MATERIALS AND METHODS
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Biology of the Experimental animal

After the onset of North East Monsoon in November, the Tiger butterfly, Danaus chrysippus (L.) lays eggs on the floral parts of the main host plant, Calotropis gigantea (L.) (Fig.1). The eggs hatch into larvae (I instar) which feed on floral parts of the host plant (Fig.2). From the second instar onwards they feed on the leaves of the host plant (Figs.3 to 6). The total larval period lasts 9 or 10 days. At the end of the larval period, as indicated by cessation of feeding, they undergo pupation. The pupa is generally light green or whitish in colour and affixed to the lower part of the host leaf (Fig.7). The period of pupation is six days. The adult is orange with black and white markings on the wings (Fig.8). Sex could be distinguished at the adult stage (Nayar et al., 1986).

Collection and maintenance of the test animal

For experiments, freshly hatched/moulted Danaus chrysippus (L.) larvae of the second instar were collected during the months of October - November from Calotropis gigantea in our University campus where they are available in abundance. In order to avoid genetic and size variations, larvae were collected from the same locality and host plant for each experimental series. They were weighed and reared individually in plastic containers. During this period they were fed with fresh Calotropis gigantea leaves. The larvae were acclimatised to 30°±1° C, 75±10 % rh and 10 h photoperiod.
Fig. 1. The eggs of *Danaus chrysippus* on the floral parts of *Calotropis gigantea*.
Fig. 2. A 1 instar larva of Danaus chrysippus feeding on a flower of Calotropis gigantea.
Fig. 3. A II instar larva of Danaus chrysippus on a leaf of Calotropis gigantea.
Fig. 4. A 3rd instar larva of Danaus chrysippus on a leaf of Calotropis gigantea.
Fig. 5. A IV instar larva of Danaus chrysippus on a leaf of Calotropis gigantea.
Fig. 6. A V instar larva of Danaus chrysippus on a leaf of Calotropis gigantea.
Fig. 7. A pupa of Danaus chrysippus affixed to the lower side of a leaf of Calotropis gigantea.
Fig. 8. An adult female (A) and adult male (B) of *Danaus chrysippus*.
Alkaloid

In the present investigation an alkaloid, piperine received from the Sigma, Chemical company St. Louis, USA has been used. Piperine is the constituent of black pepper, *Piper nigrum* (L.) and in the form of pale white crystals. It is insoluble in water but highly soluble in acetone. Piperine has a sharp burning taste.

Structural formula

\[
\begin{align*}
\text{O} & \\
\text{CH} = & \\
\text{CHCH} = & \\
\text{CH} - & \\
\text{C} - & \\
\text{N} & \\
\end{align*}
\]

Empirical formula

\[C_{17}H_{19}O_3N\]

Method of application

Freshly moulted IV instar larvae were isolated and reared in separate plastic containers. They were fed daily with fresh leaves of *Calotropis gigantea* soaked in sublethal concentration (0.125 ppm) of the alkaloid piperine. The soaking of leaves in piperine is done for 10 seconds. The Controls were similarly fed with leaves soaked in distilled water (mixed with 2 ml of acetone) for 10 seconds. The experiment was maintained till pupation. The LC$_{50}$ (1.25 ppm) value for piperine applied on IV instar larvae has been determined by probit analysis method of Finny (1971). Safe sublethal dose has been selected from LC$_{50}$ values determined for the application of the alkaloid piperine. Morphological changes
Fig. 9. A. Control larva of *Danaus chrysippus* at 10% acetone.

B. Deformed larva of *Danaus chrysippus* treated with piperine (1.25 ppm).
Fig. 10. A. Control pupae of *Danaus chrysippus* at 10% Acetone.

B. Pupal deformation of *Danaus chrysippus* treated with piperine (0.5ppm).
Fig. 11. A. Control adult of *Danaus chrysippus*.

B. Deformed adult of *Danaus chrysippus* due to sublethal (0.125 ppm) piperine treatment.
were also observed in the treated larvae, pupae and adult emergence (Fig. 9 to 11).

