MATERIALS AND METHODS
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Materials:

The silkworm, Bombyx mori L. strain used throughout this study was F₁ hybrid between two varieties L and M₄D₂. Egg were obtained from the State Government Sericulture Department, Coimbatore, Tamil Nadu. After hatching, the larvae were reared on mulberry leaves at 25°C ± 2°C, 75% ± 5% R.H. and a photoperiod of 12 hours following Krishnaswami et al (1973).

Dimilin®, a registered commercial trade mark containing 25% diflubensuron (N-[4-chlorophenyl) amino] carbonyl] 2,6-difluorobenzenamide), a moult inhibiting insecticide was used in the present investigation. The insecticide used was obtained from Mysore Insecticide Company (P) Ltd., Madras. Aqueous suspension of Dimilin was prepared by dissolving in distilled water. Concentrations of Dimilin ranging from 0.015 ppm to 0.5 ppm were prepared for oral administration through mulberry leaves.

From the culture of each instar, five replicates of 50 larvae per replicate were used for each concentration. The larvae were fed with chopped mulberry
leaves treated with Dimilin. A minimum of five concentrations of Dimilin were used in each stage. Controls for all the batches in each instar were also maintained by feeding them with mulberry leaves sprayed with distilled water. Both the experimental and control batches were fed at six hourly intervals until they enter into their respective moulting stages.

For histological, histochemical and biochemical studies, cuticles of Bombyx mori larvae of all the instars both from the control batches and from the larvae treated with their respective EC50 concentrations of Dimilin were used. Quantitative estimations of protein, chitin and lipid were made after treatment for every 24 hrs.

**Calculation of Regression line:**

The percent effect of Dimilin on the moulting of silkworm larvae in each instar was corrected for natural response according to Abbott (1925).

Busvine (1971) has suggested that the critical doses or susceptibility can be estimated with sufficient accuracy from a probit/log. concentration graph. The two
variables are plotted on a plain paper or the original data (percentage failed to moult and dose) can be plotted on a logarithmic probability paper. A straight line is fitted by eye and the critical doses determined by inspection. Values determined graphically are often remarkably close to calculated results but they give no precise information on limits of accuracy (Busvine, 1971).

$EC_{50}$ and $EC_{95}$ values were arrived at using probit analysis method of Finney, (1978). According to Busvine (1971), the homogeneity of the population used in this investigation was verified employing $\chi^2$ test.

**Materials:**

Dlinilin treated silkworm larvae of all the 5 instars which failed to moult in each stage were fixed for histological and histochemical studies immediately after 72nd hr (1st instar larvae), 48th hr (2nd instar larvae), 72nd hr (3rd instar larvae), 96th hr (4th instar larvae) and 168th hr (5th instar larvae) respectively, along with their controls when they enter into their respective moult ing stage (Akagushi, 1978). For biochemical and enzyme assays fresh tissue from the above larvae were used.
Innumerable kinds of fixatives were employed by previous workers for the histochemical studies of insects. These include Bouin's fluid (Richards, 1952, 1958; Dennell and Malek, 1954; Taylor and Richards, 1963; Mouse, 1972), Carnoy's fluid (Richards, 1952; Dennell and Malek, 1954; Ishii, 1971), Dietrich's fluid (Clausen and Richards, 1951; Pipe and Cook, 1958; Richards, 1963), Gilson's mercuronitric mixture (Dennell and Malek, 1954), Susa (Pipe and Cook, 1958), Denslay's fluid (Richards, 1952), and Zankler's acetic acid mixture (Clausen and Richards, 1951; Taylor, 1963).

The present investigation involves both histology and histochemistry of the cuticle. Hence, alcoholic fixatives were avoided as these would affect the protein (Pearse, 1968). Of the other fixatives tried, Susa gave consistently reproducible results.

Sectioning methods:

Paraffin sections measuring 7-8 μ thickness for histological studies were made by conventional wax embedding method. Frozen sections for histochemical tests were prepared in the following manner. The fixed materials were washed thoroughly in running tap water to remove the
fixative. Small bits of the material measuring 5 mm x 5 mm were embedded in gelatin. For gelatin embedding, the above materials were successively infiltrated with 5% and 10% solutions of gelation for two days and finally the blocks prepared with 10% gelatin solution. Sections of 20 micron thickness were prepared by using a freezing microtome.

