Chapter 3

Material and Methods
INSECTICIDES

Dicofol

Dicofol (2, 2, 2-trichloro-1, 1-bis (4-chlorophenyl) ethanol), is an organochlorine miticide used on a wide variety of fruit, vegetable, ornamental and field crops. It is sold under a number of trade names, including Acarin, Benzenemethanol, Carbax, Cekudifol, Colonel, Dichlorokelthane, Decofol, Hifol, Kelthane, Milbol and Miltigan.

*Structural formula*

![Structural formula of dicofol](image)

*Physiochemical properties of dicofol*

- **CAS name**: 4-chloro-*alpha*-(4-chlorophenyl)-*alpha*-(trichloromethyl) benzene-methanol
- **Molecular Formula**: C\textsubscript{14}H\textsubscript{9}Cl\textsubscript{5}O
- **Molecular Weight**: 370.49 g/mol
- **Physical state**: Colorless crystalline solid
- **Odour**: Fresh-cut hay
Relative density : 1.45 g/cm³ (at 25 °C)

Water solubility : 0.8 mg/l (25 °C)

Solubility in organic solvents : Soluble

Melting point : 78.5 - 79.5 °C

Boiling point : 180 °C

Vapor Pressure : Negligible at room temperature

Stability : Stable to acids but not to alkaline media.

Slightly corrosive to metals

**Deltamethrin**

Deltamethrin, chemically known as \((S)-\alpha\)-cyano-3-phenoxybenzyl \((1R,3R)-3-(2,2\text{-dibromovinyl})-2,2\text{-dimethylcyclopropane carboxylate}\) is a synthetic pyrethroid ester insecticide mostly used on cotton (45% of the consumption) and on crops such as coffee, maize, wheat, rapeseed, soya beans, fruits, vegetables and hops. It is also used in public health programmes and to protect stored crops, primarily cereal grains. It is used in animal facilities and against cattle infestation. The active ingredient deltamethrin is found in a variety of commercial insecticide products such as Butoflin, Butoss, Butox, Cislin, Crackdown, Cresus, Decis, Decis-Prime, K-Othrin, and K-Otek.

*Structural formula*
Physiochemical properties of dicofol-

CAS name : (S)-cyano(3-phenoxyphenyl)methyl (1R,3R)-3-(2,2-dibromoethenyl)-2,2-dimethylcyclopropanecarboxylate

Molecular Formula : C_{22}H_{19}Br_{2}NO_{3}

Molecular Weight : 505.2 g/mol

Physical state : Colorless crystalline powder

Odour : Odorless

Relative density : 0.5 g/cm³ (at 25 °C)

Water solubility : < 0.002 g/litre (20 °C)

Solubility in organic solvents : Soluble

Melting point : 98 – 101 °C

Boiling point : decomposes >300 °C

Vapor Pressure : 1.5 \times 10^{-8} \text{ mmHg}
Material and Methods

Stability: Stable to light, heat, and air, but not to alkaline media.

For the present study, dicofol 18.5 % EC (Emulsifiable Concentrate) with commercial name Colonel–S, manufactured by Indofil Chemicals Company, Mumbai, India and deltamethrin 2.8% EC with commercial name Decis®, manufactured by Bayer CropScience Limited, Gujarat, India were used.

EXPERIMENTAL SUBJECT

Fertilized eggs of BV 300 breed (*Gallus domesticus*) were collected from a commercial hatchery (Kewalramani Hatcheries, Ajmer, India). All eggs were cleaned and were kept in an incubator with capabilities of maintaining and monitoring temperature, humidity and turning the eggs periodically. The temperature in the incubator was maintained at 38 ±0.5°C and relative humidity was kept between 70-80%.