Statistical analysis

Results obtained in the present investigation has been subjected to the following statistical analysis.

i. Standard Deviation

ii. Analysis of variance (two way)

i. Standard Deviation ($\pm$SD)

\[
\text{SD} = \sqrt{\frac{\Sigma d^2}{N - 1}} \tag{1a-1}
\]

ii. Analysis of variance

\[
\text{Correction Factor (CF)} = \frac{(\text{Grand sum of all } \Sigma x)^2}{N} \tag{1a-2}
\]

\(X\) and \(X^2\) were calculated. Sum of \(X\) for all the columns were squared and divided by the number of tabulated values and a Correction Factor (CF) was obtained.
Total sums of Squares = Sum of \((\sum X)^2\) for all columns - CF

(la-3)

Between column sum of Squares = Sum of \((\sum X)^2\) of each column - CF

-----------------------------------------------
Number of values in a column

(la -4)

Between row sum of Squares = Sum of \((\sum X)^2\) of each row - CF

-----------------------------------------------
Number of values in a column

(la -5)

Interaction sum of squares = Sum of \((\sum X)^2\) for all blocks

-----------------------------------------------
Number in each block
- CF - between column SS
- between row SS

(la -6)

Within a cell SS = Total sum of (between column SS+) squares (between row SS)

Considering the degree of freedom for each source of variance, mean square was calculated.
Degree of freedom (df)

Total degrees of freedom = Number of values in the table - 1

Between column degrees of freedom = Number of columns - 1

Between row degrees of freedom = Number of rows in column - 1

Interaction degrees of freedom = Between column degrees of freedom \times Between row degrees of freedom

Within cell degree of freedom = Total degrees (between column of freedom df + between rows df + interaction df)

F Value for the variance between columns = \frac{\text{Means square between columns}}{\text{within cell mean squares}}

F Value for the variance between rows = \frac{\text{Means square between rows}}{\text{within cell mean squares}}
\[ F \text{ Value for the Interaction means square due to Interaction} = \frac{\text{interaction within cell mean squares}}{(la - 15)} \]

Significant level at the corresponding df was read from table D.11 given in Zar (1974).

**Biochemical Analysis**

Fourth instar larvae of *Danaus chryippus* (L.) were reared separately in plastic containers fed with fresh leaves of *Calotropis gigantea* soaked in sublethal concentration of the alkaloid piperine (Treated group). Control larvae were reared in separate plastic containers and fed with fresh leaves of *Calotropis gigantea* (Control group). The larvae completes the process of pupation within 8 to 10 h. Pupae of Control and treated groups were removed on 1st, 2nd, 3rd, 4th, 5th, 6th days and also on 7th day (Imago); sacrificed, mixed with 70% alcohol, ground well and kept in hot air oven maintained at 50°C for about 10 days. Then the dried sample of each group was thoroughly homogenised with the aid of mortar and stored in the freezer at -20°C for biochemical analysis.

**Preparation of material for enzyme assay**

The fresh material was homogenized with a mixture of butanol: benzene:acetone (2:1:1 v/v/v) by grinding in a chilled mortar (Morton quoted by Colowick and Kaplan, 1955). The homogenate was stored in a refrigerator for 24 h and later dried using an air drier. The dry powder was stored in a refrigerator for further work.

The required amount of dry powder was extracted with cold buffer (specific for each enzyme assay), kept for 24 h in a refrigerator and then filtered and centrifuged at
3000 rpm for 5 min. The supernatant was used for enzyme assay.

Collection of haemolymph

The pupae were punctured in the dorsal side with the help of a microneedle and then pressed gently. The haemolymph was collected in a graduated micropipette. Syringe was also used to collect the haemolymph. Then the haemolymph was suspended in a centrifuge tube and centrifuged at 1000 rpm. The haemolymph, thus tapped in the centrifuge tubes, was also utilized for biochemical estimations and electrophoresis.

METHODS OF BIOCHEMICAL ESTIMATIONS

Total reducing sugars

This was estimated by phenol sulphuric acid method of Dubois et al. (1956).

An aqueous extract of the dried material was prepared for the estimation of reducing sugars. A solution of 0.01% strength of glucose was used as standard. The colour developed was read at 490 nm in spectronic 20D+ Milton Roy, USA and the values are expressed in mg/100mg dry weight of haemolymph.

Estimation of glycogen

This was estimated by the anthrone method of Seifter et al. (1950).

The dried material was digested in KOH at 100°C for 15 min and washed twice with alcohol to collect sediment. The aqueous sediment was subjected to reaction with
0.2 % anthrone. The developed green colour was read at 630 nm in Spectronic 20D+Milton Roy, USA. The values were compared with that of known quantity of glycogen standard. The glycogen content was expressed as mg/100 mg dry wt. of haemolymph.

**Total fat**

Lipid content (mg/100mg dry weight of haemolymph) was estimated by the Potassium dichromate-Sulphuric acid method of Bragdon (1951).

