Some of the results of this chapter have already been published and some more have been communicated for publication.
INSECTICIDES

Organophosphate Compounds

1. MONOCROTOPHOS (Nuvacron®)

Monocrotophos is a commonly used organophosphate pesticide having both insecticidal and acaricidal properties. Its trade name is Nuvacron®. It has both systemic and residual contact properties. Chemically it is known as O,O-diethyl-O-(2-methyl-carbamoyl-1-methyl-vinyl)-phosphate and the structure is as follows.

\[
\text{CH}_3\text{O} \quad \text{P} \quad \text{O} \quad \text{CH} \quad \text{CO} \quad \text{NH} \quad \text{CH}_3
\]

(Structure of monocrotophos)

It is recommended for use in various crops like cotton, sugar cane, potatoes and peanuts for controlling bullworms, weevils, loopers, thrips, mites and aphids. Monocrotophos has been reported to be genotoxic in prokaryotes, eukaryote animal cells both in vivo and in vitro (Waters et al., 1982; Garrett et al., 1986; Tripathy and Patnaik, 1992) and in plants (Jagannmohan et al., 1985) but negative in the Drosophila sex-linked recessive lethal test (Sandhu et al., 1985). There is no report in the literature on the mutagenicity of monocrotophos in an avian test system. It was thought worthwhile to study the genotoxic effect of monocrotophos in the chick cytotogenic test system using two bioassays namely the chromosome aberration (CA) and micronucleus test (MNT).

MATERIALS AND METHODS

Test chemical: Technical grade monocrotophos (96% pure) (Nuvacron®) was donated by Bharat Pulverising Mills Ltd. Bombay (India). Glass double distilled (g.d.d.) water served as vehicle.
Dose: Three different doses (1.25, 2.5 and 5 mg/kg) were selected by trial since LD\textsubscript{50} values of monocrotophos for chicks were not available.

Experimental protocol: The details of the experimental design of BMCA, MNT and statistical analyses have been presented in the "General materials and methods".

RESULTS

General toxicity: The general toxicity symptoms included loss of appetite, ataxia and the inactivation of the entire body with the highest dose for the first 4-6 h after the administration of the chemical. The symptoms were milder with the lower doses and using the p.o. route. During chronic treatment these symptoms were not present in comparison to the acute treatment. However, in 30 days chronic treatment group, after sacrifice, a tumour like growth was noted in one animal and white patches in the liver in another animal were observed.

Genotoxicity:

Chromosome aberration: (Figs. 21a-h, 22 a-b, 23 and 27; Table-8)

Qualitative: Monocrotophos induced chromosomal aberrations including chromatid and isochromatid gaps and breaks, ring chromosomes, deletion etc. The chemical also induced varieties of chromosomal anomalies such as differential digestion of the chromatid arms, uneven accumulation of chromatin material, woolly appearance and centromeric separations. With the highest dose the microchromosomes were not distinct, with a tendency for individual chromosomes to clump together.

Quantitative: The highest dose induced the highest percentage of aberration (5.00 %) after 24 h of exposure. Regarding the route sensitivity, the chemical was found to be most effective when injected
i.p.. The fractionated dose produced a lesser effect (2.00%) than the equivalent acute dose (5.00%). In the case of dose-response studies, all the doses induced higher frequencies of aberrations than the control, but only the highest dose induced a statistically significant effect ($t = 5.89; \text{df} = 6; \ p < 0.01$). Significant variations were obtained (ANOVA) among time-response analyses ($F = 3.61; \text{df} = 3,12; \ p < 0.05$). Only the results at 24 h differed significantly from control, but not at the other time points (6, 48 and 120 h). However, no significant variation was observed (ANOVA) in the dose-response analyses ($F = 1.69; \text{df} = 2,9; \ p > 0.05$). The effect produced by the p.o. route was lower than with the i.p. route. However, the result did not differ significantly ($t = 1.41; \text{df} = 6; \ p > 0.05$) from the control. The result in the 30 days (chronic) treatment series with the fractionated dose regimens was more (8.00%) than the equivalent acute dose treated once and differed significantly ($t = 5.42; \text{df} = 6; \ p < 0.01$) over the respective control value. Monocrotophos induced a dose-response linearity in chromosome aberration test.

**Micronucleus:** (Figs. 24 a-n, 25, 26, 28 and 29; Tables 9-11)

The MN were mostly dot shaped and generally occurred one per cell, but infrequently even three micronuclei occurred within a cell. Throughout the entire course of investigation both small and large size MN were observed in the treated individuals. In bone marrow cells, all three doses using the i.p. route induced significant increase of MN over the control but not by the p.o. route. In case of peripheral blood erythrocytes, the two lower doses in the i.p. and only the highest dose in the p.o. route gave significant results. Besides the induction of MN, monocrotophos also induced different types of nuclear anomalies. No significant variations were obtained (ANOVA) among the doses of bone marrow ($F = 2.314; \text{df} = 2, 9; \ p > 0.05$) and peripheral blood ($F = 1.068; \text{df} = 2, 9; \ p > 0.05$) micronucleus tests. No dose response linearity was observed for both bone marrow and peripheral blood micronucleus test.
EXPLANATION OF FIGURE

Fig. 21. Photomicrographs of bone marrow metaphase spreads showing different types of chromosomal aberrations induced by monocrotophos:

(a) woolly appearance of macrochromosomes.
(b) uneven accumulation of chromatin material in the q-arm of chromosome 1.
(c) -(d) differential digestion of chromatin material.
EXPLANATION OF FIGURE

Fig. 21 (contd.)

(e) chromatid break (thick arrow) and iso-chromatid gap (thin arrow) in chromosome 1.
(f) chromatid break in chromosome 4.
(g) chromatid break in chromosome 2.
(h) chromatid break in chromosome 4.
EXPLANATION OF FIGURES

Fig. 22. Histograms showing the frequencies of chromosome aberrations in the bone marrow cells of chicks:
(a) after different time intervals.
(b) by acute and chronic exposures.

Fig. 23. Histograms showing the frequencies of route-response chromosome aberrations in bone marrow cells of chicks.
Fig. 22 (a,b) FIXATION

Fig. 23 ROUTES

Acute
Chronic (5 days)
Chronic (30 days)

% OF CHROMOSOMAL ABERRATION

Fig 22(a,b)  FIXATION TIMES (HOURS)

Fig. 23  ROUTES
Fig. 24. Photomicrographs showing micronuclei and nuclear anomaly in bone marrow and peripheral blood cells of chicks:

(a) peripheral erythrocyte with two micronuclei.
(b) - (d) peripheral erythrocytes with one micronucleus in each.
(e) - (h) bone marrow erythrocytes with one micronucleus in each.
Fig. 24. (contd.)

(i) bone marrow cell showing anaphase division with chromatin bridge.

(j) bone marrow erythrocyte with MN.

(k) peripheral blood erythrocyte with MN.

(l) peripheral blood erythrocyte with three MN.

(m) peripheral blood erythrocyte with large MN.

(n) peripheral blood erythrocyte with dot-shaped MN adjacent to the nucleus.
EXPLANATION OF FIGURES

Fig. 25. Histograms showing the frequencies of monocrotophos induced MN in bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks exposed to different doses through different routes.

Fig. 26. Histograms showing the frequencies of monocrotophos induced micronuclei in bone marrow and peripheral blood erythrocytes after 30 days (chronic) exposure by i.p. route.
EXPLANATION OF FIGURES

Fig. 27. Linear regression analysis of chromosome aberration frequencies in the bone marrow cells of chicks. C in abscissa indicates control.

Fig. 28. Linear regression analysis of micronuclei frequencies in chick bone marrow (BM) and peripheral blood (PB) erythrocytes. C in abscissa indicates control.
\begin{align*}
\text{Fig 27} & \quad r = 0.92 \ (p < 0.05) \\
b & = 0.914
\end{align*}

% OF ABERRATION

\begin{align*}
\text{Fig 28} & \quad r = 0.832 \ (p > 0.05) \\
b & = 0.26
\end{align*}

% OF ABERRATION
EXPLANATION OF FIGURE

Fig. 29. Weekly increase in body weight of control and treated groups of chicks over a period of 30 days. Each point represents the mean body weight of four chicks plus or minus the standard deviation.
Fig 29

- Control
- Treated

Body Weight (g)

WEEKS
Frequency of chromosome aberrations induced by monocrotophos in the bone marrow cells of chicks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Fixation time (h)</th>
<th>No. of cells studied</th>
<th>Chromatid gaps</th>
<th>Chromatid breaks</th>
<th>Isochromatid gaps</th>
<th>Isochromatid breaks</th>
<th>Deletions</th>
<th>Rings</th>
<th>Total</th>
<th>% of aberration ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.p.</td>
<td>6</td>
<td>200</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>2(5)</td>
<td>1.00 ± 0.58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>10(15)</td>
<td>5.00 ± 0.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>1.p.</td>
<td>48</td>
<td>200</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>4(8)</td>
<td>2.00 ± 1.41&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.5</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>3</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>9(12)</td>
<td>4.50 ± 1.89</td>
</tr>
<tr>
<td>1.25</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>4(10)</td>
<td>2.00 ± 0.82&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 X 5</td>
<td>1.p.</td>
<td>120</td>
<td>200</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>4(7)</td>
<td>2.00 ± 0.82</td>
</tr>
<tr>
<td>0.167 X 30</td>
<td>1.p.</td>
<td>24 X 30</td>
<td>200</td>
<td>12</td>
<td>11</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>16(28)</td>
<td>8.00 ± 1.15&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>p.o.</td>
<td>24</td>
<td>200</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>3(6)</td>
<td>1.50 ± 0.50</td>
</tr>
</tbody>
</table>

Results in the parentheses are including of chromatid and isochromatid gaps.

Values are excluding of chromatid and isochromatid gaps.

Result is significantly different from the control at *p < 0.01 (Student's t-test)

<sup>a,b</sup> Results with the same superscripts do not differ significantly from each other at p > 0.05 (ANOVA)
Table - 9

Incidence of micronucleated bone marrow cells of chicks induced by monocrotophos.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>i.p.</td>
<td>16,000</td>
<td>24</td>
<td>1.50 ± 0.31**</td>
<td>3</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>2.5</td>
<td>i.p.</td>
<td>16,000</td>
<td>25</td>
<td>1.56 ± 0.12**</td>
<td>2</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td>1.25</td>
<td>i.p.</td>
<td>16,000</td>
<td>15</td>
<td>0.94 ± 0.21*</td>
<td>2</td>
<td>0.12 ± 0.12</td>
</tr>
<tr>
<td>0.167 X 30</td>
<td>i.p.</td>
<td>16,000</td>
<td>62</td>
<td>3.87 ± 0.46**</td>
<td>5</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>5</td>
<td>p.o.</td>
<td>16,000</td>
<td>08</td>
<td>0.50 ± 0.23</td>
<td>0</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p < 0.05; ** p < 0.01 (Students' t-test)
### Table - 10

**Incidence of micronucleated peripheral blood cells of chicks induced by monocrotophos**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>i.p.</td>
<td>16,000</td>
<td>11</td>
<td>0.69 ± 0.21</td>
<td>6</td>
<td>0.37 ± 0.16</td>
</tr>
<tr>
<td>2.5</td>
<td>i.p.</td>
<td>16,000</td>
<td>16</td>
<td>1.00 ± 0.10**</td>
<td>4</td>
<td>0.25 ± 0.10</td>
</tr>
<tr>
<td>1.25</td>
<td>i.p.</td>
<td>16,000</td>
<td>12</td>
<td>0.75 ± 0.14*</td>
<td>5</td>
<td>0.31 ± 0.19</td>
</tr>
<tr>
<td>0.167 x 30</td>
<td>i.p.</td>
<td>16,000</td>
<td>61</td>
<td>3.81 ± 0.45**</td>
<td>24</td>
<td>1.50 ± 0.31</td>
</tr>
<tr>
<td>5</td>
<td>p.o.</td>
<td>16,000</td>
<td>9</td>
<td>0.56 ± 0.16*</td>
<td>5</td>
<td>0.31 ± 0.19</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p<0.05; ** p < 0.01 (Student's t-test)
Table - 11

Mean of the weekly recorded weight (in grams) of 4 chicks ± SD

<table>
<thead>
<tr>
<th></th>
<th>0 week</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.75 ± 2.50</td>
<td>44.50 ± 3.12</td>
<td>66.0 ± 3.74</td>
<td>99.0 ± 6.05</td>
<td>129.50 ± 13.67</td>
</tr>
<tr>
<td>Treated</td>
<td>33.25 ± 2.75</td>
<td>39.50 ± 1.91</td>
<td>55.75 ± 4.35</td>
<td>85.75 ± 9.43</td>
<td>112.50 ± 16.05</td>
</tr>
</tbody>
</table>
In 30 days chronic treatment the incidence of micronuclei both in bone marrow and peripheral blood were more in comparison to the acute treatment and differed significantly from the respective control value. In addition to the induction of MN, other nuclear anomalies such as vacuolated nucleus, binucleated cells and disintegrated nucleus were also observed. A lone case of anaphase bridge was also recorded.

