Materials and methods.
For the detailed studies on the influence of osmotic stress several tropical poikilotherms commonly occurring in the fresh water ponds and streams have been chosen. The following are the representative species, from the different phyla and major groups occurring in fresh water, that have been used in the present study.

1. The fresh water field crab, *Paratelphusa* sp.
2. The common Indian cattle leech, *Hirudinaria granulosa*
3. The fresh water apple snail, *Pila globosa*, and
4. The fresh water mussel, *Lamellidens marginalis*

*Paratelphusa* sp.: *Paratelphusa* sp. occurs in abundant numbers on the outskirts of Tirupati about seventy miles from the sea. These crabs live in burrows in the paddy fields and tankbinds and live close to the water’s edge. It is completely cut off from the sea and there is nothing in the ecology of the crab that takes it to the saline medium. The crab is amphibious and reproduces in fresh water itself. The crabs were kept in the laboratory in a large earthenware tank with water just enough to submerge half of the body of the crabs. Mortality was great in the first few days till the crabs had become adjusted to laboratory conditions. Water was changed on alternate days and a day before experimentation the crabs were transferred in batches of twelve into glass troughs.
The crabs were fed with earthworms on alternate days and the crabs that have been used in the experiments were starved twenty four hours before the start of experiment, to avoid the discrepancy and variation in metabolism and blood composition due to differential diet. Gravid females and injured animals were not used in the experiments.

**Hirudinaria granulosa:** The leeches were collected from ponds in and around Madras and were transported to the Zoological laboratories of Sri Venkateswara University in earthenware pots with some amount of water. The leeches lived for long periods (from 4 to 5 months) in the laboratory. Once they were brought to the laboratory they were not given any blood meal.

**Pila globosa:** The snails were collected from local ponds and kept in glass troughs in the laboratory. Some detritus and aquatic vegetation was kept in the glass troughs on which these animals feed.

**Lamellidens marginalis:** The mussels were collected from a small local fresh water pond called 'Mangala gunta'. They were placed in groups of twelve in glass troughs containing water. Pondwater was mixed up with tap water to enable the animals to feed. Water was renewed daily.
In all the above cases animals of the widest size range available were collected. The animals were weighed before experimentation. The crabs were weighed on a Pelouze metric balance nearest to 0.20 gms. Leaches were weighed, after carefully blotting out the water adhering to their surface, in a chemical balance. Shelled forms like Pila and freshwater mussel were weighed entire before experimentation. The weight of the empty shells was taken after the conclusion of the experiments so as to get the weight of the soft parts which was taken as the standard body weight.

Sea water was collected from the Madras coast. It was filtered and kept in glass corboys. Record of salinity of the sea water has been kept since it varies with seasons. Different grades of sea water (10 percent, 25 percent, 37.5 percent, 50 percent, 62.5 percent, 75 percent) were prepared in the laboratory by diluting the 100 percent sea water with tap water.

For the measurement of oxygen consumption under water, the method reported by Saroja (1959) was used. To determine the dissolved oxygen content of water samples, the standard winkler's procedure as given in Welsh and Smith (1953) was followed. Blood was collected from all the animals listed above for analysing various ions like
chlorides, sulphates, sodium, potassium, calcium, magnesium and also for the determination of total osmotic pressure and the free aminoacid content.

The procedures followed for withdrawing the blood were different for different animals.

In the case of crabs the blood was withdrawn with the help of a hypodermic syringe through a small puncture made in the arthrodeal membrane at the base of the fourth walking leg. Bamberger and Olmstead (1933) have shown that there is a considerable variation in the calcium composition of the blood during moult cycle and Robertson (1939) has shown that there is a great increase in the calcium content of blood just before moult. For these reasons all the determinations have been carried out using the animals in the intermoult stage. The animals just after moult which could be recognised by the softness and pale color of the carapace, and the animals yielding blood of dirty brown colour were discarded.

In the case of leeches body fluid was taken for the analysis. To avoid mixing up of the host blood that is present in the alimentary canal of the leech, the animal was dissected open and the entire digestive tract was removed. The rest of the tissues were cut into small bits and were squeezed between two metal plates using a 'G' clamp. 0.25 to 0.35 ml of fluid was obtained in
this way which was used for the estimation of various ions.

The same procedure as above was followed for *Pila*. The alimentary canal of the animal was removed and the rest of the tissues were squeezed, yielding from 0.5 to 0.3 ml of fluid.

In the case of freshwater mussels, one of the valves of the shell was removed and the water present inside was drained off. About 0.5 to 1 ml of blood could be withdrawn from the heart with the help of a hypodermic syringe. It has been found that certain samples of the blood of *Paratellina* clot immediately and certain others do not. Heparin was used in the case of osmotic pressure determinations only. The effect of Heparin as an anticoagulant for the blood of crabs was very inconsistent. It has been noticed that the addition of a minute quantity of Heparin to blood samples (2 to 2 ml) does not effect the osmotic pressure of the fluid. The following are the methods followed for the blood analysis.

**Blood chloride:** Chloride content of the blood was estimated using 1.0 ml of the sample in the case of crab and 0.5 ml in the case of other animals following the Sendroy's method modified by Robertson and Webb (1939). The silver iodate was prepared in the laboratory as suggested by Robertson and Webb (1939). In leeches the chloride was estimated by direct titration with AgNO₃ using
Gallenkamp electrical (potentiometric) microtitrator. Blood sulfate: The method followed for the estimation of sulfate content was that of Catherton and Tomsett (1931) as suggested in Hilton and Waters (1955).

The principle involved is that the penzidine sulfate which is insoluble in acetone is diazotised in precipitate, coupled with Thymol and estimated colorimetrically.

Free amino acids: For the determination of free amino acid content of the blood and body fluids, Folin and Danielson's method (Hawk, Oser and Summerson 1954) was employed.

Osmotic pressure: Osmotic pressure of blood and body fluids was determined by the Raizer's vapour pressure method modified by Trogh (as described by Indira 1960). The osmotic pressure was expressed in terms of percentage Sodium chloride.

Sodium: Method of Weinbeck (as given in Hawk, Oser and Summerson 1954) was employed for the estimation of Sodium content in all the animals except leeches. After deproteinization the Sodium in the filtrate (precipitated in alcoholic medium as a triple salt, Uranyl Zinc Sodium acetate) is washed, dissolved in water and titrated with standard Sodium hydroxide. In the body
fluids of leeches the Sodium content was estimated using the Flame photometer.

Potassium: The Potassium content in the blood of crab, mussel and in the body fluids of \textit{Pila} was estimated using the spectrophotometer by the method of Meyer, Anderson and Swanson (\textit{Comparative Laboratory Plant Physiology}, 1955). The potassium content in the body fluids of leeches was estimated using the Flame photometer by the method suggested in Hawk, Oser and Summerson (1954).

Calcium: Clark-Collip modification of the Kramer-Tisdall method as given in Hawk et al (1954) was followed. Calcium is precipitated from the blood as oxalate and the latter is titrated with potassium permanganate.

Magnesium: The method of Koesan (1937) as given in Hilton and Waters (1955) was followed. Magnesium is estimated after separation as a salt of 3 Hydroxiquinoline.