CHAPTER - III

MATERIALS AND METHODS
(A) EXPERIMENTAL STUDY

Animals

Pure inbred Swiss male mice of 6-8 weeks of age were used.

Test Chemical

Technical grade BHC (hexachlorocyclohexane), obtained from M/s Hindustan Insecticides Ltd., Delhi, India, was used. The composition of the test chemical for alpha, beta and gamma isomers was as follows: alpha 72.5%; beta 8.3% and gamma 13.5%.

Diet

The composition of basal diet consisted of cracked wheat (70%), cracked bengal gram (20%), fishmeal (55%), yeast powder (4%), groundnut oil (0.5%) and shark liver oil (0.5%) in the form of dry mesh. Control animals were given this diet. The animals of experimental groups were given this diet containing 500ppm of BHC. This dose approximates the highest tolerable limit causing insignificant mortality in test animals. The insecticide BHC was weighed and dissolved in alcohol and homogeneously mixed in the diet with an electrically operated mixer to make up 500ppm concentration, and alcohol was allowed to evaporate. The concentration of BHC was checked periodically by soxhlet extraction followed by Gas Liquid Chromatographic quantification using electron
capture detector and was found to be within range of 10% variability. Diet was prepared once in a week and kept in cold room.

**Experimental Design**

The experimental design is given in Table-6. Group 0 served as control group for the experimental groups A, B, C and D. Each of the experimental groups consisted of six subgroups for 0, 2, 4, 6, 8 and 10 months of discontinuation following BHC administration. Control group 0 contained 20 animals, of which 10 were taken for histochemical study. Each experimental group contained 72 animals with 12 animals in each subgroup. Out of these 12 animals, 6 animals were taken for histochemical study.

The animals were housed in cages in group of four in an airconditioned room under natural lighting conditions and were given diet and water ad libitum. After the completion of the scheduled treatment, animals were sacrificed by cervical dislocation after 12-16 hours of fasting. The morphological, histochemical and biochemical studies were carried out as follows:

(I) Morphological study: The animals of different experimental and control groups were weighed and sacrificed as per schedule outlined in the experimental design. Necropsy was conducted and liver and blood were collected. The whole liver was weighed after cleaning and part of it was fixed in 10% formal saline for histological examination. Sections of
### Table-6
Design for Experimental Study

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Duration of BHC feeding (in months)</th>
<th>Duration after completion of BHC feeding (in months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control:</td>
<td>0 48</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Experimental: A</td>
<td>72</td>
<td>2</td>
<td>0,2,4,6,8,10</td>
</tr>
<tr>
<td>B</td>
<td>72</td>
<td>4</td>
<td>0,2,4,6,8,10</td>
</tr>
<tr>
<td>C</td>
<td>72</td>
<td>6</td>
<td>0,2,4,6,8,10</td>
</tr>
<tr>
<td>D</td>
<td>72</td>
<td>8</td>
<td>0,2,4,6</td>
</tr>
</tbody>
</table>

Each experimental group was divided into 6 subgroups of 12 animals each, of which 6 were taken for histochemical study.

Control group: 0 - contained 48 animals, with 12 animals each for experimental groups A, B, C and D.

BHC: Technical grade BHC (500ppm in diet).
liver tissue with 5 um thickness were cut and stained with haematoxylin and eosin (HE) and observed under the light microscope. The remaining part of the liver was preserved in cold for biochemical studies as given below. The blood was allowed to clot and serum was separated and preserved in cold till use.

(II) Histochemical study: Three to four days before sacrifice of the animals, 0.2ml of iron dextran complex, containing 10mg of ferric hydroxide, was administered intravenously to animals of control and different experimental groups. The animals were then sacrificed and liver tissues were collected. The liver tissues were cut into small pieces and fixed separately in carnoy's solution for localization of glycogen and in 10% formal saline for iron distribution. Sections of the liver tissues with 5 μm thickness were cut and spread in a 70% alcohol bath to keep the glycogen intact without dissolving during the process of spreading the sections in the bath. For glycogen, sections were stained by periodic acid schiff's method (Pearse, 1968). For iron, sections were stained with Pearl's stain as described by Pearse (1972).

(III) Biochemical study: The liver tissue preserved in cold as mentioned above, were taken to prepare 5% liver homogenate in cold 0.25 M sucrose medium. For preparation of homogenate at 2000 rpm scientronic homogenizer with glass tube and teflon pestle was used. The whole homogenate as such was kept in cold till use. The analysis of protein,
gamma glutamyl transpeptidase, leucine amino peptidase, S' nucleotidase, alkaline phosphatase and isoenzymes of lactate dehydrogenase was carried out from this whole homogenate.

The serum samples preserved in cold were used for analysis of serum proteins, alpha fetoprotein and LDH isoenzymes.