For the separation of fat, benzene and butanol mixture in 2:1 volume was used. 100 mg. of dried material was homogenised with butanol-benzene mixture. To this homogenate equal volume of 5% trichloro acetic acid solution was added and thoroughly shaken well to displace the protein into aqueous phase and the protein was pipetted out. The butanol-benzene phase containing lipids was transferred to another clean test tube and the solvent was completely evaporated under reduced pressure. To this test tube, 2 ml of potassium dichromate reagent and an equal volume of distilled water were added (Bragdon, 1951). The colour developed was read at 580 nm in within 15 to 20 min. Spectronic 20D+Milton Roy, USA. Palmitic acid was used as a reference standard.

**Lactic acid**

This was estimated by the method of Barker and Summerson (1941).

To 100 mg of dried material, 5 ml of 10% TCA was added. Then the material was ground well, centrifuged and filtered. To the supernatant, 1 ml of 20% copper sulphate solution was added and made upto 10 ml by adding distilled water. To this 1 g of calcium hydroxide was added, shaken well, centrifuged and filtered. To 1 ml of this supernatant, 0.5 ml of 4% copper sulphate and 6 ml of sulphuric acid were added and kept in a
waterbath for 90 seconds, removed and then cooled. To this 0.1 ml of hydroxydiphenyl reagent was added. The colour developed was read at 560 nm Spectronic 20D+Milton Roy, USA. Lithium lactate was used as standard. The obtained result was expressed in mg/100 mg dry weight of haemolymph.

Citric acid

The penta bromo acetone method of Natelson et al. (1948) was adopted.

To 1 ml of extract, 1 ml of 10% trichloroacetic acid was added and shaken well. This was centrifuged and with 1 ml of supernatant solution, 0.5 ml of 18 N sulphuric acid was added and kept in an oil bath for 10 min and then cooled. To this 0.04 ml of 5% potassium bromide solution, 2 drops of 6% hydrogen peroxide and 1.3 ml of n-Heptane were added. This was centrifuged and the upper layer was transferred to a test tube. To this 3.5 ml of thiourea solution was added, shaken well and centrifuged inorder to decant the heptane phase. The aqueous phase was read at 490 nm in Spectronic 20D+Milton Roy, USA. and the result was expressed in mg/100mg dry wt. of haemolymph. Citrate was used as standard.

Pyruvic acid

The Pyruvic acid (mg/100mg dry wt. of haemolymph) was estimated by the 2,4-Dinitrophenyl hydrazine method of Friedmann and Haugen (1943).

To 2 ml of the extract of dried material, 10 ml of 10% trichloroacetic acid was added and centrifuged well and the supernatant was used for estimation. To 3 ml of
supernatant, 1 ml of dinitrophenyl hydrazine and 3 ml xylene were added and shaken well. Lower layer in the test tube was removed and to the upper layer, 6 ml of 10% sodium carbonate was added and mixed well. 5 ml of aqueous layer was mixed well with 5 ml of 1.5 N sodium hydroxide solution. The colour developed was read at 520 nm Spectronic 20D+Milton Roy, USA. Lithium pyruvate was used as standard.

Nucleic acids

Extraction of nucleic acids was done by suspending the material in 5% TCA at 0°C and extracting with 80% ethanol (hot ethanol) and 0.3 N Potassium hydroxide solution. The supernatant was adjusted to pH 1.5 with cold perchloric acid to precipitate DNA.

RNA

The total ribonucleotides in the supernatant was estimated by the orcinol method (Webb and Hitton, 1955).

To 5 ml of supernatant, 5.0 ml of orcinol reagent was added immersed in boiling water for 40 min and then cooled. The colour developed was extracted with 5 ml of isoamyl alcohol and read at 620 nm in Spectronic 20D+Milton Roy, USA. Quantification was done by referring to the standard curves prepared with standard yeast RNA. and the obtained values are expressed in µg / mg dry wt. of haemolymph.

DNA Estimation

DNA content was determined by reaction with diphenylamine (Burton, 1956).
The precipitated DNA and KClO₄ was hydrolysed in hot HClO₄ and incubated for 7 min at 90° C. The supernatant was collected. Excess perchlorate was removed by adding KOH and eliminating the insoluble KClO₄ by centrifuging. The supernatant containing deoxy-ribo nucleotides was then mixed with modified diphenylamine reagent. After incubation at 30°C for 16h, the colour developed was measured at 620 nm inSpectronic 20D+Milton Roy, USA. The quantities were arrived at by comparing with standard curve prepared with calf thymus DNA solution and the results are expressed in μg/100mg of dry wt. of haemolymph.

