PART - II
INTRODUCTION
Introduction:

Different environmental agents have been found to induce alterations in the genetic systems even in subtoxic doses, when administered for prolonged periods of time. Since environmental factors are often involved in the production of abnormal conceptuses (Hook 1981), the effects of these agents singly and in combination have gained great importance in the past decades following rapid industrialisation and urbanisation (see Berg 1979, Hsu 1982, Sharma 1984a,b, Sugimura et al. 1982).

The metals form a major environmental pollutant as they enter the biogeochemical cycle through different sources. Following the disasters in Minamata and Niigata extensive researches have been carried out on the toxic effects of mercury on biological systems (see Das et al. 1982, Harada 1968).

The present investigation was therefore undertaken to observe the foetotoxic effects of the inorganic form of mercury on a single mammalian test system viz. Rattus norvegicus. In order to analyse the action of this toxic environmental agent, the following parameters were studied:

1. In the pregnant female rats, study of the bone marrow chromosomes following oral administration of different doses of HgCl₂.

2. In the foetal tissue obtained after 11 days from these rats, (a) study of micronuclei from foetal liver cells,
(b) study of the teratogenic and embryotoxic effect, (c) histological studies and in situ DNA estimation from different organs of the foetus.
MATERIAL AND METHODS
2. MATERIALS AND METHODS

Experimental test system

Animals - Female and male albino inbred rats - *Rattus norvegicus* were used to study the teratogenic and embryotoxic effects of mercuric chloride (HgCl₂).

Chemical used - Mercuric chloride (HgCl₂) (BDH, Glaxo Laboratories India Ltd.). Mercuric chloride is said to be highly toxic to mammalian systems. It is a divalent compound - melting point 277°C, molecular weight 277.50.

Properties - It is a crystalloid or powder in nature, white in colour, soluble in water; **LD**₅₀ of rat orally is approximately 37 mg/kg body weight (vide *The Merck Index*, 1976). In case of human systems 1-2 gms is frequently fatal. It is used in different industries in drugs or antiseptics etc.

Breeding and maintenance

A laboratory strain of albino rats was selected for this experiment. Adult male and female rats were segregated before mating. In female rats the estrous cycle is generally completed in 4-5 days, although the timing of the cycle may be influenced by external factors like light, temperature, nutritional and social relationships. The estrous cycle is completed in the following four phases:

(a) **Estrus** is the period of heat when copulation is permitted. This condition lasts for 9-15 hours and is chara-
cterised by high rate of running activity. During late estrus stage cheesy masses of loose cornified cells with degenerated nuclei are observed. Very few leucocytes are found.

(b) Metestrus occurs shortly after ovulation and lasts for 10-14 hours; mating is usually not permitted. Some leucocytes and a few cornified cells are observed in the vaginal smear. Different cell sizes are also observed.

(c) Diestrus lasts for 60 to 70 hours during which functional regression of corpora lutea occurs. The vaginal mucosa is thin and leucocytes migrate to it. A vaginal smear consists almost entirely of these cells.

(d) In proestrus, vaginal smear predominantly consists of nucleated epithelial cells which occur singly or in sheets.

Ovulation takes place in the early hours of estrus phase. Each phase can be identified by vaginal smear technique. By a small pasteur pipette 0.1 ml of normal saline solution was injected into the vagina and then again drawn out. The smear was made on a grease free slide and air dried. The dried slide was stained by haematoxylin and studied under the microscope. The rats in estrus phase were immediately transferred to a mating chamber with a healthy sexually active male rat. In one mating chamber one male rat and two female rats were kept together in dark. Next day the two sexes were separated and the vaginal smear of the female rats were checked for estrus, sperm positive stage.
The gestation period of female rat is 22-25 days. The pregnant rat was kept on normal diet, milk and vitamin.

The adult rats were raised on normal balanced diet prepared fresh daily both before and after treatments. It contained wheat flour as carbohydrate, oil as fat, milk protein or casein and salt mixture in the following proportions:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat flour</td>
<td>10 kg</td>
</tr>
<tr>
<td>Fish meal</td>
<td>2 kg</td>
</tr>
<tr>
<td>Sugar</td>
<td>500 gms</td>
</tr>
<tr>
<td>Oil</td>
<td>1 kg</td>
</tr>
<tr>
<td>Salt mixture</td>
<td>400 gms</td>
</tr>
</tbody>
</table>

The salt mixture of different proportions being essential for the laboratory animal contained:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCO₃</td>
<td>300 gms</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>322 gms</td>
</tr>
<tr>
<td>CaHPO₄·2H₂O</td>
<td>197 gms</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>102 gms</td>
</tr>
<tr>
<td>NaCl</td>
<td>167.5 gms</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>27.5 gms</td>
</tr>
<tr>
<td>KI</td>
<td>0.8 gms</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>5 gms</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.25 gms</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.3 gms</td>
</tr>
</tbody>
</table>

(Phillips and Hurt 1935)

The ingredients were homogenized into a fine powder form separately mixed thoroughly and kept in cold.
The standard balanced diet was mixed with adequate water, the mixture was kneaded well to form a thick dough and given to rats. Vitamin B tablets were dissolved in water corresponding to the treatment dose, once a week. The animals were provided with separate drinking water. However, during treatment the rats were kept only on standard diet and drinking water but no vitamin or milk was given.

Treatment

After mating, the day on which estrus sperm positive was seen was taken as day 1. On day 7 a single dose of HgCl₂ dissolved in distilled water in different concentrations was orally fed to the pregnant female rat.

(a) Treatment 1 - 0.185 mg (1/20th of LD₅₀ orally) HgCl₂/100 gms body weight were force fed. (0.37 mg was dissolved in 10 ml of distilled water). Then the rat was kept in a separate cage and given only standard diet and drinking water. It was sacrificed on the 18th day of gestation.

(b) Treatment 2 - 0.247 (1/15th of LD₅₀ orally) HgCl₂/100 gms body weight was force fed (0.454 mg was dissolved in 10 ml of distilled water). The next stage was same as in treatment 1.

(c) Treatment 3 - 0.37 mg HgCl₂ (1/10th LD₅₀ orally)/100 gms body weight of rat was force fed (0.74 mg HgCl₂ was dissolved in 10 ml of distilled water). The next step was same as in treatment 1.
For each set a corresponding control set was maintained on distilled water.

**Mitotic chromosome preparation**

**Preparation of reagents**

**Colchicine** - 40 mg of colchicine powder (Sisco, India) was dissolved in 100 ml of distilled water to give 0.04% solution and stored at 4°C.

**Sodium citrate solution** - 1% Trisodium citrate solution was prepared in distilled water to be used as a hypotonic solution.

**Acetic acid : ethanol (1:3)** - used as a fixative.

**Giemsa solution** was prepared as described in Method III (pages 122 to 123).

**Method for study of mitotic chromosome**

**Pretreatment** - 0.04% colchicine, at a dosage of 1 ml/100 gms body weight was injected intraperitoneally in the abdomen after cleaning the site with ethyl alcohol.

**Extraction of tissue** - 1½ hours after pretreatment the rat was sacrificed. The abdomen was cut open and the flesh and muscle from the two hind legs were removed. The femur was dissected out with bone cutter and was held with forceps. The hypotonic solution, kept at 37°C, was taken in a syringe fitted with a fine needle and injected through the femur to flush out the bone marrow. The procedure was repeated twice. The
bone marrow was thoroughly suspended in the hypotonic solution with a pasteur pipette and kept at 37°C for 15 minutes.

**Fixation** - After removing the hypotonic solution by centrifugation, fixative was added to the cell pellet, thoroughly mixed and kept for 20 minutes. Two more changes were given after 10 minutes each.

Slides were prepared by flame drying technique described in Method II (page 94). Slides were stained in Giemsa as described in Method III (pages 122 to 123). The stained slides were made permanent by mounting in DPX.

**Method of observation**

The cells were scanned in Olympus microscope Model No. KIC 27489.

The normal karyotype, prepared according to Mori and Sasaki (1973), contains 21 pairs of chromosomes, arranged serially according to size. 20 pairs of autosomes and one pair are sex chromosomes, XX in female and XY in male. The autosomes are classified according to their general morphological characters including size and position of centromere, into 7 pairs of metacentrics, 5 pairs of submetacentrics and 8 pairs of telo- or acrocentrics. Mitotic indexes of each set were calculated.
Different types of chromosomal abnormalities were also scanned which are categorised as follows:

(a) **Stickiness** - Metaphase chromosomes were joined with one another showing a sticky nature.

