CHAPTER II
PLANNING, EXPERIMENTAL PROTOCOL, MATERIALS AND METHODS PERTAINING TO THE STUDY.
A] PLANNING AND EXPERIMENTAL PROTOCOL

It is known that studies on the specific responses of biological systems to varying levels of exposure can provide a great deal of information on the nature of the responses, their underlying causes and the possible consequences of various levels of exposure. In order to increase the chances of recognizing possible toxic properties of the insecticides, the dose was occasionally raised above the therapeutically useful range, the duration of treatment was often lengthened and the pesticides were administered to various species of birds. The rationale was to imitate the field use of the pesticide with respect to dose and duration of treatment. Also the future field application potential of insecticides guided the planning of our toxicity/physiological studies.

Acute and chronic experiments

Acute experiments are quick and quite sensitive analytical assay methods for the determination of minimal symptomatic or toxic doses, emetic doses and an approximate maximum tolerated doses. However, acute experimental data alone cannot fully represent the ecological hazards of any insecticide. The general relationship of acute and chronic toxicities in terms of killing effects is that cumulative effects are observed for generally persistent pesticides while in case of the degradable pesticides, the toxic effects do not carry over. Thus for chronic administration the final pattern of accumulation at equilibrium is expected to be different from the acute dosing distribution.

In nature, the sudden (usually accidental) poisoning of wildlife by a single exposure to an insecticide generally does not create long-lasting and widespread ecological problems since such exposures are generally limited in geographical
area and duration. What generally causes ecological threat is the long-term build up of pesticides by environmental concentration. A low ratio can be regarded as the sign of non-cumulative poison (e.g. zectran) since in such a case, the animal is effectively eliminating the poison through detoxification or excretion mechanisms at the end of each day. In contrast, high ratios for organochlorines such as DDT, HCH, dieldrin and endrin indicate the cumulativeness of chlorinated hydrocarbon insecticides (Matsumura, 1976). Also in acute dosing, lipophilic insecticides gradually settle in fatty tissues after a certain period of time for redistribution, while in continuous daily dosing the general pattern is a gradual accumulation in the fat approaching a plateau after some months.

Therefore, both short and long term experiments were performed with occasionally varying doses of the insecticides. In this connection we were particularly guided by Zbinden's (1963) observation regarding chronic experiments:

'three or atmost four dose levels and a control group are sufficient (for an experiment), ...... the highest level must produce some toxic symptoms, the lowest should be within the range of 2-10 times the proposed therapeutic dose and the remaining doses should lie in between'

Parameters studied

In accordance to the above experimental design, the following parameters were studied:
1. Hematology:

For hematological study the following indices were specially considered: Total count of Red blood corpuscles (RBC), White blood corpuscles (WBC) and Reticulocytes from peripheral circulation, total count of cells from spleen and bone marrow, differential count of cells both from bone marrow (femur) and peripheral blood smears, Hemoglobin content (Hb), Hematocrit (Hct), Erythrocyte sedimentation rate (ESR), Bleeding time (BT), Clotting time (CT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH) and Mean corpuscular hemoglobin concentration (MCHC). Besides, plasma calcium, osmotic fragility tests of erythrocytes were also performed along with Pearl's reaction to detect iron in the erythrocytes. For details, see Methodologies in this chapter and chapter III.

2. Histopathology:

Histopathological studies of liver, kidney, spleen, duodenum and lungs of both the pigeon and the domestic duck fed lindane were undertaken. For details see this chapter and chapter IV.

3. Clinical chemistry and organ function tests:

Acid- and alkaline phosphatase, aspartate aminotransferase (GOT), alanine aminotransferase (GPT), glucose, cholesterol, total lipid, total protein and bilirubin content of serum were estimated biochemically along with whole blood urea and plasma non-protein nitrogen (NPN). Glucose-6-phosphate dehydrogenase (G6PDH) activity, total
lipid, total protein, glycogen and nucleic acids (DNA and RNA) content of liver were estimated. G6PDH was also estimated from RBC.

4. **Brain neurotransmitters**:

Catecholamines (dopamine, noradrenaline and adrenaline) and acetylcholine (ACh) together with measurement of acetylcholinesterase (AChE) activity of the whole brain extract.

5. **Residue analyses**:

Analyses of lindane residues in liver, kidney, abdominal fat, pectoral muscle and brain were performed in both the pigeon and the duck. Lindane residues in serum was estimated only in the duck (both non-laying and laying).

The whole experimental protocol is outlined in the following table:
### Table 2.1 Experimental protocol for the study undertaken

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Insecticide</th>
<th>Bird species</th>
<th>Dose (per kg body weight)</th>
<th>Duration (week)</th>
<th>Parameters studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comparative, short-term :</td>
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</tr>
<tr>
<td>I</td>
<td>Lindane</td>
<td>Sparrow, baya weaver, myna, parakeet, pigeon, duck</td>
<td>5 mg, twice/week</td>
<td>1</td>
<td>Hematology only (Same as above)</td>
</tr>
<tr>
<td>II</td>
<td>Chlordane</td>
<td>Myna, pigeon, duck</td>
<td>5 mg, twice/week</td>
<td>1</td>
<td>Hematology only (Same parameters plus total count of reticulocytes, total and differential count of bone marrow cells from femur)</td>
</tr>
<tr>
<td>III</td>
<td>Fenitrothion</td>
<td>Pigeon, duck</td>
<td>0.1 mg, twice/week</td>
<td>1</td>
<td>Hematology only (Same as above)</td>
</tr>
<tr>
<td>IV</td>
<td>Carbaryl</td>
<td>Pigeon</td>
<td>0.1 mg, twice/week</td>
<td>1</td>
<td>Hematology only (Same as above)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Insecticide</th>
<th>Bird species</th>
<th>Dose (per kg body weight)</th>
<th>Duration (week)</th>
<th>Parameters studied</th>
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<tbody>
<tr>
<td>Long term:</td>
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<tr>
<td>I</td>
<td>Lindane</td>
<td>Duck, juvenile</td>
<td>10 mg, daily</td>
<td>4</td>
<td>Hematology only (Total count of RBC and WBC, Hb, Hct, ESR, MCV, MCH, MCHC, BT, CT, differential count of WBC)</td>
</tr>
<tr>
<td>II</td>
<td>Fenitrothion</td>
<td>Pigeon</td>
<td>0.1 mg, twice/week</td>
<td>2,4,6</td>
<td>Hematology only (Above parameters plus total count of splenic cells)</td>
</tr>
<tr>
<td>III</td>
<td>Chlordane</td>
<td>Pigeon</td>
<td>2 mg, daily</td>
<td></td>
<td>Hematology only (Total count of RBC, WBC, Reticulocytes and splenic cells, Hb, Hct, ESR, MCV, MCH, MCHC, BT, CT, differential count of WBC, total and differential count of bone marrow cells from femur, cellular fragility of RBC)</td>
</tr>
<tr>
<td>Experiment No.</td>
<td>Insecticide</td>
<td>Bird species</td>
<td>Dose (per kg body weight)</td>
<td>Duration (week)</td>
<td>Parameters studied</td>
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<tr>
<td>IV</td>
<td>Lindane</td>
<td>Pigeon</td>
<td>5 mg, daily</td>
<td>4,8</td>
<td>Hematology (Above parameters plus plasma calcium)</td>
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<td></td>
<td></td>
<td></td>
<td>Histopathology (Liver, kidney, spleen, duodenum, lungs)</td>
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<td></td>
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<td></td>
<td></td>
<td>Clinical chemistry and organ function tests (Acid and alkaline phosphatase, GOT, GPT, glucose, glycogen, G6PDH, total lipid, cholesterol, bilirubin, total protein, urea, NPN, DNA, RNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain neurotransmitters and AChE activity (Catecholamines viz. dopamine, noradrenaline, Adrenaline; Acetylcholine, Acetylcholinesterase activity from whole brain extract)</td>
</tr>
</tbody>
</table>

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## Table 2.1 (Contd...)  