DOSES

Different dose concentrations of two commercial formulations of each insecticide (deltamethrin and dicofol) were prepared according to their recommended dose used for the field application. There were three doses for each insecticide-

1. Low dose: Concentration of insecticide that was half of the recommended dose.

   \(\text{(250 mg L}^{-1}\text{ for dicofol and 12.5 mg L}^{-1}\text{ for deltamethrin)}\)

2. Medium dose: Concentration of insecticide that was equal to the recommended dose.

   \(\text{(500 mg L}^{-1}\text{ for dicofol and 25 mg L}^{-1}\text{ for deltamethrin)}\)

3. High dose: Concentration of insecticide that was double of the recommended dose.

   \(\text{(1000 mg L}^{-1}\text{ for dicofol and 50 mg L}^{-1}\text{ for deltamethrin)}\)
Different doses of each insecticide were prepared in distilled water (vehicle).

**MODE OF ADMINISTRATION OF DOSE**

Experimental eggs were exposed to different dose concentrations of each toxicant or vehicle by immersion technique (dipping for 1 hour at 37°C temperature).

**GROUPS OF EXPERIMENTAL SUBJECT**

To estimate the insecticide induced teratogenicity in the chick, fertilized eggs were marked, numbered and divided into following three groups:

1. **Group I (Untreated):** A predefined number of non manipulated eggs were served as Control Group I to study background toxicity.
2. **Group II (Vehicle treated):** Alike number of eggs were immersed in vehicle (distilled water). This group was served as Control Group II.
3. **Group III (Insecticide treated):** A predefined number of eggs were immersed in different doses (low, medium and high) of each insecticide.

**EXPERIMENTAL PROTOCOL**

For estimating the teratogenic effect of dicofol and deltamethrin respectively in developing chick embryo, three experimental plans were proposed which were based on exposure of fertilized eggs to different dose levels of each insecticide formulation (Colonel and Decis) on three “critical period” of embryogenesis. Use of the chick embryos was in conformity with the policies of Institutional Animal Care and Use Committee (IACUC, 2008). All the experiments were carried out according to the guidelines of Animal Ethical Committee of Institute.

**EXPERIMENT PLAN I**
A predefined number of unincubated fertilized eggs of *Gallus domesticus* were obtained and exposed on day “0” of incubation with low, medium and high doses of each insecticide, that is, 250, 500, 1000 mg L\(^{-1}\) of dicofol and 12.5, 25, 50 mg L\(^{-1}\) of deltamethrin. The day “0” was selected for the treatment since the blastoderm in the unincubated egg is on the threshold of gastrulation and may thus be more susceptible to any toxicant (Sahu and Ghatak, 2002). Also, the exposure of eggs on embryonic day “0” more closely resembles an environmental exposure scenario (DeWitt *et al.*, 2005). The two groups; vehicle control and control with same number of fertilized eggs were respectively immersed in distilled water and no treatment. All the eggs were kept for incubation and candled daily. Those which were infertile were discarded. Chick embryos were sacrificed on day 4 (stage 24, Hamburger and Hamilton, 1951), day 7 (stage 31, Hamburger and Hamilton, 1951) and day 16 (stage 42, Hamburger and Hamilton, 1951) of incubation for teratological study with following teratological parameters:-

**Teratological parameters** (On embryonic day 4 and 7)

1. Number of surviving embryos, Number of embryo with malformations and wet body weight of embryo.

2. Incidence of gross external malformations (head, eye, neck, limb and lower body).

3. Biochemical estimation of whole embryo (Total protein, cholesterol, glycogen, DNA and RNA contents and activities of enzymes ALP, ACP, GOT and GPT).

**Teratological parameters** (On embryonic day 16)

1. Number of surviving embryos, Number of embryo with malformations and wet body weight of embryo.

2. Incidence of gross external malformations (head, eye, neck, limb and lower body).
Material and Methods

3. Histopathological study of liver.

4. Biochemical estimation of liver (Total protein, cholesterol, glycogen and GSH contents and activities of enzymes ALP, ACP, GOT and GPT)

5. Acetylcholinesterase assay of brain.


Fertilized eggs

⇓

Day “0” of incubation (Insecticide exposure)

⇓

Incubation (at 38 ±0.5°C and 70-80% humidity)