(b) **Erosion** - Chromosomes were woolly with blurred outlines.

(c) **Fuzziness** - Later phase of erosion where chromosomes were very narrow.

(d) **Pulverization** - Gross effects on chromosomes.

(e) **Dicentric** - Occurs due to fusion of chromosomes resulting in two centromeres on one chromosome.

(f) **Deletion** results from a loss of intercalary segment of a chromosome (Sharma 1985).

(g) **Centric fusion** results from reciprocal translocation across the centromeric region of acrocentric chromosomes or telomeric fusion of telocentric chromosomes to give to metacentric ones.

(h) **Gap** is an unstained part of a chromatid within a connection (Manna and Gupta 1979).

(i) **Polyploidy** - Doubling of the diploid set. It may be tetra or octaploid or more.

(j) **Break** - All types of breaks are chromosomal aberrations. According to the position and types of break these are mainly two types:
(a) **Chromatid break** - When the break or complete separation of a part of a chromatid occurs on a single chromatid indicating action at $G_2$ phase. When there is a thin connection it is called subchromatid break. Break may again be proximal, middle and distal according to the position (Manna 1975).

(b) **Isochromosome break** - When the break occurs on both chromatids in the same position indicating action in $G_1$ phase.

(k) **Ring** - When two acrocentrics unite to form a ring like structure.

All the abnormal types were scanned from well scattered metaphase plates and observed under the oil immersion objective. Different categories of abnormalities and their percentage were calculated and statistical analysis was done to determine the test of significance. The categories were as follows:

- **Group I**: Direct effects on chromosome only
  - Breaks and gaps, centric fission, centric fusion, telomeric fusion, translocation, inversion, erosion, fuzziness.

- **Group II**: On spindle - polyploidy and diplochromatid.

- **Group III**: Combination of groups I and II on moderate levels

- **Group IV**: Other effects
  - Micronuclei, premature chromatin condensation.
Group V : Lethal effects

Pycnosis, extrusion of chromatin matter, pulverisation and cell death.

Statistics

(a) Mean \( \bar{X} = \frac{X_1}{n} \)

\( X_1 \) = Value of each sample of a group.
\( n \) = Total number of samples of a group.

(b) Standard Deviation \( = \sqrt{\frac{\sum (X_1 - \bar{X}_2)^2}{n-1}} \)

\( \bar{X} \) = Mean value of a group.

(c) Test of significance or 't' test :

\( t = \frac{\bar{X}_1 - \bar{X}_2}{s \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \)

\( s^2 = \frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{(n_1 + n_2) - 2} \)

\( s = \sqrt{\frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{(n_1 + n_2) - 2}} \)

where, \( t \) = 't' value

\( \bar{X}_1 \) = Mean value of control
\( \bar{X}_2 \) = Mean value of treated
\( S_1 = S.D. \) of control
\( S_2 = S.D. \) of treated
\( s^2 = \) Variance
Teratological studies

The foetuses were dissected out from the mother rat and were preserved in phosphate formalin buffer following the method II described in page 102.

From the preserved foetuses - (a) histological studies (Method II) by haematoxylin and eosin staining (Method II) and in situ DNA estimation (Method II) were done (see pages 105 to 108). Morphological abnormalities (if any) were studied from a preserved foetus with a hand lens.

Micronucleus test from foetal liver cells

The liver was dissected out from a foetus, minced, fixed in 1:3 acetic ethanol, and thoroughly flushed. Three changes were given and slides were prepared by flame drying schedule (Method II - pages 94 to 98), stained in Giemsa (Method III, pages 122 to 123) and observed.

The cells were observed under the oil immersion objective of the Olympus microscope (KIC 27489). Two types of hepatic cells were seen, large and small. Percentages of large cells with micronuclei and small cells with micronuclei were calculated. The data were statistically analysed following method IV (see page 234).
OBSERVATIONS
Clastogenic, teratogenic, and embryotoxic effects of mercuric chloride were tested on animal systems.

Pregnant rats were given orally HgCl₂ on the 7th day of gestation and kept on normal diet until 18th day and sacrificed. Three doses viz., 1/20th LD₅₀ (1.85 mg/kg b.w.), 1/15th LD₅₀ (2.47 mg/kg b.w.) and 1/10th LD₅₀ (3.70 mg/kg b.w.) were administered. The following parameters were studied:

1) Chromosomes from bone marrow of the mother rat, showing effects of different doses of HgCl₂ on -
   (a) Mitotic indices (Tables 1 to 4, Fig.III, Plate - 39).
   (b) Changes in chromosome number and structure (Tables 5 to 13; Figs.III to VIII, Plate - 39).

2) Formation of micronuclei in foetal liver cells (Tables 14 to 16; Figs.IX to XI, Plate - 39).

3) Teratogenic and embryotoxic effects of HgCl₂ (Table 17, Fig.XI).

4) Histological studies from the sections of foetuses (Pages 250 to 252, Plates 40 to 44).

5) In situ DNA estimation from different organs from sections of foetuses (Table 18, Figs.XII to XIV).
Table 1: Effect of 1.85 mg/kg b.w. of HgCl₂ (1/20th LD₅₀) on the mitotic index

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Dose given</th>
<th>Total cells counted</th>
<th>Dividing cells</th>
<th>Mitotic index</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Single</td>
<td>Control</td>
<td>1021</td>
<td>44</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1052</td>
<td>41</td>
<td>3.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1045</td>
<td>36</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1122</td>
<td>39</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1075</td>
<td>24</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1028</td>
<td>29</td>
<td>5.16</td>
</tr>
</tbody>
</table>

df = 8

Table 2: Effect of 2.47 mg/kg b.w. of HgCl₂ (1/15th LD₅₀) on the mitotic index

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Dose given</th>
<th>Total cells counted</th>
<th>Dividing cells</th>
<th>Mitotic index</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Single</td>
<td>Control</td>
<td>1021</td>
<td>44</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1030</td>
<td>11</td>
<td>3.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1045</td>
<td>36</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1022</td>
<td>18</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1075</td>
<td>24</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1000</td>
<td>27</td>
<td>5.16</td>
</tr>
</tbody>
</table>

df = 8; P = <.05

Table 3: Effect of 3.70 mg/kg b.w. of HgCl₂ (1/10th LD₅₀) on the mitotic index

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Dose given</th>
<th>Total cells counted</th>
<th>Dividing cells</th>
<th>Mitotic index</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Single</td>
<td>Control</td>
<td>1021</td>
<td>44</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1047</td>
<td>11</td>
<td>3.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1045</td>
<td>36</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1064</td>
<td>32</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1075</td>
<td>24</td>
<td>2.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>1033</td>
<td>17</td>
<td>5.16</td>
</tr>
</tbody>
</table>

df = 8; P = <.05
Table 4: Effect of different doses of HgCl₂ on mitotic index

<table>
<thead>
<tr>
<th>Different sets studied</th>
<th>Total cells counted</th>
<th>Dividing cells counted</th>
<th>Mean ± S.D.</th>
<th>'P' value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5581</td>
<td>200</td>
<td>3.48 ± 1.27</td>
<td></td>
</tr>
<tr>
<td>1/20th LD₅₀</td>
<td>5436</td>
<td>188</td>
<td>3.40 ± 0.54</td>
<td></td>
</tr>
<tr>
<td>(single dose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/15th LD₅₀</td>
<td>5076</td>
<td>96</td>
<td>1.89* ± 0.77</td>
<td>P &lt; .05</td>
</tr>
<tr>
<td>(single dose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/10th LD₅₀</td>
<td>5593</td>
<td>101</td>
<td>1.82* ± 0.78</td>
<td>P &lt; .05</td>
</tr>
<tr>
<td>(single dose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Inference

Mercuric chloride appeared to reduce mitotic index and increase chromosomal abnormalities. These effects were seen even at a low concentration and following a single application with 1.85 mg/kg b.w. mitosis was reduced but not significantly. However, at higher concentrations, that is at 1/15th LD₅₀ (2.47 mg/kg b.w.) and 1/10th LD₅₀ (3.70 mg/kg b.w.) the mitotic index of the treated set was significantly lower than that of the control.
Fig. III COMPARISON OF MITOTIC INDEX BETWEEN THE CONTROL AND THE TREATED SETS