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Insecticide</th>
<th>Bird species</th>
<th>Dose (per kg body weight)</th>
<th>Duration (week)</th>
<th>Parameters studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>Lindane</td>
<td>Duck, laying</td>
<td>20 mg, daily</td>
<td>4</td>
<td>Tissue residue analysis (Liver, kidney, abdominal fat, pectoral muscle, brain)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>Hematology (same as above)</td>
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<td></td>
<td>Clinical chemistry and organ function tests (same as above)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain neurotransmitters and AChE activity (same as above)</td>
</tr>
<tr>
<td>VI</td>
<td>Lindane</td>
<td>Duck, non-laying</td>
<td>10 mg, once/week</td>
<td>8</td>
<td>Hematology (same as above)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 mg, daily</td>
<td></td>
<td>Histopathology (same as above)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clinical chemistry and organ function tests (same as above)</td>
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<td></td>
<td></td>
<td></td>
<td>Brain neurotransmitters and AChE activity (same as above)</td>
</tr>
</tbody>
</table>

Contd....
Table 2.1 (Contd....)

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Insecticide</th>
<th>Bird species</th>
<th>Dose (per kg body weight)</th>
<th>Duration (week)</th>
<th>Parameters studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>VII</td>
<td>Lindane</td>
<td>Duck, laying</td>
<td>20 mg, twice/week</td>
<td></td>
<td>Tissue residue analysis (same as above + serum)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 mg, thrice/week</td>
<td></td>
<td>Hematology (same as above)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 mg, daily</td>
<td></td>
<td>Histopathology (same as above)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clinical chemistry and organ function tests (same as above)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain neurotransmitters and AChE activity (same as above)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tissue residue analysis (same as above + serum)</td>
</tr>
</tbody>
</table>

Note: Reported LD$_{50}$ values of insecticides (Tucker and Crabtree, 1970)

a) Lindane: Mallards > 2,000 mg/kg (orally)
b) Chlordane: Mallards 1,200 mg/kg (orally)
c) Carbaryl: Mallards > 2,179 mg/kg (orally)
   Pigeons 1,000 to 3,000 mg/kg (orally)
B] SELECTION OF EXPERIMENTAL ANIMALS

Several birds of agricultural and economic importance were generally selected for the study with the assumption that their chances of exposure to pesticides are greater than the other birds. Besides, recommendations of Tucker and Leitzke (1984) were specially considered in selecting the test animals for toxicity studies.

The following birds were selected for the study:

i) House sparrow (Passer domesticus Linnaeus, Order Passeriformes).

ii) Baya weaver bird (Ploceus philippinus Linnaeus, Order Passeriformes).

iii) Common myna (Acridotheres tristis Linnaeus, Order Passeriformes).

iv) Rose-ringed parakeet (Psittacula krameri Scopoli, Order Psittaciformes).

v) Blue-rock pigeon (Columba livia Gmelin, Order Columbiformes).

vi) Domestic duck (Anas platyrhynchos domesticus, Order Anseriformes).

Excepting the domestic duck the other birds are considered as agricultural pests (Fitzwater and Prakash, 1978). The domestic ducks have commercial importance as good layer birds. In India they form about 10% of the total poultry population and constitute about 5% of the total egg production.
production (Banerjee, 1982). In the seventh five-year plan of India considerable importance has been given to duck-improvement schemes. Also in the free range, this species finds about 75% of its food in the form of insect life, snails, fishes, earthworms, natural grass and weeds (ibid). The indigenous breed of the domestic duck was therefore used for this study. All the birds (excepting the duck) were trapped from the natural populations. Control and experimental birds which were healthy and free from diseases, were always taken from the same population. The birds selected for the study (excepting the duck) were sexually mature and of either sex. For the ducks, both juvenile (2-2½ months old) and sexually mature (1-1½ years old) female birds were procured from a local animal dealer. Also, both laying and non-laying ducks formed the experimental groups. The birds were acclimatized to uniform laboratory condition for at least seven days before the onset of experiments. For comparative hematological studies, several species were collected during the same months (generally March and April) to conduct experiments simultaneously and therefore to negate variations in climatological influence, if any and from the same geographic location.

Animal care:

1) Housing condition

The birds were housed in a well ventilated room, diffusely lighted during the day and darkened at night. Temperature of the room was usually 28°C with 5°C fluctuation and the humidity being 45 to 50 per cent. Frequent washing and flushing of walls and floors were made. Extermination of insects such as ticks, mites, fleas, cockroaches, flies etc., was
achieved by occasional spraying (when rooms were cleared of animals). Excreta was removed almost every day. Bird cages were cleaned every week, scrapped and scrubbed with water. The birds were kept in wire-cages of various designs and sizes according to their size so as to allow them reasonable freedom of movement.

The ducks on the other hand, were kept in outdoor pens provided with shelters and well-drained soil, proper ventilation and free access to drinking water and food. The birds were also allowed swimming at a fixed time in a pond very close to the enclosure. The pens were cleaned after every 3-4 days.

ii) Diet

The birds were generally fed with grains, seeds, insects, worms and fruits. In long-term experiments the ducks were supplied with commercial chicken food (Maharaja Poultry Feed, Ballygunge Fodder Mills, Calcutta). 'Chick-mash', 'Grower-mash' and 'Layer-mash' brand food was supplied to the juvenile, non-laying and laying ducks respectively. The layer-mash is composed of:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>18%</td>
</tr>
<tr>
<td>Fat</td>
<td>3%</td>
</tr>
<tr>
<td>Fibre</td>
<td>8%</td>
</tr>
<tr>
<td>Calcium</td>
<td>2.5%</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.6%</td>
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</table>

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8000 I.U./kg</td>
</tr>
<tr>
<td>D</td>
<td>800 I.U./kg</td>
</tr>
<tr>
<td>E</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td>B</td>
<td>45 mg/kg</td>
</tr>
</tbody>
</table>

In cases of 'chick'- and 'grower'-mash the percentage of protein was 22% and 20% respectively. For the breeding ducks, straw and saw dust were provided as litter.
iii) Symptomatological study

All the experimental birds were watched regularly for changes of behavior, appetite and general appearance. They were weighed twice weekly during the course of experiment. Brief physical examination including simple neurological checkup of muscular tonus, gait, and pupillary, corneal, attitudinal and postural reflexes were made. The eyes were examined regularly for cataract and changes of the fundus.

Selection of insecticides

India, as pointed out earlier, is one of the largest users of agricultural insecticides, mostly organochlorines. Of the organochlorines used in India, hexachlorocyclohexane (HCH) and its derivatives constitute more than 50% (Anon, 1984). The effect of lindane (γ-HCH), therefore was elaborately studied (both hematological and physiological). Along with lindane, another organochlorine viz. chlordane (a cyclodiene compound) is also used (use being 50 Metric Tonnes in 1984-85, Anon, 1984). Therefore hematological effects of this insecticide were also studied.

Among the organophosphates, fenitrothion (Sumithion) is a very selective insecticide (Matsumura, 1976). This insecticide is used in India in a modest way (about 150 Metric Tonnes in 1984-85, Anon, 1984). Of the carbamates, carbaryl (sevin) accounts for highest use (about 2,000 Metric Tonnes in 1984-85). Hematological effects of these two insecticides were therefore also studied.

The insecticides used for hematological investigations were:
i) Lindane (99.8%, granular),  
ii) Chlordane (20%, emulsion),  
iii) Fenitrothion (50%, emulsion) and  
iv) Carbaryl (90%, granular).

While for physiological studies and residue analyses, lindane was selected.

The insecticides were dissolved in olive oil (Olio Sasso, Italy) and administered to birds at various dose levels (Table 2.1), by a stomach tube. Control birds received the vehicle only. Ten birds were evenly distributed between control (5) and treated (5) groups in every experiment.

**Route of insecticide administration:**

The oral route was preferred, since oral ingestion is expected to be the most likely route of entry for insecticidal compounds. Also, the use of a stomach tube was preferred rather than adding the insecticidal compound to the animal's diet because the latter method is less accurate for estimation of the actual oral dose taken by the animal. At the same time, homogenous preparation of pesticide impregnated feed is difficult.

C) **MATERIALS AND METHODS**

**Collection of blood**

Blood was collected from the brachial vein into clean glass tubes previously rinsed with disodium ethylene diamine tetraacetic acid (EDTA: 1.5 mg/ml of blood) solution as anticoagulant. For the differential counts of leukocytes,
however, freshly drawn blood from the brachial vein (without anticoagulant) was used. For collection of serum, blood (without anticoagulant) was allowed to clot for one hour at room temperature and the serum was preserved at -80°C whenever necessary.