Stains employed:

Three principal stains were employed by previous workers on the cuticle of insects, viz: Mallory's triple stain, Masson's trichrome stain and Heidenhain's iron haematoxylin (Clausen and Richards, 1951; Richards, 1952; Dennell and Malek, 1954; Pipe and Cook, 1958; Taylor and Richards, 1963, 1965; Sharan and Srivastava, 1972; Hepburn and Farr, 1975). In the present investigation also these stains were used on the paraffin sections and they yielded good results consistently.

Histochemical techniques:

The details of the histochemical tests for protein, chitin, lipid and other substances, along with the references and relevant details have been given in the tables and text in the sequel in the appropriate contexts.
The relative intensity of the reactions by different layers of the cuticle has been indicated in the tables by the number of pluses.

Information concerning the types of forces involved in stabilizing the structural protein of different layers of the cuticle was obtained by the techniques of Trim (1941) and Brown (1930) which consisted of treating the protein under test with various reagents. The effect of the reagents on the different layers of the cuticle was examined by cutting fresh frozen sections, 20 μ thick, and putting them with the reagents in welled slides with coverslips rimed with petroleum jelly to prevent evaporation, and also by placing pieces of cuticle and reagents in stoppered bottles. After 24 hours the surviving cuticular materials were washed rapidly in distilled water and fixed in 9% neutral formaldehyde solution. Frozen sections of 20 μ thick were cut and examined.

Electrophoretic analyses of cuticle proteins:

As proteins show characteristic mobilities in the electrical field, electrophoresis had been a most important tool in protein analysis (Bier, 1959). This method was used Hackman (1953) to fractionate different proteins of the cuticle in many species of invertebrates.
A horizontal type electrophoretic set-up (Biochem) with a power pack and a separate chamber was used. A platinum electrode ran along the whole length of the electrophoretic troughs to ensure supply of identical voltage of current to all the paper strips when more than one were used.

The following precautions were taken during operation. The troughs of the electrophoretic chamber were cleaned before every run and filled with a fresh buffer solution. The paper strips were fixed in such a way that they reached into the trough at uniform depth. The levels of buffer solution at both ends of the strips were kept equal to avoid siphoning effect. The strips were held in horizontal position lightly tensioned with two glass rods equipped with lifting handles. Care was taken to close the chamber air-tight. To ensure that the atmosphere within the chamber has the necessary vapour pressure, the recesses between the brackets holding the glass rods were filled with the buffer solution.
The extraction of protein for the electrophoretic study from the cuticular material was done according to Heckman (1953). Both control and treated silkworm larvae of different instars were cut longitudinally on the mid-dorsal line and the underlying tissues removed in a washing medium of 65% (v/v) aqueous ethanol to free the cuticle. With aqueous ethanol the adhering epidermis and muscles were removed by scraping with a scalpel under a stereo-binocular microscope. After a final rinse in aqueous ethanol, the cuticles were dried in a desiccator in vacuo to constant weight.

The dried cuticles were powdered in a porcelain mortar and extracted with chloroform-ether (1:1 v/v) to remove lipid material. The residue was extracted for 36 hours at 50°C with a borax buffer at pH 9.2 (5:5:4:1 parts by volume of borax solution:water:ethanol:ether) until there was no further material precipitated by cold aqueous 5% trichloroacetic acid. The borax extract was filtered, acidified between pH 3 and 4 with 2% hydrochloric acid and the protein was precipitated by one third saturated ammonium sulphate solution.

The precipitated protein was collected by centrifugation, resuspended in distilled water and
subsequently dialyzed 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. Any remaining lipoid 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 protein was kept between 2 and 3%.

Stripes of Whatman No. 1 filter paper cut into bits of size 2 cm x 45 cm and a fine pencil line was made breadthwise at the centre. Prior to the application of the sample, the paper strips were soaked in the same buffer solution and the excess of buffer blotted by pressing the soaked strips in between two sheets of filter papers with the help of a rubber roller. By means of a smooth-tipped pipette taking care not to scratch the surface of the paper the protein sample was applied along the line marked.

The electrophoretic runs were carried out under the following conditions: Phosphate buffer pH 5.8 and ionic strength 0.2 (0.167 M K₂HPO₄ and 0.0167 M KOH), constant voltage 8 V/cm and duration of run 4 hours.
After completion of electrophoretic run, the strips were taken out, dried at 40°C for 30 minutes and stained in 0.05% bromophenol blue in saturated mercuric chloride solution and 3% acetic acid in ethanol. Subsequently washing in 1% glacial acetic acid was done until the blue bands became distinct against the white background.