- CONTROL
- \(1/20\) \(\text{LD}_{50}\) (1.85 mg/kg b.w.)
- \(1/15\) \(\text{LD}_{50}\) (2.47 mg/kg b.w.)
- \(1/10\) \(\text{LD}_{50}\) (3.70 mg/kg b.w.)
Explanation of microphotographs

Plate No. 38

1. Photograph of a normal mitotic metaphase (2n = 42, XX) of *Rattus norvegicus* and Karyogram made from it (X 6220)
Table 5: Total percentage of abnormality with 1.85 mg/kg b.w. (1/20th LD₅₀) of HgCl₂

<table>
<thead>
<tr>
<th>Total cells counted</th>
<th>Percentage of abnormal cells</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>60</td>
<td>85</td>
<td>3.33</td>
</tr>
<tr>
<td>60</td>
<td>85</td>
<td>1.66</td>
</tr>
<tr>
<td>60</td>
<td>82</td>
<td>3.33</td>
</tr>
<tr>
<td>60</td>
<td>77</td>
<td>10.00</td>
</tr>
<tr>
<td>62</td>
<td>75</td>
<td>3.33</td>
</tr>
</tbody>
</table>

df=8; P = <.02

Table 6: Total percentage of abnormality with 2.47 mg/kg b.w. (1/15th LD₅₀) of HgCl₂

<table>
<thead>
<tr>
<th>Total cells counted</th>
<th>Percentage of abnormal cells</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>3.33</td>
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<tr>
<td>60</td>
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<td>60</td>
<td>10.00</td>
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<tr>
<td>62</td>
<td>61</td>
<td>3.33</td>
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df=8; P = <.01

Table 7: Total percentage of abnormality with 3.70 mg/kg b.w. (1/10th LD₅₀) of HgCl₂

<table>
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<tr>
<th>Total cells counted</th>
<th>Percentage of abnormal cells</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>60</td>
<td>82</td>
<td>3.33</td>
</tr>
<tr>
<td>60</td>
<td>85</td>
<td>1.66</td>
</tr>
<tr>
<td>60</td>
<td>84</td>
<td>3.33</td>
</tr>
<tr>
<td>60</td>
<td>84</td>
<td>10.00</td>
</tr>
<tr>
<td>62</td>
<td>74</td>
<td>3.33</td>
</tr>
</tbody>
</table>

df=8; P = <.001
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<thead>
<tr>
<th>Different sets studied</th>
<th>Dividing cells counted</th>
<th>Abnormal cells</th>
<th>Mean ± S.D.</th>
<th>'p' value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>302</td>
<td>13</td>
<td>4.33 ± 3.24</td>
<td></td>
</tr>
<tr>
<td>1/20th LD₅₀ (single dose)</td>
<td>407</td>
<td>53</td>
<td>13.10** ± 5.33</td>
<td>P &lt; .02</td>
</tr>
<tr>
<td>1/15th LD₅₀ (single dose)</td>
<td>301</td>
<td>47</td>
<td>15.62*** ± 4.37</td>
<td>P &lt; .01</td>
</tr>
<tr>
<td>1/10th LD₅₀ (single dose)</td>
<td>409</td>
<td>70</td>
<td>17.11**** ± 3.64</td>
<td>P &lt; .001</td>
</tr>
</tbody>
</table>

**df=8**

**Inference**

Mercuric chloride induced a high percentage of chromosomal abnormalities leading to lethality in the bone marrow cells of the treated pregnant rats.

The total percentage of abnormalities showed a dose dependent relationship. Even at the lowest dose applied i.e., at 1/20th LD₅₀ the percentage of abnormal cells was significantly higher than that of the control. With the next two higher doses 1/15th LD₅₀ and 1/10th LD₅₀ the increase in the total percentage of abnormalities was highly significant.
Different abnormal cells have again been grouped according to their nature as follows:

**Group I:** Direct effect on spindle only includes polyploidy, diplochromatid formation, irregular condensation of chromosomes.

**Group II:** Direct effect on chromosomes includes breaks, gaps, fragments, centric fission and fusion.

**Group III:** Combination of groups I and II involving one or more chromosomes.

**Group IV:** Micronuclei and premature chromosome condensation.

**Group V:** Gross abnormalities involving all chromosomes, leading to pycnosis and lethality.

Table 9: Effect of HgCl₂ (1/20th LD₅₀ = 1.85 mg/kg b.w.) on group I type of abnormality in mitotic chromosomes

<table>
<thead>
<tr>
<th>Different sets studied</th>
<th>Total cells counted</th>
<th>Abnormal cells belonging to group I</th>
<th>Mean ± S.D.</th>
<th>'P' value</th>
<th>Df.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>302</td>
<td>2</td>
<td>0.666 ± 0.912</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/20th LD₅₀ 407 (single dose)</td>
<td>13</td>
<td>13</td>
<td>3.178*** ± 1.307</td>
<td>P = &lt;.01</td>
<td>8</td>
</tr>
<tr>
<td>1/15th LD₅₀ 301 (single dose)</td>
<td>8</td>
<td>8</td>
<td>2.666*** ± 0.647</td>
<td>P = &lt;.01</td>
<td></td>
</tr>
<tr>
<td>1/10th LD₅₀ 409 (single dose)</td>
<td>2</td>
<td>2</td>
<td>0.473 ± 0.647</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 10: Effect of different doses of HgCl₂ on group II type of abnormality in mitotic chromosomes

<table>
<thead>
<tr>
<th>Different sets studied</th>
<th>Total cells counted</th>
<th>Abnormal cells belonging to group II</th>
<th>Mean ± S.D. value D.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>302</td>
<td>2</td>
<td>0.666 ±0.912</td>
</tr>
<tr>
<td>1/20th LD₅₀ (single dose)</td>
<td>407</td>
<td>3</td>
<td>0.792 ±1.188</td>
</tr>
<tr>
<td>1/15th LD₅₀ (single dose)</td>
<td>301</td>
<td>5</td>
<td>1.666* P = &lt;.05</td>
</tr>
<tr>
<td>1/10th LD₅₀ (single dose)</td>
<td>409</td>
<td>14</td>
<td>3.379*** P = &lt;.01</td>
</tr>
</tbody>
</table>

Table 11: Effect of different doses of HgCl₂ on group III type of abnormality in mitotic chromosomes

<table>
<thead>
<tr>
<th>Different sets studied</th>
<th>Total cells counted</th>
<th>Abnormal cells belonging to group III</th>
<th>Mean ± S.D. value D.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>302</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1/20th LD₅₀ (single dose)</td>
<td>407</td>
<td>1</td>
<td>0.227 ±0.508</td>
</tr>
<tr>
<td>1/15th LD₅₀ (single dose)</td>
<td>301</td>
<td>2</td>
<td>0.666 ±0.912</td>
</tr>
<tr>
<td>1/10th LD₅₀ (single dose)</td>
<td>409</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### Table 12: Effect of different doses of HgCl₂ on group IV type of abnormality in mitotic chromosomes

<table>
<thead>
<tr>
<th>Different sets studied</th>
<th>Total cells counted</th>
<th>Abnormal cells of group IV</th>
<th>Mean ± S.D.</th>
<th>'P' value</th>
<th>D.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>302</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1/20th LD₅₀ (single dose)</td>
<td>407</td>
<td>16</td>
<td>4.038*</td>
<td>&lt;.05</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±3.770</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/15th LD₅₀ (single dose)</td>
<td>301</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1/10th LD₅₀ (single dose)</td>
<td>409</td>
<td>38</td>
<td>9.355****</td>
<td>&lt;.001</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±4.656</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 13: Effect of different doses of HgCl₂ on group V type of abnormality in mitotic chromosomes

<table>
<thead>
<tr>
<th>Different sets studied</th>
<th>Total cells studied</th>
<th>Abnormal cells of group V</th>
<th>Mean ± S.D.</th>
<th>'P' value</th>
<th>D.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>302</td>
<td>9</td>
<td>2.988</td>
<td>2.181</td>
<td></td>
</tr>
<tr>
<td>1/20th LD₅₀ (single dose)</td>
<td>407</td>
<td>18</td>
<td>4.382</td>
<td>2.025</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±2.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/15th LD₅₀ (single dose)</td>
<td>301</td>
<td>17</td>
<td>4.172</td>
<td>2.059</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±2.059</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/10th LD₅₀ (single dose)</td>
<td>409</td>
<td>32</td>
<td>10.638**</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>±5.492</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Inference

The action of mercuric chloride in different groups of abnormalities showed considerable variation.

