (\text{ml of alkali} \times \text{normality of alkali}) \times 100.$$
The values are represented as millimoles/g wet weight of the tissue.

Chemicals used: All the chemicals (fine and bulk) used in this investigation have been obtained from standard companies viz., Sigma (USA) E.Merrck (Germany), Loba (Austria) and BDH (England).

Enzyme Nomenclature: The nomenclature of the enzymes was according to the reports of International Union of Biochemists (Oxford, Pergamon Press, 1961).

Validity of experimental procedures:
Aliquots for assay: Aliquots were selected for the assay such that the initial rates were approximated as nearly as possible, yet providing sufficient product to fall in a convenient range for the spectrophotometric measurement.

Enzyme units: Enzyme activities were expressed in standard units i.e., \( \mu \) moles of product formed/protein/h.

Lambert-Beer Law: Almost all the products of the reactions were measured by the colorimetric procedures in which the optical density (absorbance) of the resulting coloured complex was proportional to the concentration of reaction products.
Assay of dehydrogenases using INT: Karmarker et al. (1959) have reported that INT is superior to most of the tetrazolium salts as an electron acceptor for the assay of dehydrogenases. The advantages of using tetrazolium salts as electron acceptors are:-

(1) these salts give a stable colour upon reduction.
(2) they are highly soluble in aqueous solution.
(3) they can be reduced both aerobically and anerobically.
(4) they have high redox-potential which makes the reduction easier; and
(5) they are freely permeable through membranes.

Statistical treatment of the data: Standard deviation (SD); Percent change (%) and 't' test were calculated by standard procedures (Pillai and Sinha, 1968). Significant assessment level is 0.05 and not significant values are mentioned as NS.