⇓

Day 4 of incubation (ED 4)  •••••••• Teratological study

⇓

Day 7 of incubation (ED 7)  •••••••• Teratological study

⇓

Day 16 of incubation (ED 16)  •••••••• Teratological study

Experiment plan I

EXPERIMENT PLAN II

Prior to dosage, fertilized eggs were placed in an incubator to initiate embryonic development. On 4th day of incubation, all the eggs were observed by candling with high
intensity light source and the unfertilized eggs were excluded from the study. All the eggs were immersed in different suspensions of each insecticide, that is, 250, 500, 1000 mg L\(^{-1}\) of dicofol and 12.5, 25, 50 mg L\(^{-1}\) of deltamethrin respectively. Vehicle control and untreated control eggs were immersed in distilled water and with no treatment respectively. Eggs exposure to toxicant was done on 4\(^{th}\) day of embryonic development because the day 4 of incubation is a period in which organogenesis and differentiation of various organ rudiments are established (DeWitt et al., 2005). All the treated and control eggs were kept for reincubation and candled daily to determine the survivability of embryos, and the infertile eggs as well as dead embryos were discarded. All the eggs from these three groups were sacrificed on embryonic day (ED) 7, 10 (stage 36, Hamburger and Hamilton, 1951) and 16 of incubation and were examined for teratological effect.

**Teratological parameters** (On embryonic day 7 and 10)

1. Number of surviving embryos, Number of embryo with malformations and wet body weight of embryo.
2. Incidence of gross external malformations (head, eye, neck, limb and lower body).
3. Biochemical estimation of whole embryo (Total protein, cholesterol, glycogen, DNA and RNA contents and activities of enzymes ALP, ACP, GOT and GPT).

**Teratological parameters** (On embryonic day 16)

1. Number of surviving embryos, Number of embryo with malformations and wet body weight of embryo.
2. Incidence of gross external malformations (head, eye, neck, limb and lower body).
3. Histopathological study of liver.
4. Biochemical estimation of liver (Total protein, cholesterol, glycogen and GSH contents and activities of enzymes ALP, ACP, GOT and GPT)

5. Acetylcholinesterase assay of brain.


\[
\text{Fertilized eggs} \quad \downarrow
\]

\[
\text{Incubation (at 38 \pm 0.5^\circ C and 70-80\% humidity)} \quad \downarrow
\]

\[
\text{Day “4” of incubation (Insecticide exposure)} \quad \downarrow
\]

\[
\text{Reincubation} \quad \downarrow
\]

\[
\text{Day 7 of incubation (ED 4)} \quad \rightarrow \quad \text{Teratological study} \quad \downarrow
\]
Day 10 of incubation (ED 7)  ↓ Teratological study

Day 16 of incubation (ED 16)  ↓ Teratological study

**Experiment plan II**

**EXPERIMENT PLAN III**

Fertilized eggs were obtained and incubated in incubator until embryonic day 7 of the incubation period. Embryos were observed during the incubation and, dead and growth retarded embryos at the time of treatment were excluded from further study. On embryonic day 7, eggs were exposed to 250, 500 and 1000 mg L\(^{-1}\) doses of dicofol and 12.5, 25 and 50 mg L\(^{-1}\) doses of deltamethrin respectively. Vehicle control and untreated control eggs respectively immersed in distilled water and with no treatment. By the day 7 of development the chorio-allantoic membrane is well developed with its vascular network lying next to shell membrane facilitating uptake of toxicants (Karnofsky, 1965). Most of the organ formation also completes by this embryonic day. The eggs were returned to incubator until the time of sampling. Chick embryos were taken out from the eggs on 16\(^{th}\) day of incubation for teratological study.

**Teratological parameters**

1. Number of surviving embryos, Number of embryo with malformations and wet body weight of embryo.

2. Incidence of gross external malformations (head, eye, neck, limb and lower body).
3. Histopathological study of liver.