The group I type of abnormalities, which included those abnormal cells where the action was directly on spindle only, showed a highly significant increase at 1/20th LD$_{50}$ and 1/15th LD$_{50}$ than that of the control set. However, at the highest dose (1/10th LD$_{50}$) the group I abnormality was much lower.

At 1/20th LD$_{50}$ the increase in group II type of abnormality was not significant. But the next dose (1/15th LD$_{50}$) showed significant increase in these cells. With the highest dose given (1/10th LD$_{50}$) the increase in group II type of abnormal cells was highly significant.

Group III type of abnormal cells included relatively mild effects on both chromosomes and spindle. This type though observed at 1/20th LD$_{50}$ and 1/15th LD$_{50}$ was not significant.

Group IV type of abnormalities including micronuclei and premature chromatin condensation, were observed both at lowest (1/20th LD$_{50}$) and highest doses (1/10th LD$_{50}$). At the lowest dose the increase was significantly higher than that of the control; while at the highest dose the increase was highly significant.

The lethal effects seen in group V type of abnormality showed a moderately significant increase at the highest (1/10th LD$_{50}$) dose.
The II, IV and V groups of abnormalities showed a dose dependence increase.

The group I showed a highly significant increase at the lowest and the next higher doses. However, at the highest dose it was not significant.

The group III did not show any significant increase.

Mercuric chloride apparently acted both at chromosome and spindle levels. It was highly cytotoxic and caused lethality.
**Fig. II** COMPARISON OF TOTAL ABNORMALITY PERCENTAGES BETWEEN CONTROL AND TREATED SETS

- Control
- 1/20th LD<sub>50</sub> (1.85 mg/kg b.w.)
- 1/15th LD<sub>50</sub> (2.47 mg/kg b.w.)
- 1/10th LD<sub>50</sub> (3.70 mg/kg b.w.)

*P* = <0.02, <0.01, <0.001
**Fig. III** COMPARISON OF GROUP I ABNORMALITIES BETWEEN THE CONTROL AND THE TREATED SETS

- **CONTROL**
- 1/20th LD$_{50}$ (1.85 mg/kg b.w.)
- 1/15th LD$_{50}$ (2.47 mg/kg b.w.)
- 1/10th LD$_{50}$ (3.70 mg/kg b.w.)

**PERCENTAGE OF CELLS**

- P = <.01
- P = <.01
Fig. IIiv  COMPARISON OF GROUP - II ABNORMALITIES BETWEEN THE CONTROL AND THE TREATED SETS

□ CONTROL

1/20th LD50 (1.85 mg/kg b.w.)

1/15th LD50 (2.47 mg/kg b.w.)

1/10th (3.70 mg/kg b.w.)

PERCENTAGE OF CELLS

P = < 0.05  P = < 0.01
Fig. IIx COMPARISON OF GROUP-III ABNORMALITIES BETWEEN THE CONTROL AND THE TREATED SETS

□ CONTROL

I 1/20th LD50 (1.85 mg/kg b.w.)

□ 1/15th LD50 (2.47 mg/kg b.w.)

□ 1/10th LD50 (3.70 mg/kg b.w.)
Fig. IIvi  COMPARISON OF GROUP - IV ABNORMALITIES BETWEEN THE CONTROL AND THE TREATED SETS.

<table>
<thead>
<tr>
<th>Percentage of Cells</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td></td>
</tr>
<tr>
<td>1/20 th LD50 (1.85 mg/kg b.w.)</td>
<td>*</td>
</tr>
<tr>
<td>1/15 th LD50 (2.47 mg/kg b.w.)</td>
<td>*</td>
</tr>
<tr>
<td>1/10 th LD50 (3.70 mg/kg b.w.)</td>
<td>*</td>
</tr>
</tbody>
</table>

P = < .05  P = < .001
Fig. II vii  COMPARISON OF GROUP-V ABNORMALITIES BETWEEN THE CONTROL AND THE TREATED SETS

□ CONTROL

■ 1/20th LD50 (1.85 mg/kg b.w.)

□ 1/15th LD50 (2.47 mg/kg b.w.)

■ 1/10th LD50 (3.70 mg/kg b.w.)
Fig. II viii COMPARISON OF DIFFERENT GROUPS OF
ABNORMALITIES BETWEEN THE CONTROL
AND THE TREATED SETS

CONTROL, \( -\frac{1}{20}\text{th} \text{LD}_{50}(1.85 \text{mg/kg b.w.}) \)

\( -\frac{1}{15}\text{th} \text{LD}_{50}(2.47 \text{mg/kg b.w.}) \)

\( -\frac{1}{10}\text{th} \text{LD}_{50}(3.70 \text{mg/kg b.w.}) \)

- GROUP I, \( \Delta \) GROUP II, \( \bullet \) GROUP III, \( \square \) GROUP IV,

- GROUP V.
Micronuclei were observed from foetal liver cells following different doses of HgCl₂ treatment to the mother rat.

Table 14: Micronuclei in foetal liver cells following the administration of 1.85 mg/kg b.w. of HgCl₂ (1/20th LD₅₀) to the mother rat

<table>
<thead>
<tr>
<th>Total cells counted</th>
<th>% of abnormal cells</th>
<th>Mean ± S.D.</th>
<th>Affected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>277</td>
<td>291</td>
<td>0.72</td>
<td>0.36 ± 0.26</td>
</tr>
<tr>
<td>276</td>
<td>294</td>
<td>1.80</td>
<td>0.68</td>
</tr>
<tr>
<td>271</td>
<td>294</td>
<td>-</td>
<td>0.34</td>
</tr>
<tr>
<td>277</td>
<td>310</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>254</td>
<td>349</td>
<td>-</td>
<td>0.28</td>
</tr>
</tbody>
</table>

df = 8

Table 15: Micronuclei in foetal liver cells following administration of 2.47 mg/kg b.w. of HgCl₂ (1/15th LD₅₀) to the mother rat

<table>
<thead>
<tr>
<th>Total cells counted</th>
<th>% of abnormal cells</th>
<th>Mean ± S.D.</th>
<th>Affected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>277</td>
<td>176</td>
<td>0.72</td>
<td>1.13 ± 0.51</td>
</tr>
<tr>
<td>276</td>
<td>179</td>
<td>1.08</td>
<td>2.23</td>
</tr>
<tr>
<td>271</td>
<td>174</td>
<td>-</td>
<td>0.57</td>
</tr>
<tr>
<td>277</td>
<td>173</td>
<td>-</td>
<td>1.12</td>
</tr>
<tr>
<td>254</td>
<td>166</td>
<td>-</td>
<td>0.60</td>
</tr>
</tbody>
</table>

df=8; *P < .05
Table 16: Micronuclei in foetal liver following administration of 3.70 mg/kg b.w. of HgCl₂ to the mother rat (1/10th LD₅₀)

<table>
<thead>
<tr>
<th>Total cells counted</th>
<th>% of abnormal cells</th>
<th>Mean ± S.D.</th>
<th>Affected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control treated</td>
<td>Con- Trea-</td>
<td>Mean ± S.D.</td>
<td>Large</td>
</tr>
<tr>
<td>277</td>
<td>237</td>
<td>0.72 ± 0.84</td>
<td>0.36 ± 0.14</td>
</tr>
<tr>
<td>276</td>
<td>259</td>
<td>1.08 ± 0.77</td>
<td>0.65 ± 0.21</td>
</tr>
<tr>
<td>271</td>
<td>242</td>
<td>2.06</td>
<td>0.32 ± 0.23</td>
</tr>
<tr>
<td>277</td>
<td>261</td>
<td>0.76</td>
<td>0.19 ± 0.62</td>
</tr>
<tr>
<td>254</td>
<td>233</td>
<td>1.28</td>
<td>0.62</td>
</tr>
</tbody>
</table>

**Inference**

Different doses of mercuric chloride administered to pregnant rats induced micronuclei formation in foetal liver cells. At the lowest dose of 1/20th LD₅₀ (1.85 mg/kg b.w.) the percentage of micronuclei though higher than the control was not statistically significant.