**Estimation of hemoglobin (Hb) (Halaz, 1967)**

Blood was drawn by a pipette up to the 0.5 mark and excess blood was wiped off from the tip with cotton. It was then transferred to the hemometer tube containing N/10 HCl up to the mark 2 and mixed thoroughly. After 5 minutes a colour comparison was made with the standard coloured prism of hemometer. The brown solution was diluted drop by drop with distilled water until the colour precisely matched that of the standard. The final reading of the solution in the tube was noted. Hemoglobin was calculated in g/dl from the reading of the tube.

**Counting of erythrocytes (Natt and Herrick, 1952; Dacie and Lewis, 1975)**

Since avian RBC is nucleated, for counting of erythrocytes in the peripheral circulation, blood samples were diluted 1:200 with a special diluent namely Natt and Herrick's (1952) fluid with the use of a Thoma pipette. The diluting fluid (pH 7.3) is composed of:

- **Sodium chloride (NaCl)**: 3.88 g
- **Sodium sulphate (Na₂SO₄)**: 2.50 g
- **Disodium hydrogen phosphate (Na₂HPO₄·12H₂O)**: 2.91 g
- **Potassium hydrogen phosphate (KH₂PO₄)**: 0.25 g
- **Methyl violet**: 0.10 g
- **Formalin (37%)**: 7.50 ml
- **Distilled water**: 1 litre
After vigorous mixing, a drop of resulting mixture was discharged under the cover glass of an improved Neubauer hemocytometer and the corpuscles were allowed to settle for 2 minutes. The number of erythrocytes in 80 small squares were counted under light microscope. The number of cells in 1 μl of diluted blood was calculated following the standard formula:

\[
\text{R.B.C. (Erythrocyte) count/μl} = \frac{N \times \frac{1}{0.02} \times 200}{\text{number of cells in 80 small squares (i.e., 0.02 μl)}}
\]

\[= N \times 10,000\]

**Measurement of hematocrit (Hct) (Wintrobe et al., 1976)**

Hematocrit was measured using the microhematocrit tubes which are approximately 70 mm long and have a 1 mm bore. The tubes filled with uncoagulated blood were centrifuged at 2,000 X g for 30 minutes at room temperature. The hematocrit percentage was determined as:

\[
\text{Hct (x)} = \frac{\text{Height of the column of packed erythrocytes}}{\text{Total height of the column}} \times 100
\]

**Erythrocyte sedimentation rate (E.S.R.) (Washburn et al., 1957)**

E.S.R. was estimated by Westergren tube. The tube is a glass pipette, 300 mm long with an internal diameter 2.5 mm, graduated in millimeter from 0 to 200. 3.8% sodium citrate (A.R.) solution was used as anticoagulant, 0.5 ml of which was used for 2 ml of blood. By sucking, the mixture was drawn into the tube up to the 'zero' mark. Since avian erythrocytes settle rather slowly, the tube was inclined at 45° angle in a stand with a spring clip on the top and a rubber band at the
bottom to substantially improve the E.S.R. (Washburn et al., 1957). After one hour the level of the red cell column was recorded.

Red cell indices

The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined from hemoglobin, red cell and hematocrit values using the standard formulae of Dacie and Lewis (1975):

\[
\text{MCV (fl)} = \frac{Hct (\%)}{\text{RBC count (10}^6/\mu\text{l})} \times 10;
\]

\[
\text{MCH (pg)} = \frac{Hb (g/dl)}{\text{RBC count (10}^6/\mu\text{l})} \times 10;
\]

\[
\text{MCHC (g/dl)} = \frac{Hb (g/dl)}{Hct (\%)} \times 100
\]

Reticulocyte count (Kolmer et al., 1969)

Reticulocytes in the peripheral blood were counted after staining with brilliant cresyl blue. The staining solution was prepared by dissolving 1.0 g of brilliant cresyl blue (E. Merck, Germany) in 100 ml of normal saline (0.85%) and was filtered before use. Freshly drawn venous blood was mixed thoroughly with the stain and cell suspension was made thereafter on a clean glass slide. The erythrocytes were observed under oil immersion. The cells with reticulum that stain pale blue were classified as reticulocytes. Absolute reticulocyte count per microliter of blood was calculated from the following formula:
Reticulocyte count = RBC count \((10^6/\mu l)\) \times \frac{\text{Reticulocyte}(x)}{100}

**Total count of leucocytes** (Natt and Herrick, 1952; Wintrobe et al., 1976)

Blood was diluted 1:20 with Natt and Herrick's fluid (1952). The improved Neubauer hemocytometer was filled similarly with the mixture as in case of erythrocyte counting, and the number of cells in four corner blocks (each block subdivided into 16 squares) was determined. The total leucocyte count per microliter of blood was calculated by multiplying the average number of cells in four blocks by 200 (Wintrobe et al., 1976).

**Differential count of leucocytes** (Lucas and Jamroz, 1974)

Freshly drawn blood without anticoagulant was used to prepare blood films on perfectly clean grease-free slides. The smears were air dried and after a number of trials with various stains (viz., Giemsa, Eosin-Hematoxylin and Leishman's stain) were finally stained with Wright's stain at pH 6.4-6.8.

**Preparation of Wright's stain**

3.30 Gms of dry Wright's powder (BDH, England) was taken in a clean, dry, dark bottle and 500ml of acetone-free methyl alcohol (E. Merck) was added. Mixing was carried out on a magnetic stirrer for 60 min at room temperature. The mixture was filtered, kept in a dark place for at least a week and shaken daily for about 5 minutes. A phosphate-buffer solution of pH 6.4 was used during staining to maintain the pH of the stain around 6.4-6.8 (prepared by dissolving 6.63 g potassium phosphate,
monobasic and 3.20 sodium phosphate, dibasic in one liter of double distilled water).

Staining and counting procedure

1] The blood film was covered with undiluted stain and left for one minute so that methyl alcohol fixed the smear.

2] The stain was diluted with two volumes of buffer solution until a metallic scum appeared.

3] The mixture was allowed to remain on the side for 10 minutes; the slides were flooded with distilled water and washed for 30 seconds thereafter. The slides were then air dried and observed under an oil immersion objective of a light microscope. The cells were identified and counted according to their nuclear cytoplasmic characteristics. The percentage distribution of each type of leucocyte was calculated after counting at least 1,000 cells at random.

Determination of clotting and bleeding time (Kolmer et al., 1969)

i) Clotting time (CT)

Clotting time was determined by Sabraze's capillary tube method (Kolmer et al., 1969). Three capillary tubes about 8 cm in length and 0.8-1.2 mm in diameter were used. After puncturing a fine wing capillary vein of bird, the first drop of blood was discarded. The second drop was secured and the time was noted. The
tubes were then allowed to fill with the blood by capillary action. At the end of 1-2 minutes, about 1 cm length of each tube was broken off every 30 seconds. The clotting time was recorded as the interval from the time blood appeared to the time at which fibrin thread bridged the broken ends of the tubes when separated at a distance of 5 mm or more.

ii) Bleeding time (BT)

Duke's method was followed for the determination of bleeding time (Kolmer et al., 1969). Briefly, a fine wing capillary vein of bird was cleaned and punctured deeply so that blood flowed drop by drop without any effort. The time at which the first drop appeared was noted. Each drop of blood was removed by a piece of filter paper without touching the skin. When bleeding stopped spontaneously, the time was recorded. The time interval between the appearance of the first drop and the removal of the last, represented the bleeding time.

Assessment of bone marrow and splenic cellularity (Rencricca and Coleman, 1979)

The spleen and two intact femurs of each bird were removed after killing the animals by decapitation. The organs were washed in Ca\(^{2+}\) and Mg\(^{2+}\)-free cold Hank's balanced salt solution (HBSS). Bone marrow was obtained from the femurs by snipping the ends of these bones. A 25 gauge needle mounted on a 1 ml syringe containing Ca\(^{2+}\) and Mg\(^{2+}\)-free HBSS was inserted into one end of the bone and the marrow was discharged by forcing the solution through the marrow cavity. The marrow plug was flushed back and forth through the
syringe (without needle) several times. The resultant
cell suspension was free of cell clumps. The cells were
washed twice with HBBS and the washed cells were used for
total and differential cell counts. Nucleated cells were
counted in hemocytometer under light microscope after
staining the immature cells with 1% brilliant cresyl blue
in 0.85% NaCl.