The difference in body weight between the control and the treated groups at the end of the fourth week (30 days chronic treatment) was found to be insignificant, indicating that the daily administration of such a low quantity of pesticide had no significant effect on the general growth rate of the chicks.

**DISCUSSION**

It is evident from the present investigation that monocrotophos is clastogenic in the chick in vivo system. These results are in agreement with the earlier findings of monocrotophos-induced mutagenicity in the Ames Salmonella test (Moriya et al., 1983) and gene mutation in bacteria (Hanna and Dyer, 1975), recombination and reverse mutation in yeast (Simon et al., 1976), clastogenesis in plants (Rao et al., 1987), chromosome damage in mouse bone marrow cells (Bhunya and Behera, 1988) and human leukocytes (Vaidya and Patankar, 1982). Monocrotophos was also found to induce sister-chromatid exchanges in rat tracheal epithelial (RTE) and Chinese hamster ovary (CHO) cells (Wang et al., 1987; Lin et al., 1987).

In the chromosome aberration assay the highest dose induced the highest percentage of aberrations (5.00%) after 24 h of exposure. It has been reported that chemicals in general produce the highest frequency of chromosomal aberration in rodents 24 h after single exposure, since this timing coincides roughly with the normal length (22-24 h) of the cell cycle (Schmid, 1973). In chicks the normal cell
cycle time is 17.5 h (Bianchi and Molina, 1967), but B-lymphocytes in perinatal chicks have cell kinetics of 9-10 h (Wilmer and Bloom, 1991). Monocrotophos is known to perturb cell cycle kinetics in human lymphoid cells in vitro (Sobti et al., 1982). The non-significant results for 6 h and 48 h treatments may indicate that at the early time point (6 h) the test chemical or its metabolites probably could not reach the target molecule (DNA or protein) and at the late time (48 h) the damaged cell may have died and the metabolites have been eliminated from the body. The lower extent of chromosome damage using the p.o. route might be due to poor absorption from the gut or greater elimination of the chemical from the body. During chronic treatment (5 times at 24 h intervals) the lower mutagenic effect may be due to less accumulation of the chemical. Similar results have also been described for irradiation (Committee, 17, 1975) and obtained in case of some pesticides in the mouse test system (Bhunya and Behera, 1987; Bhunya and Pati, 1990). The higher incidence of chromosomal aberrations after 30 days chronic treatment (30 times at 24 h intervals) may be due to either the accumulation of the chemical in the bone marrow with a low concentration over a long time period in the target tissue or the accumulation of aberrant cells which produced between the successive doses of administration.

The micronucleus test is one of the most dependable cytogenetic bio-assays to screen environmental agents for mutagenicity (Schmid, 1976; Savage, 1988). Micronuclei indicate acentric chromosome fragments or whole chromosomes that have not been incorporated into the main nuclei at cell division. In the present study in bone marrow cells, significant induction of MN was observed at all three doses and in peripheral blood only at the two lower doses. The significant increase of monocrotophos induced MN in the erythrocytes of bone marrow and peripheral blood demonstrates its genotoxicity in the chick. In the present study the peak incidence of micronuclei in bone marrow and peripheral blood erythrocytes after a chronic exposure (30 times at 24 h intervals) was higher than that obtained after single acute exposure.
Henderson et al. (1993) observed a higher incidence of micronuclei in the rat bone marrow and peripheral blood with a 28 days sub-chronic exposure than that obtained after a single acute exposure with azathioprine. The significant increase of MN in the 30 days chronic study with the multiple treatments of the chemical demonstrates that these micronuclei are either derived from structural chromosomal aberrations or due to the lagging of chromosomes which arise due to the interaction of the chemical with the spindle fibres of the cell. The existence of large micronuclei suggests, the tested chemical can also be an aneugen.

The exact mechanism of action of monocrotophos at the molecular level is not clearly known. The general metabolism of organophosphates occurs principally by oxidation, hydrolysis by esterases and by the transfer of a portion of the molecule to glutathione (WHO, 1986). The oxidation of organophosphorus insecticides may result in more or less toxic products. Klopman et al. (1985) suggested that chemicals having methoxy-phosphynyl groups have an elevated potential for being mutagenic. In general the phosphorus in organophosphorus moiety appears to be a good substrate for nucleophilic attack which may cause the phosphorylation of DNA and result in the ultimate DNA damage. However, the major reactions of pentavalent phosphorus esters are phosphorylation and alkylation. The phosphorylation is responsible for the inhibition of acetylcholinesterase while mutation appears to be due to alkylation properties (Eto and Ohkawa, 1970). The alkylation reaction is more likely with the methyl ester group than the ethyl and higher alkyl ester group and phosphate esters are more reactive than the phosphorothionate esters (Eto, 1974). So the mechanism of action of monocrotophos may be explained by methylation reaction of the phosphate esters.
2. ASATAF (Acephate®)

Acephate is a broad spectrum organophosphorus insecticide active on moths and aphids and is used for the protection of a variety of vegetables and fruits. It has both contact and systemic actions on a wide variety of sucking and biting insects. It contains 97 % of acephate (technical) as active ingredient. The chemical name of acephate is \( \text{O, S-dimethyl-acetylphosphoramidothioate} \) or \( N\)-ethoxy (methylthio) phosphonoylacetamide having chemical formula \( C_4H_{10}NOS \).

\[
\text{CH}_3S\text{O} \quad \text{O} \quad \text{O} \\
\text{CH}_3\text{O} \quad \text{P} \quad \text{NHC} \quad \text{CH}_3
\]

(Structure of acephate)

The genotoxic and embryotoxic effects of acephate have been reported in a mouse in vivo system (Behera and Bhunya, 1989). The mutagenicity of acephate has been evaluated in several test systems such as the \textit{Salmonella typhimurium} histidine reversion assay (SAL), the thymidine kinase gene mutation assay using mouse lymphoma L5178Y cells (L5T), a prokaryotic DNA repair assay and mitotic recombination in strain D3 of the yeast \textit{Saccharomyces cerevisae} (YE3) (Klopman et al., 1985). There is no record in the literature regarding the mutagenicity of acephate in an avian test system, and the present results provide evidence of the genotoxic potential of acephate in the chicken in vivo system.

**MATERIALS AND METHODS**

**Test chemical:** Technical grade acephate (purity 97 %) was donated by Rallis India Ltd. (Bombay). Glass double distilled (g.d.d.) water was used as vehicle.

**Dose:** Toxicity data (LD\textsubscript{50}) of acephate for chicks were not available. The maximum tolerable dose determined by trial was 100 mg/kg body weight and the two other lower doses selected were 50 and 25 mg/kg respectively.
Experimental protocol: The details of BMCA, MNT and statistics have been described in "General materials and methods".

RESULTS

General toxicity: After acute treatment with acephate, toxicity symptoms such as loss of appetite, restricted movement of eyelids and limbs, etc. were observed. During the study with 5 and 30 days chronic treatment these symptoms were not as obvious as in acute treatment.

Genotoxicity:

Chromosome aberration: (Figs. 30 a-f, 31 a-b, 32 and 36; Table-12).

Qualitative: In the metaphase analysis of bone marrow cells aberrations such as chromatid-type gaps, breaks, deletions, rings and structural rearrangements such as pericentric inversion were observed in the treated series. Gaps occurred more frequently than breaks and chromatid deletions. In thirty days (chronic) treatment with the fractionated dose regimens of the highest acute dose, aberrations like chromatid separations and stretching in the centromeric regions were also observed.

Quantitative: Among the three different doses, the dose 50 mg/kg b.w. of acephate induced significant bone marrow chromosome aberrations (t = 3.16; df = 6; p < 0.05) after 24 h of exposure. No significant variation was observed (ANOVA) among dose-response (F = 3.71; df = 2, 9; p > 0.05) analyses. In the time-response studies the highest effect was observed after 48 h of exposure. An analysis of the results of single dose (100 mg/kg) and repeated exposures (5 days chronic treatment) of the fractionated doses (5 X 20 mg/kg b.w.) revealed that the frequency of aberrations produced by acute treatment (2.00 %) was slightly higher than the chronic treatment (1.5 %) and not significant over the
respective control value. However, when the same acute dose was fractionated into thirty equal parts and each part was administered over a period of 30 days (30 times with 24 h intervals), more number of frequencies (5.00 %) were obtained in comparison to single acute treatment and differed significantly ($t = 4.90; \text{df} = 6; p < 0.01$) over the control value. The result by the p.o. route was not significant and was less than by the i.p. route.

Regarding the regional sensitivity to aberrations, induced by acephate, the middle region of the first two pairs of macrochromosomes were most vulnerable to gaps and breaks than the other chromosomes considered for aberration scoring.

Micronucleus: (Figs. 33 a-h, 34, 35, 37 and 38; Tables 13-15)

The frequencies of micronucleated erythrocytes in bone marrow and peripheral blood were significantly higher than the respective control values using the i.p. route but not the p.o. route. Further, only significant variations were noted between the results of 25 versus 100 mg/kg doses in the bone marrow micronucleus assay ($F = 4.84; \text{df} = 2, 9; p < 0.05$) but no significant variations were observed among the results of the peripheral blood micronucleus assay ($F = 3.36; \text{df} = 2, 9; p > 0.05$). Moreover, a linear increase in the frequency of micronuclei with the dose of acephate was observed for bone marrow ($b = 0.010; r = 0.977; p < 0.01$) and peripheral blood ($b= 0.013; r = 0.964; p < 0.01$) erythrocytes as revealed by linear regression analysis. A significant increase of micronuclei were observed both in bone marrow and peripheral blood erythrocytes after 30 days repeated treatment with the fractionated dose regimens of the highest acute dose. Besides the induction of micronuclei, the chemical also induced other nuclear anomalies such as vacuolated nuclei and binucleated cells with unequal nuclear material, in the treated series. In some cases karyorhexis and disintegration of chromatin were also observed.
EXPLANATION OF FIGURE

Fig. 30. Photomicrographs of bone marrow metaphase spreads showing different types of chromosomal aberrations induced by acephate:

(a) isochromatid gap in the p-arm of chromosome 1.
(b) chromatid break in the q-arm of chromosome 2.
(c) chromatid break (and deletion) in the q-arm of chromosome 2.
(d) chromatid break in the q-arm of chromosome 1.
(e) chromatid break in chromosome 3.
(f) chromatid break in the q-arm of chromosome 1.
Fig. 31. Histograms showing the frequencies of acephate induced chromosome aberrations in the bone marrow cells of chicks.
(a) after different time intervals.
(b) by acute and chronic exposures.

Fig. 32. Histograms showing the route response chromosome aberration frequencies in the bone marrow cells of chicks.
7% OF CHROMOSOMAL ABERRATIONS

Fig 31 (a,b)  FIXATION TIMES (HOURS)

Fig 32  ROUTES
EXPLANATION OF FIGURE

Fig. 33. Photomicrographs showing acephate induced micronuclei in the bone marrow and peripheral blood erythrocytes of chicks:

(a)-(d) bone marrow erythrocytes with one micronucleus in each.