With the next higher dose 1/15th LD₅₀ (2.47 mg/kg b.w.) the total percentage of micronuclei was not significantly higher than control but the large cells affected were higher.

With the highest dose 1/10th the LD₅₀ (3.70 mg/kg b.w.) both the total percentage of affected cells and that of large cells affected were significantly higher.

Mercuric chloride when administered at different doses to pregnant rats induced different degrees of micronuclei formation in the foetal liver cells. The large cells were more affected. Micronuclei formation showed a direct dose dependence.
Fig IIix  MICRONUCLEI INDUCED IN FOETAL LIVER CELLS FOLLOWING ADMINISTRATION OF HgCl₂ TO PREGNANT RATS

□ CONTROL
■ 1/20th LD₅₀ (1.85 mg/kg b.w.)
□ 1/15th LD₅₀ (2.47 mg/kg b.w.)
■ 1/10th LD₅₀ (3.70 mg/kg b.w.)

PERCENTAGE OF CELLS WITH MICRONUCLEI

P = <0.05
Fig. IIx MICRONUCLEI INDUCED IN LARGE AND SMALL FOETAL LIVER CELLS FOLLOWING ADMINISTRATION OF Hg Cl₂ TO PREGNANT RATS

- CONTROL LARGE CELL
- CONTROL SMALL CELL
- TREATED LARGE CELL
- TREATED SMALL CELL

PERCENTAGE OF CELLS WITH MICRONUCLEI

1/20th LD₅₀ (1.85 mg/kg b.w.)
1/15th LD₅₀ (2.47 mg/kg b.w.)
1/10th LD₅₀ (3.70 mg/kg b.w.)

P = <0.05
Explanation of microphotographs

Plate No. 38

1. Metaphase plate showing a dicentric chromosome (Ca X 7130)

2. Metaphase plate showing diplochromatid condition (Ca X 7130)

3. Metaphase plate showing polyploidy (Ca X 6220)

4. Metaphase plate showing erosion (Ca X 7130)

5. Tc-Lal liver cells showing a micronucleus and nuclear fusion with dumb-bell shaped appearance (Ca X 4670)
The teratogenic and embryotoxic effects of mercuric chloride on pregnant rats were studied and tabulated as follows:

Table 17: Teratogenic and embryotoxic effects following administrations of HgCl₂ to pregnant rats

<table>
<thead>
<tr>
<th>Sets studied</th>
<th>Doses given</th>
<th>Day of treatment</th>
<th>Day of sacrifice</th>
<th>No. of Corpora lutea</th>
<th>No. of foetuses on each side</th>
<th>No. of resorbed foetuses</th>
<th>Percent of foetal loss</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>7</td>
<td>18</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>All fully formed foetuses were normal</td>
</tr>
<tr>
<td>2</td>
<td>1/20th LD₅₀</td>
<td>7</td>
<td>18</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>All fully formed foetuses were normal</td>
</tr>
<tr>
<td></td>
<td>(1.85 mg/kg b.w.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 foetal losses. 3 resorption sites were located showing small, dark structures. Rest of the fully formed foetuses were normal.</td>
</tr>
<tr>
<td>3</td>
<td>1/15th LD₅₀</td>
<td>7</td>
<td>18</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>43.33</td>
</tr>
<tr>
<td></td>
<td>(2.47 mg/kg b.w.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 foetal losses. No resorption sites were located. Rest of the fully formed foetuses were normal.</td>
</tr>
<tr>
<td>4</td>
<td>1/10th LD₅₀</td>
<td>7</td>
<td>18</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>33.33</td>
</tr>
<tr>
<td></td>
<td>(3.70 mg/kg b.w.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Inference

Different doses of mercuric chloride administered orally to pregnant rats namely, 1/20th LD50 (1.85 mg/kg. b.w.), 1/15th LD50 (2.47 mg/kg. b.w.) and 1/10th LD50 (3.70 mg/kg. b.w.) gave different degrees of teratogenic and embryotoxic effects. The lowest dose applied was not sufficiently high to cause foetal loss or defect.

However, in the next higher dose (1/15th LD50) foetal losses had occurred. Resorption sites could be located, characterised by the presence of small dark coloured, placenta with multicellular structures. None of the fully formed foetuses had any congenital defects.

Foetal losses also occurred in the highest dose applied without discrete resorption site. Here also none of the fully formed foetuses had any congenital defects.

However, the percentage of foetal loss is same in both cases, and the losses have occurred in the post-implantation stage.
TERATOGENIC AND EMBRYOTOXIC EFFECTS OF DIFFERENT DOSES OF MERCURIC CHLORIDE GIVEN TO PREGNANT RATS

- CONTROL
- 1/20th LD50 (1.85 mg/kg b.w.)
- 1/15th LD50 (2.47 mg/kg b.w.)
- 1/10th LD50 (3.70 mg/kg b.w.)

PERCENTAGE OF FOETAL LOSS
Histological observations on foetus from the control and treated pregnant rats

Control: No defect of the foetus has been observed. All the cells of different organs were intact and even. No damage of brain cells was observed. Placenta was prominent with regular sized nuclei.

Following 1/20th LD50 (1.85 mg/kg b.w.): Foetus: No defect of the foetus was observed.

The cortical cells of the brain showed disruption of normal structure. The neuroglial cells of the entire cerebral cortex, cerebellum and spinal cord were markedly disrupted with degeneration at places.

The liver also showed marked disruption of the tissue. There appeared to be haemorrhage with engorgement of sinusoidal spaces which were filled with blood.

The kidney showed a few RBC and subnuclear vacuolation of the tubular cells.

The adrenal gland was normal.

The placenta was large, multicellular with prominent blood vascular spaces. The syncytial cells showed aneuploidy; there were some very large cells with large, hypochromatic nuclei and prominent nucleoli in the chorionic tissue next to the uterine wall. In this area multiple mitosis and binucleate cells were also observed.
With 1/15th LD<sub>50</sub> (2.47 mg/kg, b.w.): Resorped site - This zone showed complete resorption of foetal tissue in three areas. A few groups of syncytial cells remained with areas of necrosis and haemorrhage. In some areas the large nucleated cells were seen in the uterine chorial spaces. In other areas there was complete degeneration and vacuolation of cells with small pyknotic nuclei.

**Foetus:** - The brain area was increased in the number of cortical cells with disrupted arrangement. In the cerebellum the nuclei were irregularly arranged. The neuroglial cells of the cerebral cortex were markedly disrupted.

The liver showed degeneration with haemorrhage.

The kidney showed marked swelling of tubular cells with vacuolation and haemorrhage.

The placenta showed cellularity with attempts of villus formation. On the maternal aspect, there was proliferation of cells and multinucleate giant cells were observed. There were also areas of foamy cells (?) pigment filled macrophages.

Following 1/10th LD<sub>50</sub> (3.70 mg/kg, b.w.):

**Foetus:** - The brain showed decrease in the number of cortical cells and cerebellar cells. The neuroglia were markedly disrupted. The neuroglial cells were degenerated both in cortex and cerebellum and in some areas there was degeneration to the point of cyst formation.
The liver showed large areas of congestion with spaces filled with blood cells.

The kidney showed some tubular swelling and some degeneration.

The placenta showed marked increase in the cells in between the placenta and the maternal tissue. The large nucleated cells were very markedly increased as also the degenerated foamy cells in between. There appears to be intense tissue reaction in this area and some of the nuclei were extremely large with prominent nucleoli. Marked increase in aneuploidy was also observed in this area.
None of the foetuses from all the pregnant rats subjected to the three treatments showed any congenital defect. But at cellular level varying effects were observed in different organs.

The brain cells in all cases showed marked effects, which was directly dose dependent showing maximum damage at the highest dose (1/10th LD$_{50}$).

With all three treatments the liver showed degeneration and areas of haemorrhage.

The kidney showed tubular swelling and marked vacuolation and haemorrhage in all cases.

The placenta, like the brains, showed a dose dependent effect. At the lowest dose the effect on the placenta was mainly observed on the maternal surface of the chorion. At the next higher dose (1/15th LD$_{50}$) more marked reaction was observed in the placenta specially in areas close to the maternal interphase. The resorbed sites show mainly placental elements indicating loss at the post-implantation stage. The placenta was most damaged with the highest dose (1/10th LD$_{50}$) showing reactive and degenerated cells extending through a large part.