Spleen cell suspensions were made by mincing the organ in
cold HBSS. The possibility of cell clumps was minimized
by passing the cell suspension sequentially through 20-,
22- and 23-gauge needles. The total number of nucleated
cells in the spleen was counted in hemocytometer similarly
as in medullary measurements.

**Counting of erythroid cells**

The differential distribution of bone marrow cells in
normal and treated birds was determined using 0.1 ml
aliquot of marrow cells suspended in HBSS. A thin smear
of cells was prepared on a clean, grease-free, glass
slide and fixed for 10 minutes in absolute methanol. The
cells were stained with benzidine and May-Grunwald Giemsa
(MGG) stain.

**Benzidine staining for hemoglobin (LoBue et al., 1963)**

The methanol-fixed slides were flooded with 1% solution of
benzidine (Reanal, Budapest, Hungary) in absolute methanol
and allowed to stand for 1 minute. The benzidine solution
was then tipped off and a mixture of 30% H₂O₂ and 70%
ethanol (1:3, v/v) i.e., 7.5% H₂O₂ in ethanol was added to
the slides and left for 1 minute. The slides were then
washed with tap water and counter stained with MGG stain.
The cytoplasm of the erythroid cells gives a golden-yellow
colour owing to the presence of hemoglobin. Generally, four slides were used to estimate differential cell count with 250 cells per slide for a total count of about 1,000 cells per bird.

Method of counter staining with MGG (Lucas and Jamroz, 1974)

The slides were covered with MGG stain (0.25% in methanol) for 5 minutes. The stain was diluted with an equal quantity of distilled water and mixed well for 1-2 minutes. The stain was then tipped off and without rinsing, diluted Giemsa (40 drops of stock Giemsa solution to 40 ml of distilled water) was added to it and kept for 15-20 minutes. The slides were then dipped (1 or 2 times) in methyl alcohol, blotted immediately and mounted with cover glass.

Measurement of osmotic fragility of erythrocytes (Dumont et al., 1977; Buetler et al., 1982)

Osmotic fragility measurements were performed using a series of dilutions of a phosphate buffer containing 13 g NaCl, 2.73 g Na₂HPO₄ and 0.374 g NaH₂PO₄ in 200 ml of distilled water. This buffer is osmotically equivalent to 10% NaCl and was diluted with water to produce hypotonic solutions of 0.15% to 0.90% sodium chloride equivalents in increments of 0.025%.

0.5 ml of heparin mixed blood samples were collected and centrifuged at 2,000 X g for 5 minutes at 4°C. The plasma and buffy coat were removed and the erythrocytes were washed thrice with phosphate buffer saline (pH 7.2). The total number of erythrocytes in the samples were counted with the help of a standard hemocytometer. Aliquots of 1 X 10⁷ washed red cells were added to a series of 15 ml
centrifuge tubes containing 4 ml of graded hypotonic buffered salt concentrations. The contents of the tubes were mixed well and allowed to stand for one hour at a constant temperature (30°C) because the hemolytic response of the erythrocytes is temperature dependent. After this the tubes were shaken vigorously and centrifuged for 5 minutes at 1,000 X g. The supernatant was transferred into a test tube and the optical density (O.D.) was measured at 540 mp in a spectrocolorimeter (E. Leitz, Inc. N. York, Model No. 26875). The sample from the tube containing 0.85% NaCl was used as blank and the supernatant of the tube containing 0.15% NaCl to which blood was added represented the standard i.e., 100% hemolysis. The percentage of hemolysis at different salt concentrations was calculated from the following equation (Dumont et al., 1977):

\[
\text{Hemolysis (\%)} = \frac{\text{O.D. of sample}}{\text{O.D. of sample from 0.15}\% \text{NaCl}} \times 100
\]

Osmotic fragility was expressed as the mean corpuscular fragility (MCF) determined by extrapolating the saline concentration at which 50% of the erythrocytes hemolyze.

Iron staining by Pearl's prussian blue reaction (Pearse, 1972)

Tissue (spleen) iron stores in the form of hemosiderin was evaluated by subjective grading after staining for prussian blue reaction. Only impression smear was studied for hemosiderin.

Reagents used:
1. 2% HCl (A.R.) in iron-free distilled water.
2. 2% K-ferrocyanide (E. Merck, India) in iron-free distilled water.
3. 1% aqueous solution of neutral red (BDH, England).
Procedure: Methanol fixed (for 10 min) impression smear was brought to water and exposed to a fresh mixture of equal parts of 2% K-ferrocyanide and 2% HCl for 45 minutes. The slide was washed in distilled water and counterstained with 1% neutral red for 3 min. thereafter. After washing in running tap-water, and dehydration in graded ethanol, the slide was mounted in DPX. The ferric iron gives deep prussian blue colour reaction while the nuclei are red.

Estimation of total lipid (Bligh and Dyer, 1959)

Fresh tissue (200 mg) or serum (0.5 ml) was homogenized in a 6 ml mixture of chloroform and methanol (1:2 v/v) followed by addition of 4 ml of chloroform and thorough mixing for 30 seconds. 3.2 Ml of distilled water was then added to the contents and mixed for another 30 seconds. The mixture was filtered through Whatman No. 1 filter paper and the filtrate was transferred to a 10 ml graduated test tube. After allowing a few minutes for complete separation, the volume of the chloroform layer was recorded and the alcoholic layer removed by aspiration. The chloroform layer contained the purified lipid.

The lipid withheld in the tissue residue was recovered by blending the residue and filter paper with 4 ml chloroform. The mixture was filtered and the blender and residue were rinsed with 2 ml chloroform. The filtrate was mixed with the original filtrate prior to removal of the alcoholic layer.

A portion of the lipid extract (1 ml) was evaporated to dryness in a previously weighed microbeaker by keeping it
overnight in an incubator (37°C). The amount of lipid was determined as follows:

\[
\text{Total lipid} = \frac{\text{Weight of lipid in aliquot} \times \text{volume of chloroform layer}}{\text{Volume of aliquot}}
\]

**Estimation of liver glycogen** (Carrol *et al.*, 1955)

Immediately after killing the animal, about 200 mg of tissue was taken from a fixed lobe of the liver, weighed accurately and transferred to a homogenizing tube containing 6 ml of cold 5% trichloroacetic acid (TCA). After homogenizing, the content was transferred into a centrifuge tube and centrifuged for 15 minutes at 1,000 X g. 2 ml supernatant was taken into a separate centrifuge tube containing 5 ml of 95% alcohol. The contents were mixed thoroughly, stoppered tightly and kept overnight at room temperature. Next day, the contents were again mixed and centrifuged for 15 minutes at 1,000 X g. The supernatant was discarded and the precipitate was washed with 4 ml absolute alcohol (2 to 3 times) followed by ether (4 ml, 2 to 3 times) with subsequent centrifugation. Finally the precipitate (i.e., glycogen) thus obtained was dried at room temperature and dissolved in 10 ml of distilled water. To the diluted sample (1 ml), 5 ml of anthrone reagent (25 mg anthrone and 500 mg thiourea dissolved in 50 ml of 72% cold \(\text{H}_2\text{SO}_4\)) was added. The mouth of the tube was covered with a marble and placed upright in a boiling water-bath for 15 minutes. Simultaneously a blank and a standard tube were prepared by carrying out the same procedure but without glucose. Optical densities of the standard and unknown were measured at 620 m\(\mu\) in a colorimeter (Klett Summerson, N.Y., Model No. 800-3). From the readings the amount of glycogen present per sample was calculated.
Estimation of serum glucose (Nelson, 1944; Somogyi, 1945)