(e) peripheral blood erythrocyte with one MN.

(f)-(g) peripheral blood erythrocyte with large MN.

(h) two erythrocytes of peripheral blood with one MN in each.
EXPLANATION OF FIGURES

Fig. 34. Histograms showing the frequencies of micronuclei induced by acephate in the bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks exposed to different doses through different routes.

Fig. 35. Histograms showing the frequencies of micronuclei induced by acephate in BM and PB erythrocytes after 30 days (chronic) exposure.
**Fig 34**

- **Y-axis:** MN per 1000 cells
- **X-axis:** Dose (mg/kg)
- **Legend:**
  - BM
  - PB

**Fig 35**

- **Y-axis:** MN per 1000 cells
- **X-axis:** Tissue
- **Legend:**
  - Control
  - Treated
EXPLANATION OF FIGURES

Fig. 36. Linear regression analysis on chromosome aberration frequencies in the bone marrow cells of chicks. C in abscissa indicates control.

Fig. 37. Linear regression analysis on micronuclei frequencies in bone marrow (BM) and peripheral blood (PB) of chicks. C in abscissa indicates control.
Fig. 36

% of aberration vs. dose (mg/kg)

$r = 0.347 (P > 0.05)$

$b = 0.01$

Fig. 37

% of aberration vs. dose (mg/kg)

$r = 0.964 (P < 0.01)$

$b = 0.013$

$r = 0.977 (P < 0.01)$

$b = 0.01$
EXPLANATION OF FIGURE

Fig. 38. Weekly increase in body weight of control and treated groups of chicks over a period of 30 days. Each point represents the mean body weight of the four chicks plus or minus the standard deviation.
Table - 12

Frequency of chromosome aberrations induced by acephate in the bone marrow cells of chicks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Fixation time (h)</th>
<th>No. of cells studied</th>
<th>Chromatid gaps</th>
<th>Isochromatid gaps</th>
<th>Deletions</th>
<th>Rings</th>
<th>Exchange</th>
<th>Total</th>
<th>% of aberration ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.p.</td>
<td>6</td>
<td>200</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3(4)</td>
<td>1.50 ± 0.96</td>
</tr>
<tr>
<td>100</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>6</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>4(10)</td>
<td>2.00 ± 0.82</td>
</tr>
<tr>
<td>100</td>
<td>1.p.</td>
<td>48</td>
<td>200</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>-</td>
<td>6(13)</td>
<td>3.00 ± 0.58</td>
</tr>
<tr>
<td>50</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>3</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>11(14)</td>
<td>5.50 ± 1.50*</td>
</tr>
<tr>
<td>25</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>3(6)</td>
<td>1.50 ± 0.96</td>
</tr>
<tr>
<td>20 X 5</td>
<td>1.p.</td>
<td>120</td>
<td>200</td>
<td>3</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>3(6)</td>
<td>1.50 ± 0.96</td>
</tr>
<tr>
<td>3.333X30</td>
<td>1.p.</td>
<td>24X30</td>
<td>200</td>
<td>4</td>
<td>8</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>10(14)</td>
<td>5.00 ± 0.58**</td>
</tr>
<tr>
<td>100</td>
<td>p.o.</td>
<td>24</td>
<td>200</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3(5)</td>
<td>1.50 ± 0.96</td>
</tr>
</tbody>
</table>

Results in the parentheses are including of chromatid and isochromatid gaps.

+ Values are excluding of chromatid and isochromatid gaps

Result is significantly different from the control at * p<0.05; ** p<0.01 (Student's t-test)
Table - 13

Incidence of micronucleated bone marrow cells of chicks induced by acephate.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>i.p.</td>
<td>16,000</td>
<td>20</td>
<td>1.25 ± 0.18**a</td>
<td>5</td>
<td>0.31 ± 0.26</td>
</tr>
<tr>
<td>50</td>
<td>i.p.</td>
<td>16,000</td>
<td>12</td>
<td>0.75 ± 0.10**b</td>
<td>5</td>
<td>0.31 ± 0.12</td>
</tr>
<tr>
<td>25</td>
<td>i.p.</td>
<td>16,000</td>
<td>10</td>
<td>0.62 ± 0.16*a</td>
<td>8</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>3.333X30</td>
<td>i.p.</td>
<td>16,000</td>
<td>31</td>
<td>1.94 ± 0.12*</td>
<td>7</td>
<td>0.44 ± 0.12</td>
</tr>
<tr>
<td>100</td>
<td>p.o.</td>
<td>16,000</td>
<td>04</td>
<td>0.25 ± 0.14</td>
<td>3</td>
<td>0.19 ± 0.06</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from control at * p<0.05; ** p<0.01 (Student’s t-test).

a,b Results with same superscript differ significantly from each other at p<0.05 (ANOVA).
Table - 14

Incidence of micronucleated peripheral blood cells of chicks induced by acephate.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.p.</td>
<td>16,000</td>
<td>26</td>
<td>1.62 ± 0.22**</td>
<td>11</td>
<td>0.69 ± 0.19</td>
</tr>
<tr>
<td>50</td>
<td>1.p.</td>
<td>16,000</td>
<td>18</td>
<td>1.12 ± 0.24*</td>
<td>10</td>
<td>0.62 ± 0.16</td>
</tr>
<tr>
<td>25</td>
<td>1.p.</td>
<td>16,000</td>
<td>14</td>
<td>0.87 ± 0.16*</td>
<td>9</td>
<td>0.56 ± 0.21</td>
</tr>
<tr>
<td>3.333X30</td>
<td>1.p.</td>
<td>16,000</td>
<td>29</td>
<td>1.81 ± 0.19*</td>
<td>12</td>
<td>0.75 ± 0.27</td>
</tr>
<tr>
<td>100</td>
<td>p.o.</td>
<td>16,000</td>
<td>07</td>
<td>0.44 ± 0.06</td>
<td>8</td>
<td>0.50 ± 0.27</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from control at *p<0.05; ** p<0.01 (Student's t-test)
<table>
<thead>
<tr>
<th></th>
<th>0 week</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>37.75 ± 3.86</td>
<td>44.50 ± 6.85</td>
<td>56.50 ± 6.65</td>
<td>85.75 ± 7.59</td>
<td>110.50 ± 10.85</td>
</tr>
<tr>
<td>Control</td>
<td>36.75 ± 2.50</td>
<td>44.50 ± 3.12</td>
<td>66.00 ± 3.74</td>
<td>99.00 ± 6.05</td>
<td>129.50 ± 13.67</td>
</tr>
</tbody>
</table>

Table -15. Mean of the weekly recorded body weight (in grams) of four chicks ± SD.
The difference in body weight between the control and the treated groups in both the assays at the end of the fourth week was found to be insignificant, indicating that the daily administration of such a low quantity of pesticide had no significant effect on the general growth rate of the chicks.

DISCUSSION

The present results indicate the genotoxic property of acephate in chicks. The highest percentage of aberrations (5.50%) was induced by 50 mg/kg acephate after 24 h exposure. The present results on the clastogenic potential of acephate are in accordance with the findings of Behera and Bhunya (1989) in mice and Sandhu et al. (1985) in the root tip cells of Vicia faba. However, negative results for acephate-induced mutagenicity in different strains of mice (CD-1 and Swiss Webster) using analysis of chromosome aberration, sister chromatid exchange and micronucleus in bone-marrow cells, and the dominant lethal test have also been reported by Carver et al. (1985). It is possible that the negative results might be due to strain differences or specific pharmacokinetic properties of the chemical. In the time-response studies, the highest percentage of aberrations was observed 24 h after exposure. Such a result is expected since this timing coincided roughly with the normal duration (17.5 h) of cell cycle in the bone marrow cells of chicks (Bianchi and Molina, 1967). The frequencies of chromosome aberrations induced by the 5 days chronic and acute dosing did not differ significantly. However, significant number of chromosome aberrations produced by 30 days chronic dosing suggests greater cumulative effect of the chemical over a long time period.

Any agent capable of inducing micronuclei is considered as a clastogen or aneugen (Maier and Schmid, 1976; Heddle et al., 1983). The significant increases in acephate-induced small and large size micronuclei in the erythrocytes of bone marrow and peripheral blood indicate clastogenic and aneugenic potential in the chick.
In soil and plants, acephate is converted into methamidophos (O, S-dimethyl phosphoramidothioate), which is a very potent anticholinesterase agent (Bull, 1979). The insecticidal action of acephate is due to methamidophos or due to combined anticholinesterase effect of acephate and methamidophos (Kao and Fukoto, 1977; Eto et al., 1977; Magee, 1982; Hussain et al., 1984). Besides acute toxicity, methamidophos caused delayed neuropathy in man through the mechanism of inhibition of 'neuropathy target esterase' (NTE) (Senanayake and Johnson, 1982). Low levels of acephate were detected in the urine of field workers exposed to it (Pack, 1972). However, an assessment of the metabolism of acephate in rodents suggests that acephate is not degraded to methamidophos, but rather is excreted unchanged in the urine (Lee, 1972).

Acephate is a pentavalent phosphorus compound. The mechanism of action of acephate is mainly through phosphorylation and alkylation. The phosphorylation reaction (at about 460 p.p.m.) is responsible for inhibition of acetylcholinesterase (Ando and Wakamatsu, 1982), which causes corresponding neurotoxicity. However, the phosphorus moiety in organophosphates appear to be a good substrate for nucleophilic attack. This may cause phosphorylation of DNA, which is an instance of DNA damage (Wild, 1975). The alkylating properties of phosphorus esters are mainly responsible for the induction of mutations (Eto and Chkawa, 1970; Wild, 1975) and several phosphorothioate compounds can also induce mutations (Breau et al., 1985). The heterocyclic bases of nucleic acids may serve as nucleophilic agents for such a reaction, and alkylation may take place on nitrogen atoms possessing high electron density. The alkylation reaction resides with the methyl ester group rather than with the ethyl and higher alkylester groups, and phosphate esters are more reactive than phosphorothioate esters (Eto, 1974). It has been proposed that the reactivity of acephate is due to the methylation reaction of phosphate esters.
Considering the present results from chicks, along with the in vivo results in mice (Behera and Bhunya, 1989) systems and other in vitro results (Waters et al., 1982), it is suggested that acephate is a potential mutagen which also confirms the inclusion of acephate under 'pesticides with largely positive results' by Garrett et al. (1986). Hence, the restricted use and cautious handling of acephate are recommended.
Phosphamidon is an organophosphorus pesticide used on a wide range of temperate and tropical crops including rice, cotton, soya bean and vegetables. It is a systemic insecticide having special activity against sucking pests such as aphids, leaf hoppers, plant hoppers, white flies and bugs. It also kills other pests by means of its strong stomach poisoning action. It rapidly penetrates into the treated plant tissue such as roots, stems or leaves, where it is translocated into all other parts of the plant (Voss and Geissbuhler, 1971). Its residue in various crops are found to disappear within 4-6 days after spraying (Menzer and Ditman, 1963). Phosphamidon was first manufactured in 1952 by the Perkow reaction which produced a mixture of 30 % trans-isomer and 70 % cis-isomer, with the latter being biologically more active (CIBA,1968). Its trade name is Dimecron and the chemical name is O-(2-chloro-2-diethylcarbamoyl-1-methyl-vinyl)-o, o-dimethyl-phosphate. The structural formula of phosphamidon is as follows:

\[
\begin{align*}
\text{CH}_3 \text{O} & \quad \text{O} \\
\text{CH}_2 \text{O} & \quad \text{P} \quad \text{O} \quad \text{C} \quad \text{C} \quad \text{N} \\
& \quad \text{C} \quad \text{N} \\
& \quad \text{C}_2 \text{H}_5 \\
& \quad \text{C}_2 \text{H}_5
\end{align*}
\]

(Structure of phosphamidon)

Wuu and Grant (1966) reported the chromosome aberration in plants by phosphamidon. Lakhani and Pandy (1985) studied a variety of anomalies induced by phosphamidon in spermatogonia, spermatocytes and sperm cells in fish. Cheng et al. (1989) reported the chromosome aberration by phosphamidon in female Wister rats after oral administration. Another study reported the mutagenic potential of phosphamidon basing upon the results of sister chromatid exchange (SCE) of bone marrow cells of rats and of the Ames test (Zhang, 1984). Behera and Bhunya (1987) conducted a study on the genotoxicity of phosphamidon in mice by three different assays: chromosomal aberration, micronucleus and sperm shape abnormality and reported that phosphamidon is a potent mutagen in mice in vivo.
somatic cells and may be a potential germ cell mutagen. Viswanath and Jamil (1986) reported phosphamidon as a base change mutagen in Ames Salmonella assay. Patankar and Vaidya (1980) reported its mutagenic activity in 3 test systems like (i) human leucocyte culture, (ii) mice bone marrow chromosome and (iii) mice bone marrow micronucleus assay. These results confirmed the earlier report that phosphamidon had mutagenic activity verified in both in vivo and in vitro studies by Georgian (1975) who observed that minimal dose induced chromosome aberrations in bone marrow cells of rats and mice by intraperitoneal injection, and a wide range of dose caused chromosome aberrations in the cultures of human peripheral blood lymphocytes. The results obtained by Rani et al. (1980) and Adhikary and Grover (1988) also provided consistent results with the above observation. The examination of chromosomes of lymphocytes in peripheral blood of the phosphamidon-poisoned workers indicated that phosphamidon may possess mutagenic activity in humans (See Zhuang et al., 1993).