The details of assay conditions for each of the enzymes are given in Table-7. The principles of the methods of analysis for each parameter are briefly described below.

(1) Gamma Glutamyl Transpeptidase:– Gamma glutamyl transpeptidase (GGT E.C.2.3.2.2) was assayed as described by Naftalin et al (1969). According to this technique, L-γ-glutamyl-p-nitroanilide serves as the substrate and glycylglycine as the acceptor molecule for the glutamyl group released by the action of GGT. The liberated p-nitroaniline is diazotized and measured at 550nm.

(2) Leucine Amino Peptidase:– Leucine amino peptidase (LAP E.C.3.4.1.1) was assayed as described by Martinek et al (1964). In this procedure the substrate L-Leucyl-β-Naphthylamide is hydrolysed by the action of LAP. The liberated β-Naphthylamine after reduction with sodium nitrite, produces Diazo reagent which in turn reacts with N-(1-Naphthyl)-Ethylene diamine to form blue azo dye complex which is read at 580nm.

(3) S'Nucleotidase:– S'nucleotidase (S'ND E.C. 3.1.35) was determined as described by Gerlach and Hibi (1974).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Substrate</th>
<th>Cofactors</th>
<th>Buffer</th>
<th>Incubation temp, time</th>
<th>Estimation</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L-γ-glutamyl-p-nitroanilide</td>
<td>Glycylglycine (acceptor) Trizma</td>
<td>37°C, 20 mins</td>
<td>p-nitroaniline (at 550 nm)</td>
</tr>
<tr>
<td>GGT</td>
<td>(E.C. 2.3.2.2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S'ND</td>
<td>(E.C. 3.1.3.5)</td>
<td>Adenosine-N5'-monophosphate</td>
<td>Mn++</td>
<td>Veronal (40 mM, pH 7.5)</td>
<td>37°C, 30 mins</td>
<td>Inorganic Phosphorus (at 578 nm)</td>
</tr>
<tr>
<td>ALP</td>
<td>(E.C. 3.1.3.1)</td>
<td>p-nitrophenyl phosphate-disodium salt</td>
<td>Mg++</td>
<td>Glycine (0.1M, pH 10.5)</td>
<td>37°C, 30 mins</td>
<td>p-nitrophenol (at 400 nm)</td>
</tr>
<tr>
<td>OCT</td>
<td>(E.C. 2.1.3.3)</td>
<td>Carbamoyl phosphate-dilithium salt, Ornithine</td>
<td>-</td>
<td>Veronal (70 mM, pH 7.0)</td>
<td>37°C, 30 mins</td>
<td>Citrulline (at 460 nm)</td>
</tr>
</tbody>
</table>
According to this method, 5'ND activity was assayed in the presence and absence of nickel ion as selective complete inhibitor of the enzyme. In the presence of these ions the activity of non-specific phosphatase is determined, while in their absence the sum of the activities of non-specific phosphatases and 5'Nucleotidase is measured. The difference in the amount of phosphate ion liberated per unit time in the two assays, as determined by Fiske and Subbarrow method (1925), is a measure of 5'Nucleotidase activity.

(4) Alkaline Phosphatase:— Alkaline phosphatase (ALP E.C.3.1.3.1) was estimated as described by Berger and Rudolph (1965). The substrate p-nitrophenyl phosphate-disodium salt is hydrolysed by ALP to produce p-nitrophenol. The amount of p-nitrophenol liberated per unit time, as determined in alkaline solution at 400 nm, is a measure of the enzyme activity.

(5) Lactate Dehydrogenase Isoenzymes:— Lactate dehydrogenase (LDH E.C.1.1.1.27) isoenzymes were determined by the method of Dietz and Lubrano (1967). The polyacrylamide disc gel electrophoresis was carried out using 7.5% gel and tris-glycine buffer (5mM, pH 8.3). The separated LDH isoenzymes in association with nicotinamide adenine dinucleotide (NAD) as a coenzyme, catalyze the dehydrogenation of substrate lactate to produce pyruvate. The freed H\(^+\) then reduces the NAD to NADH, which in turn is oxidized back to NAD by the phenazine methosulphate. The freed H\(^+\) from the NADH now reduced the colourless tetrazolium salt to the blue
coloured insoluble formazan. The formazan is quantitatively related to the levels of LDH isoenzymes present. The isoenzymes are then scanned by densitometer at 600 nm.

(6) Serum Proteins and Alpha Fetoprotein (AFP):- Serum proteins were analysed by the method of Davis (1964). Electrophoresis of proteins in polyacrylamide disc gel was carried out using 7.5% gel and Tris-glycine buffer (50 mM, pH 8.3). The proteins are separated on the basis of charge density and molecular size. After running, the gel is stained with 1% amido black dye solution and scanned by densitometer at 600 nm.