Apart from causing foetal loss mercuric chloride also showed transplacental permeability causing damage to the organs of fully formed foetuses.
Explanation of Microphotographs

Plate 40

1. Section through the uteroplacental junction of rat foetus showing degeneration of cytotrophoblast cells to form small pyknotic nuclei specially in the region next to the uterine wall. There are also some cells with very large nuclei which in this area could either be of uterine (maternal) or syncytial (foetal) origin (ca X80).

2. Section under higher magnification showing the degenerated cells below and large nucleated cells in the upper part. (ca X120)

3. Section showing large phagocytic cells and fibrin formation with a single villus structure. (ca X80)

4. Section of the same under higher magnification showing nucleated cells and cells with prominent nucleoli in the cytotrophoblast. (ca X120)
1. Section through chorionic plate showing degeneration of the placenta. (ca X80)

2. Section through chorionic plate under higher magnification showing the pycnotic nuclei and pale cytoplasm of the degenerated cells. The remaining cytotrophoblast cells show dark nuclei some of which are kidney shaped like Hofbauer cells. (ca X120)

3. Section showing areas of degenerated cells at the junction of the uterus and placenta. The large nucleus on the lower left hand corner appears to be of foetal origin. (ca X120)

4. Section showing sheets of dark staining syncytial cells with patchy fibrinoid degeneration at places. (ca X80)
Explanation of Microphotographs

Plate 42

1. Section through brain tissue of foetal rat. (ca X60)

2. Section through brain tissue of foetal rat under higher magnification showing neurohal cells and nerve fibres. (ca X80)

3. Section through brain tissue of treated foetal rat showing degeneration of neural tissue. (ca X60)

4. Section through brain tissue of treated foetal rat showing extensive degeneration. (ca X80)
Explanation of Microphotographs

Plate 43

1. Section through brain tissue of treated foetal rat showing degeneration of neural cells. (ca X80)

2. Section under higher magnification showing degeneration of neural cells. The nuclei are very few and fibres have lost their normal structure. (ca X120)

3. Section of the same through another region showing nuclei of various shapes and disruption of the fibres. (ca X120)

4. Section through brain tissue of treated foetal rat showing degeneration of nerve fibres. (ca X80)
Explanation of microphotographs

Plate W

1. Section through brain tissue of foetal rat under higher magnification showing degeneration with cyst-like formation. (ca X120)

2. Section through area around the eye-ball of the treated foetus showing disruption of the cellular tissue with cyst formation. (ca X80)

3. Section through a portion of area of the uterus where foetal death has occurred. In the lower part can be seen the uterine cells while the degenerated mass above is of foetal origin showing a few ghost villi. (ca X60)

4. Section under higher magnification of the foetal tissue showing degenerated vacuolated cells. (ca X80).
Table 18: **In situ** nuclear DNA contents (mean ± S.D.) of different organs of the foetus following treatment of pregnant rats with HgCl₂.

(Values are given in absorbance X10² in arbitrary units)

<table>
<thead>
<tr>
<th>Experimental sets used</th>
<th>Brain</th>
<th>Liver</th>
<th>Kidney</th>
<th>Placenta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large cell</td>
<td>Small cell</td>
<td>Large cell</td>
<td>Small cell</td>
</tr>
<tr>
<td>Control</td>
<td>17.50 ± 1.65</td>
<td>17.50 ± 1.64</td>
<td>12.00 ± 0.89</td>
<td>17.16 ± 1.94</td>
</tr>
<tr>
<td>1/20th LD50 (1.85 mg/kg b.w.)</td>
<td>16.83 ± 0.98</td>
<td>15.00 ± 1.75</td>
<td>11.66 ± 1.03</td>
<td>15.66 ± 1.04</td>
</tr>
<tr>
<td>1/15th LD50 (2.47 mg/kg b.w.)</td>
<td>15.33 ± 0.82</td>
<td>18.16 ± 2.14</td>
<td>11.83 ± 1.94</td>
<td>18.83 ± 0.98</td>
</tr>
<tr>
<td>1/10th LD50 (3.70 mg/kg b.w.)</td>
<td>16.66 ± 0.98</td>
<td>18.00 ± 1.55</td>
<td>11.66 ± 1.86</td>
<td>16.50 ± 1.22</td>
</tr>
</tbody>
</table>

**Inference**

**In situ** nuclear DNA estimation of different organs of the foetus following treatment of pregnant rats with different doses of HgCl₂ showed considerable variations.

In the brain variation in DNA content was lower in different experimental sets.

In liver the DNA content of small cells (taken as 2n) differed widely from that of the larger ones.

Kidney showed variation from 2n to just below 3n.
In placenta the variation was from 2n to just below 3n with considerable aneuploidy.

The large cells of the liver had a lower DNA content than the control at the lowest dose but the next two higher doses led to increased DNA content. But the small cells showed higher DNA content in control than the sets given the minimum and maximum doses.

The intermediate dose led to an increased DNA content, higher than that of the control. The small cells showed more or less same values in both control and treated set.

In placenta minimum DNA values were in control as compared to the treated set. The small cells however showed variations where minimum DNA content was observed with 1/15th LD50 and maximum with 1/10th LD50.

In all cases including control, there was considerable variation in DNA content from organ to organ.
Fig. IIxii VARIATIONS IN FOETAL NUCLEAR DNA CONTENT INDUCED BY ADMINISTRATION OF HgCl₂ (1.85mg/kg b.w.) TO PREGNANT RATS
Fig. II.xiii VARIATIONS IN FOETAL NUCLEAR DNA CONTENT INDUCED BY ADMINISTRATION OF HgCl₂ (2.47 mg/kg.b.w.) TO PREGNANT RATS

- BRAIN
- LIVER (Large cell)
- LIVER (Small cell)
- KIDNEY (Large cell)
- KIDNEY (Small cell)
- PLACENTA (Large cell)
- PLACENTA (Small cell)
- CONTROL
VARIATIONS IN FOETAL NUCLEAR DNA CONTENT INDUCED BY ADMINISTRATION OF Hg Cl₂ (3.70mg/kg b.w.) TO PREGNANT RATS

- BRAIN
- LIVER (Large cell)
- LIVER (Small cell)
- KIDNEY (Large cell)
- KIDNEY (Small cell)
- PLACENTA (Large cell)
- PLACENTA (Small cell)
- CONTROL

DNA CONTENT IN ARBITRARY UNITS

BRAIN  LIVER  KIDNEY  PLACENTA
DISCUSSION
Discussion:

1. Effects of HgCl₂ on bone marrow cells of pregnant female rats:

In this part of the work the effects of different doses of inorganic mercury were observed, following its administration orally to pregnant female rats. The criteria recorded included foetotoxicity and embryotoxicity as well as clastogenic effect.

Mercury is a protoplasmic poison which has been shown to be lethal to all living species in higher concentrations. It has been recorded to induce chromosomal alterations and to cause abnormal cell division (Das et al. 1982, 1985). Both organic and inorganic mercury derivatives are known to affect the spindle. Teratogenic effects had been reported earlier (Harada 1968, Khera 1973).

Chronic oral administration of HgCl₂ in laboratory animals inhibited cell division in bone marrow. This effect could be avoided if the animals were allowed to recover following short periods of treatment. Higher doses were, however, both mitostatic and strongly clastogenic. A single acute dose was more drastic than chronic exposure to the same amount (Das et al. 1982).