In carcinogenicity testing, phosphamidon displayed equivocal results in rats and negative results in mice (Di Carlo and Fung, 1984). Again the carcinogenicity of phosphamidon was tested in rats and mice by the National Cancer Institute of the United States of America (NCI, 1979) and there was no evidence of carcinogenicity of the substance.

MATERIALS AND METHODS

Test chemical: Technical grade of phosphamidon (93.1 % pure) was supplied by Hindustan Ciba-Geigy (Agrochemical Division), Bombay. The solvent was glass double distilled (g.d.d.) water.

Dose: Three different doses; 2.3 and 4 mg/kg b.w. were employed. Toxicity data (LD_{50}) of phosphamidon for chicks was not available. The maximum tolerable dose determined by trial was 4 mg/kg body weight and the two other lower doses selected were 3 and 2 mg/kg respectively.
Experimental protocol: The experimental design and statistics for the two assays like bone marrow chromosome aberration (BMCA) and micronucleus test (MNT) in both bone marrow and peripheral blood have been described in "General materials and methods".

RESULTS

General toxicity: After the administration of the chemical, inactiveness of the body and restricted movements were observed. These symptoms were prominent when the chemical was administered in i.p. route than in p.o. route. The animals resumed to be normal just after few minutes (15-20 minutes) of treatment.

Genotoxicity:

Chromosome aberration: (Figs. 39 a-d, 40, 41 and 44; Table-16)

Qualitative: The treatment of phosphamidon induced different types of aberrations. The aberration spectrum included chromatid gaps, breaks, deletions and rings. Very rare plates with centromeric separations were observed. In addition to these effects, uneven accumulation of chromatin material and poor staining of some chromosome in the same metaphase plate were also observed. In some metaphase plates partial G-banded like appearance in the larger chromosome has been observed.

Quantitative: The highest dose 4 mg/kg applied through i.p. route produced highest frequency (4.50 %) of aberrations after 24 hours of exposure. The yield of aberrations with the two other lower doses, 3 and 2 mg/kg was 2.50 % and 1.50 % respectively. The frequency of aberration after 6 and 48 hours of exposure was 0.50 % and 1.00 % respectively. Regarding the route sensitivity, the chemical was found to be most effective in i.p. route. In chronic treatment the percentage of aberration (1.50 %) was lower than the percentage of aberration produced...
by the equivalent acute dose treated once. In dose-response studies all the doses induced higher frequencies of aberrations than the respective controls, but only the highest dose induced a statistically significant effect \((t = 5.65; \, \text{df} = 6; \, p < 0.01)\). However, no significant variations were observed (ANOVA) in the dose-response analyses \((F = 3.62, \, \text{df} = 2,9; \, p > 0.05)\). Significant variations were obtained (ANOVA) among time-response analyses \((F = 11.96; \, \text{df} = 3,12; \, p < 0.001)\). The effect produced by the p.o. route was lower than the i.p. route and was statistically insignificant from the control \((t = 1.73; \, \text{df} = 6; \, p > 0.05)\).

Regarding the regional sensitivity of the chromosomes to phosphamidon, aberrations were observed randomly in all the chromosomes considered for scoring except Z chromosomes, in which no aberration was observed. So from the present investigation no special claim can be made for the regional sensitivity of the chromosomes to the chemical assault.

**Micronuclei:** (Figs. 42 a-h, 43 and 45; Tables 17-18)

The MN were mostly dot shaped and generally occurred one per cell. In bone marrow MN test all the doses in i.p. route gave significant results over the control but not in p.o. route. Only the two highest doses in peripheral blood MN test gave significant results in the i.p. route. Significant variations were obtained among the doses of bone marrow MN test \((F = 5.56; \, \text{df} = 2,9; \, p < 0.05)\) but not for peripheral blood micronucleus test \((F = 3.25; \, \text{df} = 2,9; \, p > 0.05)\). A linear increase in the frequency of micronuclei with the dose of phosphamidon was observed for both bone marrow \((b = 0.271; \, r = 0.994; \, p < 0.001)\) and peripheral blood \((b = 0.180; \, r = 0.963; \, p < 0.01)\). Besides the induction of micronuclei the chemical also induced other nuclear anomalies such as fragmented nuclei, stretching of the nuclear material having very thin connection etc. in the treated series.

**DISCUSSION**

In the present investigation phosphamidon induced a statistically significant result in the bone marrow chromosome aberration only with the
EXPLANATION OF FIGURE

Fig. 39. Photomicrographs showing phosphamidon induced chromosome aberrations in the bone marrow metaphase plates of chicks:

a) ring chromosome.
b) chromatid break in the p-arm of chromosome 2.
c) chromatid deletion in the p-arm of chromosome 4.
d) chromatid break in the p-arm of chromosome 2.
EXPLANATION OF FIGURES

Fig. 40. Histograms showing the frequencies of phosphamidon induced chromosome aberrations in the bone marrow cells of chicks:

(a) after different time intervals.
(b) by acute and chronic exposures.

Fig. 41. Histograms showing the route-response chromosome aberration frequencies in the bone marrow cells of chicks.
EXPLANATION OF FIGURE

Fig. 42. Photomicrographs showing phosphamidon induced micronuclei in the bone marrow and peripheral blood erythrocytes of chicks:

(a) bone marrow cell with 'v' shaped nucleus.
(b)-(c) bone marrow erythrocytes with one micronucleus in each.
(d) bone marrow erythrocyte with one large micronucleus adjacent to the nucleus.
(e) peripheral blood erythrocyte showing the stretching and thinning at the middle of the nucleus alongwith the cytoplasm.
(f) peripheral blood erythrocyte containing one large micronucleus.
(g) peripheral blood erythrocyte showing one small micronucleus.
(h) peripheral blood erythrocyte showing one micronucleus adjacent to the nucleus.
EXPLANATION OF FIGURE

Fig. 43. Histograms showing the frequencies of phosphamidon induced micronuclei in the bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks exposed to different doses through different routes.
Fig. 43

DOSE (mg/kg)

MN PER 1000 CELLS

BM
PB

0
0.2
0.4
0.6
0.8
1.0
1.2
1.4

2
3
4
4

IP
PO

Fig. 43
EXPLANATION OF FIGURES

Fig. 44. Linear regression analysis on chromosome aberration frequencies in the bone marrow cells of chicks. C in abscissa indicates control.

Fig. 45. Linear regression analysis on micronuclei frequencies in bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks. C in abscissa indicates control.
Fig 44

% of aberration

DOSE (mg/kg)

Fig 45

MN per 1000 cells

DOSE (mg/kg)
Frequency of chromosome aberrations induced by phosphamidon in the bone marrow cells of chicks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Fixation time (h)</th>
<th>No. of cells studied</th>
<th>Chromatid gap</th>
<th>Chromatid break</th>
<th>Deletion</th>
<th>Ring</th>
<th>Total</th>
<th>% of aberrations ± SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>i.p.</td>
<td>6</td>
<td>200</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1(4)</td>
<td>0.50 ± 0.50a</td>
</tr>
<tr>
<td>4</td>
<td>i.p.</td>
<td>24</td>
<td>200</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>9(14)</td>
<td>4.50 ± 0.50b</td>
</tr>
<tr>
<td>4</td>
<td>i.p.</td>
<td>48</td>
<td>200</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>2(5)</td>
<td>1.00 ± 0.58a</td>
</tr>
<tr>
<td>3</td>
<td>i.p.</td>
<td>24</td>
<td>200</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>5(10)</td>
<td>2.50 ± 0.96</td>
</tr>
<tr>
<td>2</td>
<td>i.p.</td>
<td>24</td>
<td>200</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>3(6)</td>
<td>1.50 ± 0.96</td>
</tr>
<tr>
<td>0.8X5</td>
<td>i.p.</td>
<td>120</td>
<td>200</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>3(5)</td>
<td>1.50 ± 0.50a</td>
</tr>
<tr>
<td>4</td>
<td>p.o.</td>
<td>24</td>
<td>200</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2(5)</td>
<td>1.00 ± 0.50</td>
</tr>
</tbody>
</table>

Results in the parentheses are including gaps.
* Values are excluding of gaps.
+ Result is significantly different from the control p < 0.01 (Student's t-test)
a,b Results with same superscript donot differ significantly from each other at p > 0.001 (ANOVA)
<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with nuclear anomalies</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>1.p.</td>
<td>16,000</td>
<td>19</td>
<td>1.19 ± 0.16**a</td>
<td>8</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>3</td>
<td>1.p.</td>
<td>16,000</td>
<td>15</td>
<td>0.94 ± 0.12**b</td>
<td>7</td>
<td>0.44 ± 0.19</td>
</tr>
<tr>
<td>2</td>
<td>1.p.</td>
<td>16,000</td>
<td>9</td>
<td>0.56 ± 0.17*</td>
<td>5</td>
<td>0.31 ± 0.24</td>
</tr>
<tr>
<td>4</td>
<td>p.o.</td>
<td>16,000</td>
<td>6</td>
<td>0.38 ± 0.16</td>
<td>2</td>
<td>0.13 ± 0.07</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p < 0.05; ** p < 0.01 (Student's t-test)

a, b Results with same superscript differ significantly from each other at p < 0.05 (ANOVA).
Table - 18

Incidence of micronucleated peripheral blood cells of chicks induced by phosphamidon.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>i.p.</td>
<td>16,000</td>
<td>14</td>
<td>0.88 ± 0.16 *</td>
<td>4</td>
<td>0.25 ± 0.18</td>
</tr>
<tr>
<td>3</td>
<td>i.p.</td>
<td>16,000</td>
<td>12</td>
<td>0.75 ± 0.10 *</td>
<td>8</td>
<td>0.50 ± 0.18</td>
</tr>
<tr>
<td>2</td>
<td>i.p.</td>
<td>16,000</td>
<td>6</td>
<td>0.38 ± 0.16</td>
<td>3</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>4</td>
<td>p.o.</td>
<td>16,000</td>
<td>7</td>
<td>0.44 ± 0.21</td>
<td>5</td>
<td>0.31 ± 0.19</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p < 0.05 (Student's t-test)
highest dose after 24 hours of intraperitoneal exposure. A linear increase in the frequency of anomalies by the different doses was observed in both the assays. In chromosome aberration assay gaps and breaks occurred more frequently than other types of aberrations. Decrease of anomalies with lower doses and in late hours was probably due to the non-availability of the critical concentration of the test compound or its metabolites at the target molecules (DNA and chromosomal protein) and elimination of the metabolites from body with time (Matter, 1976, Tates and Natarajan, 1976). The highest effect after 24 hours, might be due to the completion of one cell cycle. In the bone marrow cells of chick the normal duration of cell cycle is 17.5 hours (Bianchi and Molina, 1967). Chemicals in general induced highest effects in the bone marrow cells of mice 24 hours (very close to one cell cycle time) after the treatment. In case of mammals it has been reported that the rate of detoxification of organophosphate compounds is very fast (Degraeve et al., 1984). Again relatively lower mutagenic effects after fractionated (sub-acute) dosing could be anticipated in the event of daily lower intake and less cumulative effect resulting in the non-availability of the equivalent concentration at the target molecules.