Alpha fetoprotein (AFP) was detected in serum by the counter immuno electrophoretic technique as described by Bapat et al (1973) with some modification. Standard murine AFP and antibodies against AFP, supplied by Prof. Engelhardt from U.S.S.R., were used. Agarose (0.8%) in veronal acetate buffer (0.1 M, pH 8.4) was used for preparation of gel. Two sets consisting of a single row or wells of nearly 2mm diameter were prepared with a blunt liver biopsy needle. The known antigen with the help of pasture pipette was filled in the cathodic well while the unknown antibodies in the serum were put in the anodic well. The slides were run in an electrophoretic tank using veronal acetate buffer (0.1 M, pH 8.9). After the run, the slides were taken out and washed with distilled water to remove untreated proteins and dried at 37°C. The dried slides were stained with 0.5% amido black solution
for 15 mins. to observe the precipitin lines.

(B) HUMAN STUDY

(I) Patients with Liver Cancer

Serum samples were obtained from 37 hospital patients with liver cancer. Out of these 37 cases, 6 were primary liver cancer cases and 31 were metastasized liver cancer cases as revealed by the clinical and histological examinations of percutaneous liver biopsy.

Sera of 24 healthy subjects were collected for comparison.

The enzymes, gamma glutamyl transpeptidase (GGT), leucine amino peptidase (LAP), 5' nucleotidase (5' ND) and alkaline phosphatase (ALP) were investigated in all these serum samples by the methods described earlier under 'Experimental Study'.

(II) Workers exposed to BHC

Examination of 38 male workers at a BHC producing plant was carried out. Special attention was paid to detailed investigations of total protein, A/G ratio, protein electrophoresis, immunoglobulins and liver specific enzymes GGT, LAP, ALP and ornithine carbamoyl transferase (OCT) from blood sera of these workers.

The workers were divided into two groups: (1) Non-handler group which consisted of 22 subjects working at BHC plant but were not directly exposed to BHC, (2) The second
group comprised of 16 BHC handlers who had direct BHC exposure while handling and packing the chemical.

For comparison purposes, an external control group of 18 male subjects of the same age distribution having no contact with BHC was examined in the same way.

(1) Total Protein:— Total protein and A/G ratio were determined by the biuret method (Reinhold, 1953). The serum protein was treated with alkaline biuret solution containing \( \text{Cu}^{2+} \) ions and coloured chelate complex, formed between \( \text{Cu}^{2+} \) ion and the carbonyl \((-c=O)\) and imine \( (=N-H)\) groups of the protein, was read at 540 nm against reagent blank. The protein standard solution (6g%) was used for calibration.

(2) Protein Electrophoresis:— Electrophoresis of serum protein was carried out by Microzone method using cellulose acetate membrane obtained from Beckman Instruments, U.S.A. The samples were charged with the help of applicator and electrophoresis was done for 20 minutes using Beckman B-2 barbital buffer (0.1 M, pH 8.6). The membrane was stained with Ponceau-S dye solution (0.2%) for 10 minutes and rinsed with 5% acetic acid solution. The membrane was dried with absolute ethanol and cleared with 30% cyclohexanone in ethanol. Finally it was dried and scanned at 520 nm using Beckman Microzone Densitometer.

(3) Immunoglobulins:— The immunoglobulins IgG, IgA and IgM were determined by single radial immunodiffusion technique using 'Tri-partigen' plates supplied by Hoechst
These plates contained a prepared agar gel in which H-chain specific antiserum to the respective immunoglobulin is incorporated. The antiserum is produced by immunization of sheep and goats.

IgA and IgM were determined using undiluted serum, but for IgG and control, serum was diluted 1:10 with isotonic saline. Control serum was a stabilized pooled serum from healthy adults with negative HBsAg.

Wells were filled with 5 μl test serum sample along with control and plates were kept in moist chamber at room temperature for immunodiffusion for 50 hours for IgG and IgA and 80 hours for IgM. At the end of the given diffusion time, the diameter of the precipitin rings were measured using Partigen ruler. The immunoglobulin concentrations, related to the measured diameters, were read directly from the table of reference values. For IgG, values found were multiplied by the dilution factor 10.

(4) Enzymes:- The enzymes GGT, LAP and ALP were assayed by the methods described under 'Experimental Study'. The enzyme ornithine carbamoyl transferase (OCT E.C.2.1.3.3) was assayed by the method described by Ceriotti (1973) with modification.

The substrate carbamoyl phosphate, with ornithine formed citrulline by the action of OCT. The amount of citrul-line formed per unit time is a measure of the OCT activity. The urea present in the sample interferes with citrulline
determination, so it is destructed by the urease added. The citrulline is then determined by diacetylmonoxime-antipyrine reaction.