Estimation of enzyme activities

In the present studies, triphenyl tetrazolium chloride was employed to determine the activities of various enzymes investigated. The method of Kun and Abood (1949) was adopted. The intensity of the reduced red coloured formazon (indicating the liberation of the hydrogen ion from substrate and the absorption of hydrogen by the colourless TTC which was reduced to red coloured formazon) indicative of the enzyme activity was matched with artificially reduced formazon standards. The obtained values are expressed in mg of TTC reduced / 100 mg dry wt. haemolymph.

100 mg of TTC was reacted with 5 ml of 10% sodium hydroxide to produce the formazon. The formazon was then dried, weighed and known concentration, ranging from 1 mg to 2 mg were prepared by dilutions of the formazon in toluene. These were then subjected to Spectrophotometry at 420 nm for the preparation of standard curve. The obtained values are expressed in mg of TTC reduced / 100 mg dry wt. haemolymph.
Glycerol dehydrogenase (EC 1.1.1.6. glycerol : NAD oxidoreductase)

0.1 g of dried material was extracted with 5 ml of sodium pyrophosphate buffer. To the entire supernatant obtained, 1 ml of 1 M aqueous solution of glycerol and 1 ml of a freshly prepared solution of 0.1% triphenyl tetrazolium chloride (TTC) were added. The mixture was incubated at 45°C for 30 min and the intensity of red colour, due to the formazon formation was read at 420 nm in Spectrophotometer, along with the artificially reduced standards as per the method of Kun and Abood (1949). The results are expressed in mg of TTC reduced / 100 mg dry wt. haemolymph.

Glucose dehydrogenase

0.1 g of dried material was homogenised in 5 ml of citrate phosphate buffer, centrifuged and filtered. To this supernatant, 1 ml of 0.1% glucose and 1 ml of 0.1% TTC were added and incubated at 45°C for 30 min. The developed colour was estimated at 420 nm in spectrophotometer along with artificially reduced TTC standards and the results are expressed in mg of TTC reduced / 100 mg dry wt. haemolymph.

Alcohol dehydrogenase (EC 1.1.1.1., alcohol: NAD oxidoreductase)

0.1 g of dried material was extracted with 5 ml of 0.02 m sodium pyrophosphate buffer (pH 8.6). To the entire extract, 1 ml of 0.1 methanol and 1 ml of 0.1% TTC were added and the mixture incubated at 45°C for 30 min. The colour developed was read at 420 nm Spectronic 20D+Milton Roy, USA along with artificially reduced TTC standards. The obtained values are expressed in mg of TTC reduced / 100 mg dry wt. haemolymph.
Glutamate dehydrogenase

The method of Colowik and Kaplan (1955) was adopted. To 0.1 g of dried material, 10 ml of potassium phosphate buffer was added, ground, centrifuged and filtered. To 5 ml of this supernatant, 1 ml of 0.05 N Potassium glutamate and 1 ml of 0.1 % TTC were added and incubated at 45°C for 30 min. The developed red colour was measured at 420 nm in Spectronic 20D+Milton Roy, USA along with artificially reduced TTC standards. The results are expressed in mg of TTC reduced / 100 mg dry wt. haemolymph.

Succinate dehydrogenase (EC 1.3.9.9.1, Succinate (acceptor) oxidoreductase)

The method of Kun and Abood (1949) was followed. 0.1 g of dried material was extracted with 5 ml of 0.1 % pyrophosphate buffer. To this supernatant, 1 ml of 0.2 M sodium succinate and 0.1% TTC were added and the mixture was incubated at 45°C for 30 min. The developed red colour was read at 420 nm along with artificially reduced TTC standards. The obtained values are expressed in mg of TTC reduced / 100 mg dry wt. haemolymph.

Malate dehydrogenase (EC 1.1.1.37, L. malate : NAD oxidoreductase)

The method of Kun and Abood (1949) was adopted. To 5 ml of the pyrophosphate extract of 0.1 g of dried materials, 1 ml of 0.2 M solution of sodium malate and 0.1% TTC were added and incubated at 45°C for 30 min. The developed red colour was read at 420 nm in Spectronic 20D+Milton Roy, USA along with artificially reduced TTC standards. The
results are expressed in mg of TTC reduced / 100 mg dry wt. haemolymph.

