C-H-A-P-T-E-R : II

MATERIAL AND METHODS

I. MATERIAL

A. Breeding of Chrysomyia rufifacies :

Various species of blowflies have long been favourite forms for a wide variety of experimental problems. Methods of culture, however, have not been satisfactory, and while much remains to be done in this field, to make the technique of blowfly culture more precise. Conditions of temperature and humidity, oviposition media, and larval foods all require additional investigation. A rather detailed study of this sort has been made on some species but there is no work on Indian blowfly Chrysomyia rufifacies .

The Chrysomyia rufifacies mate and ovipesit, and larvae mature successfully, at temperatures between 25° to 30°c. At temperatures much below or above these limits oviposition is greatly reduced or completely inhibited. In our laboratory we have successfully maintained a culture of these blowflies for last five to six years. The method of rearing the blowflies as described by Munich (1959) with some modifications was followed. The adults were housed in a screen wire cage and fed crystalline sucrose and water. Sheep meat was placed in the cage to stimulate egg production. The eggs hatched within 20 hrs. after laying, such early embryonic developmental

stages at 0, 5, 10, 15, 20 hrs. were taken. The larval growth was measured from the mean time of hatching (± 1.0 hr.) to the prepupal stage. The larval stages lasted for 4 days. The stage at puparium formation is called the pharate pupa (Prepupa). The prepupation and pupation (Pharate adult development) lasted for one and four days respectively, in each case of larval growth stage and prepupation and pharate adult development the studies were carried out at an interval of 24 hr.

For the study of Free Amino Acids following stages were selected.

I. Embryonic development

1) E_0 : Eggs just laid.
2) E_1 : Eggs 5 hr. development.
3) E_2 : Eggs 10 hr. development.
4) E_3 : Eggs 15 hr. development.
5) E_4 : Eggs 20 hr. development.

II. Larval growth stages

1) L_1 : 1st day larvae.
2) L_2 : 2nd day larvae.
3) L_3 : 3rd day larvae.
4) L_4 : 4th day larvae.

III. Prepupae

1) PP : Prepupae (Pharate pupa)

IV. Pupation

1) P_1 : 1st day Pharate adult development.

2) P_2 : 2nd day Pharate adult development.

3) P_3 : 3rd day Pharate adult development.

4) P_4 : 4th day Pharate adult development.

V. Adult

1) A : Freshly emerged adults.

VI. Empty puparia

Puparium membranes which are left behind after the emergence of adults.

B) Isolation of fat body

Fat bodies from various life stages of Chrysomyia were isolated under ice-cold Ringer's solution similar to that used by Stevenson and Wyatt (1962) adjusted to pH 6.5-7.0 with NaBH.

C) Preparation of haemolymph

The anterior end of the larva was pricked with a fine pin and the haemolymph released was collected in an ice-cold tube. By this method enough quantity of haemolymph was collected and centrifuged to remove the haemocytes. To prevent coagulation, prior chilling of the larvae and glassware and rapid dilution into saline was generally effective. The action of polyphenol oxidase, which causes darkening and eventual formation

of melanic precipitation was inhibited by addition of a few crystals of phenylthiourea.

D. Chemicals

All solvents were reagent grade and were obtained from E. Merck & Co., Rahway; U.S.A. and B.D.H. England, unless otherwise indicated. They included n-Butanol, M Methanol, acetic acid, phenol, ninhydrin.

Reference amino acids

The following main reference amino acids were used; β-Alanine,βAmino-n-butyric acid, Arginine, Aspargine, Aspartic acid, Glutamic acid, Glutamine, Glycine, Histidine, Proline, Hydroxy proline, Leucine, Isoleucine, Lysine, Methionine, Phenyl alanine, Serine, Threoine, Tyrosine, Tryptophan, Valine were obtained from B.D.H.England.

II. METHODS

Extraction, chromatographic separation,identification, and quantitative analysis of free amino acids.

One obvious reason that paper chromatography has been most widely used for the study of insect amino acids is to its high sensitivity, Owing to the small size of most insects, the available material is limited, especially when the analysis of individual tissues or organs is required. Sensitivity of ninhydrin reaction for detection of amino acids on paper is considerably high and the results of reasonable accuracy can be obtained with quantities of amino acids as little as 0.05 mg.

Under normal conditions the experimental procedure is simple. The Rf-Values of virtually all known amino acids and derivatives are available and may serve as a guide to the choice of the chromatographic system. It is, however, not always possible to predict on the basis of these data of which system is most suitable of the analysis of the sample in question. This is especially the case for insects which show the largest qualitative and quantitative diversities amino acids and related compounds.

The eggs, larvae, pharate pupae and stages of pharate adult development and freshly emerged adults, and puparia were isolated and after thorough cleaning were homogenized in pre-cooled 80% methanol, the homogenates were centrifused at 0°C for 10-15 min. at Ca 6000g. The supernatant fractions were carefully pipetted out, the residues washed with another volume of 80% methanol and again centrifused. These pooled methanolic extracts were preserved at-20°C.

In the present work the technique of horizontal and decending paper chromatography was used for separation of free amino acids and their quantative determination. Methanolic samples prepared from the various embryonic developmental stages, larval growth stages, pharate pupae and pharate adult developmental stages as well as a freshly emerged adults and empty puparia were transfered

to a Whatman No.1 filterpaper. In order to visualize comparative pattern of free amino acids of various life stages of Chrysomyia all the samples were placed simultaneously on the same sheet. The paper was run in n-Butanol: Acetic acid: Water (80:20:20), until the moving phase reached the end of the paper. After drying in air, the chromatogram was dipped into 0.3% ninhydrin in absolute acetone and kept at 60°C for 30 min. to develop the ninhydrin color. Following this, the paper was cut into strips and the color intensity of the individual spots measured on Spectronix-20. The total concentration was also determined by eluting the ninhydrin-positive components on each strip in an acidic copper nitrate solution, and the extinction value of the copper complex was estimated by a spectronix-20 at 510 Mμ. Substances which occur in the same spot on the one dimentional chromatogram as well as on horizontal chromatogram were checked by two dimentional chromatography using 70% n-propanol (ascending) and water saturated phenol (descending) as the first and second solvent respectively. Presence of serine phosphatide and ethanolamine phosphatide was confirmed by thin layer chromatography using silica gel G as a absorbant. The thin layer chromatography was carried out in the manner previously described (Mankapure and Sawant, 1980).