The micronucleus test is more practical, reliable and sensitive for screening chemicals for mutagenic potential and recognised as a short term method for screening mutagens and aneugens (Maier and Schmid, 1976; Lynch and Parry, 1993). The significant effect in the MN test gives an evidence for chromosome breakage and/or spindle poisoning effect induced by phosphamidon.

Regarding the route sensitivity, the higher effect in i.p. route for both the assays than in p.o. route might be due to the inactivation and/or detoxification of the chemical in the liver, because in oral administration a higher quantities of chemical has to pass through liver (Charbonneau et al., 1979). Lower absorption and greater excretion of the chemical from the body may be another criteria for the insignificant result in p.o. route.
The exact mechanism of action of phosphamidon at molecular level is not clearly known. However, the organophosphorus pesticides are also known for their alkylating properties (Wild, 1975) and they react with the nucleophilic sites in proteins as well as on DNA and thus may produce aneuploidy by the alkylation of spindle apparatus proteins (Dellarco et al., 1986) or guanosine triphosphate which is required for tubulin polymerization (Olmsted and Dorisy, 1975). The potential electrophilic (DNA reactive) site of phosphamidon is the presence of CH$_2$O-group (Ashby and Tennant, 1988) which might be responsible for DNA methylation. Methylation of DNA by DDVP (an organophosphorus insecticide) was reported by Lawly et al. (1974) and chromosome aberration due to methylation by Lawly and Brookes (1961). Loveless (1969) suggested that multiple methylation of DNA induces miscoding of bases and persistence of these methylation results in many somatic mutations of the GC-AT type. Phosphamidon is a pentavalent phosphorus ester and its methylation reaction is probably similar to that of the other phosphate esters like acephate and monocrotophos (see acephate and monocrotophos in the present thesis). In the present experiment, effects like uneven accumulation of the chromatin material, differential digestion and non-staining area indicate that the chemical has a strong affinity for interaction with chromosomal proteins than the constituent DNA.

The present results revealed genotoxic property of phosphamidon in the chick in vivo test system. It is further concluded that the clastogenic action of phosphamidon with such a low doses in vivo, indicates the genetic peril for the occupational workers.
4. TAFETHION (Ethion®)

Tafethion is an organophosphate pesticide and is used both as an insecticide and acaricide (Smith, 1975). It has very quick knockdown and long-lasting residual activity against mites. Its technical name is ethion. Chemically it is known as O,O,O,O-tetraethyl S-S-methylene-bis-phosphorodithioate ($C_{9}H_{22}O_{4}P_{2}S_{4}$). The chemical structure of ethion is as follows:

![Structure of ethion]

Although organophosphates are less persistent than organochlorines (Osweiler et al., 1985) the problem of residues still exists, if they are not properly used in the environment. Ethion is mainly used in tea, cotton and vegetables for controlling spider, mites, thrips and flush worms. Domestic animals are usually exposed to ethion during dipping or spraying to remove ectoparasites (Mosha et al., 1990). Poultry may be exposed to ethion if their feed is contaminated and such exposure has been ably documented by Keating (1977).

Most of the mutagenicity tests on ethion have been done in in vitro assays, both in prokaryotes and eukaryotes. Garrett et al. (1986) in their review paper considered ethion to be non-mutagenic in Salmonella and other in vitro test systems. Similar results were also obtained by other workers (Moriya et al., 1983; EMSI, 1987). However, the mutagenicity of a commercial grade ethion (50 % purity) has been reported in mice in vivo test system by Bhunya and Behera (1989). There is no record in the literature regarding the genotoxic effect of ethion in chick system. So it was thought worthwhile to study the genotoxic effect of ethion in chick test system.
MATERIALS AND METHODS

Test chemical: Technical grade ethion (purity 95%) donated by Rallis India Ltd., Bombay, served as the test chemical. Glass double distilled water was used as vehicle. The application volume was 10 ml/kg b.w.

Doses: Three different doses; 10, 15 and 20 mg/kg b.w. were used in the present investigation.

Experimental protocol: Detailed description of the experimental design for BMCA, MNT and statistics have been described in "General materials and methods".

RESULTS

General toxicity: After the administration of the chemical, toxicity symptoms such as loss of appetite, salivation from the mouth, mild ataxia of the legs and incoordinated movement were observed. These symptoms were less obvious in chronic treatment and with lower doses.

Genotoxicity:

Chromosome aberration: (Figs. 46 a-f, 47, 48 and 52; Table - 19)

Qualitative: The chemical induced aberrations like chromatid gaps, breaks, deletions and exchange in the treated series. Gaps and breaks were more frequent than other types of aberrations.

Quantitative: In dose-response studies the chemical produced highest percentage (5.50%) of aberrations by the highest dose (20 mg/kg) in the i.p. route. In p.o. route the aberration was less (3.00%) in comparison with the i.p. route. Repeated exposures (5 days chronic) of fractionated dose induced less aberration (1.50%) than the equivalent acute dose treated once. In 30 days repeated treatment with the
fractionated dose regimens of the highest acute dose, the chemical produced higher frequency of aberrations (6.00%) and was significantly different ($t = 5.003; df = 6; p < 0.01$) from the respective control. No significant variations were observed (ANOVA) among the three different doses used in the dose-response study ($F = 0.942; df = 2, 9; p > 0.05$). However, significant variations were obtained (ANOVA) among time-response analyses ($F = 6.222; df = 3,12; p < 0.05$).

Regarding distribution of gap and break points within the different regions of the chromosomes it was found that the middle and the distal portion of the chromosomes were relatively more sensitive to the aberrations. A single aberration was observed also in the proximal region of chromosome 1.

**Micronuclei:** (Figs. 49 a-h, 50, 51, 53 and 54; Tables- 20-22)

The incidence of MN in both bone marrow and peripheral blood erythrocytes was significantly higher than the respective control value with all the three doses and in both routes. However, highest frequency of aberration was observed in both the assays after 30 days chronic treatment with the fractionated doses of the highest acute dose. No significant variations were observed among the results of different doses in bone marrow ($F = 1.214; df 2,9; p > 0.05$) and peripheral blood erythrocyte ($F =0.759; df = 2,9; p > 0.005$) micronucleus test. Besides the induction of MN, the chemical induced different types of nuclear anomalies.

The insignificant difference in the body weight between the control and the treated animals at the end of fourth week indicates that the daily administration of such a low quantity of pesticide had no significant effect on the general growth rate of the chicks.

**DISCUSSION**

Ethion has been reported to be non-mutagenic in a number of prokaryotic and eukaryotic test systems (Garrett et al., 1986;
EXPLANATION OF FIGURE

Fig. 46. Photomicrographs of bone marrow metaphase plates showing different types of chromosomal aberrations induced by ethion:

(a) chromatid gap in the q-arm chromosome 4.
(b) chromatid break in the q-arm of chromosome 4.
(c) chromatid break in the q-arm of chromosome 2.
(d) chromatid break in the q-arm of chromosome 1.
(e) chromatid break in chromosome 3.
(f) chromatid break in the q-arm of chromosome 2.
EXPLANATION OF FIGURES

Fig. 47. Histograms showing the frequencies of ethion induced chromosome aberrations in the bone marrow cells of chicks:

(a) after different time intervals.
(b) by acute and chronic exposures.

Fig. 48. Histograms showing the frequencies of chromosome aberrations in the bone marrow cells of chicks induced by ethion through different routes.
EXPLANATION OF FIGURE

Fig. 49. Photomicrographs showing ethion induced micronuclei and nuclear anomalies in the bone marrow and peripheral blood erythrocytes of chicks:

(a) bone marrow erythrocyte showing separation of cytoplasm but not nuclear material.
(b) bone marrow erythrocyte with one micronucleus.
(c) two bone marrow erythrocytes with one small and large micronucleus respectively.
(d) bone marrow erythrocyte with one micronucleus.
(e) peripheral blood erythrocyte having dumb bell shaped nuclear material along with cytoplasm.
(f) peripheral blood erythrocyte with one micronucleus.
(g)-(h) peripheral blood erythrocytes with one large MN adjacent to the nucleus.
EXPLANATION OF FIGURES

Fig. 50. Histograms showing the frequencies of ethion induced micronuclei in the bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks exposed to different doses through different routes.

Fig. 51. Histograms showing the frequencies of micronuclei induced by ethion in BM and PB erythrocytes after 30 days chronic exposure.
Fig 50

Fig 51
EXPLANATION OF FIGURES

Fig. 52. Linear regression analysis on chromosome aberration frequencies in the bone marrow cells of chicks. C in abscissa indicates control.

Fig. 53. Linear regression analysis on micronuclei frequencies in bone marrow (BM) and peripheral blood (PB) erythrocyte of chicks. C in abscissa indicates control.
Fig 52

% OF ABERRATION

DOSE (mg/kg)

r = 0.974 (P < 0.01)
b = 0.288

Fig 53

% OF ABERRATION

DOSE (mg/kg)

r = 0.972 (P < 0.01)
b = 0.05

r = 0.979 (P < 0.01)
b = 0.04

BM

PB
EXPLANATION OF FIGURE

Fig. 54. Weekly increase in body weight of control and treated groups of chicks over a period of 30 days. Each point represents the mean body weight of four chicks plus or minus the standard deviation.
Body weight (g) vs. weeks

- Control
- Treated

Fig 54
Table 19

Frequency of chromosome aberrations induced by ethion in the bone marrow cells of chicks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Fixation time (h)</th>
<th>No. of cells studied</th>
<th>Chromatid gaps breaks</th>
<th>Isochromatid gaps breaks</th>
<th>Deletions</th>
<th>Exchange</th>
<th>Total</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.p.</td>
<td>6</td>
<td>200</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>1(2)</td>
<td>0.50 ± 0.50b</td>
</tr>
<tr>
<td>20</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>5</td>
<td>7</td>
<td>-</td>
<td>1</td>
<td>3</td>
<td>11(16) 5.50 ± 0.96a</td>
</tr>
<tr>
<td>20</td>
<td>1.p.</td>
<td>48</td>
<td>200</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>5(11)</td>
<td>2.50 ± 0.96b</td>
</tr>
<tr>
<td>15</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>8(11)</td>
<td>4.00 ± 2.16</td>
</tr>
<tr>
<td>10</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>1</td>
<td>5(6)</td>
<td>2.50 ± 1.26</td>
</tr>
<tr>
<td>4 X 5</td>
<td>1.p.</td>
<td>120</td>
<td>200</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>3(10)</td>
<td>1.50 ± 0.96b</td>
</tr>
<tr>
<td>0.667X30</td>
<td>1.p.</td>
<td>24X30</td>
<td>200</td>
<td>9</td>
<td>12</td>
<td>1</td>
<td>-</td>
<td>12(22)</td>
<td>6.00 ± 0.82*</td>
</tr>
<tr>
<td>20</td>
<td>p.o.</td>
<td>24</td>
<td>200</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>6(8)</td>
<td>3.00 ± 0.58*</td>
</tr>
</tbody>
</table>

Results in the parentheses are including of chromatid and isochromatid gaps.

+ Values are excluding of chromatid and isochromatid gaps.

