CHAPTER III

MATERIALS AND METHODS
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Collection and Rearing of Insects:

The insect selected for the present study is *Poekilocerus pictus* (Fabr) Orthoptera: Acrididae.

The nymphs were collected from the calotropis plants from the fields around Sagar during June to August, and were kept in glass fronted cages. Fresh calotropis leaves were provided daily for feeding. Various stages of nymphs (1st to 6th) were separated in bottles. Newly emerged adult insects were used for experimental work. After treatment, the male and females were kept together in pairs and their tissues were taken out at different time intervals according to the experimental stages selected.
Chemicals Used and Methods of Treatment:

The chemosterilants aphpolate and hempa were received as gift samples from Prof. W.E. Weidhaas, USDA, Florida (U.S.A.) and Dr. A.B. Borkovec, USDA, Maryland (U.S.A.) as 100% pure crystals of aphpolate and 100% redistilled solution of hempa.

Both the chemosterilants are soluble in acetone methanol and water (Borkovec, 1972). However, acetone and methanol (Saha and Khudabaksh, 1974) are known to induce chromosomal aberrations in insects, as such they could not be given to the control series of insects. Distilled and water containing glucose was, therefore, used either to dissolve or dilute the chemosterilants. The aphpolate was dissolved in distilled water. Saturated solution of aphpolate as well as of hempa in distilled water was made in percentage of 3.12% concentration for the chemosterilants, used in present investigation. .065 ml and .125 ml of aphpolate and hempa were injected to the experimental insects.

The newly emerged adult Poekilocerus pictus were selected for treatment. The chemicals were administered into the abdominal segment of body of this insect by injection of various amounts of aphpolate and hempa in distilled water with a microlitre syringe. By trial and error the desired dose of .065 ml and .125 ml of 3.12% of aphpolate and hempa were selected as it served as a sublethal dose and the insects survived after treatment with these doses for their full life span, that is upto 50 days and
more. Usually 6-8 grasshoppers were used for each experiment and those injected with distilled water only, were taken as the control insects.

The adult insects treated with apholate and hempa of doses viz. .065 ml. and .125 ml. were vivisected after 3, 7, 14, 21, 28, 35, 42 and 50 days of the treatment.

**Histological Studies of Cuticle:**

The cuticle from the controls as well as treated insects were fixed in 70% alcohol for overnight. After dehydration, the paraffin blocks prepared in usual way were cut at 8 μ thickness. The sections of the cuticle were stained with (Periodic acid Schiff's reaction (PAS) according to the method of McManus (Davenport, 1960) and were mounted in DPX.

**Collection of Haemolymph Samples:**

The base of metathoracic leg was pierced with a sharp needle and drops of haemolymph so exuded was collected on a clean glass slide.

(a) **In Vitro Procedure:**

For in vitro studies, haemolymph drops were directly transferred to a drop of 2% glacial acetic acid on a glass slide and were covered with a clean cover slip and the haemocytes were studied according to the method of Jones (1962).

(b) **Blood Film Preparation:**

(i) **Fixation by Heat:** The insect was immersed in a hot water at 60°C for 2 to 4 minutes and the blood from an antenna was
spread on slide, which was dried at 32°C and stained in Giemsa stain for about 20 min and was covered with the cover-glass.

(iii) **Fixation in Formalin Vapour**: A drop of haemolymph spread on a clean glass slide which was immediately placed in vapour of 40% formalin in a covered container and left for one hour.

It was rinsed in distilled water and stained in Giemsa stain and covered with a clean coverslip.

**Staining**:

The following stains were used for histomorphological and histochemical observations:

(a) **Giemsa Staining**: It is considered as the most reliable according to Arnold and Hinks (1979). It can be used directly for rapid staining for differential of more detailed results.

(i) **Rapid Staining**: Air dried slides are directly placed in the Giemsa solution (1 drop of concentrate stain per millilitre distilled water) for 5 minutes then rinsed in distilled water for one minute. It is blotted dry, and mounted as a permanent slide in DPX. Temporary slides were prepared using glycerol according to method of Arnold and Hinks (1979).

(ii) **Differential Giemsa Staining**: Air dried films of heat fixed haemolymph were immersed in Giemsa solution (1 drop of concentrate per millilitre distilled water) from 20 minutes to 2 hours. They were rinsed in distilled water, then immersed in alkaline water (distilled water to which a few drops of lithium
carbonate have been added). They were rinsed again in distilled water and then immersed briefly in acid water rinsed in distilled water and mounted in glycerine (Arnold and Hinks, 1979).

**Total Haemocyte Count (THC):**

The total haemocyte count was done by using a haemocytometer with improved double Neubauer rulings. The haemolymph was directly drawn from unfixed and live individuals of known sex and age through the pipette (used for the counting of WBC of mammal).

The metathoracic leg of *P. pictus* was cut with sharp scissors. The pipette was filled upto 0.5 mark in a white blood cell dilution pipette. The pipette was then filled with 2% glacial acetic acid upto the 11 mark. After this the pipette was shaken for several minutes to distribute haemocyte evenly. The first five drops from pipette were discarded and the rest of the diluted haemolymph sample was used to fill the haemocytometer. Cells in the four corners were considered in counting.

Haemocyte count/cubic mm of blood was done by the following formula as per Shapiro (1979):

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\text{Haemocyte in four square} \times \text{dilution} \times \text{depth factor of chamber} \div \text{Number of Square Counted}
\]

**Differential Haemocyte Count (DHC):**

This was done in *vitro* preparations and 100 cells per preparation were classified according to Jones (1962). Cells in mitosis were also counted. Some haemocytes could not be
placed with certainty in any of the classes according to the method of Jones (1962) and these cells were designated as unclassified cells. Minimum of 5 insects of each stage and physiological status were used in this study.

**Histopathological Studies:**

The tissues selected for the present study are — Pharynx, Oesophagus, Gizzard, Gastric caeca, Stomach (fore part), Stomach (mid part), Stomach (hind part) and Rectum.

Desired pieces of alimentary canal from the control as well treated insects were fixed in Carnoy's solution (Davenport, 1960). After 1/2 hour fixation the material was transferred to absolute alcohol giving two changes of half an hour. Then the material was kept in methyle benzoate for over night. From methyle benzoate the material was transferred to bensene for 15 minutes and three changes were given in paraffin wax of half an hour. The paraffin blocks were made and the sections were cut at 6 U thickness and were stained by Delafield's Haematoxylin/Eosin and mounted in DPX.