**THE INVESTIGATION OBJECT**

*Dysdercus koenigii* (Hemiptera: Pyrrhocoridae) is commonly known as the red cotton bug. It is a serious pest of cotton plant chiefly and thrives well also on the alternate host plant, *Hibiscus esculentus*.

**Rearing method and life-cycle:**

The insects were reared in a culture room at 26 ± 1°C, RH-70 ± 5%, 14:10 LD period. These insects were maintained on soaked cotton seeds in glass troughs. Additional water requirement was provided by keeping water bottles containing 0.05% L-ascorbic acid. Transfer to fresh culture troughs was done on every third day. At this time, the eggs were collected and kept in petridishes with wet cotton swab in a corner to maintain the humidity and left for hatching. The eggs hatch in about 5–6 days. These newly hatched nymphs were transferred to another jar with fresh, soaked cotton seeds. The post-embryonic development in this bug passes through five nymphal instars and the duration of first to fourth instars is around 3–4 days. The fifth instar has a slightly longer duration ranging between 5–6 days, after which they moult.
into adults. The adults start mating 2–3 days after their emergence and the mating continues up to egg-laying. Almost immediately after cessation of the prolonged copulation, the females start laying eggs on 7th or 8th day after emergence. The first reproductive cycle lasts for 7–8 days. This is followed by more reproductive cycles under laboratory conditions. Males have a longer life-span than females.

**Insects used for the experiments:**

Newly emerged adult males as well as females were collected at regular intervals and maintained separately. These were used at different times, according to the experimental requirements.

**CHEMICALS**

Acrylamide, actinomycin-D, bovine serum albumin (fraction V), brilliant blue R, DNA (calf-thymus), JH-III (cis-10,11-epoxy-3,7,11-trimethyl-trans-trans-2,6-dodecadienoic acid methylester), N N'-methylene-bis-acrylamide, POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene), PPO(2,5-diphenyloxazole), RNA (yeast), TEMED (N,N,N',N'-tetra...
methylethlenediamine), trizma base[Tris(hydroxymethyl)-amino methane] were purchased from Sigma Chemical Company, St. Louis, MO, USA. Isotopes \( \text{H}^3 \)-leucine (3,300 mCi/mmole) and \( \text{H}^3 \)-uridine (13,800 mCi/mmole) were purchased from Bhabha Atomic Research Centre, Trombay, India. Hyamine hydroxide (tissue solubilizer) was supplied by Beckman Company, England. Precocene II (6,7-dimethoxy-2,2-dimethyl-2-\( \text{H} \)-l-benzopyran) was obtained from Ega-Chemie, W. Germany. Ilford K₂ emulsion was purchased from Ilford Ltd., England. Chemicals for electron microscopy were supplied by Taab Laboratories, England. Stains were purchased from Serva Chemical Company, Heidelberg, W. Germany. All other chemicals used were obtained from commercial sources in India and were of analytical grade.

METHODS

Preparation of homogenate:

The fat body and ovaries were dissected out and freed from the surrounding tissues. All the subsequent operations were carried out at 0-4°C, unless otherwise specified. Tissues taken from several insects were pooled, weighed and homogenized in cold distilled water to make ly
to 10% homogenates as required, in a glass homogenizer with a teflon pestle (Potter Elvejhem type). The aliquots were used for the extraction of nucleic acids and estimation of proteins.

Extraction of nucleic acids:

The nucleic acids were extracted according to the procedure of Schmidt and Thannhauser (1945) slightly modified, as suggested by Munro (1966).