Five replicates were used for each of the enzyme study. The average of the 5 replicates were given in the respective tables.

AMINO ACIDS

Extraction of Free Amino Acids (FAA)

100 mg of dried material was ground well in a mortar with sufficient quantity of 80% ethanol and the homogenate was filtered. Then 3 volumes of chloroform was added to each volume of ethanol extract. After thorough shaking and centrifuging, the remaining aqueous layer (upper layer) was removed to a china dish (Awapara, 1948, quoted by Block et al., 1958) and evaporated to dryness at temperature below 40°C (Thompson and Steward, 1951). The sedimented thin layer of free amino acids was dissolved in sufficient quantity of 10% isopropanol and the sample was stored at 4°C.

Extraction of Bound Amino Acids (BAA)

After the removal of the free amino acids with ethanol, the residue was hydrolysed with sufficient quantity of 6 N hydrochloric acid for 20h in a water bath at 100°C (Thompson and Steward, 1951). The hydrolysate was centrifuged and undissolved material was removed. The hydrolysate was then adjusted to pH 7.0 by adding enough ammonia solution and evaporated to dryness in a water bath at 100°C. The dried bound amino acids were dissolved in sufficient quantity of 10% isopropanol and stored at 4°C).
Extraction of Bound Tryptophan

The sediment, remaining after ethanol extraction from 100 mg dried material, was hydrolysed with sufficient quantity of 14% barium hydroxide under reflux at 100°C for 20 h. Barium was removed with a slight excess of 1 N sulphuric acid and the precipitate was thoroughly washed with hot water containing a drop of acetic acid. The filtrate was concentrated to a small volume in vaccum and then evaporated to dryness in a desiccator over calcium chloride. The dried amino acid was dissolved in sufficient quantity of 10% isopropanol and stored at 4°C (Block et al., 1958).

Application of the sample

Since the free and bound amino acids were very much less in quantity, they were dissolved in 0.1 ml of 10% isopropanol. For the free amino acids, the entire sample and for the bound amino acids half of the sample was applied onto the Whatman No.1 paper, little by little with the help of micropipette and the spots were dried by means of a hot air blower.

Chromatographic procedure

The free and bound amino acids were studied by two-dimensional ascending paper chromatographic techniques. The chromatograms were made on Whatman No.1 Paper 12 X 12 cms without any pretreatment. 20 X 20 X 8 cms size museum jars were used as chromatography chambers. The first solvent consisting of Butanol, acetic acid and water (4:1:1, V/V/V) (Reed 1950, quoted by Block et al., 1958) was used for the first direction. Buffered phenol (Berry and Cain, 1958 quoted by Block et al., 1958) was used as solvent for the second direction. To the Buffered phenol 0.04% B-hydroxy quinoline was added to prevent decomposition.
Chromatogram and detection of amino acids

The chromatograms were run for 12 h in the first solvent at room temperature 30°C - 32°C. After drying overnight, they were run in the second solvent for 6 h and dried overnight at room temperature.

Chromatograms were run with fresh 1% solution of ninhydrin in acetone and heated to 45°C for one hour in an oven (Hanks and Feldman, 1963) to develop the colour. The amino acids of the samples were identified by comparing their Rf values with those of standard amino acids, chromatographed simultaneously along with unknown, under identical conditions. The spots were marked and cut out at the end of an hour except proline, which was cut at the end of first 5 min of heating according to Hanks and Feldman (1963).

Preparation of Standard Amino Acids

The following amino acids were prepared as 0.1 % solution in 10% isopropanol.

1. Leucine 12. Hydroxyproline
2. Isoleucine 13. Glycine
4. Valine 15. Aspartic acid
5. Methionine 16. Citrulline
6. Tyrosine 17. Arginine
7. Tryptophan 18. Histidine
8. Proline 19. Lysine
Quantitative estimation of amino acids

The developed colours of the amino acids were eluted according to the method of Giri (1952) (quoted by Block et al., 1958). The intensity of the eluted colour of the various amino acids were read at 550 μm except for proline and hydroxyproline which were read at 530 μm. A blank determination was made on an uncoloured area of the same paper and a blank correction for each spot was calculated from the weight of the paper.

As per the method of Thompson and Steward (1951) standard curves were prepared under the same conditions for each amino acid in different amounts and referred for agreement by running two mixed standard solution, containing two sets of amino acids in various amounts.

Sample readings were converted to absolute amounts of micro milligram and calculated to micro milligram/100 mg dry weight. The amount below 5 micromilligram were recorded as traces. The reported data were based on atleast 5 chromatograms.

