3. MATERIALS AND METHODS

3.1 Experimental Animals

Young healthy male albino rats (150 ± 20 gm weight) of Druckrey strain were drawn from the animal breeding facility of Industrial Toxicology Research Centre, Lucknow. The animals were bred on closed brother-sister mating. Young ones were weaned from the mother after 1½ months. The separated rats showing any kind of disease symptoms were not kept for the breeding as well for the experimental work. To check the infections in the colony, some usual antibiotics were given with the water. The boiled water was given to the animals for drinking to avoid any type of infection. The walls of the animal rooms are cleaned regularly and the floors are cleaned with disinfectant twice a day. Every care is taken to keep the animal colony of rats healthy. The rats were kept in steel cages. The animals were maintained on usual animal husbandry conditions and fed standard pellet diet (Hindustan Lever, India) or the synthetic diet and the water ad libitum, the latter with the help of glass bottle and nozzels attached.
A temperature of $28^\circ C \pm 2^\circ$ was maintained during the experiments.

3.2 Chemical Profile of Pesticides

3.2.1 Endosulfan: Endosulfan is a chlorinated hydrocarbon pesticide of cyclodiene group. It was developed and introduced by Farbwerke Hoechst A.G. in 1954 under the registered trade mark "Thiodan" (Maier-Bode, 1968). The other synonyms of endosulfan are Cyclodan, Thimol, Thiofar and Malix. It is chemically known as $6,7,8,9,10,10$-hexachloro-$1,5,5a,6,9a$-hexachydro-$6,9$-methano-$2,4,3$-benzodioxathiepine-$3$-oxide, or $\alpha,\beta-1,2,3,4,7,7$-hexachlorobicyclo-$2,2,1$-heptene-$2$-bis-hydroxy-methylene-$5,6$sulfite (Fig. 4). The insecticide endosulfan is obtained by the action of thionylchloride on the addition product from hexachlorocyclopentadiene and cis-butene-diol-$1,4$ (Frensch, 1958). The structural formula of endosulfan is given in Fig. 5. It is a mixture of two stereoisomers, the alpha of m.p. $108-110^\circ C$, the beta of $280-210^\circ C$, having a mol. wt of 407. The structure of endosulfan has been deduced with divergent results by Riemschneider (1963) and Forman et al. (1956). It is stable to sunlight but subject to
slow hydrolysis by alcohol and sulphur dioxide. It is compatible with non-alkaline pesticides.

The technical grade endosulfan consists of $\alpha$- and $\beta$-isomers in the ratio of 70:30. It is a pure mixture (90-95%) of isomers of cream to brown coloured flakes with a terpene like odour (spec. grav. 1.745, vapour pressure $9 \times 10^{-3}$ mm Hg at 80°C). Endosulfan melts between 80°C and 90°C and is practically insoluble in water. At 20°C the solubility in acetone is 33%, in benzene 37%, xylene 45%, carbon tetrachloride 29%, chloroform 50%, ethanol 5%, methanol 11% and kerosene 20%. Under normal conditions it is stable on storage, non-inflammable and can be hydrolysed slowly by aqueous alkali and acids (Frensch, 1958).

3.2.2 Malathion: Malathion is a non-systemic organophosphorous pesticide. Its chemical name (IUPAC) is diethyl (dimethoxythiophosphorylthio) succinate of S-1, 2bis(ethoxycarbonyl)ethyl 0,0-dimethyl phosphorodithioate (CA) (Fig. 5). Technical grade malathion (c.95% pure) is a clear amber liquid. Solubility at room temperature: 145 mg/l water; miscible with most organic solvents; of limited solubility in petroleum
Fig. 4  Structural Formula of Endosulfan

$(\text{CH}_3\text{O})_2\text{P-S-CHCOOC}_2\text{H}_5$

$\text{S CH}_2\text{COOC}_2\text{H}_5$

Fig. 5  Structural Formula of Malathion
oils (350 gm light petroleum/1 malathion). It is stable in natural fresh and saline waters but gets rapidly hydrolysed at pH above 7.0 or below 5.0. It is stable in aqueous solution buffered at pH 5.26.

In addition to a wide range of agricultural and horticultural uses, it is used to control animal ectoparasites, flies, household insects, human head and body lice and mosquitoes.