The pherograms were scanned by a BIOCHEM densitometer. The curve obtained was traced on a graph sheet and the relative percentage of the individual proteins out of the total concentration was estimated by counting the squares enclosed in the peaks for the protein fractions.

Analyses of amino acids by chromatography:

The amino acid composition of electrophoretically separated protein components was analysed as fellows. An electropherogram stained with bromophenol was kept as a guide in which the positions of the different proteins were marked. By comparison with the guide strip the positions of different proteins were marked in other strips of unstained pherograms prepared under identical conditions as the former. The protein zones were cut and pooled separately. These were made into fine bits by a
scissors and hydrolysed with 6N hydrochloric acid under reflux for 12 hours at 105°C. The hydrolysate was concentrated to a syrupy fluid over a boiling water bath and dried completely by keeping in a desiccator in vacuo over potassium hydroxide. The dried material was dissolved in n-butanol to give a 3% concentration.

Two dimensional paper-partition chromatographic method of Block et al (1950) was followed for the analyses of amino acids on Whatman No. 1 papers cut into squares of 12 cm x 12 cm. The solvent for the first run was 2-butanol : 3% ammonia (150 : 60 v/v) and for the second run at right angle was 2-butanol : formic acid : water (150 : 30 : 20 v/v/v). After the second run, the chromatograms were dried completely at room temperature. The amino acids were visualized by spraying 0.1% ninhydrin in n-butanol (v/v) on the chromatograms and they were identified by measuring their Rf values as well as by comparison with standard chromatograms, prepared under similar conditions using authentic samples of amino acids. The differences in the intensity of the colour of the spots were taken as a criteria for their relative intensity which had been denoted by the number of pluses.
Quantitative estimation of cuticle protein:

Cuticle protein was extracted according to Hackman (1953) until they did not give positive reaction to the tests specific for proteins. The protein content was determined following the method of Lowry et al. (1951).

Cuticles of known weight of the control and treated larvae were homogenised separately and the homogenates in distilled water (100-fold of the cuticle weight) were centrifuged for 15 minutes at 3000 rpm. The protein content in the supernatant and the precipitate was determined by Folin-Ciocalteu reagent.

Preparation of standard graph:

Dissolved 25 mg of bovine serum albumin crystals in a little amount of 1M NaOH in a 5 ml standard flask and made upto 5 ml with 1M NaOH. This served as standard protein solution.

The protein solution of known concentrations were taken in separate test tubes. Individually all the solutions were made upto 2 ml with 1 N NaOH. Then 8 ml of biuret reagent was added and kept at room temperature. Blank was set up with 2 ml of 1 N NaOH and 8 ml of biuret reagent. After 30 minutes the absorbance value at 530 nm
was measured in a spectrophotometer. The concentrations of protein in x-axis and the absorbance values in y-axis were plotted to draw the slope.

**Determination of Protein:**

To 1 ml of sample 5 ml of reagent C (Alkaline copper solution) was added, mixed well and allowed to stand for 10 minutes at room temperature. 0.5 ml of reagent P (diluted Folin reagent) was added very rapidly and mixed within a second or two. After 30 minutes the absorbancy of the blue colour developed by the sample was measured at 530 nm. By comparing these values with that of known protein standards the total protein content in the cuticle was calculated.

**Estimation of Chitin:**

Chitin was estimated from the cuticle of both the control and treated larvae of different instars according to Hedman and Goldberg (1971). Silkworm larvae were washed briefly in water, drained and chilled to render them immobile. Pieces were cut from each end of the larvae and the body contents as well as muscles removed by opening the larvae longitudinally in 70% aqueous
ethanol and scraping the cuticles (Hackman, 1953). Each cuticle was examined under the microscope to see that it did not contain non-cuticular material.

Lipids were extracted from the cuticle by repeated extraction with boiling chloroform-methanol (2:1 v/v). After each extraction the cuticles were collected on a funnel. The cuticle was then air dried and powdered by hand in a mortar. Chitin was estimated from the powdered cuticle as follows.