Estimation of inorganic ions

Sodium

To 0.1 ml of haemolymph, 7 volumes of Distilled water and 2 volumes of 20% of TCA were added, mixed and centrifuged. Filterate was collected as per the method of
Weinbach (1955) to a centrifuge tube and then 5 ml of Ur.Zn. Acetate reagent was added. Then 0.3 ml of 95% ethanol was mixed, centrifuged and decanted. Precipitation was done by adding 2 ml of ethyl acetate:acetic acid mixture. Ether was used to wash the precipitate. To the precipitate 4 ml of distilled water, 1 drop of acetic acid and 5.0 ml Potassium ferrocyanide were added and diluted to required quantity. The developed colour was read at 660 nm in Spectronic 20D+Milton Roy, USA as per the method of Snell and Snell (1954) and the obtained values are expressed in m-equiv/l of haemolymph.

**Magnesium**

Determination of Magnesium was done by titan yellow method of Neill and Neely (1956). Protein free filtrate was prepared by adding 0.1 ml of haemolymph to 5 ml of distilled water 2 ml of 10% sodium tungstate and 2 ml of 2/3 N H₂SO₄. 5 ml of filtrate was transferred to a tube containing 1 ml of distilled water, 1 ml of polyvinyl alcohol, 1 ml of titan yellow and 2 ml of 4 N NaOH. Standard was prepared by using 0.1 ml of working standard with other additions. After adding the NaOH, the colour developed was read at 540 nm in Spectronic 20D+Milton Roy, USA and the obtained values are expressed in m-equiv/l of haemolymph.

**Calcium**

Method of Snell and Snell (1954) was adopted. To 0.1 ml of haemolymph, 2 ml of distilled water and 1 ml of 4% ammonium oxalate solution were mixed. Overnight standing was permitted. The solution was centrifuged and the supernatant was poured off. The precipitate was washed twice with 3 ml of dilute ammonia. The remaining calcium
oxalate precipitate was diluted with distilled water and mixed with 0.35 ml of alizarin reagent. The colour developed was read at 620 nm in Spectronic 20D+ Milton Roy, USA. The calcium level was expressed in m-equiv/l of haemolymph.

Iron

Method of Jackson (1962) was adopted. 0.1 ml of haemolymph was digested with a mixture of nitric acid, sulphuric acid and perchloric acid (9:2:1, v/v/v). Initial digestion was done in a cold state and digested over a sand bath until a white digest was obtained. This digest was diluted and filtered. To 1 ml of extract, 0.5 ml of concentrated HCl, 2 ml of hydroxylamine hydrochloride and 1 ml of orthophenanthroline were added. The colour developed was measured in a spectrophotometer using 420 nm. This was compared against the standard. The iron level was expressed in m-equiv/l of haemolymph.

Manganese

Method of Snell and Snell (1954) was adopted. Extraction was done as shown in the case of extraction of iron. To 1 ml of extract 2 ml of HNO$_3$ and 0.2 ml of concentrated H$_2$SO$_4$ were added. Then 0.5 ml of 85% orthophosphoric acid was added and boiled. Finally 0.5 g of Potassium or Sodium periodate was mixed and boiled for 15 min. The colour developed was read at 530 nm in a spectrophotometer. The result was expressed in m-equiv/l of haemolymph.

Phosphorus

Method of Oser (1965) was followed. To 0.1 ml of haemolymph, 1 ml of 8.1% NaCl and 2.5 ml of 5 N H$_2$SO$_4$ were mixed and heated to get brown material and cooled. The
fused material was mixed with a drop of 30% hydrogen peroxide and diluted to required level with distilled water. That was followed by the addition of 1.25 ml of 2.5% ammonium molybdate and 0.5 ml of amino naphthol sulfonilic acid reagent. The colour developed was measured at 530 nm in a colorimeter. The phosphorus content was expressed in m-equiv/l of haemolymph.

**Sulphur**

Method of Snell and Snell (1954) was followed. Haemolymph is deproteinized with uranium acetate. The protein free filtrate was mixed with 1:1 HCl and diluted to required quantity with distilled water. Then 1.0 ml of 10% BaCl₂ solution was added and shaken well. The transmittance of white colour was measured at 530 nm in a spectrophotometer. The sulphur content was expressed in m-equiv/l of haemolymph.

**Polyacrylamide gel electrophoresis**

Modified Methodology by Davis, 1964

The following stock solutions were prepared, filtered and refrigerated in dark coloured bottles.