* Result is significantly different from the control at p < 0.01 (Student’s t-test)

a, b Groups with same superscript do not differ significantly from each other at p > 0.05 (ANOVA).
Table - 20

Incidence of micronucleated bone marrow cells of chicks induced by ethion.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.p.</td>
<td>16,000</td>
<td>19</td>
<td>1.19 ± 0.16**</td>
<td>4</td>
<td>0.25 ± 0.18</td>
</tr>
<tr>
<td>15</td>
<td>1.p.</td>
<td>16,000</td>
<td>16</td>
<td>1.00 ± 0.10**</td>
<td>3</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>1.p.</td>
<td>16,000</td>
<td>14</td>
<td>0.88 ± 0.16*</td>
<td>5</td>
<td>0.38 ± 0.16</td>
</tr>
<tr>
<td>0.667X30</td>
<td>1.p.</td>
<td>16,000</td>
<td>51</td>
<td>3.19 ± 0.28**</td>
<td>12</td>
<td>0.75 ± 0.31</td>
</tr>
<tr>
<td>20</td>
<td>p.o.</td>
<td>16,000</td>
<td>17</td>
<td>1.06 ± 0.12**</td>
<td>3</td>
<td>0.19 ± 0.12</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p < 0.05; ** p < 0.01 (Student's t-test)
Results are mean ± SE of four chicks/group. Results are significantly different from the control at * p < 0.05; ** p < 0.01 (Student's t-test).
### Table - 22

<table>
<thead>
<tr>
<th></th>
<th>0 week</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>38.50 ± 3.11</td>
<td>44.00 ± 3.56</td>
<td>60.75 ± 5.06</td>
<td>94.00 ± 7.35</td>
<td>120.25 ± 6.85</td>
</tr>
<tr>
<td>Control</td>
<td>36.75 ± 2.50</td>
<td>44.50 ± 3.11</td>
<td>66.00 ± 3.74</td>
<td>99.00 ± 6.05</td>
<td>129.50 ± 13.67</td>
</tr>
</tbody>
</table>

Mean of the weekly recorded weight (in grams) of 4 chicks ± SD
EMSI, 1987), while it failed to induce mutation in S. typhimurium either in the presence or absence of microsomal induction (Viswanath and Jamil, 1986). The mutagenicity of a commercial grade ethion has been reported in mice in vivo test system employing chromosome aberration, micronucleus and sperm abnormality assays (Bhunya and Behera, 1989). In the present investigation ethion induced significant (p < 0.01) chromosomal aberrations in both the routes by the highest tolerable dose (20 mg/kg) after 24 hours of exposure. The probable reasons for the induction of such type of effects by the chemical in bone marrow after 24 h of exposure have been discussed elsewhere (Please see the discussion of monocrotophos). Furthermore in the present study highest effect was induced by the highest dose. It has been reported that structural determinants of the chemicals are responsible in order to exert their effects at the maximum tolerated dose (MTD) (Rosenkranz and Klopman, 1993). In the present investigation ethion produced significant chromosome aberrations by the maximum tolerable dose (20 mg/kg b.w.). So it is envisaged that the structural determinants of the pesticide might also be responsible for such type of result. Again at the highest dose the concentration of the chemical and/or its metabolites might have reached the optimum concentration for the production of significant effects. The insignificant results after 48 h acute and 5 days chronic studies with fractionated dosing indicate the poor availability of the chemical in the target tissue. The rate of detoxification of organophosphate pesticides is very fast in mammals and these substances do not accumulate in the bone marrow and testis and are excreted rapidly (Degraeve et al., 1984). The elimination and tissue distribution of $^{14}$C ethion laying hens and eggs after oral exposure was studied by Mosha et al. (1990) and they have reported that concentration of the chemical was highest in liver and kidney and less in fatty tissue and skeletal muscle. This might be the reason for less effect after 48 h and 5 days (chronic) exposure in which the chemical could not raise the optimum concentration in the target tissue. The induction of significant aberration after 30 days chronic exposure with the repeated dosing suggests the cumulative effect of the chemical after a long time exposure with a low dose.
The induction of micronuclei in both bone marrow and peripheral blood erythrocytes in response to treatment with test chemicals was recognised as a sensitive indicator of genotoxic potential (Schmid, 1976; Maier and Schmid, 1976; MacGregor et al., 1980). The significant increase of ethion induced MN in the erythrocytes of bone marrow and peripheral blood demonstrates its genotoxicity in the tested system.

The exact mechanism of action of ethion in molecular level is not yet properly known. However, it is assumed that it might have acted in a manner like that of other organophosphate compounds possessing alkylating properties (Wild, 1975; Natarajan et al., 1983). Again several phosphorothioates can lead to mutagenesis (Breau et al., 1959). It is envisaged that ethion being a phosphorodithioate, may produce its mutagenic effect in the similar way like that of other phosphorothioates. Again ethion being an ethyl phosphorodithioate ester, produced less effect than other methyl phosphate esters (EtO, 1974). This may be one of the plausible reasons for the inconsistent results obtained so far in different test systems.
GENERAL COMMENTS

The effects of the four organophosphorus insecticides (monocrotophos, acephate, phosphamidon and ethion) in the present chick in vivo test system have been discussed individually earlier. In the present chapter an attempt has been made to generalise their effects.

Organophosphates are usually absorbed rapidly by inhalation, ingestion and through the skin but do not accumulate in the body (Matsumura, 1976; IARC, 1983). Apart from possible genetic risk, the organophosphate compounds have similar signs and symptoms of acute systemic poisoning and are related to the inhibition of acetylcholinesterase. After the administration of these insecticides, different toxicity symptoms were observed in the treated series. The detailed general toxicity symptoms have been described under individual chemicals. The magnitude of toxicity symptoms varied according to the doses, routes and durations of exposure. Generally, the maximum acute toxicity symptoms were observed by the highest dose administered intraperitoneally. The acute toxicity of organophosphorus insecticides is due to their anticholinesterase action. It has been reported that for single exposure, a dose-effect relationship exists between the dose and the severity of symptoms and also the degree of acetylcholinesterase (AChE) inhibition in the nervous tissue (WHO, 1986). In chicks organophosphorus ester like desbromoleptophos (DBL) inhibited acetylcholinesterase (AChE) and neuropathy target enzymes (NTE) exposed on day 3 or day 15 of incubation or 10 days post hatching (Farage-Elawar et al., 1991). Chick embryos and young chicks showed post hatching paralysis and altered muscle morphology after in ovo exposure to leptophos (Misawa et al., 1982; Sheets and Norton, 1985).

Dose response studies are important in the evaluation of mutagenicity: the confidence with which a mutagenic effect can be established is strengthened by the observation of an increasing
incidence of mutagenic effects with increasing dose. In chromosome aberration assay the mutagenic effects induced by each chemical increased with dose except acephate in which the highest dose failed to induce the highest percentage of aberration (Fig. 55). Whereas, the middle dose of acephate (50 mg/kg b.w.) produced significant effect and which is equivalent to the aberration produced by the highest dose of ethion in the acute treatment. Except acephate, a positive linear dose-dependent effect was observed in the rest three insecticides. The information on metabolic transformation and excretion of these pesticides in chicks and consequent cytogenetic effects are lacking in the literature for which a further comparison among themselves is not possible. The induction of significant effects by these four insecticides in acute treatment, revealed the clastogenic potential of the tested chemicals in the present test system. However, on the basis of the frequency of chromosome aberration produced by the highest dose after 24 hours, the clastogenic potential of the chemicals can be graded as follows:

\[
\text{ethion} > \text{monocrotophos} > \text{phosphamidon} > \text{acephate}.
\]

Organophosphates are thought to be rapidly absorbed by body through different routes of administration. So the study of these pesticides in different routes (i.p. and p.o.) is quite important. In the present investigation higher percentage of aberration was produced in i.p. than oral route. Only ethion produced significant effect in both the routes while monocrotophos, acephate and phosphamidon were inactive (insignificant) in p.o. route (Fig. 56). This type of route response effects of the chemicals are quite expected since absorption, retention and elimination of a test compound in in vivo system are intricately related to the path of the entry of the chemicals into the body (Nuebert, 1974 and Matter, 1976). Again body metabolism plays important role for activation or detoxification of a particular compound which in turn is also related to route of administration. For example, relatively higher quantities of orally administered chemical has to pass through liver where activation or detoxification of xenobiotics takes place.
Fig. 55. Composite graph showing chromosome aberrations induced by different doses of four organophosphate insecticides (E = Ethion, M = Monocrotophos, P = Phosphamidon and A = Acephate).
Fig. 56. Histograms showing the frequencies of chromosome aberrations induced by the highest dose of four organophosphate insecticides treated in different routes (i.p. and p.o.).
Fig. 56
It has been shown that radiolabelled bromophos at a dose of 10 mg/kg body weight, approximately 96% of the radiolabel was absorbed from the gastrointestinal tract and excreted in the urine within 24 hours of oral administration. There is also evidence of comparatively inefficient absorption in hens administered large doses of insoluble organophosphorus pesticides like haloxon and leptophos (WHO, 1986). So the insignificant result in p.o. route may be due to the detoxification of organophosphate chemical in liver. It may also be due to less absorption from the alimentary tract and/or higher rate of elimination with faecal matter.

In time-response analyses the highest dose was tested for three different (6, 24 and 48 h) exposure times. The histograms(Fig. 57) show that the highest frequency of aberration was produced after 24 hours of treatment. But the yields of chromosomal aberrations after 6 and 48 hours of treatment were not uniform for every chemical. However, apparently a trend of time-dependent effects induced by the chemical was noted. The results induced by these four insecticides after 6 and 48 hours of exposure were found to be statistically insignificant.

A comparative genotoxicity assessment of the insecticides was also made for acute and chronic dosing (Fig. 58). Besides short term (5 days) chronic treatment, three insecticides (monocrotophos, acephate and ethion) were also tested for 30 days (long term) exposure (see Results). In five days chronic treatment with the fractionated doses of the highest acute dose, all the tested insecticides induced lower frequencies of aberrations than the equivalent acute dose treated once. Committee-17 (1975) stated that chronic or low dose irradiation produces only about 1/3rd as many mutations as does acute dosing. It is assumed that very likely the same also holds true for environmental chemicals. Such results have also been reported earlier by Bhunya and Pati (1990) and Behera and Bhunya (1989) for other pesticides tested in mouse in vivo system.
EXPLANATION OF FIGURE

Fig. 57. Histograms showing the time-response frequencies of chromosomal aberrations induced by the highest dose of four organophosphate insecticides treated i.p.
Fig. 57

% of aberration

Fixation times (Hours)

Monocrotophos  | Acephate  
Phosphamidon   | Ethion
EXPLANATION OF FIGURE

Fig. 58. Histograms showing the frequencies of chromosome aberrations induced by the four organophosphate insecticides after acute and chronic exposures.
Fig. 58
In 30 days chronic treatment with the fractionated dosing all the three pesticides produced significant effects over the respective control values (Fig. 59). Acephate and ethion produced a nearly same effect to that of equivalent acute dose treated once while monocrotophos produced higher frequency of aberrations. Sram (1976) reported that the fractionated application of TEPA, thio-TEPA, EMS and cyclophosphamide produced same or higher frequency of dominant lethals and chromosome aberrations in the bone marrow cells of mice than produced by the equivalent dose treated once. However, Degraeve et al. (1984) reported that the organophosphate insecticides applied in the small doses for several weeks induced low (insignificant) frequency of chromosome aberrations. Besides genotoxicity, chemicals have also different types of adverse effects in both somatic and germinal tissues upon exposure to a low level chronic treatment. In a 90 days toxicity study with dichloroacetate (DCA), degeneration of testicular germinal epithelium and formation of syncytial giant cells were noted in male beagle dogs (Cicmanec et al., 1991). Another 90-days sub-chronic toxicity study with chloroacetic acid revealed variable degrees of alternations in the lung and liver of rats (Bhat et al., 1991). The significant results produced in chronic study indicate a potent cumulative effect of the chemical in the target organ. Considering the results of the previous workers and those of the present study it is envisaged that probably the relationship between acute and chronic exposure in mutagenicity studies varies from chemical to chemical.