4. Biochemical estimation of liver (Total protein, cholesterol, glycogen and GSH contents and activities of enzymes ALP, ACP, GOT and GPT)

5. Acetylcholinesterase assay of brain.


Fertilized eggs

\[ \downarrow \]

Incubation (at 38 ±0.5°C and 70-80% humidity)

\[ \downarrow \]

Day “7” of incubation (Insecticide exposure)

\[ \downarrow \]

Reincubation

\[ \downarrow \]

Day 16 of incubation (ED 16) Teratological study

**Experiment plan III**

All the experiments were repeated three times maintaining identical conditions.
BIOCHEMICAL PARAMETERS

Estimation of Total Protein

**Principle**

Total protein content of tissue was estimated according to method described by Lowry *et al.* (1951). Protein, in alkaline medium, bind with the cupric ion present in biuret reagent. The aromatic amino acid, tryptophan and tyrosine in the treated protein sample react with peptide nitrogen of phosphomolybdic-phosphotungstic acids (component chemical substances of Folin ciocalteau reagent) to give final colour complex. The intensity of the colour formed is directly proportional to amount of protein present in the sample. Total protein was expressed in mg/g wet tissue.

**Sample Preparation**

10% homogenate of tissue (whole embryo/ liver) was prepared in ice cold PBS-Phosphate Buffer Saline (pH 7.4) and centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was used for estimation of total protein content and various enzymes.

**Reagents**

A. 0.1 N NaOH

B. Folin- ciocalteau reagent

Commercial available reagent was diluted with an equal volume of 0.1 N NaOH just prior to use.

C. 4% Na2CO (w/v) in distilled water.

D. 0.5% CuSO4. 5H2O in 1% potassium sodium tartrate.

E. Alkaline copper solution

Prepared fresh by mixing 50 ml of reagent C with 2 ml of reagent D.
F. Standard protein solution

0.25 g of bovine serum albumin was dissolved in 50 ml of distilled water. From this, 2 ml was taken and made up to 100 ml with distilled water.

Procedure

1 ml of sample was dissolved in 1 ml of 0.1 NaOH. 2 ml of alkaline copper solution was added, mixed and allowed to stand for 15 minutes. Then 0.2 ml of Folin-ciocalteau reagent was added and kept undisturbed for 30 minutes. The absorbance was read at 650 nm in UV-spectrophotometer against blank.

Estimation of Total Cholesterol

Principle

The Liebermann and Burchardt reaction (Henry and Henry, 1974) was used for estimating total cholesterol content of tissue. Cholesterol has got a ring structure which is cyclopentene perhydro phenanthrene. When cholesterol residue is dissolved in glacial acetic acid, this ring reacts with ferric chloride-acetic acid and sulfuric acid reagents to produce a coloured derivative. The intensity of colour formed is directly proportional to the amount of cholesterol present in the given sample of tissue. Cholesterol level was expressed as mg/g wet tissue.

Sample Preparation

70 mg of tissue was homogenized with 7 ml of acetic acid and centrifuged at 1000-3000 rpm for 20 minutes. The supernatant was used for analyzing total cholesterol content.

Reagents

A. Glacial acetic acid
B. Ferric chloride reagent
10 g of FeCl\(_3\) was dissolved in 100 ml of glacial acetic acid. 1 ml of it was taken and made up to 100ml with Conc. H\(_2\)SO\(_4\). Prepared fresh at the time of estimation.

C. Standard cholesterol solution

200 mg of commercially available cholesterol was dissolved in 100 ml of glacial acetic acid. From this, 1ml was taken and made up to 100 ml with glacial acetic acid.

**Procedure**

0.5 ml of tissue homogenate was taken and mixed with 6 ml of glacial acetic acid. 4 ml of Ferric chloride reagent was added and mixed well. The tubes were allowed to cool in dark for 30 minutes. The absorbance was measured at 550 nm.

**Estimation of Total Glycogen**

**Principle**

Glycogen content were extracted and estimated according to procedure described by Montgomery (1957). Carbohydrates treated in the presence of sulfuric acid and phenol undergoes dehydration with the formation of hydroxyl aldehydes which are subsequently converted to furfural derivatives. The reaction gives rise to a pink colour, the intensity of which is proportional to the amount of glycogen present. Total glycogen content was expressed as mg/g wet tissue.

