MATERIALS AND METHODS
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The materials investigated are millipedes of the species *Phyllocomastagus nigrolabiatus* commonly available in Milgiris, South India. The identification of the species is based on the recent revised account of the genus *Phyllocomastagus* by Demange (1973). Largest individuals measure more than 15.0 cm in length and more than 1.4 cm in diameter. Hence they could give large quantities of haemolymph, which is an advantage for exhaustive analyses of the different components.

These diplopods are very sensitive to changes in the humidity in the atmosphere and to the moisture content of the soil. They lead active life in the monsoon season in the humid conditions. With the onset of summer, when the environment becomes less humid, these millipedes are less active and during summer, they disappear to aestivate deep in the soil.

The individuals, during aestivation, are found in a tightly coiled condition. Any amount or kind of disturbance does not restore them to normal active condition.
Millipedes in the active and aestivating phases of life were studied for the composition of the haemolymph, as this fluid tissue is the integrator of all the internal organs and this is the first tissue to be affected in adverse conditions (see Jauniaux, 1971). As it is known from the work of Sundara Rajulu (1974) that sex has an influence on the haemolymph components, only females have been studied throughout in the present investigation.

Collection Schedule:

The millipedes were collected at monthly intervals from January 1980 to December 1981 in a particular locality in Nilgiris and such specimens were used for the study of the haemolymph components, except for the electrophoretic studies of the proteins for which quarterly collections were found appropriate.

Collection of haemolymph:

Twenty five millipedes were taken from the collection and the haemolymph from the above animals was collected and pooled. The haemolymph was aspirated by means of an 18-gauge hypodermal needle and syringe through a puncture made in the addorsal line of a trunk...
Segment. The coagulation of the plasma was prevented by adding citrate salts. The haemolymph sample was centrifuged for 20 minutes at 2000 rpm to separate the haemocytes. From the supernatant (plasma) aliquots have been used for the appropriate studies.

**Study of free amino acids:**

The plasma was deproteinised following the method of Stein and Moore (1954). The deproteinised sample was centrifuged to separate the precipitated proteins, if any. The clear supernatant was decanted and used for estimations. The Folin method as delineated in Hawk et al. (1954) was adapted for quantitative estimations of total free amino acids content.

**Two-dimensional paper partition method**

of Block et al. (1958) was adopted for the qualitative analyses of amino acids on Whatman No.1 papers cut into squares of 12 cm x 12 cm size. The solvent for the first run was 2-butanol-3X ammonia (130:60 v/v) and the same for the second run at right angle was 2-butanol-formic acid-water (150:30:20 v/v/v). After run, the chromatograms were dried completely at room temperature. The amino acids in the chromatograms
were visualised by spraying 0.1% ninhydrin in n-butanol (w/v). The amino acids were identified by measuring their Rf values as well as by comparison with standard chromatograms prepared under similar condition using authentic samples of amino acids. The differences in the intensity of the colour of the bands were used as criteria for quantitative assessment and the relative intensity had been denoted by the number of pluses.

**Electrophoretic analyses of haemolymph proteins:**

Since proteins show characteristic mobilities in the electrical field, electrophoresis had been a most valid procedure in protein studies (Mier, 1959). This method was used to fractionate the different proteins of plasma.

The plasma protein was prepared as follows for electrophoretic studies. The protein was first precipitated by 10% TCA after separating the haemocytes by centrifugation at 2000 r/min for 30 minutes. The precipitated protein was collected by centrifugation, resuspended in the distilled water and subsequently dialysed in a cellophane bag against running distilled
water for 24 hours at 10°C. The material remaining within the cellophane bag was transferred to a clean china dish and dried in vacuo over phosphorus pentoxide and calcium chloride.

Any remaining lipid material was removed by again extracting successively with ethanol and ether. The residue was finally dried to constant weight. The dried protein precipitate was dissolved in an appropriate amount of phosphate buffer of pH 5.8, so that the concentration of the protein was kept between 2 and 5%.