Micronucleus test is an important assay for the detection of mutagens and carcinogens in vivo (CSGMT, 1990). Experimental data on micronucleus test have proved that this test is a more sensitive and reliable method than metaphase scoring in the screening of chemical agents for chromosome aberration (Hedde, 1973; Matter and Grauwiler, 1974; Schmid, 1976; Maier and Schmid, 1976). The micronucleus assay also provides an indirect measure of the induction of structural and numerical chromosomal changes (Mavournin et al., 1990). In the present experiment all the four organophosphate insecticides were tested for the induction
EXPLANATION OF FIGURE

Fig. 59. Histograms showing frequencies of chromosome aberrations (CA) and micronuclei in bone marrow (BM) and peripheral blood (PB) erythrocytes induced by three organophosphate insecticides after 30 days chronic exposures.
Fig. 59

Insecticides

- Monocrotophos
- Acephate
- Ethion

CA %
BM %
PB %
of MN in both bone marrow and peripheral blood erythrocytes. For dose-response analysis, different doses of the chemicals were administered through i.p. route (Fig. 60-61). Only the highest dose of each chemical was tested in p.o. route for route-response studies.

In bone marrow micronucleus assay all the four insecticides induced significant MN over the control by all the three doses treated intraperitoneally. Only ethion induced significant effect in p.o. route (Fig. 62). In 30 days chronic treatment with the fractionated doses of the highest acute dose, all the three insecticides induced significant yield of MN over the control (Fig. 59). Monocrotophos, failed to induce highest frequency of MN by the highest dose treated i.p.. A linear increase in the frequency of micronuclei with the dose was observed for the rest three (acephate, ethion and phosphamidon) insecticides. Besides the induction of MN, all the four insecticides induced different types of nuclear anomalies in the treated series.

In the peripheral blood micronucleus assay acephate and ethion produced significant MN by all the three doses treated i.p.. Monocrotophos failed to induce significant effect by the highest dose, whereas phosphamidon produced significant effect only by the two higher doses in i.p. route. Except monocrotophos, all the insecticides produced dose-dependent effects by all the three doses treated intraperitoneally (Fig. 63). Besides the induction of MN, other nuclear anomalies were also induced by all the four insecticides which have been summarized elsewhere (see results).

The exact mechanism of action of organophosphorus insecticides at the molecular level is not clearly known. The general metabolism of organophosphates occurs principally by oxidation, hydrolysis by esterases and by the transfer of a portion of the molecule to glutathione (WHO, 1986). The oxidation of organophosphorus insecticides may result in more or less toxic products. In general the phosphorus in the organophosphorus moiety appears to be a good substrate for nucleophilic
attack which may cause the phosphorylation of DNA and result in the ultimate DNA damage. All the four organophosphate insecticides tested in the present investigation belong to pentavalent phosphorus ester group. So their major reactions are phosphorylation and alkylation. The phosphorylation is responsible for the inhibition of acetylcholinesterase while mutation appears to be due to alkylation properties (Eto and Ohkawa, 1970). The alkylation reaction is more likely with the methyl ester group than with the ethyl and higher alkyl ester groups and phosphate esters are more reactive than the phosphorothionate esters (Eto, 1974). Thus the reactivity of acephate, monocrotophos and phosphamidon may be explained in the light of methylation reaction of phosphate esters. Since ethion is an ethyl ester it might act by a different mechanism. Considering all assay results in the present study the relative genotoxicity of the four insecticides has been determined to be ethion > phosphamidon > monocrotophos > acephate.
EXPLANATION OF FIGURE

Fig. 60. Composite graph showing frequencies of micronuclei in the bone marrow cells induced by four organophosphate insecticides (M = Monocrotophos, P = Phosphamidon, E = Ethion and A = Acephate).
Fig 60
Fig. 61. Composite graph showing frequencies of micronuclei in the peripheral blood erythrocytes induced by four organophosphate insecticides (A = Acephate, E = Ethion, P = Phosphamidon and M = Monocrotophos).
Fig 61
EXPLANATION OF FIGURE

Fig. 62. Histograms showing route-dependent frequencies of micronuclei in bone marrow erythrocytes induced by four organophosphate insecticides.
Fig. 62

% of aberration

Routes

- Monacrotophos
- Phosphamidon
- Acephate
- Ethion
EXPLANATION OF FIGURE

Fig. 63. Histograms showing route-dependent frequencies of micronuclei in peripheral blood erythrocytes induced by four organophosphate insecticides.
Fig. 63
Organochlorine Compound

5. LINDANE (γ-BHC®)

Lindane (γ-isomer of benzene hexachloride (BHC)), is a potent insecticide employed for agricultural and medicinal purposes (Solomon et al., 1977). The present organochlorine pesticide lindane is the technical grade (99% BHC) containing 23% γ-isomer. Its chemical name is 1,2,3,4,5,6-hexachlorocyclohexane.

In our country lindane is recommended to be used as broad spectrum insecticide for apples, beans, peas, cole crops, cucurbits, tomatoes and other vegetables. It has also been recommended as a general or multipurpose insecticide in many countries (Singh et al., 1988). Like other organochlorine compounds it enters animal tissues through the food chain, inhalation into lungs or by direct diffusion through the skin and then accumulates in the adipose tissue and in the membrane lipid bilayer of all cells (Zhu et al., 1986; Lopez-Aparicio et al., 1989). Literature on the neurotoxicity of lindane is on record (see Smith, 1991) and it also possesses properties for mutagenicity, carcinogenicity and teratogenicity (Wolff et al., 1987). Lindane has been studied inadequately epidemiologically for cancer incidence, but the technical grade lindane and the α-isomer are carcinogenic to experimental animals, causing liver and lymphoreticular neoplasm (IARC, 1979; 1987). Degenerative changes in the testis and decreased sexual receptivity caused by lindane in mammals have been reported (U'house, 1987; Srinivasan et al., 1988). Parent-Massin et al. (1994) evaluated the haematotoxicity of lindane by the in vitro tests on human and rat bone marrow progenitors.
Studies have been carried out with different isomers of BHC to evaluate its mutagenic potential in various test systems. A clastogenic property of lindane in a number of plant root tip cells has also been reported (Epstein and Ligator, 1973; Jain and Sarbhoy, 1987a; Dhingra et al., 1987. It gave negative mutagenic activity in bacteria (Shirasu et al., 1975) or in yeast (Shahin and Von Borstel, 1977). Negative effects of lindane have been reported in unscheduled DNA synthesis (UDS) (Ahmed et al., 1977), micronucleus test and Ames test (Jenssen and Ramel, 1980), while it could induce dominant lethals in mice (Lakkad et al., 1982). It was also reported to be negative for gene mutations in V79 cells and for SCEs in in vivo mouse bone marrow after both oral and i.p. administration (see Dearfield et al., 1993). Its genotoxic effect has been reported in cultured human lymphocytes by Rupa et al. (1989). However, there is no information in the literature on the mutagenic effect of lindane in birds. Considering these facts, it was thought imperative to study the genotoxic effect of lindane, employing chicken in vivo bioassays like bone marrow chromosome aberration and the micronucleus test in both bone marrow and peripheral blood erythrocytes.

MATERIALS AND METHODS

Test chemical: A technical formulation of lindane (γ-isomer of BHC), CAS NO. 58-89-9, donated by Southern Pesticide Corporation Ltd. (India), served as the test chemical. Dimethyl sulphoxide (DMSO) was used as the vehicle.

Dose: Three different doses (50, 75 and 100 mg/kg) were used. Negative controls received only 0.1 ml of the solvent (DMSO) per animals (25-30 g body weight).

Experimental protocol: The details of the experimental design of BMCA, MNT and statistical analyses have been mentioned in "General materials and methods".
RESULTS

General toxicity: General toxicity symptoms like shivering, convulsive seizures and increased spontaneous neuronal activity were exhibited by the animals just after the administration of the chemical and the intensity increased with dose and decreased with time. Further, the toxic symptoms were less in p.o. than in i.p. administration.

Genotoxicity:

Chromosome aberration: (Figs. 64 a-d, 65, 66 and 70; Table 23).

Qualitative: Aberrations produced by the chemical included chromatid gaps and breaks, isochromatid gaps and breaks and exchange like dicentric and rings. Some cases of chromatin stretching were also recorded.

Quantitative: Quantitatively the highest dose induced the highest percentage of aberrations after 24 hours of treatment. Chronic dosing induced less effect than the equivalent acute dosing. Significant variations were obtained among the results of time-response analyses (F = 5.29; df = 3, 12; p < 0.05). The results for 6 versus 120h and 24 versus 48 h respectively, do not differ significantly from each other. However, significant variations were noted between the highest (100 mg/kg) and the lowest (50 mg/kg) doses (p < 0.05). The result for the p.o. route was lower than that for the i.p. route, but was not significantly elevated over the respective control value (t = 1.68; df = 6, p > 0.05).

Regarding the regional sensitivity of chromosomes to lindane, the first two pairs of macrochromosomes were more vulnerable to the chemical assault, than the rest chromosomes considered for aberration scoring.

Micronucleus: (Figs. 67 a-h, 68, 69, 71 and 72; Tables 24-25)

The size as well as the location of MN within the cytoplasm varied from cell to cell. In general one MN per cell was recorded. But a few
Fig. 64. Photomicrographs of bone marrow metaphase spreads showing different types of chromosomal aberrations induced by lindane:

(a) chromatid break in the p-arm of chromosome 1.
(b) iso-chromatid gap in the q-arm of chromosome 2.
(c) ring chromosome.
(d) dicentric chromosome.
Fig. 65. Histograms showing the frequencies of lindane induced chromosome aberrations in the bone marrow cells of chicks:
(a) after different times of exposures.
(b) by acute and chronic exposures.

Fig. 66. Histograms showing the route-response chromosome aberration frequencies in the bone marrow cells of chicks.
Fig 65 (a,b) FIXATION TIMES (HOURS)

Fig 66
Fig. 67. Photomicrographs showing lindane induced micronuclei in the bone marrow and peripheral blood erythrocytes of chicks:

(a)-(d) bone marrow erythrocytes with one relatively large micronuclei in each, having largest one in C.

(e) peripheral blood erythrocytes with MN extruded with a chunk of cytoplasm.

(f)-(g) peripheral blood erythrocytes with large MN.

(h) peripheral blood erythrocytes with one MN in each.
EXPLANATION OF FIGURES

Fig. 68. Histograms showing the frequencies of micronuclei in bone marrow (BM) and peripheral blood (PB) erythrocytes induced by different doses in i.p. route.

Fig. 69. Histograms showing the frequencies of micronuclei in bone marrow (BM) and peripheral blood (PB) erythrocytes induced by different doses in p.o. route.
Fig. 70. Linear regression analysis on chromosome aberration frequencies in the bone marrow cells of chicks. C in abscissa indicates control.
Fig. 70

% OF ABERRATION

DOSE (mg/kg)

\[ r = 0.974 \quad (P < 0.01) \]
\[ b = 0.062 \]
EXPLANATION OF FIGURES

Fig. 71. Linear regression analysis on micronuclei frequencies in bone marrow (BM) erythrocytes of chicks induced by i.p. and p.o. routes.

Fig. 72. Linear regression analysis on micronuclei frequencies in peripheral blood (PB) erythrocytes of chicks induced by i.p. and p.o. routes.
cells with two micronuclei were also seen. In some rare cells extrusion of the cytoplasm along with a micronucleus was also observed. Additionally, a few anaphase bridges, irregular shaped nuclei and some binucleated cells were also observed in the treated series.

The incidence of MN in bone marrow erythrocytes was significantly higher than the control with all three doses and both routes and only with the two higher i.p. doses for peripheral blood erythrocytes. No significant variations were obtained among the dose-response analyses for i.p. (F = 1.44; df = 2,9; p > 0.05) and p.o. (F = 2.62; df = 2,9; p > 0.05) treatments. The variations for peripheral blood results were also not significant for either the i.p. (F = 0.967; df = 2,9; p > 0.05) or the p.o. (F = 0.541; df = 2,9; p > 0.05) routes.