**Reagents**

A. 30 % KOH (w/v) in distilled water

B. 80 % phenol (w/w) in distilled water

C. Conc. H\(_2\)SO\(_4\)

D. Standard Glycogen solution

100 mg of commercially available glycogen was dissolved in 100 ml of distilled water.
Procedure

20- 40 mg of tissue was taken in 2ml of reagent A and boiled in water bath till the tissue was digested. After cooling at room temperature, 2-4 ml of absolute alcohol was added and kept for 20 minutes and then centrifuged for about 15 minutes at 1500 rpm. Supernatant was discarded and procedure was repeated after adding 2-4 ml of absolute alcohol again. Then the tubes were kept in dessicator for overnight. The precipitated glycogen was dissolved in 4 ml of distilled water and mixed well. To 2 ml of it, 0.1 ml of reagent B and 5 ml of reagent C was added. The mixture was cooled for 30 minutes at dark and absorbance was read at 620 nm.

Estimation of Nucleic acid

Isolation of nucleic acids

Reagents

A. 5 % TCA in distilled water
B. 10 % TCA in distilled water
C. 0.9 % of NaCl in distilled water

Procedure

5 % homogenate of the tissue was made in reagent C. A known volume of it was treated with equal volume of 10 % TCA. The solution was mixed well and centrifuged. The supernatant was discarded. The precipitate was washed twice with 10 % TCA and 5 ml of absolute alcohol followed by ether extraction. 2 ml of 10 % TCA was added and centrifuged and supernatant was saved in a test tube (I). Remained residue was treated with 5 ml of 5 % TCA, kept at 100°C for 15-20 minutes, cooled and centrifuged. The supernatant was added in test tube (I) and used from estimation of DNA and RNA.
Estimation of DNA

Principle

Estimation of DNA was performed by using diphenylamine reagent (Schneider, 1957). This method is based on the property of deoxyribose moiety of DNA to form \( \alpha \)-hydroxy laevulaldehyde in TCA solution. This reacts with diphenylamine to give a coloured complex. The intensity of colouration is presumed to be proportional to the pentose (deoxyribose) concentration in the DNA hydrolyzate. Total DNA content was expressed as mg/100g wet tissue.

Reagents

A. Dische’s diphenylamine reagent

1 g of diphenylamine was dissolved in 100 ml of glacial acetic acid. After adding 2.75 ml of Conc. \( \text{H}_2\text{SO}_4 \), the solution was mixed and stored in dark.

B. Standard DNA Solution

Freshly prepared by dissolving 100 mg of commercially available DNA with 100 ml of distilled water.

Procedure

1 ml of isolated nucleic acid TCA hydrolyzate was taken and mixed well with 0.5 ml of perchloric acid. The solution was kept for boiling after adding 2 ml of reagent A. Boiling was done in water bath for 10 minutes. The solution was then cooled under tap water and optical density was measured at 565 nm against blank.

Estimation of Total RNA

Principle

Total RNA content was estimated by the methods of Schneider (1957) using orcinol reagent. The ribose moiety of RNA in the TCA extract is converted to furfural which condenses with orcinol to yield a blue coloured complex. The colour intensity is presumed
Material and Methods

to be proportional to RNA concentration in the extract. Total RNA content was expressed as mg/100g wet tissue.

Reagents

A. Orcinol reagent

500 ml of 30 % HCl was taken and 1 g of orcinol was dissolved well in it. 4-5 ml of 10 % ferric chloride was added and mixed in it. The solution was stored in a brown bottle.

B. Standard RNA Solution

Dissolved 100 mg of RNA in 100 ml of distilled water

Procedure

2 ml of nucleic acid TCA extract was taken and added with 2 ml of orcinol reagent. The solution was mixed and kept for boiling for 20 minutes and cooled thereafter. Absorbance was read at 670 nm in UV- spectrophotometer.