Serum (0.1 ml) was taken in a test tube and deproteinized with 0.95 ml of zinc sulfate (5%) and barium hydroxide (4.5%) solution. The contents were shaken vigorously and filtered through a dry filter paper (Whatman No. 1). From the filtrate, 0.5 ml of sample was taken into a test tube containing 1 ml of alkaline copper reagent (Alkaline copper reagent: Solution A - 50 g of anhydrous sodium carbonate, 50 g of sodium potassium tartarate, 40 g of sodium bicarbonate and 400 g of anhydrous sodium sulfate was dissolved in 1,000 ml of distilled water, and finally diluted to 2,000 ml. Solution B - 150 g of copper sulfate was dissolved in 900 ml of distilled water and finally diluted to 1,000 ml. 0.5 ml of concentrated sulfuric acid was added and mixed well. Before use 4 ml of solution B was mixed with 96 ml of solution A). The contents were mixed by tapping. The mouth of the tube was covered with a marble and placed upright in a boiling water-bath for 20 minutes. After bringing to room temperature the sample was treated with 1 ml arsenomolybdate colour reagent (prepared by dissolving 100 g of ammonium molybdate in 1,800 ml of distilled water and thereafter adding 84 ml of concentrated sulfuric acid with stirring. Then 12 g of disodium ortho-arsenate was dissolved in 100 ml of distilled water and added with stirring to the acidified molybdate solution. The mixture was placed in an incubator at 37°C for 1 or 2 days before use). Finally, the contents were mixed by tapping and then diluted to 10 ml with distilled water. A blank and a standard tube were simultaneously prepared by carrying out the same procedure. The colorimeter (E. Leitz, Inc., N. York, Model No. 26875) was set at 'zero' density with the blank, and the optical densities of standard and unknown were measured at 540 μm.
Estimation of protein (Lowry et al., 1951)

Protein was estimated both from the serum and hepatic tissue.

a. From serum

0.1-Ml of sample was taken in a centrifuge tube and the volume was made up to 1 ml by adding physiological saline (0.85%). The contents were mixed thoroughly and centrifuged (1,000 X g) for 15 minutes. The supernatant was discarded and the precipitate was successively treated with 4 ml of absolute ethyl alcohol and 3 ml of ether, with subsequent mixing, centrifuging and discarding of the supernatants. The final precipitate was dried at room temperature for 10 minutes, dissolved in 5 ml 2(N) NaOH solution and boiled at 70°C for about 20 minutes. The mixture was then cooled to room temperature and 0.2 ml of sample solution was taken in a separate test tube. The volume was made up to 1 ml with glass distilled water. The contents of the tube were mixed properly and kept at room temperature for 15 minutes after adding 5 ml of alkaline-copper sulfate solution (Solution A - 20 g of sodium carbonate was dissolved in 1 liter of 0.1 N NaOH containing 590 mg of sodium-potassium tartarate. Solution B - 5 g of copper-sulfate was dissolved in 1 liter of distilled water. The final solution was prepared by mixing 100 ml of solution A and 2 ml of solution B). Finally 0.5 ml of diluted Folin phenol (1:1 v/v) was mixed with the sample solution and kept at room temperature for 30 minutes.
The blank and the standard tubes were simultaneously run and after colour development readings were taken at 750 μ in a Klett Summerson colorimeter (N. York, Model No. 800-3).

b. From tissue

Immediately after killing the animal, required amount (200 mg) of tissue (liver) was taken, weighed accurately, homogenized in 5 ml of 0.25 M cold sucrose solution and centrifuged at 1,000 X g for 30 minutes. The lipids, phosphates and nucleic acids were subsequently removed from the sample by treating with chloroform: absolute ethyl alcohol: ether (1:2:1 v/v) mixture and 5% cold perchloric acid. The final precipitate thus obtained was dissolved in 5 ml of 2(N) NaOH and hydrolysed at 70°C for 20 minutes. The same procedure was followed for successive steps as in case of serum protein using Lowry's method.

Estimation of blood urea (Levine et al., 1964)

3-Ml of blood was mixed with 12 ml of 10% trichloroacetic acid, shaken vigorously and allowed to stand at room temperature for 5 minutes. The solution was filtered through Whatman No. 40 filter paper. To the filtrate 500 mg of charcoal was added, mixed well and filtered. 5-Ml of charcoal treated filtrate was added to 1 ml of modified Ehrlich's reagent (prepared by dissolving 5 g of p-dimethyl-aminobenzaldehyde in 20 ml of concentrated HCl. To it 80 ml of distilled water was added carefully. The solution was stirred and cooled before use) and allowed to stand at room temperature for 5 minutes. A blank and a standard tube were simultaneously prepared following the same procedure. Between
5 and 60 minutes, spectrocolorimetric (E. Leitz, Inc., N. York, Model No. 26875) readings were taken at 420 m\(\mu\). The amount of urea present per sample was calculated from the readings of the standard and unknown.

**Estimation of non-protein nitrogen (NPN)** (Folin and Wu, 1919; Bell *et al.*, 1959)

One ml of uncoagulated blood or plasma was added to 9 ml of 10% (w/v) TCA solution in a test tube, mixed thoroughly and filtered after keeping it at room temperature for 15 minutes. The filtrate (5 ml) was mixed with 1 ml diluted acid mixture (Regular acid mixture: 300 ml of 85% phosphoric acid, A.R., was added to 50 ml of 5% copper sulfate solution. 100 ml of concentrated H\(_2\)SO\(_4\) A.R., was then added to it and mixed thoroughly. The diluted acid mixture was prepared by diluting the regular acid mixture with an equal volume of distilled water). The mouth of the tube was closed with a marble and placed in an upright position in a boiling water-bath for 10 minutes. It was allowed to stand at room temperature for 5 minutes and after that 10 ml of distilled water was added. A blank and a standard tube suitable for colorimetric measurement were simultaneously prepared. Then 5 ml of Nessler's reagent was added to each of the tube followed by 1 ml of distilled water. The contents were mixed thoroughly by inverting several times. The tubes were allowed to stand at room temperature for 10 minutes after which spectrocolorimetric (E. Leitz, Inc., N. York, Model No. 26875) measurements were taken at 540 m\(\mu\). The amount of non-protein nitrogen present per sample was calculated from the standard curve.

**Estimation of serum alkaline phosphatase** (Bessy *et al.*, 1946; Bergmeyer, 1963)

Two tubes were taken for each set of sample. One for
experimental and the other for blank. To both the tubes 1 ml of substrate buffer (375 mg glycine, 10 mg MgCl₂, 6H₂O A.R. and 165 mg Na-P-nitrophenyl phosphate, E. Merck, Germany, was dissolved in 50 ml of 0.1 N NaOH and diluted to 100 ml with doubly distilled water after adjusting the pH to 10.5) was taken and kept at 37°C for 10 minutes for temperature equilibration. Then 0.1 ml of non-hemolyzed serum was added to the experimental tube, mixed by inversion several times and incubated (37°C) for 30 minutes. No serum was added to the blank tube and no incubation was done. After incubation, 10 ml of 0.02(N) NaOH was added to both the tubes, followed by addition of 0.1 ml of serum to the blank tube. The contents of both the tubes were mixed well by inverting several times. The optical density was measured at 420 μm in a spectrocolorimeter (E. Leitz, Inc., N. York, Model No. 26875) after setting the blank at 'zero'. The phosphatase unit present per sample was calculated from the standard curve. The specific activity was expressed as μmoles of paranitrophenol liberated per 30 minutes per microgram of enzyme protein.

Estimation of serum acid phosphatase (Bergmeyer, 1963)

Two tubes were taken for each set of sample. One for experimental and the other for blank. To both the tubes 1 ml of substrate-buffer (410 mg citric acid, A.R., 1125 mg sodium citrate, A.R., and 165 mg Na-P-nitrophenyl phosphate, E. Merck, Germany, was dissolved in 50 ml doubly distilled water and the volume was made up to 100 ml after adjusting the pH to 4.8) was taken and kept at 37°C for 10 minutes for temperature equilibration. Then 0.2 ml of non-hemolyzed serum was added to the experimental tube, mixed by inverting several times and incubated (37°C) for 30 minutes. No serum was added to the blank tube and no incubation was done. After
the incubation 4 ml of 0.1 N NaOH solution was added to both the tubes, followed by addition of 0.2 ml of serum to the blank tube. The contents of both the tubes were mixed thoroughly by inverting several times. The optical density was measured at 420 μm in a spectrocolorimeter (E. Leitz, Inc., N. York, Model No. 26875) after setting the blank at 'zero'. The phosphatase unit present per sample was calculated from the standard curve. The specific activity was expressed as μmoles of paranitrophenol liberated per 30 minutes per microgram of enzyme protein.