During the course of the present investigation three doses were administered orally to pregnant female rats. The bone
marrow cells showed that the mitotic index decreased proportionately with increase in the concentration of the chemical used (Tables 1 to 4; pages 237 to 238 from observation). The abnormalities included both effects on chromosome as well as spindle disturbances. A comparison of three doses indicated that the abnormalities involving the chromosomes increased significantly with increased concentrations (Tables 5 to 13; pages 249 to 245 from observation of this thesis). On the other hand the spindle disturbances were significantly high with the two lower doses but fell abruptly with the highest one given. The group III abnormalities, which involved more than one chromosome in a single plate did not alter significantly with enhanced concentrations of the chemical. On the other hand the group IV abnormalities, which included micronuclei, were dose dependent. This effect is expected since the formation of micronucleus indicates chromosome breaks which increased with enhancement in the dosage of the chemical administered. In view of the mitostatic action of HgCl₂ the decrease in number of spindle disturbances with higher dosages can be attributed to the drastic reduction in the divisional cells which would show such abnormality. The common spindle disturbances like diplochromatid formation and polyploidy could not be assessed in the absence of sufficient number of divisional plates. On the other hand the increase in micronuclei indicates continuing chromosome breaks with higher dosages even after 11 days of recovery.
2. Effects of HgCl₂ on foetus following maternal administration during pregnancy:

HgCl₂ has been found to cross the placental barrier and to affect the foetus during gestation. Following the oral administration of this chemical in subtoxic doses to the pregnant females after 11 days effects were observed on different foetal tissues.

a) Formation of micronuclei in foetal liver: Micronuclei were recorded in the liver cells of the foetus recovered after 11 days of administration of HgCl₂ to the pregnant mothers. The percentage of micronuclei ranged from 0.21 in control to 1.14 in the foetus recovered from the mother subjected to the maximum dose. The difference is statistically significant, indicating that HgCl₂ induces chromosomal breaks resulting in micronuclei with higher doses in the foetus. Of the two types of liver cells the number of micronuclei was significantly higher in the larger ones.
Teratological studies:

The effect of mercury on the developing foetus had been highlighted after the studies on Minamata disease (Harada 1968). Organic mercurials and methyl mercury had been found to be the most toxic forms and it has also been shown that organic mercurials reach higher levels in foetal tissues. Comparatively, inorganic mercury is observed in the foetus in much lower doses after being given to the mother (Ullberg et al. 1982). It had also been stated that placenta was more discriminating in early and late gestations, at least in rodents (Dencker 1979). In late pregnancy mercury was found mainly in the placenta and the foetal concentration was demonstrated to be much lower than the maternal one (Dencker 1982, Ullberg et al. 1982). Nevertheless the administration of inorganic mercury to the mother was found to be transmitted through the placenta to reach the foetal brain, liver and other tissues. There was also demonstrably higher accumulation of mercury in the visceral yolk sac and placenta (Ullberg et al. 1982). They had also demonstrated that the visceral yolk sac epithelium, which later developed into the placenta as well as the foetal gastrointestinal tract, would be responsible for concentration of the metal which would produce teratogenic effects. It has been suggested that this accumulation in selective tissues and its consequent effect varies depending upon the presence of other nutrients, metals and also the time of the closure
of the vitelline duct (Beck et al., 1967). The presence of congenital defects like cleft palate used in studying teratogenic mechanism has been attributed to multifactorial causes. Therefore, in animal experiments a single study involving the presence or absence of congenital defects of the palate, foot, tail or gastrointestinal tract cannot be taken to be conclusive.

In the present study the effect of mercuric chloride in causing teratogenicity of the rat foetus has shown a dose-related response. There was no effect observed after a single dose of 1/20th LD50 (1.85 mg/kg, b.w.) given on the 7th day of gestation. The litters obtained on the 18th day were morphologically and histologically normal. 1/15th LD50 (2.47 mg/kg, b.w.) given as a single dose, on the other hand, showed loss of 4 foetuses out of 10 (33.33%). 1/10th LD50 (3.70 mg/kg, b.w.) also showed the same degree of resorption.

The remaining fully formed foetuses of both the sets, when examined histologically, showed disruptive influence, specially on brain and in the liver. The degree of disruption of neuroglial cells, as observed in the cerebral cortex, cerebellum and spinal cord, was dose related. 1/10th LD50, being the highest dose used, induced the most marked disruption of neuroglial cells through cyst formation. The liver also showed areas of congestion and degeneration. The effect on kidney was most prominent, specially on kidney tubules which showed
vacuolation and haemorrhage.

Inorganic mercurials produce a neuropathic effect by a direct action on the neuroglial cells. Similarly after absorption the concentration of these highly soluble salts in the liver and its excretion by the kidneys cause direct degenerating effects (Chang 1979).

The effect on the placenta shows most marked activity in the areas close to the maternal interface. The placenta acts as a concentrating site and specially in rodents does not allow the substance to pass through. Here the effect observed was dose-dependent and involved increased cellularity with the presence of foamy cells and also of large nucleated cells in the decidua.

Mercury, specially methyl mercury, has been demonstrated to induce minimal visible alterations in the nervous system producing defects in locomotion and sensory functions characteristic of Minamata disease. In the human beings also, it is expected that this defect in the tissue, affecting adversely the behaviour may be caused (Vorhees and Butcher 1982). It has been suggested that teratogenic responses may be studied as producing three primary manifestations, namely, embryo lethality, malformation and functional teratogenesis.
e) **DNA content**: DNA contents of individual cells, estimated following *in situ* cytophotometry, indicate that effects of HgCl$_2$ in the foetal tissue differ in different organs.

In the brain, for example, all the nuclei were of equal size and the DNA contents of the control did not differ significantly from those treated with different concentrations of HgCl$_2$ although extensive neuronal dysrupts was observed.

In the other three tissues studied, two types of cells, large and small, were observed. The DNA contents however showed considerable variation which could not always be related to the amount of HgCl$_2$ administered to the mother. In the liver and kidney, the amount of DNA in the large cells was always significantly higher than in the small ones, presumably due to the fact that the two were in two different phases of replication cycle, G$_2$ and G$_1$ respectively. However the administration of mercury did not affect the DNA contents of the individual cells or the ratio between the large and small cells.

In the placenta, on the other hand, the observations indicated (Table 18; pages 254 to 255) a slight influence of HgCl$_2$ on DNA content. It showed a slight decrease or increase from the control in the small cells, which could not be related to the dose of HgCl$_2$ administered and was not
significant. The DNA contents of the large cells, as in the case of liver and kidney, were appreciably higher than those of the corresponding small cells showing that they were in different phases of DNA replication. The DNA contents of these large cells, however, showed a slight but steady increase with increase in dosage of HgCl₂ from the control. This increase is appreciable though not statistically significant. This relationship of DNA content of large placental cells with the dose of HgCl₂ administered to the pregnant females thus indicates that the placenta is affected by any external agent given to the female and this effect is maintained for a relatively long period, that is, even 11 days after administration. Since the placenta is able to influence the well-being of the foetus, the effects of any external agent on the placenta is liable to affect the foetus, as shown by the observation of micronuclei and histological changes in the foetal tissues discussed earlier.

Thus the investigation shows that the effect of a chemical agent on the pregnant female may be transmitted through the placenta to the foetus leading to harmful results.
SUMMARY
Summary:

In order to study the role of external agents in the induction of abnormal conceptuses, mercuric chloride was used as a test chemical since mercury is a known poison with teratogenic and cytotoxic effects.

The effect of different doses of mercuric chloride was tested on pregnant female laboratory bred rats (*Rattus norvegicus* L.), maintained on balanced diet.

Three subtoxic doses were used equivalent to $\frac{1}{20}$, $\frac{1}{15}$ and $\frac{1}{10}$ of the lethal dose $L_D^{50}$. Pregnant female rats on the seventh day of gestation were administered orally a single oral dose and then maintained on normal standard diet without milk and additional vitamins. They were sacrificed on the 18th day of gestation. The following parameters were studied:

a) **Effect on bone marrow chromosomes of the mother rat**:

The slides were prepared according to colchicine-hypotonic-fixative flame dry schedule and were observed for

1) **Mitotic index**: The bone marrow cells showed that the mitotic index decreased proportionately with increase in the concentration of the chemical used. At $\frac{1}{15}$ and $\frac{1}{10}$th $L_D^{50}$ this decrease was statistically significant as compared to the control.
ii) The abnormalities observed included both effects on chromosome as well as spindle disturbances. A comparison of three doses showed the abnormalities involving the chromosomes increased significantly with increased concentrations. The spindle disturbances were significantly high for the two lower doses but fell abruptly with the highest one. The abnormalities involving more than one chromosome in a single plate did not alter significantly with the enhanced concentrations. On the other hand the cells with micronuclei showed a dose dependent increase. The effect was in accordance with the highly significant chromosomal aberrations observed. On the other hand the increase in micronuclei was however correlated with highly significant chromosomal aberrations.

b) Effects of HgCl₂ on foetus:

i) Percentage of foetal loss: After sacrificing the female rat, the total number of corpora lutea on each side and the corresponding number of foetuses were counted.