One ml of 10% homogenate (w/v) was mixed with 1.25 ml of 10% ice cold TCA and centrifuged to remove acid soluble compounds. The sediment was washed once with 1.25 ml of ice cold TCA. After the removal of acid soluble compounds, the sediment was extracted twice with 2.5 ml of 95% ethanol and the extract was removed by centrifugation. An ethanol-ether (3:1) wash was given to the sediment to remove the lipids present. The lipid-free pellet was suspended in 1 ml of 1 N Potassium hydroxide and incubated for 2 h at 37°C. This incubation with 1N Potassium hydroxide was sufficient to hydrolyse the RNA of the ovary and fat body. DNA and protein were then precipitated by the addition of 0.2 ml of 6N hydrochloric
acid and 1.3 ml of 5% TCA and allowed to stand in ice for 10 min and centrifuged. The supernatant fraction was collected separately to estimate RNA content. The sediment was suspended in 1.25 ml of 5% TCA at 90°C for 15 min with occasional shaking. The mixture was centrifuged and the supernatant was collected in a test tube. Now, the sediment was washed with 0.75 ml of 5% TCA and both the supernatants were taken for estimation of DNA.

**Extraction of RNA by the method of Fong and Fuchs (1976):**

The fat body was dissected out and homogenized in 1 ml of cold 95% ethanol containing 10% potassium acetate (w/v). The homogenate was centrifuged at 27,000 g for 15 min at 4°C and the pellet was washed with 3 ml of cold 2% perchloric acid (PCA). Hydrolysis of RNA in the pellet was accomplished by adding 1 ml of 0.5 N Potassium hydroxide and incubating at 37°C for 24 h. After the incubation, the hydrolysate was acidified by the addition of 0.27 ml of 7% PCA. The hydrolysate was then centrifuged and the supernatant was used either for RNA estimation or for radiolabelled counting using Bray’s mixture (see further below).
Estimation of DNA and RNA:

DNA was estimated by diphenylamine method (Burton, 1956). For estimation of DNA, 1 ml of DNA extract was mixed with 2 ml of diphenylamine reagent and heated for 10 min in boiling water. The intensity of blue colour developed, was read at 600 nm in a Systronics spectrophotometer. The amount of DNA present in a sample was determined from a standard curve using calf-thymus DNA as standard. RNA was estimated by the orcinol reaction (Schneider, 1957). For estimation of RNA, 1 ml of RNA extract was diluted to 2.5 ml with 5% TCA and heated for 30 min after adding 2.5 ml of orcinol reagent in a boiling water bath. The intensity of the resultant green colour was then read at 700 nm. A standard curve was prepared using purified yeast RNA as the standard.

Diphenylamine reagent:

This was prepared by dissolving 1 g of purified diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of concentrated sulphuric acid.
Orcinol reagent:

One gram of purified orcinol was dissolved immediately before use in 100 ml of concentrated hydrochloric acid containing 0.5 g of Ferric chloride.

Preparation of standards:

DNA (calf-thymus) and RNA (yeast) were first dissolved in water at a concentration of 2 mg/ml. Then a portion of this solution was diluted with 5% TCA and heated for 15 min at 90°C. The volume of solution thus obtained was made up with 5% TCA in such a way that the final concentration was adjusted to 200 μg/ml.

Protein estimation:

To a fraction of the homogenate, an equal volume of cold 10% TCA was added and kept at 0-4°C for 10 min. The sample was then centrifuged and the pellet thus obtained was washed twice with cold 5% TCA, followed by a wash with an ethanol-ether (3:1) mixture. The pellet was dissolved in 0.1 N sodium hydroxide and used for protein estimation by the method of Lowry et al., (1951).
An aliquot of the sample was made up to 1 ml with distilled water. To this, 5 ml of alkaline copper reagent was added (1 ml of 2% sodium potassium tartrate and 1 ml of 1% copper sulphate mixed with 100 ml of 2% sodium bicarbonate in 0.1 N sodium hydroxide) and the contents of the tube were mixed in a cyclomixer. After 15 min, 0.5 ml of Folin-Cio calteau reagent was added and the contents were stirred immediately. The colour was allowed to develop and after 30 min, the optical density readings were taken at 670 nm against a blank developed with 1 ml of distilled water. The protein concentration of the samples were determined from a standard curve drawn, using 20-200 µg of bovine serum albumin (fraction V).