3.3 Equipment and Apparatus

The following instruments were used for chemical estimations and histopathological studies:
- Spectrophotometer 20 (Baush & Lomb, U.S.A)
- Mechanical Shaker water bath (Scientronic-India)
- Refrigerated centrifuge (IEC-Bombay)
- Single pan balance, Ovalabor (Germany)
- Potter Elvehjem homogenizer with teflon pestle
- Microtome (Rotating) (India)
- Olympus Research Binocular Microscope (Japan)

3.4 Preparation of Synthetic Diet

The synthetic diet (low protein cereal diet), was prepared according to Krishna Murti & Subrahmanian(1949)
as below. Low protein cereal diet consisted of the ingredients given in Table 4.

The constituents as given in Table 4 were mixed at a total quantity of 2-5 kg, cooked in a pressure cooker for 20 min. dried at 50-55°C, and powdered. Groundnut oil was heated at 120-140°C for 40-45 min. in a stainless steel vessel and then added to the dry powder. The dry powder was made into a paste in water and was fed to the rats ad libitum. The diets were supplemented with one per cent salt mixture (McCollumn and Davies, 1918), the ingredients of which are given in Table 5. Each rat in addition received 6 μg/day of multivitamin solution (ABDEC Drops; Parke-Davis, Bombay).

3.5 Chemical Estimations

3.5.1 Acetylcholinesterase: (Acetylcholine acetylhydrolase EC.3.1.1.7.): Enzyme activity was estimated by the method of Ellman et al. (1961). The method is based on the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed. This is accompanied by the continuous reaction of the thiol with 5,5-dithiotris-2-nitrobenzoate ion to
<table>
<thead>
<tr>
<th>Contents</th>
<th>Percentage</th>
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<tr>
<td>Whole milk powder</td>
<td>0.9</td>
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<tr>
<td>Common salt</td>
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</tr>
<tr>
<td>Ground nut oil</td>
<td>0.5</td>
</tr>
<tr>
<td>(Soybean meal, corn meal)</td>
<td>5.0</td>
</tr>
<tr>
<td>(Potatoes, vegetables)</td>
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</tr>
<tr>
<td>(Lettuce, vegetables)</td>
<td>0.0</td>
</tr>
<tr>
<td>(Pulses, legumes)</td>
<td>0.0</td>
</tr>
<tr>
<td>Polished rice</td>
<td>78.8</td>
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Table 4: Components of the Low Protein Cereal Diet
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<tr>
<th></th>
<th>3.19</th>
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<th>4.69</th>
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<td>NaCl</td>
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<td>NH₄NO₃</td>
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<td>CaCl₂(Po₄)(H₂O)ζ²</td>
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</tbody>
</table>

| Table 5: Constituents of salt mixture in grams |
produce the yellow ion of 5-thio-2-nitrobenzoic acid.

Tissue was homogenized in phosphate buffer 0.1 M pH 3.0. In cuvettes containing 2.6 ml of phosphate buffer was added 0.4 ml of homogenate (20 mg/ml). To this was added 0.1 ml of dithiobisnitrobenzoic acid (DTNB) 0.01 M (prepared in phosphate buffer 0.1 M pH 7.0). Reaction was started by addition of 0.02 ml of acetylthiocholine iodide 0.075 M. Changes in optical density were recorded at 412 nm after every 30 seconds. Enzyme activity was expressed as moles of acetylthiocholine hydrolysed/min/gm x 10^-6.

3.5.2 Blood Glucose: Blood glucose was estimated by the method of Somogyi as modified by Nelson (1944). Blood was collected in heparinized tubes from the tips of the tail or from jugular vein. 0.1 ml of blood was pipetted into centrifuge tubes. To this was added 4.5 ml of distilled water, 0.2 ml of ZnSO_4 (5%) followed by 0.2 ml of Ba(OH)_2 (4.5%). Tubes were shaken vigorously and centrifuged for 10 minutes. In 1.0 ml of the supernatent, 2.0 ml of alkaline copper reagent was added and tubes were kept in boiling water bath for 20 minutes. In the tubes, 2.0 ml of arsenomolybdate
reagent was added. Tubes were shaken and volume made up to 12.5 ml with distilled water. The colour intensity was read at 540 nm. The concentration of blood glucose was expressed as mg/100 ml blood.

3.5.3 Glycogen: Glycogen was extracted by the method of LeBaron (1955) and estimated colorimetrically as described by Montgomery (1957).