**Solution A**

In HCl : 48.0 ml

Tris (Hydroxylmethyl amino methane) : 36.6 gm

N, N, N', N' tetramethyl ethylene diamine : 0.46 ml

Glass distilled water : 100 ml

pH : 8.8
Solution B

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>30.0 gm</td>
</tr>
<tr>
<td>Crystallised N methylene bisacrylamide (BIS)</td>
<td>0.8 gm</td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Solution C

<table>
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<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate</td>
<td>140 mg</td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>100 ml</td>
</tr>
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Buffer solution for reservoirs

<table>
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<tbody>
<tr>
<td>Tris salt</td>
<td>600 mg</td>
</tr>
<tr>
<td>Glycine</td>
<td>28.8 gm</td>
</tr>
<tr>
<td>Glass distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>8.6</td>
</tr>
</tbody>
</table>

100 ml of stock solution was made upto 1 litre with glass distilled water and was used as tank buffer.

Preparation of working solution

Electrophoresis was carried out with the small pore gel and the gel concentration was kept at 7.5%.
Small pore gel solution

Solution A : 3 ml
Solution B : 6 ml
Solution C : 12 ml
Water : 3 ml

Acrylamide polymerization

Eight open ended glass tubes (4 mm I.D. X 100 mm long) were stoppered with rubber caps at one end placed with the open end up. The running gel (small pore gel) solution was pipetted into each tube to a depth of 90-95 mm taking care to avoid air bubbles. With a pasteur pipette distilled water was added slowly to avoid mixing up to a height of 5 mm. A sharp refractive boundary was visible between the gel solution and water. The entire set-up was kept undisturbed at 28°C for 30 min. After polymerization, the water layer was removed with the help of pasteur pipette.

Electrophoresis

After polymerization of gels in the glass tubes, samples (20 μl of haemolymph in each tube) in 10% sucrose were layered directly on top of the gel.

The upper ends of the tubes inserted into the rubber grommets at the bottom of the upper container. Tris - Glycine buffer with a few drops of tracer dye (Bromophenol blue 0.01% aqueous) was filled into each tube without disturbing the sample solution, the level reached the grommets. After filling the upper and lower containers with 500 ml of Tris - Glycine buffer, the power supply was connected, with the cathode on top and anode at the
bottom reservoirs. The current was adjusted to give 2 to 2.5 mA per tube for about 2.5 hours until the tracer dye had migrated to a distance of about 90-95 mm into the running gel. 

Electrophoresis was performed at 5-10°C.

**Protein staining and destaining of gels**

At the end of electrophoresis, the gel columns were removed from their tubes by trimming them with a blunt needle in cold distilled water. They were stained immediately after removal.

The gels were stained with 0.02% coomassie brilliant blue G 250 in a mixture of methanol, acetic acid, water (25:7:68 V/V/V). After staining for 24 hours, the gels were destained with the same solvent mixture without the stain until the bands were clear.

**Rm value was calculated by the following formula**

\[
R_m = \frac{\text{distance travelled by the protein fraction}}{\text{distance travelled by the Bromophenol blue}}
\]

from the Rm value, the slow moving, middle moving and fast moving fractions were identified and the data were presented in Table [64].
Histological studies

For histopathological studies, the IV instar larvae were fed with fresh leaves of the host plant soaked with LC50 doses of piperine solution, larvae which reached in moribund stage after 24 hours of feeding of treated leaves were dissected along with the control and its foregut, midgut and hindgut of D. chrysippus was dissected in insect ringer using thin micro needles, fixed in Bouin's fixative.

The classic paraffin sectioning and Haematoxylin-eosin staining technique were used for histological studies.

The preparations of tissues for the histological studies involved the following steps.

Fixation

In order to avoid tissue autolysis by the lysosomal enzyme and to preserve its physical and chemical structures, pieces of tissue not exceeding 2 mm in thickness, were cut and fixed in Bouin's solution as soon as possible. The Bouin's fixative was prepared by the mixing of 75 ml picric acid (Saturated aqueous) with 25 ml of formaldehyde and 5 ml of glacial acetic acid. The tissues were fixed in the Bouin's solution overnight and transferred to 30% alcohol.

Dehydration

For dehydration, ethyl alcohol was used. The tissues were kept in the following acending alcohol series.
1. 30% alcohol for an hour
2. 50% alcohol for an hour
3. 70% alcohol for half an hour
4. 90% alcohol for an hour
5. 100% alcohol for half an hour, twice.