Estimation of Total Reduced Glutathione (GSH)

Principle

The glutathione content in the liver was determined by the method of Moron et al. (1979). Reduced glutathione in the sample reacts with DTNB (5, 5-dithiobis nitro benzoic acid) and produces a yellow coloured product. The intensity of the yellow colour developed was measured in a spectrophotometer and the total glutathione concentration in the sample was expressed as µg/g tissue.

Reagents

A. 25 % of TCA in distilled water

B. Tris KCl buffer (pH 7.4)

6 g of Tris buffer and 11.5 g of potassium chloride was dissolved in 1000 ml of distilled water.
C. 0.2M NaP buffer (pH 8)

Solution A- 17.7 g of Na$_2$HPO$_4$ was dissolved in 500 ml distilled water.

Solution B- 15.6 gm of NaH$_2$PO$_4$ or KH$_2$PO$_4$ was dissolved in 500 ml of distilled water.

Solution A was mixed with equal volume of Solution B.

D. Standard GSH solution (10 nmoles/ml of 25% TCA)

**Procedure**

A homogenate was prepared with 0.5 g of liver tissue with 4.5 ml of Tris KCl buffer and centrifuged at 1000 rpm for 10 minutes. About 100 µl of supernatant was mixed with 2 ml of DTNB. The final volume of mixture was made to 3 ml by adding 900 µl of NAP buffer. Absorbance was read at 412 nm in UV-spectrophotometer against blank.

**Assays of Phosphatases (ALP and ACP) activities**

**Principle**

The activities of alkaline phosphatase (ALP, orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3:1:3:1) and acid phosphatase (ACP, monoester phosphohydrolase, acid optimum, EC 3:1:3:2) were estimated according to method of Kind and King (1954). Disodium phenyl phosphate is hydrolyzed by these enzymes present in given tissue sample, into sodium phosphate and phenol. Phenol liberated makes a coloured derivative with 4 amino antipyrene-ferricyanide reagents. Activities of these enzymes were measured in King Armstrong unit (KAU/g tissue). One King Armstrong unit corresponds to the amount of enzyme that transforms one mg of phenol in 15 minutes.
Estimation of ALP activity

Reagents

A. Buffered substrate

Solution A- 2.18 g of disodium phenyl phosphate was dissolved in 1 litre of distilled water.

Solution B- 6.36 g of sodium carbonate and 3.36 g of sodium bicarbonate was dissolved in 800 ml of distilled water. pH was adjusted to 10 and made a final volume to 1 litre with distilled water.

Solution A was mixed with equal volume of solution B at the time of enzyme assay.

B. Standard Phenol reagent

100 mg of phenol was dissolved in 100 ml of distilled water. And 1 ml of it was diluted again with 100 ml of distilled water.

C. 0.5N NaOH

20 g of NaOH was dissolved in 1 litre of distilled water.

D. 0.5M Sodium bicarbonate

Dissolved 4.2 g of sodium bicarbonate in 100 ml of distilled water.

E. 4- Aminoantipyrine (0.6 %) in distilled water

F. Potassium ferricyanide (2.4 %) in distilled water.

Procedure

2 ml of buffered substrate was taken and incubated at 37°C for 10 minutes. 0.1 ml of sample was added and kept again for incubation. After 15 minutes 0.8 ml of reagent C and 1.2 ml of reagent D was added and mixed well. Each of 1.0 ml of reagent E and reagent F was added in the solution and mixed well again. The absorbance was read at 520 nm.
Estimation of ACP activity

Reagents

All the reagents were same as that of ALP, except solution B of buffered substrate.

Buffered substrate

Solution A- 2.18 g of disodium phenyl phosphate was dissolved in 1 litre of distilled water.

Solution B- 42 g of crystalline citric acid was dissolved in distilled water and 376 ml of 1N NaOH solution was added to make volume to 800 ml with water. pH was adjusted to 5.

Solution A was mixed with equal volume of solution B at the time of enzyme assay.

Procedure

The procedure remained same as that of ALP assay except the incubation time which was increased to 60 minutes.