Tissue slices were transferred to centrifuge tubes containing 1.0 ml of ethanolic KOH (2 vol absolute ethanol + 1 vol 60% KOH). The tubes were kept in hot water bath at 30°C for 10 min, shaken and cooled. The glycogen was precipitated by addition of chloroform and methanol (1:4 V/V). The tubes were kept in hot water bath and centrifuged for 10 min. The supernatant fluid was decanted. The residue was dried in boiling water bath for 10 min. Dried residue was dissolved in 10 ml of distilled water. In 2 ml of aliquot was added 0.1 ml of 80 per cent phenol; and 5 ml of concentrated H₂SO₄ was added rapidly. The tubes were shaken and kept for 30 min. at room temperature. Readings were recorded at 490 nm. The concentration of glycogen is expressed as mg/100 gm wet tissue.
3.5.4 **Proteins:** Protein content of tissues was estimated by the method of Lowry et al. 1951 using bovine serum albumin as standard.

Tissues were homogenized in distilled water. To 1.0 ml of 10% homogenate, 1.0 ml or 10% TCA was added. Tubes were kept for one hour and centrifuged. Residue was dissolved in 1.0 ml of 0.1 N NaOH and the volume made up to 10.0 ml. To 1.0 ml aliquot, 5.0 ml of alkaline copper reagent was added. After 10 min. 0.5 ml of Folinphenol reagent was added. Tubes were shaken and kept for 30 min. at room temperature. The intensity of colour was measured at 660 nm. Protein concentration was expressed as mg/gm wet tissue.

3.5.5 **Glutamate Oxaloacetate Transaminase (GOT):** The enzyme activity was measured by the method of Umbreit et al. (1957).

The assay system consisted of 0.5 ml of 200 mM DL-Aspartic acid, 2 mM -Ketoglutarate in 0.1 M phosphate buffer pH 7.4 and 0.2 ml of 10% (w/v) rat liver homogenate or serum. The reaction mixture was incubated at 25°C for 20 minutes. The reaction was
stopped by the addition of one drop of 100% TCA solution followed by one drop of aniline citrate. The reaction mixture was kept at room temperature for 10 minutes. An addition of 0.5 ml of 0.1% 2,4-dinitrophenyl hydrazine (in HCl) converted the pyruvate into dinitrophenyl hydrazine pyruvate, which was extracted by 2 ml of toluene solution. A red colour complex was developed by the addition of 3.0 ml of 2.5% KOH in absolute alcohol to 1.0 ml of supernatant toluene solution. The colour intensity was read at 520 nm. The activity was expressed as μ mole of pyruvate liberated/min/gm wet tissue, or/ml serum.

3.5.6 Glutamate Pyruvate Transaminase (GPT): The enzyme activity was determined by the method of Umbreit et al. (1957). The assay system consisted of 0.5 ml of 200 mM DL-alanine, 2mM -ketoglutarate in 0.1 M phosphate buffer pH 7.4 and 0.2 ml of 10% (w/v) rat liver homogenate or serum. The reaction was stopped by the addition of one drop of 100% TCA followed by one drop of aniline citrate. The reaction mixture was kept at room temperature for 10 minutes. An addition of 0.5 ml of 0.1% 2,4-dinitrophenyl hydrazine (in HCl)
converted the pyruvate into dinitrophenyl hydrazone pyruvate, which was extracted by 2 ml of toluene solution. A red colour complex was developed by the addition of 3.0 ml of 2.5% KOH in absolute alcohol to 1.0 ml of supernatant toluene solution. The colour intensity was read at 520 nm. The activity was expressed as µ moles of pyruvate formed/min/gm wet tissue or/1 ml of serum.

3.6 Histopathological Studies

For histopathological studies, at the termination of the experiments, the animals were sacrificed with ether anaesthesia. The organs were examined for gross pathological changes; liver, kidney and testis were removed. Pieces of these tissues were fixed in 10% neutral formalin. Liver pieces were also fixed in Bouin's fluid for histochemical demonstration of glycogen.

The tissues were processed in the following way for histopathological studies.

3.6.1 Dehydration: After allowing an adequate time for fixation, the 2-5 mm thick slices of tissues were
dehydrated in various grades (30 to absolute) of ethyl alcohol.

3.6.2 **Clearing**: After dehydration, the tissues were cleared in the xylene for about four hours.

3.6.3 **Impregnation**: Impregnation with paraffin wax was carried out in oven maintained at 54 to 66°C for 6 hours.

3.6.4 **Sectioning**: Section (5 μ thick) of the tissues were cut with the help of microtome.

3.6.5 **Staining**: The sections were stained in haematoxylin and eosin and with PAS (Mc Manus & Mowry, 1965). The sections were dehydrated in alcohol, cleared with xylene and mounted with D.P.X. and were studied under the high power Olympus research binocular microscope. The histological changes in the tissues of treated and untreated animals were noted.

3.7 **Statistical Analysis**

The data were analysed by student 't' test (Fischer, 1950) and significance of differences calculated as p values are shown in Tables and figures.