The electrophoretic apparatus employed in the present work was a horizontal type (BIOCHEM) instrument with a power pack and a separate chamber. A platinum electrode runs along the whole length of each of the electrophoretic troughs to ensure supply of identical voltage of current to all the paper strips when more than one were used.

The precautions observed were as follow:
The troughs of the electrophoretic chamber were cleaned before every run and filled with a fresh sample of buffer solution. The electrophoretic strips were fixed
in such a manner that they reached into the trough at uniform depth. The buffer solution levels at both ends of the strips were kept similar to avoid siphoning effect. Care was taken to close the chamber air-tight.

The electrophoretic runs were carried out under the following conditions: Phosphate buffer pH 5.8 and ionic strength 0.2 ($0.167 \text{ M-K}_2\text{H}_2\text{PO}_4 = 0.0167 \text{ M-KOH}$), constant voltage 8 v per centimeter length of the strip and duration of run 4 hours. After completion of electrophoretic run, the electrophoretic strips were taken out, dried at 40 to 45°C for 25 to 30 minutes and stained in bromophenol blue.

The pherograms were scanned by means of a BIOCHEM densitometer.

**Quantitative estimation of plasma protein:**

The protein constituent of the plasma was precipitated by 10% trichloroacetic acid. The protein thus precipitated was separated by centrifugation and purified by dialysis. The purified and dried material was subjected to estimation by the biuret method.
of Cornell et al (1949). Bovine serum albumin was used as the standard.

**Study of carbohydrates of the hemolymph:**

The free sugar values of the plasma were estimated after deproteinizing by the method of Stein and Moore (1954). The free sugar in the deproteinized sample was estimated by the method outlined by Oser (1965) using 'Spectronic-20'.

Protein-bound sugar was assessed as follows. The protein was precipitated by adding three volumes of absolute alcohol to one volume of the plasma. The protein thus precipitated was hydrolyzed in N-HCl for 15 hours to liberate bound sugars (Dall, 1964). The liberated sugar was estimated by the anthrone method described by Oser (1965).

For qualitative analyses of the sugars, about 10 ml of the plasma was hydrolyzed with three volumes of 6N hydrochloric for 6 hours at 100°C and analyzed by paper chromatography (Block et al 1958).
**Analyses of sugars by chromatography:**

The principle of chromatography of sugar was the same as that for amino acids. The procedure followed was the ascending paper partition method. The different kinds of solvent systems used have been given in the text. The chromatograms were developed following the silver nitrate dipping technique of Trevelyan et al. (1950); the strip was dipped into a reagent solution prepared by adding 0.1 ml of saturated silver nitrate solution in 20 ml of acetone and then dried thoroughly at room temperature. The same was subsequently dipped in a mixture of 0.5 ml of sodium hydrosulfo in 25 ml of ethanol, and again completely dried under a fan at room temperature before treating with 6N ammonium hydroxide solution for 30 minutes to remove the excess silver oxide formed. Finally the ammonia was washed by keeping the chromatograms in running water for 30 minutes. Sugars appeared as dark brown bands. They were identified based on their Rf values and also by comparison with standard chromatograms prepared simultaneously in identical manner using authentic samples of sugars.
Study of the lipids of the haemolymph:

For the study of the lipids the initial operations were carried out at very low temperature condition (5 to 8°C). The plasma was transferred to a tube held in ice and containing 1 to 2 μM reduced glutathione per ml of plasma. The mixture was then centrifuged at 3000 rev/min for 15 minutes and the supernatant used for lipid estimations.

5 ml of the supernatant was extracted with 50 ml of a mixture of isopropanol - heptane - 1M sulphuric acid (40:10:1 v/v) as suggested by Dole (1956). The extract was evaporated to dryness in vacuo and the residue was dissolved in 10 ml of scintillation fluid (5.5 gmes packard in 1 litre of toluene). Scintillation spectrophotometer (Model 3375) was used for quantitative estimation.