Assays of Transaminases (GPT and GOT) activities

Principle

Glutamate oxaloacetate transaminase (GOT; L-aspartate, 2-oxoglutarate aminotransferase; EC 2.6.1.1) and Glutamate pyruvate transaminase (GPT; L-alanine 2-oxoglutarate aminotransferase; EC 2.6.1.2) were estimated according to method of King (1965). Two types of transaminases, catalyzing the inter-conversion of amino acids and α-keto acids by the intermolecular transfer of amino groups, are present in human tissues.

Reactions catalyzed by GOT

\[
\text{GOT B6} \\
\text{L- Aspartate + \(\alpha\)-ketoglutarate \(\leftrightarrow\) Oxaloacetate + L-Glutamate}
\]

Reactions catalyzed by GPT

\[
\text{GPT B6} \\
\text{L- Alanine + \(\alpha\)-ketoglutarate \(\leftrightarrow\) Pyruvate + L-Glutamate}
\]
Pyruvate formed in the GPT catalyzed reaction, is made to react with DNPH to give a coloured derivative under alkaline conditions. Oxaloacetate, formed in GOT catalyzed reaction, also get decarboxylated to pyruvate spontaneously, producing the same coloured derivative with DNPH. Activities of these enzymes were expressed in µmol/min/g tissue which is micromoles of pyruvate formed per minute per gram of tissue.

**Estimation of GPT activity**

**Reagents**

A. **Phosphate buffer (pH 7.4)**

   11.3 g of disodium hydrogen phosphate and 2.7 g of potassium dihydrogen phosphate was dissolved and made volume to 700-800 ml with distilled water. pH was adjusted and finally made the volume to 1 litre.

B. **Substrate (0.2M, L-alanine, 0.002M α-Ketoglutaric acid)**

   9 g of L-alanine was added in 90 ml of water followed by addition of about 4 ml of 1N NaOH. 0.146 g of α-Ketoglutaric acid and 2-3 ml of NaOH was added. pH was adjusted to 7.4 and finally volume was made up to 500ml with phosphate buffer.

C. **Standard Pyruvate solution**

   Prepared freshly by dissolving 220 mg of sodium pyruvate in 100 ml of phosphate buffer and finally diluted 5 times with phosphate buffer.

D. **2, 4- Dinitrophenylhydrazine (DNPH)**

   20 mg of DNPH was dissolved in 10 ml conc. HCl and made up volume to 100 ml with distilled water.

E. **0.4N NaOH**

   16 g NaOH was dissolved in 1 litre of distilled water.
Procedure

Pipetted out 0.5 ml of buffer substrate in each test tube labeled as test and control, standard and blank. 0.1 ml of the sample was added in both the tubes labeled as test and control. In standard, 0.1 ml of standard pyruvate solution was added, while in blank 0.1 ml of distilled water was added. After that, 0.5 ml of DNPH was added in all the tubes except test. All the tubes were incubated for 30 minutes at 37°C. After termination of incubation, 0.5 ml of DNPH was added in test also and kept at room temperature for 20 minutes. Finally 5 ml of 0.4N NaOH was added in all the tubes to read the extinction at 520 nm against blank.

Estimation of GOT activity

Reagents

All the reagent were same as for GPT, except for its substrate.

Substrate (0.2M, L-aspartate acid, 0.002M α- Ketoglutaric acid)

13.3 g of L-aspartic acid was added in 90 ml of 1N NaOH. 0.146 g of α- Ketoglutaric acid was added and dissolved by adding another 10 ml of 1N NaOH. pH was adjusted to 7.4 and finally volume was made up to 500 ml with phosphate buffer.

Procedure

Procedure was almost similar to that of GPT assay. All the reagents were taken in same quantities except for that of substrate and incubation period was enhanced to 60 minutes.