Inadequately dehydrated tissues cannot be satisfactorily infiltrated with paraffin. At the same time, over dehydration results in making the tissues brittle, which would be difficult for sectioning. So the tissues have to be carefully dehydrated.

Clearing

For de-alcoholization, xylol was used as the clearing agent. Since the clearing agent is miscible with both the dehydrating and embedding agents, it permits paraffin to infiltrate the tissues. So, the clearing was carried out as the next step after dehydration, to permit the tissue spaces to be filled with paraffin in the next step. The tissues were kept in the clearing agent till they become transparent and impregnated with xylol.

Infiltration or Impregnation

It means the impregnation of paraffin wax by replacing the clearing agent xylol. The tissues were taken out of xylene and were kept in a molten paraffin wax bath. This was carried out in a paraffin embedding bath, which contains metal pots filled with molten wax. The temperature of the molten wax was maintained at 50°-58°C. The tissues were given 3 changes in the molten wax at half an hour intervals.
Embedding

The paraffin wax used for embedding should be fresh and heated up to the optimum melting point at about 50°-58°C. The melting point was maintained.

A clean glass plate was smeared with glycerine. L-shaped mould was placed on it to form a rectangular cavity. The molten paraffin wax was poured and the air bubbles were removed by using a hot needle. The tissue was placed in the paraffin and oriented with the surface to be sectioned, pressed the tissue gently towards the glass plate to make the tissues settle uniformly with a metal pressing rod and allowed the wax to settle and solidify at room temperature. Then the paraffin block was kept in cold water for cooling.

Section cutting

Section cutting is done with rotary microtome. The excess of paraffin around the tissue was removed by trimming, leaving ½ centimeter around the tissue. Then the block was attached to the gently heated object holder. Additional support was given by some extra wax, which was applied along the slides of the block. Before sectioning, all set screws holding the object holder and knife were hand tightened to avoid vibration. To produce uniform sections the microtome knife-holder with only the cutting edge comes in contact with the paraffin block. The tissues were cut in 7 micron thickness.

Flattening and mounting of section

This was carried out in a tissue floatation warm water bath. The sections were spread on a warm water bath after they were detached from the knife with the help of a hair brush. Dust free slides were coated with egg albumin over the whole surface. Required number of
sections were spread on the clear slide and kept at the room temperature.

Staining

The sections were stained with haematoxylin-eosin stain. Many tissues are colourless to observe under a light microscope. So, staining technique was applied to make various tissue components conspicuous and also permit distinction to be made between them. The tissues were cut into 7 μ - thickness, deparaffinised, hydrated, stained and mounted as described below.

Preparation of Ehrlich's Acid haematoxylin

Chemicals

Haematoxylin crystals (6g) were dissolved in absolute ethanol (300 ml) in a water bath and filtered. To the filtrate, glycerine (300 ml), distilled water (300 ml), glacial acetic acid (30 ml) and potassium alum in excess (40 g) were subsequently added. The contents were mixed thoroughly and exposed to light for six weeks to ripen the solution.

Preparation of Eosin Stain

This is an acidic stain, which was used to colour the cytoplasmic organelles. The acid eosin was dissolved in alcohol. The sections were stained as follows:

1. Deparaffinisation, treatment with xylol two times, each for 5 min.
2. Dehydration through descending grades of ethyl alcohol.

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute alcohol</td>
<td>2 min</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>1 min</td>
</tr>
<tr>
<td>70% alcohol</td>
<td>1 min</td>
</tr>
<tr>
<td>50% alcohol</td>
<td>1 min</td>
</tr>
</tbody>
</table>

3. Staining with Ehrlich's haematoxylin for 15 to 20 min.

4. Thorough washing in tap water and blowing for 10 min.

5. Rinsing with distilled water thoroughly.

6. Staining again with eosin for 2 min.

7. Dehydration again with ascending grades of alcohols

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% alcohol</td>
<td>2 min</td>
</tr>
<tr>
<td>90% alcohol</td>
<td>2 min</td>
</tr>
<tr>
<td>100% alcohol</td>
<td>2 min</td>
</tr>
</tbody>
</table>

8. Clearing in xylol for two times, each at 3 min. intervals.

**Mounting**

Mounting in DPX mountant. On the stained slide, the DPX mountant was applied uniformly and a microglass cover slide was applied.

**Microphotography**

The stained slides were viewed under a Hertel & Reuss microscope and microphotographs were taken.