**Estimation of glucose-6-phosphate dehydrogenase (G6PDH)**
(Bergmeyer, 1963; Nobrega et al., 1970)

a. **From erythrocytes**

0.5-Ml of fresh venous blood was taken in a centrifuge tube containing 0.5 ml sodium citrate, A.R. (3.8%) solution, mixed perfectly and centrifuged (1,000 X g) for 15 minutes. While discarding the supernatant the precipitate was washed three times with physiological saline (0.85%). The sediment was suspended in 1 ml of physiological saline and mixed well by rotation. In another centrifuge tube the following ingredients were successively added: 1 ml erythrocyte suspension, 1 ml distilled water, 0.7 ml triethanolamine buffer (0.93 g triethanolamine hydrochloride, E. Merck, Germany and 0.2 g EDTA-Na₂H₂O₂H₂O, E. Merck, Germany, were dissolved in 90 ml of distilled water, pH adjusted to 7.5 with 0.1 NaOH and finally diluted to 100 ml with distilled water) and 0.3 ml of 1% digitonin solution (BDH, England). The contents were thoroughly mixed and allowed to stand at 4°C for 15 minutes. The mixture was then centrifuged (1,000 X g) in a refrigerated centrifuge for at least
15 minutes and the insoluble materials were discarded. In a separate tube, 2.85 ml of triethanolamine buffer, 0.05 ml of erythrocyte hemolysate and 0.05 ml of nicotinamide adenine dinucleotide phosphate, E. Merck (2.5% solution in 1% NaHCO₃ solution) were taken and mixed with a glass rod. The tube was kept at 25°C for 5 minutes and then 0.05 ml of glucose-6-phosphate (1.3%, E. Merck, Germany) was added to it. At the same time a blank tube was prepared with all the ingredients excepting NADP solution. After an optical density increase of about 0.02 and with the help of a stopwatch, the increase in excitation at 340 mλ was recorded at an interval of 2 minutes for 10 minutes in a spectrophotometer (EC India, Model No. GS 865A). The mean optical density change per minute was used for calculations. Specific activity of the enzyme was expressed in units/min/μg protein.

b. From liver

After killing the animal, tissue (about 200 mg) was taken immediately from a fixed lobe of the liver, blotted, weighed accurately and transferred to a homogenizing tube containing 4 ml of cold EDTA-physiological saline (1%). The tissue was homogenized for 2 mins and then the homogenate was centrifuged for 20 minutes at 1,000 X g in a refrigerated centrifuge. 0.5 Ml of supernatant was collected in a separate tube containing 2.40 ml of triethanolamine buffer and 0.05 ml of NADP solution. The contents were mixed properly with a glass rod and kept at 25°C for 5 minutes. Just before measurement, 0.05 ml of glucose-6-phosphate solution (1.3%) was added into the tube. A blank tube was prepared at the same time with all the ingredients excepting the NADP solution. After an optical density increase of about 0.02 and with the help
of a stop watch, the increase in excitation at 340 m\(\mu\) was recorded at an interval of 2 minutes for 10 minutes in a spectrophotometer (EC India, Model No. GS 865A). The mean O.D. change per minute was used for calculation. Specific activity of the enzyme was expressed in units/min /\(\mu\)g protein.

Estimation of serum aspartate aminotransferase (SGOT) (Bergmeyer, 1963)

Generally two tubes were taken for each set of sample. One for experimental and the other for blank. In each of the two tubes, 1 ml substrate-buffer solution (1.50 g \(\text{K}_2\text{HPO}_4\) A.R., 0.20 g \(\text{KH}_2\text{PO}_4\), A.R., 0.03 g \(\alpha\)-oxoglutaric acid, A.R., and L-aspartic acid, A.R., were dissolved in 80 ml of double distilled water and the volume was made up to 100 ml after adjusting the pH to 7.4 with 0.4 N NaOH) was taken. 0.2 ml of diluted serum (1:10 dilution with physiological saline) was added to the substrate-buffer solution, mixed by inversion and incubated at 37°C for exactly 60 minutes. No serum was added to the blank tube and no incubation was done. After incubation, 1 ml of ketone reagent (prepared by dissolving 20 mg 2,4-dinitrophenylhydrazine, A.R., in 1 N HCl and the volume was made up to 100 ml) was added to each tube followed by addition of 0.2 ml of diluted serum to the blank tube. The tubes were allowed to stand at room temperature for 20 minutes. Finally, 10 ml of 0.4 N NaOH was added into each tube and mixed by inversion. After 5 minutes, the optical density of the experimental tube was read against the blank at 530 m\(\mu\) in the spectrocolorimeter (E. Leitz, Inc., N. York, Model No. 26875). From the standard curve, the SGOT (\(\mu\)M/ml) present in the sample was calculated.
Estimation of serum alanine aminotransferase (SGPT)
(Wroblewski et al., 1956; Bergmeyer, 1963)

Generally two tubes were taken for each set of sample. One for experimental and the other for blank. In each of the two tubes, 1 ml substrate-buffer solution (1.50 g $K_2HPO_4$, A.R., 0.20 g $KH_2PO_4$, A.R., 0.03 g $\alpha$-oxoglutaric acid, A.R., and 1.78 g DL-alanine were dissolved in 80 ml of double distilled water and the volume was made up to 100 ml after adjusting the pH to 7.4 with 0.4 N NaOH) was taken. Then 0.2 ml of diluted serum (1:10 dilution with physiological saline) was added to the substrate-buffer solution, mixed by inversion and incubated at 37°C for exactly 30 minutes. No serum was added to the blank tube and no incubation was done. After incubation, 1 ml of ketone reagent (prepared by dissolving 20 mg of 2,4-dinitrophenylhydrazine, A.R. in 1N HCl and making the volume up to 100 ml) was added to each tube followed by addition of 0.2 ml of diluted serum to the blank tube. The tubes were then allowed to stand for 20 minutes at room temperature. Finally, 10 ml of 0.4 N NaOH was added into each tube and mixed well by inversion. After 5 minutes, the optical density of the experimental tube was read against the blank at 530 nm in a spectrocolorimeter (E. Leitz, Inc., N. York, Model No. 26875). From the standard curve, the SGPT ($\mu$M/ml) present in the sample was calculated.

Estimation of serum bilirubin (Malloy and Evelyn, 1937)

Serum bilirubin was estimated both as conjugated (i.e., mostly with glucoronic acid) and total.
0.2 ml of non-hemolysed serum was diluted to 2 ml with distilled water. Six test tubes were set up marked 1, 2, 3, 4, 5 and 6 representing respectively, one-minute direct bilirubin (conjugated) and blank; total bilirubin and blank; and standard and blank. 0.5 ml of distilled water was added to the tubes 1 and 2; and 0.5 ml of methyl alcohol to the tubes 3, 4, 5 and 6. To tubes 2, 4 and 6, 0.1 ml of diazo-blank solution (prepared by diluting 60 ml of conc. HCl to 1 litre with distilled water) and to the tubes 1, 3 and 5 was added 0.1 ml of freshly prepared diazo reagent (Solution A: 5 g of sulfanilic acid, A.R. was dissolved in 50 ml of conc. HCl and then diluted to 1 litre with distilled water. Solution B: 2 g of sodium nitrite, A.R. was dissolved in distilled water and diluted to 100 ml. Diazo-reagent = 10 ml of solution A was mixed with 0.3 ml of solution B). Now 0.4 ml of the diluted serum was added to the tubes 1, 2, 3 and 4 and mixed well. To tubes 5 and 6, 0.4 ml of standard bilirubin solution (0.01 mg/ml, Sigma Chemicals) were added. Just after one minute, the optical density of the tube number 1 was recorded at 540 μm against the blank of tube number 2. Exactly 10 minutes after the addition of serum, the optical density of tube number 3 was read off against its corresponding blank (4).

Finally, the reading of tube number 5 was taken at 540 μm setting the colorimeter (E. Leitz, Inc., N. York, Model No. 26875) to 'zero' against tube number 6. The amount of bilirubin present per sample was calculated from the standard curve.

