CHAPTER II

MATERIALS AND METHODS
A. COLLECTION AND REARING OF THE INSECT:

*Dysdercus similis* (Freeman) were collected from the fields around Sagar. The adults and nymphs of *D. similis* were collected from Bhindi plant (*Abelmoscus esculentus*) and cotton plant (*Gossypium sp.*) in the months of May to December. These were then reared all the year round in the laboratory in B.O.D. temperature regulator at 27°C. The insects were fed on moist cotton seeds every day. After the final molting, the newly emerged adults were separated and kept in glass jars. Mouths of the jars were covered with muslin cloth tied with rubber bands. To obtain pure cultures, the insects were allowed to breed for four generations before taking them for experimentation. Individuals were sexed on the basis of external morphology. Newly emerged adults were separated in three groups: In the first group males and females were kept together. The second group comprised unmated males and the third group of unmated females. These three groups of insects were kept in separate jars and marked for their ages for the present investigation.

B. COLLECTION OF HAEMOLYMPH SAMPLES:

Samples of haemolymph for polyacrylamide gel electrophoresis were obtained by the following two methods:

(i) The legs, wings and tip of the abdomen were cut with sharp scissors and the digestive tract was pulled out along
with the head capsule. The insects were then placed and supported inside a centrifuge tube by a perforated glass cup, or wire-gauge. A gentle centrifugation resulted in the accumulation of the haemolymph at the bottom of the centrifuge tube. This haemolymph was free from fat body and other tissues.

(ii) The pleuron region at the base of the metathoracic coxa was pierced with a sharp pin. A drop of the haemolymph so exuded was soaked in the filter paper.

In all the experiments the haemolymph was used immediately after collection.

C. HAEMOLYMPH PROTEIN FRACTIONATION BY POLYACRYLAMIDE GEL DISC ELECTROPHORESIS;

It was carried out according to the method of Davis (1964), with a little modification that in place of sample and spacer gels, sucrose (5%) was layered on the gel and the sample in 10% sucrose was applied. The pH of the running gel had been kept between 8.8 - 9.0, while the pH of the electrolyte (Tris-Glycine buffer) was 8.3. After electrophoresis, gels were removed and proteins were located by leaving the gel for 2-3 hours in a fixing solution of 7% acetic acid containing Amido black to a concentration of 0.5%. The gel was cleared of background dye by washing in numerous changes in the fixing solution not containing the dye. In order to return the gel to its original dimensions
it was immersed and stored in aq. 10% acetic acid until required for photography.

The relative electrophoretic mobility (Rm) values of the separated protein bands were calculated by the method of Kulkarni and Mehrotra (1970):

\[
Rm = \frac{\text{Distance travelled by protein band from origin}}{\text{Distance travelled by tracking dye from origin}} \times 100
\]

D. EXTRACTION OF VARIOUS FRACTIONS:

Insects were homogenized with polytron ultrasonic vibrator in ice cold 10% TCA. The homogenate was allowed to stand for 10 minutes in ice and then centrifuged at 1500 x for 20 minutes. The sediment was washed twice with 5% ice cold TCA. The combined supernatants constitute the 'Acid-Soluble-Fraction'. All the proteins, RNA, DNA and lipids were precipitated.

(i) Removal of Lipids:

The sediment obtained after the removal of acid soluble fraction was subsequently washed with alcohol, alcohol:ether (1:3) and ether to remove all the lipids and organic solvent-soluble metabolites. The supernatant was discarded.

(ii) Nucleic Acid Fraction:

The lipid free residue was treated with 20% TCA at
80°- 90° for 20 minutes. The supernatant constituted 'Hot TCA Fraction or RNA Fraction' and contained hydrolysed RNA and DNA components.

(iii) Protein Fraction:

The post nucleic acid pellet contained proteins. It was washed twice with ether and was dissolved in 0.1% NaOH and counted.

E. PROTEIN ESTIMATION:

Total proteins were estimated by the method of Lowry et al. (1951) using photo-electric colorimeter systronic model 101. Crystalline bovine serum albumin was used as a standard. The results are expressed as mg of protein/gm wet body weight.

F. RNA ESTIMATION:

RNA was estimated according to the method of Mejbaum (1939) as described by Schneider (1957), using Sigma grade RNA (in 10% TCA) as standard. The results are expressed as mg of RNA/gm of wet body weight.

G. DNA ESTIMATION:

DNA was estimated by the method of Mejbaum (1939) as described by Schneider (1957), using Calf thymus DNA as standard. The results are expressed as mg of DNA/gm of wet body weight.
H. STATISTICAL PROCEDURES:

Statistical procedures used in the analysis of these data are detailed in Lewis (1971). Standard deviation, Standard error, Coefficient of variation and Coefficient of correlation were calculated with the help of following formulae:

\[(1) \quad \sigma = \sqrt{\frac{\sum d^2}{n - 1}}\]

Where \(\sigma\) stands for the standard deviation. \(\sum d^2\) for the sum of the squares of the deviations measured from the arithmetic average and \(n\) for the number of items.

\[(ii) \quad S.E. = \frac{\sigma}{\sqrt{n - 1}}\]

Where S.E. = Standard error of the mean

\(\sigma\) = Standard deviation

\(n\) = Number of items

\[(iii) \quad V = \frac{\sigma}{a} \times 100\]

Where \(V\) = Coefficient of variation

\(\sigma\) = Standard deviation

\(a\) = Arithmetic mean.

\[(iv) \quad r = \frac{n \sum xy - \sum x \sum y}{n^2S' S''}\]

Where \(r\) = Coefficient of correlation.

\(S'\) = Standard deviation of \(x\), the independent variable.

\(S''\) = Standard deviation of \(y\), the dependent variable.
$n = \text{Number of items.}$

In the present work, the statistical data used in the analysis of the various estimations are represented in Tables and Figures. Standard deviation, standard error, and coefficient of variation are shown in Tables 5-10 and 15-32. Graphs were plotted between age and RNA/DNA and protein content of mated (normal) and unmated (isolated) males and females of *D. similis*. The age (in days) of the insect has been shown on the 'x' axis (horizontal axis) and the values of RNA, DNA and protein content are shown on the 'y' axis (vertical). In all the figures, vertical bars represent standard deviation. The coefficient of correlation has also been calculated and the values are shown in Table 33.