H₃-uridine incorporation into fat body RNA:

The insects were injected with H₃-uridine (13,800 mCi/m mole) at a dosage of 0.5 µCi/10 mg body weight and sacrificed after 1 h incubation. The fat body was dissected out, homogenized and RNA extraction was carried out by the method of Fong and Fuchs (1976) as already mentioned above. The samples were counted in Beckman liquid scintillation counter (Model LS 3133 P–efficiency 40%) using 10 ml of Bray’s solution.
Incorporation of H³-leucine into TCA-precipitable material:

Incorporation of H³-leucine into TCA-precipitable material was determined at 1 h after the injection. The insects were injected with 1 μCi/100 mg body weight. After 1 h incubation, the haemolymph was collected with the help of microcapillaries and diluted with cold distilled water. Various organs like ovaries and fat body were dissected out and homogenized (2%) in cold distilled water. The samples were precipitated by the addition of an equal volume of cold 10% TCA. The precipitate was centrifuged at 2000 g for 10 min. The resulting pellet was washed twice with cold 5% TCA, twice with ethanol, followed by one wash with 3:1 mixture of ethanol-ether. The pellet thus obtained was dissolved in tissue solubilizer (hyamine hydroxide) and counted in Beckman liquid scintillation counter (Model LS 3133 P), using a vial containing 10 ml of toluene-based scintillation fluid (4 g PPO, 0.2 g POPOP in 1 liter of toluene).

Polyacrylamide gel electrophoresis:

Fat body and ovaries were dissected out from the adult insects and were homogenized in cold distilled water.
The haemolymph samples were collected from the clipped end of the antennae of the insects in a capillary tube and diluted with cold distilled water, according to the requirement. After protein estimation, the appropriate amounts of the same samples were used for electrophoresis.

Polyacrylamide gel electrophoresis was carried out at 4°C, using 7% gels in 0.1 M Tris–0.039 M glycine buffer (pH 8.3) at a current of 3 m amp per gel (Davis, 1964). Staining of the gels was performed with Coomassie brilliant blue and destaining was done as described by Weber and Osborn (1969).

Histological and autoradiographic techniques:

For histological studies, the tissues were dissected out in insect Ringer and fixed in Bouin's fluid. Paraffin sections were cut at 5-7 μm and stained routinely in iron alum haematoxylin-eosin.

For autoradiographic investigations, the insects were injected with H³-leucine (2 μCi/20 mg body wt.) as the precursor of protein. After varied incubation periods ranging from 30 min to 8 h, the insects were sacrificed.
The fat body and ovary were dissected out and fixed for 3 h in Carnoy's fluid. Paraffin sections (6 μm) were processed for autoradiography, using Ilford K2 emulsion. The exposure time varied between 3-5 weeks. The emulsion coated slides were then developed in Kodak D 19 B developer. Autoradiographs were mounted in Zeiss L-15 mounting medium and examined under phase optics.

Staining of neurosecretory cells:

The brain and retrocerebral complex were dissected out in insect Ringer and fixed in Bouin's fluid. The whole preparations of the brain and retrocerebral complex were stained with paraaldehyde-fuchsin (PF) technique, as modified by Bejra and Tandan (1964).

Measurement of corpus allatum volume:

The corpora allata were dissected out and taken in a drop of insect Ringer on a slide. The length and width were measured by occlometer and allatal volume was calculated using the equation $V = \frac{\pi}{6} d^2 L$, according to Goodman et al., (1968), where $V$ is volume, $d$ is diameter and $L$ is length.
Electron-microscopic studies:

Electron microscopic studies were carried out on corpora allata of experimental (precocene-treated) as well as control (acetone-treated) insects. The CA were dissected out and fixed in 2.5% glutaraldehyde in sodium-cacodylate buffer (pH 7.4) for 2 h at 4°C. After 2 h of repeated buffer wash, the materials were post-fixed in 1% osmium tetroxide for 1 h. Following dehydration, the materials were embedded in epon through propylene-oxide. Ultra-thin sections were contrasted with uranyl acetate and lead citrate, according to Reynolds (1963) and studied with Siemens Elmiscope 102 at an accelerating voltage of 60 K.V.

Statistical analysis:

All the data obtained in the study were statistically treated and the significance of difference between any two values was calculated according to Student's t-test.