No foetal loss was observed with the lowest dose given. With the two higher doses, the percentages of foetal loss were similar, though at \( \frac{1}{15} \)th \( LD_{50} \) a resorption site was located. The fully formed foetuses in all cases did not, however, bear any congenital malformations.

ii) Formation of micronuclei in foetal liver: After sacrificing the mother rat the foetuses were dissected out and
from foetal liver slides were prepared following the acetic-ethanol fraction flame dry technique. They were stained in Giemsa and observed.

The percentage of micronuclei recorded in the liver cells of the foetus recovered after 11 days of administration of HgCl$_2$ to the pregnant mothers, ranged from 0.21 in control to 1.14 in the foetus from the treated mothers subjected to the maximum dose. The difference was statistically significant indicating that HgCl$_2$ induces chromosomal breaks resulting in micronuclei with higher doses in the foetus. Of the two types of liver cells the number of micronuclei was significantly higher in the larger ones.

iii) Histological studies from the different organs of the foetus: The foetuses after dissecting out were fixed, embedded in paraffin blocks and 5 μ thick sections were prepared. The sections were suitably processed and stained in eosin-haematoxylin.

Administration of different doses of HgCl$_2$ to the mother rat was seen to affect the foetus to a considerable extent even after 11 days of recovery. The effect observed was dose dependent. The minimal dose was non-toxic, the foetuses being morphologically and histologically normal. In the next higher doses, though the fully formed foetuses were morphologically normal, they showed considerable disruptive influence histologically, specially on the brain and liver. The degree of disruption of neuroglial cells in different regions of the brain was dose related. It was
highest at $\frac{1}{10}$th LD$_{50}$ showing disruption of neuroglial cells through cyst formation. The liver was congested and degenerate. Regions of the kidney showed vacuolation and haemorrhage.

The effect on placenta was the most marked in areas close to maternal interface. Here also the effect was dose dependent and involved increased cellularity with the presence of foamy cells and large nucleated cells in the decidua.

iv) **In situ DNA estimation from different foetal organs**: Histological sections were suitably processed and stained in Feulgen. **In situ** DNA values from individual cells were estimated cytophotometrically. The results were expressed in arbitrary units.

In brain all the nuclei were of one size. The DNA content of the treated cells did not differ significantly from that of the control. In other three tissues namely liver, kidney and placenta considerable variation occurred which could not always be related to the amount of HgCl$_2$ administered to the mother. In liver and kidney the amount of DNA in the large cells was always significantly higher than the small ones, probably due to the fact that the two were in different phases of replication cycle.

The placenta showed a slight influence of HgCl$_2$ as indicated by the slight but steady increase in DNA contents of the large cells with the increasing dosage. This increase was
appreciable though not statistically significant.

Thus the placenta appears to be affected by external agents to which the mother is exposed. This effect is maintained for a relatively long period and therefore may influence the ultimate outcome of pregnancy.
EFFECTS OF CERTAIN TRANQUILLIZERS ON SOMATIC CHROMOSOMES OF HIGHER LIVING ORGANISMS

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ABSTRACT

Four common tranquillizers containing diazepam or nitrazepam as active ingredients induced chromosomal abnormalities in dividing cells and alterations in mitotic index, when applied in high concentration, to plant and animal systems in vivo.

INTRODUCTION

TRANQUILLIZERS are used very commonly in combating human ailments, being available in a variety of commercial forms, either singly or in combination with other compounds. Lately, interest in the action of drugs on genetic systems has been increasing partly due to the fact that certain hallucinogenic compounds have been shown to exert harmful effects at the levels of cell and chromosome division e.g. mescaline and LSD in mammalian test systems leading to chromosomal alterations and changes in the mitotic frequency1-2. The effects of tranquillizers on metabolic pathways have been investigated by several workers, the test systems principally being mice and rats3-5. The in vivo bone marrow chromosomal study has been recommended as an ideal protocol among the cytogenetic assays to detect potential mutagenic effects of environmental agents6-7.

Benzodiazepines are among the most commonly prescribed drugs8. More than 8000 tons of benzodiazepines were prescribed in 1977 in the United States9. Reports have also indicated a significant association between maternal intake of benzodiazepines during the first trimester of pregnancy and oral clefts10. Chronic dietary administration of benzodiazepine tranquillizers to breeding pairs of Swiss Webster mice resulted in alterations in the normal patterns of reproductive behaviour and foetal growth, which significantly depressed body weights at birth11. The irritant effects of dilute solutions of benzodiazepines on the eye and tongue of rabbit, guinea pig and man has also been established12. Chloridiazepoxide was reported to induce cytogenetic effects and dominant lethality in mice13,14 and gave a significantly high incidence of micronuclei in the bone marrow cells of mice15. The toxic effects of diazepam, chloridiazepoxide and nitrazepam on the spermatozoa of mice were studied with a daily oral dose of 0.5 mg16.

The present investigation was undertaken to assess the effects of certain commercial tranquillizers on cell and chromosome division in animal and plant test systems.

The drugs used contained either of the two compounds diazepam and nitrazepam in combination with the usual pharmaceutical ingredients in different doses.

MATERIALS AND METHOD

The actual commercial brands used were:—
(a) Nitravet (with 5 mg of nitrazepam/tablet) Mfg. by The Anglo-French Drug Co., Bangalore.
(b) Hypnotex-10 (with 10 mg of nitrazepam/capsule) Mfg. by The Pharmaceutical and Chemical Industries, Gujrat.
(c) Calmpose (with 5 mg of diazepam/tablet) Mfg. by Ranbaxy Laboratories Ltd., New Delhi and
(d) Valium-2 (with 2 mg of diazepam/tablet) Mfg. by Roche Products Ltd., Bombay.

All these chemicals were subjected to Allium test18.
Dosages applied:
(1) Dosage applied to a human adult dissolved in 100 ml distilled water as stock solution.
(2) Half dilution of the stock solution.

For plant systems, Allium cepa bulbs were placed at the mouths of tubes containing these doses for 120 hr (figure 1). Root tips were removed after the complete treatment and squashed following acetic orcein schedule.

In the animal experiments, four different doses i.e. half, normal, double and four times the normal human dose of Calmose and Nitrazepam were force fed to four sets of mice (Mus musculus) daily for one month per kg. (figure 2) of body weight, taking 50 kg as the standard human weight. A control set was fed distilled water in the same proportion. The animals were sacrificed after one month; bone marrow was aspirated and chromosomes were observed following colchicine hypotonic flame drying giemsa schedule.

Five thousand cells were scanned for each treatment for mitotic indices and 300 dividing plates were observed for percentages of abnormal cells. Statistical analysis was carried out to test for the significance of the data. Three replicates were made of each experiment and the consolidated data presented.

RESULTS

On an average both the animal and plant systems indicate that, when applied at sufficiently high concentrations these commercial products containing nitrazepam and diazepam are all capable of altering the mitotic index and increasing the number of chromosomal abnormalities in the dividing cells.

In animal experiments the mitotic indices for the four different doses of diazepam and nitrazepam were compared separately with a control. The treated series showed a suppression of mitotic index and a significant rise in total abnormalities in the chromosomes as compared to the control.

The results were slightly different with Allium cepa. The increase in abnormalities and decrease in mitotic index was dose dependent, as expected. However, nitrazepam gave an increase, at a moderate significant level in A. cepa when applied at the normal human doses.

The abnormalities observed were divided mainly into three different groups—

Group I — Abnormalities mainly due to spindle disturbances e.g. diplochromatin, C-mitosis, sticky bridge, lagging, polyploidy, multipolarity, etc.

Group II — Abnormalities due to direct action on chromosomes e.g. breaks, gaps, centric fusion, centric fission, telomeric fusion, lesions, etc.

Group III — Gross chromosomal abnormalities e.g. erosion, pulverisation, clumping, pycnosis, etc.

Abnormalities of all the three groups taken together gave the percentage of total chromosomal disorder. The histogram represents a comparison of abnormalities caused by each dose of diazepam and nitrazepam separately with the control.

It is suggested that when using tranquillizers care
should be taken not to exceed the prescribed dose and to avoid chronic treatments in order to prevent cumulative effects.

2 June 1983; Revised 26 March 1984

1. Long, S. Y., Teratology, 1972, 6, 75.