**Estimation of serum cholesterol** (Schoenheimer and Sperry, 1934)

One ml of serum was taken into a graduated centrifuge tube containing 10 ml of acetone-alcohol (1:1 v/v) mixture. The
tube was tightly stoppered and shaken vigorously for about 2 minutes. Then it was placed in a boiling water-bath until the solvent boiled. After bringing the tube to room temperature, the volume was made up to 10 ml with acetone-alcohol mixture, mixed thoroughly and then filtered.

3 ml of acetone-alcohol filtrate was transferred to a 15 ml graduated centrifuge tube followed by addition of 3 drops of potassium hydroxide (A.R.) solution (50%). The mixture was stirred vigorously with a glass rod. The mouth of the tube was tightly closed and incubated for 30 minutes at 37°C. It was then removed, cooled to room temperature and the volume was made up to 6 ml with acetone-alcohol mixture. One drop of phenolphthalein solution (1%) was added to the mixture followed by 10% acetic acid (A.R., E. Merck) drop by drop with stirring, until the red colour of phenolphthalein disappeared. One drop was added in excess, followed by 3 ml of 0.4% digitonin solution (BDH, England). The content of the tube was stirred thoroughly, stoppered tightly with a glass stopper and allowed to stand overnight at room temperature. Next day the content was mixed again and centrifuged (1,000 x g) for 15 minutes. The supernatant was decanted by placing the tube in an inverted position on a pad of filter paper and finally by wiping the mouth of the tube with a clean blotting paper. The 2 ml of acetone-ether mixture (1:2 v/v) was added along the side of the tube. The precipitate was stirred thoroughly and again centrifuged for 5 minutes. The supernatant thus obtained was removed as before and the precipitate was washed once with ether instead of acetone-ether.

The tube was then placed in a sand-bath (about 3 cm thick layer of sand in a shallow pan heated to 112°C) for about 30 minutes. After removing from the sand-bath immediately 2 ml of acetic acid (A.R., E. Merck) was added in such a way
that the acid washed down the wall of the tube and then the tube was stirred well. It was again placed in the sand-bath for 2 minutes, removed and then brought to room temperature.

After 10 minutes the tube was placed in a water-bath at 25°C and allowed to come to temperature equilibrium. A blank and a standard tube, suitable for colorimetric measurement, were simultaneously prepared. Finally, 4 ml of cold acetic anhydride-sulfuric acid reagent (prepared by mixing 20 ml of ice-cold acetic anhydride, A.R., and 1 ml concentrated sulfuric acid, E. Merck, in a glass stoppered volumetric flask) was added to the blank, standard and sample tubes, mixed thoroughly and allowed to remain in dark for 30 minutes. After colour development, the amount of cholesterol present per sample was calculated from the readings (at 620 m\u00b5m in spectrocolorimeter, E. Leitz, Inc., N. York, Model No. 26875) of unknown and standard solution.

**Estimation of serum calcium** (Kramer and Tisdall, 1921; Clark and Collip, 1925)

2 ml of non-hemolysed serum was introduced into a 15 ml centrifuge tube containing 2 ml of distilled water and 1 ml of ammonium oxalate solution (4\%). The contents were mixed thoroughly by swirling and occasional tapping at the bottom. The tube was allowed to stand overnight at room temperature for precipitation of the sample. Next day, the contents were mixed and centrifuged (500 X g) for 5 minutes. The supernatant was carefully decanted and the tube was inverted on a pad of filter paper. The precipitate was stirred up and the side of the tube was washed with 3 ml of 2% ammonia (Glaxo Laboratories, India) directed in a very fine stream, from a wash bottle. The suspension was centrifuged and drained again as before. Then 2 ml of normal sulfuric acid (28 ml concentrated acid, A.R., diluted to 1 litre with
distilled water) was added by blowing from a pipette directly upon the precipitate to break up the mat and facilitate solution. The tube was then placed in a boiling water-bath for about 1 minute. The sample was then titrated with 0.01 N potassium permanganate solution to a definite pink color. A blank containing 2 ml of concentrated sulfuric acid was simultaneously titrated. The amount of permanganate required to titrate the sample was used for calculation.

**Estimation of tissue nucleic acids**

a. **Deoxyribonucleic acid (DNA)** (Burton, 1956; Croft and Lubran, 1965)

Immediately after killing the animal, 200 mg of tissue (liver) was taken, blotted, weighed accurately and transferred into a homogenizer tube containing 0.25 M cold sucrose solution. The tissue was homogenized for 5 minutes and then centrifuged at 1,000 \(X\) g for 15 minutes. In a separate test tube 2.5 ml of the supernatant was taken and made lipid- and phosphate-free by treating with chloroform-absolute alcohol-ether mixture (1:3:1 v/v) and 5% cold perchloric acid or PCA (BDH, India) respectively. After final centrifugation the precipitate thus obtained was treated with 2 ml of 5% perchloric acid (PCA) and hydrolysed at 70°C in a water-bath for 30 minutes. The mixture was brought to room temperature and again centrifuged (1,000 \(X\) g) for 15 minutes, to separate the nucleic acid. Now 0.5 ml of the supernatant was collected in a separate tube and the volume was made up to 1 ml with 0.5 M cold PCA (prepared by diluting 7.1 ml of 70% PCA to 100 ml with distilled water). 2 ml of diphenylamine reagent (prepared by mixing 1.5 g of Diphenylamine, Glaxo Laboratories, India;
100 ml glacial acetic acid, A.R., E. Merck, India; 1.5 ml of conc. \( \text{H}_2\text{SO}_4 \), A.R. and 0.5 ml of aqueous acetaldehyde, A.R.) was added to the tube and kept at 4°C for 48 hours for colour development. The blank and the standard tubes were prepared simultaneously. Finally 1 ml of glacial acetic acid was added to all the tubes, mixed properly and readings taken at 600 m\( \mu \) in a Klett Summerson colorimeter (N. York, Model No. 800-3). The amount of DNA present was calculated from the standard curve.

b. **Ribonucleic acid (RNA)** (Mejbaum, 1939; Munro and Fleck, 1966)

After separation of nucleic acid as before, 0.5 ml of the supernatant was collected in a separate tube and the volume was made up to 1 ml with 0.5 M cold PCA (prepared as before). Then to the sample 1 ml of orcinol reagent (made by dissolving 75 mg orcinol, Sigma, England, in 0.025 ml 10\% \( \text{FeCl}_3 \) and 25 ml conc. HCl) was added and boiled in a water-bath for 30 minutes after subsequent mixing. At the same time, a blank and a standard tube suitable for colorimetric measurement were prepared. Finally, the mixture was brought to room temperature and 2 ml of 0.5 M PCA was added to it. The contents were mixed properly and readings were taken at both 600 m\( \mu \) and 660 m\( \mu \) in a Klett Summerson colorimeter (N. York, Model No. 800-3). The amount of RNA present per sample was calculated from the standard curve.

**Estimation of brain acetylcholine (ACh)** (Ritcher and Crossland, 1949)

The method used by Ritcher and Crossland (1949) was followed for extraction and estimation of brain acetylcholine (ACh).
After killing the animal the head was immersed in liquid nitrogen to produce rapid fixation and to prevent any biochemical change in the tissues and minimize post-mortem changes due to breakdown or resynthesis of ACh in the brain. Just after 2 minutes, the brain was dissected out, using chilled instruments. Further quantities of liquid nitrogen was used to keep the brain frozen. The brain was then rapidly weighed and homogenized in 4 ml of 10% TCA (cold). The homogenate was transferred to a chilled centrifuge tube and kept in ice for 30 minutes. It was then centrifuged (10,000 X g) in cold for 5 minutes. The supernatant was decanted into a separate test tube and kept in cold. The precipitate was again washed with 1 ml of 10% TCA (cold), mixed with a glass rod, centrifuged and the supernatant thus obtained was added to the previous supernatant. The combined supernatant was brought to pH 4 with normal NaOH solution. Now 1 ml of this combined solution was made to react with 2 ml of alkaline hydroxylamine solution (Solution A : 13.99 of hydroxylamine hydrochloride, Sigma, was dissolved in 100 ml of doubly distilled water to make 2 M and kept in cold at 4°C. Solution B : 14 g of NaOH was dissolved in 100 ml of doubly distilled water to make 3.5 M. Alkaline hydroxylamine solution was prepared by mixing equal volumes of solution A and B, immediately before use). After 1 minute, 2 ml of diluted hydrochloric acid solution to attain pH 1.2 ± 0.2 and 1 ml of FeCl₃ solution (10 g of FeCl₃·6H₂O was dissolved in 100 ml of 0.1 N HCl to make 0.37 M) was added. The tube was shaken vigorously after the addition of reagents to avoid bubble formation in the tube during colour absorbency reading.