For fractionation the extracted lipid was subjected to thin-layer chromatography using a solvent system of hexane-diethylether-acetic acid (80:20:2 v/v) (Beenakkers and Gilbert, 1968). The plates were sprayed with Rhodamine 6 G and visualized under ultra violet light.
Appropriate regions of thin layer plates were scraped off carefully and extracted with chloroform-methanol-water (13:8:2 v/v). The extract was evaporated to dryness in vacuo. The dry lipid fractions were dissolved in appropriate quantities of scintillation fluid and their quantities estimated by scintillation spectrophotometer (Seeakkers and Gilbert, 1968).

**Estimation of the inorganic constituents of the haemolymph:**

For the estimation of the inorganic ions also, the haemocytes were separated by centrifugation and the clear plasma was used, as mentioned earlier.

**Sodium:**

The concentration of sodium was determined by chemical analysis based on the precipitation of a metal complex. Prior to the isolation of sodium, the proteins were precipitated first and then the anion was separated in the form of uranyl zinc sodium acetate. The final estimation was by photometric method (McCane and Shipp, 1931).

Standard solutions covering the range from 100 to 160 milliequivalents of sodium per litre, at a dilution of 1:100 were used for calibration purposes.
Calibration curves were prepared by reading the standard solutions in a colorimeter. Standards were run before and after analyzing the plasma samples to make sure that the calibration was constant (Oser, 1934).

**Potassium:**

Determination of potassium was based upon precipitation as the insoluble cobalt nitrite followed by photometric estimation (Brech and Gabler, 1930; Albanese and Wagner, 1943).

Standard solutions covering a range from 3 to 7 milliequivalents of potassium per litre at a dilution of 1:100 were used for calibration. The method for the preparation of calibration curve was the same as that for sodium (Oser, 1934).

**Calcium:**

The compleximetric method of Rales (1953) was used to determine calcium content. Plasma mixed with aurexide forms a red-coloured calcium aurexide complex which was in equilibrium with free calcium ions. Titrating the complex with EDTA chelates free calcium ions, causing the aurexide complex to release calcium ions. The titration was followed photometrically at 620 µm.
Magnesium:

Magnesium in the haemolymph was precipitated as magnesium ammonium phosphate and the latter was estimated by colorimetric phosphate determination (Denis, 1922).

The standard used was 1 ml of the molybdate solution into which was added 3 ml of the standard phosphate solution (equivalent to 0.03 mg of magnesium) plus 2 ml of water (Oser, 1954).

Chloride:

The method of Sendory modified by van Slyke and Hiller (Sendory, 1937) was employed for the estimation of chlorides. The plasma was treated with phosphoric acid containing picric acid to precipitate the proteins. The mixture was then shaken with an excess of solid silver iodate and filtered. Chlorides present reacted with the insoluble silver iodate to form insoluble silver chloride and soluble iodate, which passed into the filtrate. On the addition of iodide to the filtrate, the iodide reacted to produce free iodine, which was then titrated with standard thiosulphate.
Phosphate:

The quantity of phosphate in the haemolymph was estimated by the Fiske and Subba Row method (Oser, 1954). The protein was precipitated with trichloroacetic acid and the protein-free filtrate was treated with an acid molybate solution, which forms phosphomolybdic acid from any phosphate present. The phosphomolybdic acid was reduced by the addition of 1,2, 4-aminophthol sulphonic acid reagent, to produce a blue colour whose intensity would be proportional to the amount of phosphate present.

The phosphate standard solution was a mixture of 0.4 mg of phosphorous in 50 ml of 10% trichloroacetic acid, with a 5 ml sample of this mixture was added 1.0 ml of molybate solution and 0.4 ml of aminophthol sulfonylic acid reagent. This was diluted to 10 ml with water. The resulting solution served as the standard.

Bicarbonate:

The Van Slyke's principle by titration was adopted to estimate the bicarbonate ions of the haemolymph (Hawk et al, 1954).
The values of the haemolymph components given for each month indicate the means of five analyses, in each case.

The average value of the aestivating period (January to June) in each case has been compared with that of the active period (July to December).

The data were analysed statistically by using Student's t-test (Simpson et al., 1960).