To the 100 mg powdered cuticle, 8 ml of N NaOH was added in a 15 ml centrifuge tube fitted with an air condenser. The tube and contents were heated for 24 hrs in an oil bath at 100°C, cooled the condenser removed and the insoluble material sedimented at 3,000 rpm. The supernatant was removed, care being taken not to loose insoluble material and the residue was resuspended in 8 ml alkali. The extraction was repeated twice. On completion of the extraction with alkali the insoluble residue was washed twice with water, six times with ethanol, and three times with diethyl ether. The tube and contents were dried overnight in vacuum over phosphorus pentoxide and weighed. After removal of chitin the tube was cleaned in 6N HCl at 100°C for 2 hours, washed thoroughly, dried as above, weighed, and the yield of chitin was found by difference.
Estimation of lipids:

According to Poloh et al. (1957) the total lipid content of the cuticles from both control and treated silkworm larvae was estimated. Cuticles of known weight were homogenised in a 2:1 (v/v) mixture of chloroform--methanol for 2 minutes in a varying blender at room temperature. The homogenate was allowed to stand for 2 hours and then filtered. After filtration, the chloromethanol extract was purified. The chloroform layer was evaporated in a stream of nitrogen and the residue was taken up in petroleum ether, transferred to a tared 50 ml Erlenmeyer flask, and weighed after the ether was evaporated. The total lipid content was determined gravimetrically.

Cuticle enzyme preparation:

Immediately before ecdysis the haemolymph, alimentary canal, silk glands and fat bodies were removed and the cuticles of different instars were collected separately together with their controls. Known weight of the cuticles of each instar with attached tracheae and muscles were washed in water, cut into small pieces and homogenised in distilled water in a chilled glass-Teflon tissue grinder. The homogenate was centrifuged at 3,000 rpm for 20 minutes. The resulting supernatant
is referred to as the enzyme extract which was used for the determination of phenoloxidase and chitinase activity (Ishaaya and Casida, 1974). The protein content was determined by the Lowry et al. (1951) method as given earlier. Based on the protein content, the enzyme activities were expressed per mg protein.

**Assay of phenoloxidase activity:**

This assay was based on a method described by Ishaaya (1972). The reaction mixture consisted of 2.0 ml phosphate buffer, 0.2M, 1.0 ml water, 0.3 ml aqueous catechol solution and 0.5 ml enzyme solution in water. Catechol is preferred than dopamine or 4-ethyl catechol in the phenoloxidase assay (Ishaaya and Casida, 1974). The optimum conditions for phenoloxidase assay involve a reaction mixture of 0.0125M catechol; 0.025X enzyme extract in 0.1M phosphate buffer at pH 7.0 with incubation at 25°C for 2 minutes. The reaction mixture except for the enzyme was incubated for 5 minutes at the assay temperature and the enzyme solution was added to initiate the reaction. Control was prepared with 0.2 ml of distilled water in 2 ml of substrate. The increase in absorbancy of the mixture was recorded at 410 nm in a spectrophotometer for 2 minutes at 30 seconds interval. The results were expressed in absorbancy units (A) per mg protein at 2 minutes.
Assay of chitinase activity:

Colloidal chitin was prepared from the cuticle of *Bombyx mori* larvae in different instars based on the method of Monaghan et al. (1973) in which chitin (500 mg, sigma) is added to cold concentrated HCl (40 ml). The mixture is shaken for 10 minutes and insoluble materials were removed by filtration through glass wool. The solution is then neutralised in an ice bath by gradual addition of NaOH (12.5N, 40 ml) with final adjustment to pH 7 by inclusion of dilute NaOH or HCl solution. Distilled water is then added to bring the final volume to 100 ml.

Chitinase activity was assayed colorimetrically by the method of Isahaya and Casida (1974). The optimum conditions for the cuticle chitinase assay were a reaction mixture of 0.25% chitin, 0.15% enzyme extract, 0.04M phosphate buffer at pH 6.6, with incubation at 37°C for 60 minutes using 3,5-dinitrosalicylic acid (DNSA) reagent. The reaction mixture consisted of 0.12 ml phosphate buffer 0.2M, 0.3 ml of 0.5% colloidal chitin and 0.18 ml enzyme activity was determined by adding 1.2 ml of the DNSA reagent. The reaction mixture was heated for 5 minutes at 100°C, cooled in an ice bath, and diluted with
1.2 ml water. Undigested chitin was sedimented by centrifugation for 15 minutes at 3,000 rpm and the absorbance of the supernatant was recorded at 550 nm. Direct reaction of N-acetylglucosamine with DNSA reagent gives a linear plot of absorbance versus amount of NAGA. Chitinase activity was expressed as μg NAGA released per mg protein per hour.