The optical density of the colour was measured at 540 mμ in spectrocolorimeter (E. Leitz, Inc., N. York, Model No. 26875) using the reagent blank to adjust the instrument to zero. The amount of acetylcholine was determined by using the standard curve.
solution (10 g of FeCl₃·6H₂O, E. Merck, Germany, dissolved in 100 ml of 0.1 N HCl) was added into the tubes. After each addition the tubes were shaken vigorously to avoid bubble formation in the tubes during colour absorbancy reading. Optical density of the colour in each tube was measured at 540 nm in the spectrocolorimeter (E. Leitz, Inc., N. York, Model No. 26875) using the reagent blank to adjust the instrument to zero. The amount of unreacted acetylcholine was determined by using the standard curve.

**Estimation of brain catecholamines** (Laverty and Taylor, 1968; Nagatsu, 1973)

The method used by Nagatsu (1973) was followed for extraction of brain catecholamines. After killing the animal the head was decapitated and kept in liquid nitrogen. Just after 30 minutes the brain was dissected out, using chilled instruments, weighed accurately and washed in cold physiological saline (0.85%). It was then brought to room temperature and homogenized in a glass homogenizer for three minutes with n-butanol (100 mg/ml). The tissue was then centrifuged (10,000 X g) in a refrigerated centrifuge for 15 minutes. The clear supernatant thus obtained was divided into two halves. In one half (containing 2 ml of supernatant), 10 ml of phosphate-buffer (13.61 g of KH₂PO₄, A.R. and 17.80 g of Na₂HPO₄, A.R. was dissolved in 500 ml of doubly distilled water and the volume was made up to 1 liter after adjusting the pH to 7) was added. To the other half (containing 1 ml of supernatant), 5 ml of citrate-buffer (53.65 g of Na₂HPO₄·12 H₂O, A.R. and 19.21 g of citric acid, A.R. was dissolved in 500 ml of doubly distilled water and the volume was made up to 1 liter after adjusting the pH to 4) was added. Both halves were finally added together and mixed uniformly in a cyclomixer for 1-2 minutes, and then centrifuged (6,000 X g) in cold for 1-2 minutes. As a result two layers were formed. The
bottom layer was aspirated out with the help of a pasteur pipette, divided into two equal halves and transferred to two test tubes marked 'P' and 'C'.

a. **Estimation of dopamine**

2-Ml of brain extract was taken from the tube marked 'P' and transferred to a glass-stoppered test tube. 0.1 ml of iodine solution (0.02 N iodine in 5% NaI solution, w/v) was added to the sample and shaken vigorously. After 3 minutes, 1 ml of alkaline sulfite solution (Na₂SO₃,7H₂O 2.5% w/v and Na₂EDTA 1% w/v in 2.5 N NaOH) was added to the mixture. After 5 minutes 0.2 ml of glacial acetic acid (pH 4.4) was added and mixed well. The sample was then heated in a boiling water-bath for exactly 40 minutes. After bringing it to room temperature, the sample was read in a spectrofluorometer (Turner, Model No. 430) at excitation/emission 320/375 µm. Samples with added internal standard (0.01%) and completely reversed blanks were run in parallel with the unknown sample.

b. **Estimation of norepinephrine**

2-Ml of brain extract was taken from the tube marked 'P' and transferred to a glass-stoppered tube. 0.1 ml of iodine solution was added to the sample and shaken vigorously. After 3 minutes, 1 ml of alkaline sulfite solution was added to the mixture. After 3 minutes, 0.28 ml of glacial acetic acid (pH 4.8) was added and mixed well. The sample was then kept in ice-water (20°C) for 25 minutes and then read in a spectrofluorometer (Turner, Model No. 430) at excitation/emission 380/480 µm. Samples with added internal standard and completely reversed blanks were run in parallel with the unknown sample.
c. Estimation of epinephrine

2-Ml of brain extract was taken from the tube marked 'C' and transferred to a glass-stoppered test tube as before. 0.1 ml of iodine solution was added to the sample and shaken vigorously. After 3 minutes, 1 ml of alkaline sulfite solution was added to it. Just after 1 minute, 0.2 ml of glacial acetic acid, A.R. (pH 5.0) was added to the mixture and mixed properly. Following this the sample was immediately read in a spectrofluorometer (Turner, Model No. 430) at excitation/emission 410/500 nm. Samples with added internal standard, and completely reversed blanks were run in parallel with the unknown sample.

Estimation of tissue residues of γ-HCH (Anon, 1984b)

Procedure for extraction:

Immediately after killing the animal, tissues from various organs (viz., liver, kidney, pectoral muscle, abdominal fat, brain) and also serum were collected and weighed accurately. Each tissue was macerated separately in a mortar with neutral sea-sand and a little acetonitrile (A.R., E. Merck, India), whenever required (for stiff tissue like muscle). It was then transferred to a conical flask (50 ml) containing 10 ml acetonitrile. The contents were mixed thoroughly for 5 minutes by mechanical shaker. It was then filtered through a Whatman No. 4 filter paper. The step was repeated two more times with 5 ml of acetonitrile. The filtrate was transferred to a 100 ml separating funnel containing 10 ml of n-Hexane (A.R., E. Merck, India) followed by addition of a considerable amount of double distilled water and 2 ml of saturated NaCl solution. The contents of the funnel were shaken vigorously for about 5 minutes so that the residue could pass over from the acetonitrile to the hexane.
medium. The separating funnel was then left undisturbed for the distinct separation of acetonitrile and hexane layers. After separation the lower aqueous acetonitrile layer was carefully discarded. The upper hexane layer (containing the residue) was also quantitatively collected with care into a beaker to avoid any loss or contamination. The acetonitrile layer was again extracted with n-hexane, using 10 and 5 ml of n-hexane (as described before) to extract the remaining residue, if any. Finally, the hexane fractions were collected in a beaker and allowed to concentrate to about 5 ml in a rotary vacuum evaporator.

The concentrated extract was then poured into a column-tube followed by dropwise addition of concentrated sulfuric acid (A.R., E. Merck, India) from a pasteur pipette. The acid layer after reaction, turned dark brown to yellow colour which are deposited along the lower part of the column-tube and finally discarded. Acid treatment was continued until the lower layer become completely clear. Finally, the acid layer was totally removed by regulating the stopcock of the column-tube. Following this, washing of the extract by distilled water was carried out to remove the acid from the extract. The extract was washed several times with distilled water until it became neutral to litmus paper. Lastly the tissue extract was collected in a stoppered test tube. In case of serum, 1 ml of sample was mixed with equal amount of distilled water and 8 ml of acetonitrile. The contents were mixed thoroughly for 5 minutes in a mechanical shaker and filtered through a Whatman No. 4 filter paper. The filtrate thus obtained was removed as before and the rest of the procedure was followed as described earlier with 20 ml hexane each time instead of 10 ml for tissues. The amount of residue present in the sample was determined by slight modification of the standard Gas Liquid Chromatography (GLC) technique.
Operating conditions for GLC (Pye Unicam, Model No. GCV, Cambridge, England)

- **Liquid phase**: 1.5% SP-2250/1.95% SP-2401
- **Solid support**: Supelcoport 100-120 mesh
- **Attenuation**: 32 X 10
- **Chart speed**: 1 cm/min
- **Detector**: Electron capture

**Temperature (°C)**
- **Detector**: 210
- **Column**: 200
- **Injection port**: 210

**Carrier Gas flow**
- **(ml/min)**
  - **Nitrogen**: 60

The standard pesticide under investigation (0.001% solution in acetone) was injected to GLC with a micro-syringe.

**Quantitative analysis**

The size of every peak displayed on the recorder is a measure of the amount of that particular component present in the sample. In reality, it is the area under the peak which is proportional to the quantity of the component present. The peak area was measured by triangulation method in which the area under the peak is approximated to a triangle enabling a simple calculation of total area = \( \frac{1}{2} \times \text{base} \times \text{altitude} \). Only completely resolved peaks were used for the quantitative purposes.