Assay of Acetylcholinesterase activity

Principle

The activity of the acetylcholinesterase (AChE; Acetylcholine hydrolase; EC 3.1.1.7) in brain was determined according to method of Ellman et al. (1961). It was measured by following the increase of color produced in tissue sample when acetylthiocholine is broken
down in the presence of AChE to release thiocholine, which reacts with the 5, 5'-dithio bis-2-nitrobenzoic acid (DTNB) to form thiobenzoic acid. Its unit was expressed as µmoles of substrate acetylthiocholine (AcSCh) hydrolyzed /min/g tissue.

**Reagents**

A. 0.1 M phosphate buffer (pH 7-8)

Solution A- 5.22 g of K$_2$HPO$_4$ and 4.68 g of NaH$_2$PO$_4$ were dissolved in 150 ml of distilled water.

Solution B- 6.2 g NaOH was dissolved in 150 ml of distilled water.

Solution B was added to solution A to get desired pH and then finally volume was made up to 300 ml with distilled water.

B. DTNB (dithionitrobenzoic acid) reagent

Freshly prepared by dissolving 36.6 mg of DTNB with 15 mg of NaHCO$_3$ in 10 ml of phosphate buffer.

C. Acetylthiocholine iodide

Prepared freshly by dissolving 21.67 mg of acetylthiocholine in 1 ml of distilled water.

**Procedure**

5 % homogenate of tissue was prepared in 0.1 M phosphate buffer. Added 0.4 ml aliquot of homogenate to 26 ml of phosphate buffer and 100 µl of DTNB. Finally 20 µl of Acetylthiocholine iodide was added in the solution to read the change in absorbance at 412 nm against blank of the above solution prepared at the same time. Extinction coefficient is 5.74 * 10$^{-4}$. 
SKELETAL EXAMINATION

Double staining of cartilage and bone with Alcian Blue and Alizarin Red-S by McLeod (1980).

The embryos recovered for skeletal analysis were eviscerated and fixed in absolute ethyl alcohol for 7 days. Embryos were stained for 4 days at (37-40 °C) in the following solution:

A. 1 volume 0.3% (300 mg) filtered Alcian Blue in 70% ethyl alcohol.

B. 1 volume 0.1% (100mg) filtered Alizarin Red-S in 95% ethyl alcohol.

C. 1 volume glacial acetic acid.

D. 1 volume 70% ethyl alcohol.

Solution (A) and (B) were mixed, and then (C) and (D) were added. At least 100ml of the resulting staining solution was used per embryo.

Specimens were washed for 2 hours in tap water. Embryos were placed in 1% aqueous potassium hydroxide (KOH) solution for 12- 48 hours. Macerated, stained specimens were cleared by aqueous solution of ascending gradual concentration of glycerol (20, 50, 80 %) diluted with 1% KOH, for 3 days for each step, then transferred into 100% glycerol to which a few crystals of thymol crystals were added to avoid mold proliferation. The stained skeletal elements of embryos were kept and stored in 100 % glycerol until they were examined and photographed.

HISTOLOGICAL STUDIES

The Bouin’s fixed liver tissue was cut into small pieces after 24 hours of fixation. Tissues were processed in a series of graded ethanol and xylene, embedded in the mixture of paraffin and bee wax (3:1), sections were cut at 5µm and stained with haematoxyline
Material and Methods

and eosin. The stained sections were examined and scoring of histopathological changes was done as follow: (-) absent; (+) mild; (++) moderate; (+++) severe, and (++++) extremely severe (Bancroft et al., 1996)

STATISTICAL ANALYSIS

The data obtained from the present experiments were subject to statistical analysis. These calculations were based on biological statistics. The wet body weight and all the biochemical estimations of chick embryo were analyzed with the help of Student’s “t” test and the values are expressed as mean± standard error (SE).

The teratological observations were statistically analyzed using “Mann-Whitney U-test”. The quantified data for survivability and number of abnormal survivors with external and skeletal malformations was represented in percentages.

All the statistical analysis was performed by IBM SPSS Statistics 17 analytic software (SPSS Inc, Chicago, Ilion, USA).

The value of $p$ as 0.05, 0.01, and 0.001 were considered to be significant against controls.