**DISCUSSION**

It is evident from the results that the tested pesticide is genotoxic in the present system. The chromosome aberration assay results clearly indicate its clastogenic potential in the chick in vivo system. The present results support the findings of Jain and Sarbhoy (1987a) who reported the clastogenic property of lindane in Lens and Pisum and the findings of Tzoneva-Maneva et al. (1971) who reported 7-BHC induced chromosomal aberrations in human lymphocytes cultures in vitro. Lindane induced DNA strand breaks but not UDS in rat primary hepatocytes; it did not induce UDS in human cells in vitro (see Dearfield et al., 1993). The organochlorine compounds and their metabolites have been implicated as carcinogenic and esterogenic agents (WHO, 1974; Bulger and Kupfer, 1985). Induction of micronuclei by the chemical is also indicative of clastogenicity and cytotoxicity, since micronuclei can be formed either by chromosomal fragments or by whole chromosomes lagging in anaphase.

The mechanism of action of lindane at the molecular level remains poorly understood. So far, the majority of studies have related the toxic
Table - 23

Frequency of chromosome aberrations induced by lindane in the bone marrow cells of chicks

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Fixation time (h)</th>
<th>No. of cells studied</th>
<th>Chromatid gaps breaks</th>
<th>Isochromatid gaps breaks</th>
<th>Chromatid stretching</th>
<th>Deletions</th>
<th>Rings</th>
<th>Total aberrations</th>
<th>Chromosome type exchange (Dicentric)</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>i.p.</td>
<td>6</td>
<td>200</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>3(5)</td>
<td>1.50 ± 0.96</td>
<td>d</td>
</tr>
<tr>
<td>100</td>
<td>i.p.</td>
<td>24</td>
<td>200</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>13(21)</td>
<td>6.50 ± 0.96</td>
<td>**a,c</td>
</tr>
<tr>
<td>100</td>
<td>i.p.</td>
<td>48</td>
<td>200</td>
<td>4</td>
<td>4</td>
<td>-</td>
<td>3</td>
<td>2</td>
<td>12(17)</td>
<td>6.00 ± 1.63</td>
<td>**c</td>
</tr>
<tr>
<td>75</td>
<td>i.p.</td>
<td>24</td>
<td>200</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>11(15)</td>
<td>5.50 ± 1.50</td>
<td>**b</td>
</tr>
<tr>
<td>50</td>
<td>i.p.</td>
<td>24</td>
<td>200</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>5(10)</td>
<td>2.50 ± 0.96</td>
<td>a</td>
</tr>
<tr>
<td>20X5</td>
<td>i.p.</td>
<td>120</td>
<td>200</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>4(9)</td>
<td>2.00 ± 0.82</td>
<td>d</td>
</tr>
<tr>
<td>100</td>
<td>p.o.</td>
<td>24</td>
<td>200</td>
<td>4</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>2</td>
<td>7(12)</td>
<td>3.50 ± 1.71</td>
<td></td>
</tr>
</tbody>
</table>

Results in the parentheses are including of gaps and stretches.
+ Values are excluding of chromatid, isochromatid gaps and chromatid stretches.
Result is significantly different from the control at * p<0.05, ** p<0.01 (Student's t-test)
Results with the same superscript differ significantly from each other at p<0.05 (ANOVA)
Results with the same superscript do not differ significantly from each other at p>0.05 (ANOVA).
### Table - 24

Incidence of micronucleated bone marrow cells of chicks induced by lindane

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.p.</td>
<td>16,000</td>
<td>14</td>
<td>0.87 ± 0.16**</td>
<td>8</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>75</td>
<td>1.p.</td>
<td>16,000</td>
<td>11</td>
<td>0.69 ± 0.12**</td>
<td>5</td>
<td>0.31 ± 0.06</td>
</tr>
<tr>
<td>50</td>
<td>1.p.</td>
<td>16,000</td>
<td>9</td>
<td>0.56 ± 0.16*</td>
<td>4</td>
<td>0.25 ± 0.10</td>
</tr>
<tr>
<td>100</td>
<td>p.o.</td>
<td>16,000</td>
<td>10</td>
<td>0.62 ± 0.16**</td>
<td>3</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>75</td>
<td>p.o.</td>
<td>16,000</td>
<td>4</td>
<td>0.25 ± 0.10*</td>
<td>1</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>50</td>
<td>p.o.</td>
<td>16,000</td>
<td>6</td>
<td>0.37 ± 0.07**</td>
<td>2</td>
<td>0.12 ± 0.12</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group

Result is significantly different from the control at * p < 0.05, ** p < 0.01 (Student's t-test)
Table - 25
Incidence of micronucleated peripheral blood cells of chicks induced by lindane

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>i.p.</td>
<td>16,000</td>
<td>14</td>
<td>0.87 ± 0.30*</td>
<td>10</td>
<td>0.62 ± 0.39</td>
</tr>
<tr>
<td>75</td>
<td>i.p.</td>
<td>16,000</td>
<td>10</td>
<td>0.62 ± 0.12**</td>
<td>3</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>50</td>
<td>i.p.</td>
<td>16,000</td>
<td>7</td>
<td>0.44 ± 0.21</td>
<td>4</td>
<td>0.25 ± 0.10</td>
</tr>
<tr>
<td>100</td>
<td>p.o.</td>
<td>16,000</td>
<td>7</td>
<td>0.44 ± 0.19</td>
<td>5</td>
<td>0.31 ± 0.16</td>
</tr>
<tr>
<td>75</td>
<td>p.o.</td>
<td>16,000</td>
<td>6</td>
<td>0.37 ± 0.19</td>
<td>1</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>50</td>
<td>p.o.</td>
<td>16,000</td>
<td>4</td>
<td>0.25 ± 0.10</td>
<td>2</td>
<td>0.12 ± 0.07</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p < 0.05; ** p < 0.01 (Student's t-test)
properties of hexachlorocylohexane (HCH) isomers mainly to their potential
tumorigenic effects (Wolff et al., 1987; Reuber, 1980). Previous data have
shown that \( \gamma \)-HCH is a more potent Ca\(^{2+} \) mobilizer from intracellular
stores than are the \( \alpha \)- and \( \beta \)-isomers. Lindane has been reported to
activate protein kinase C and to modulate important cell functions (Kuhns
et al., 1986). Lindane is highly lipophilic and also modifies lipid
biosynthesis (Gutierrez-Ocana et al., 1989).

In the present study the chromosome aberration frequency induced by
the highest dose differed significantly from that induced by the lowest
dose, which might indicate that the chemical was available to a greater
extent at the target tissue/cell with the highest dose than the lowest
dose. The insignificant number of chromosome aberrations produced by
chronic dosing suggests little cumulative effect of the chemical, and also
reinforces the suggestion made by Committee 17 (1975) in connection with
acute and chronic dosing with irradiation and some chemicals (Bhunya and
Pati, 1990) and Behera and Bhunya (1989) for other pesticides.

The micronucleus test is comparable to or even more sensitive and
reliable than metaphase scoring for chromosome damage (Schmid, 1976). The
significant increase in micronuclei due to the chemical treatment also
indicates the clastogenic and spindle-poisoning property of lindane. Both
in bone marrow and peripheral blood the pesticide induced more micronuclei
when administered i.p. than p.o.. The reason for such a result may be
attributed to a higher rate of elimination of the chemical from the body
when administered orally. Such route sensitive results have been obtained
with a number of other chemicals (Pati and Bhunya, 1989; Bhunya and
Behera, 1987).

In summary, the present findings demonstrate the ability of lindane
to induce cytogenetic damage in the chick in vivo. Further studies in
details employing other test systems are needed for better evaluation of
its mutagenic potential. Since lindane was found genotoxic in the present
chick in vivo test system. Its cautious handling and restricted use in
future are suggested.
Carbamate insecticides are widely used in today's agriculture due to their low persistence and broad spectra of action and ability to control pests (Wagner, 1983). Carbamates have been used as broad spectrum insecticides due to their contact and systemic toxicity and act by inhibiting acetylcholinesterase (AChE) in the nervous system of insects and other animals (Matsumura, 1985; Cramner, 1986). However, records on toxicological effects of carbamates in the biological system are not scanty (Farage-Elawar and Blaker, 1992; Anton et al., 1993).

Carbaryl (1-napthyl-N-methylcarbamate) or sevin® is widely used against insects which attack fruits, vegetables, cotton, tobacco, corn, rice, sugar beets, animal and live stock, ornamental trees and shrubs (USEPA, 1972). It gave a positive result for Ames salmonella test system and for chromosome aberration test for both in vivo and in vitro mammalian test systems (Ishidate et al., 1988). Methylcarbamate was found to be carcinogenic in both male and female rats (Fung et al., 1993). It has also been reported that anticholinesterase (anti ChE) insecticides are potential disregulators of immunity (Casale et al., 1992). Record on the genotoxicity of carbaryl in chick test system is lacking in the literature. So it was thought worthwhile to evaluate the genotoxicity of carbaryl in chick in vivo test system.
MATERIALS AND METHODS

Test chemical: Commercial grade carbaryl (sevin[®]) (50 %) pure purchased from local market served as the test chemical.

Dose: In the present study, three different doses (100, 125 and 150 mg/kg b.w.) were used.

Experimental protocol: The details of the experimental design of BMCA, MNT and statistics have been described in "General materials and methods".

RESULTS

General toxicity: After the administration of the chemical, symptoms like body shivering, ataxia or paralysis primarily in the legs were observed. In some individuals twitching of the body muscles and hyperactivity were also observed. The animals resumed to be normal after 4 to 6 hours of treatment. These symptoms were milder with lower doses. During chronic treatment these symptoms were not evident.

Genotoxicity:

Chromosome aberration: (Figs. 73 a-d, 74, 75 and 78; Table- 26)

Qualitative: Carbaryl induced only chromatid type gaps, breaks and deletions in the bone marrow cells of chicks. Chromatid gaps and breaks occurred more frequently than other types of aberrations.

Quantitative: In dose-response study the highest frequency (6.00 %) of aberration was produced by the dose 150 mg/kg b.w. and the lowest(3.00%) by the dose 100 mg/kg b.w. of carbaryl. All the three doses induced significant aberrations over the control after 24 hours of exposure. In
time-response studies all the time points induced higher percentage of aberrations except 6 hour exposure with 150 mg/kg b.w. of the chemical and the results were not significant. The result of the chronic study was very less (1.00 %) in comparison to the acute study. It was evident that in dose-response studies the results were more or less dose-dependent although the difference between the results of the doses was not significant ($F = 4.204; \, df = 2,9; \, p > 0.05$) (ANOVA). In route-response analyses the result was not significant.

A tentative analysis on the distribution of gaps and breaks within and between the chromosomes of bone marrow cells revealed that chromosome 1 and 4 were most vulnerable to the aberration induced by carbaryl. Chromosome 3 was not at all sensitive to gaps and breaks.

**Micronucleus:** (Figs. 76 a-h, 77 and 79; Tables 27-28).

The results of the incidence of micronuclei for both bone marrow and peripheral blood by the treatment of three doses of (150, 125 and 100 mg/kg b.w.) of carbaryl in i.p. and p.o. routes have been presented in tabular form. Besides the induction of micronuclei, quantitatively some of the nuclear anomalies like sickle shaped nuclei, separation of two nuclei having thin connection, constriction and thinning in the middle region of the nuclei along with anaphase laggard and bridge in bone marrow cells were also taken into account. In rare cases some binucleated cells were also observed. The MN are generally round, smooth in outer line texture and located in the cytoplasm of the cell. The location and size of the MN varied from cell to cell.

In the bone marrow cells all the three doses and in peripheral blood erythrocytes only the two higher doses could induce significantly higher percentage of MN by the i.p. route only. The result is not significant in p.o. route in both bone marrow and in peripheral blood
Fig. 73. Photomicrographs of bone marrow metaphase spreads showing different types of chromosomal aberrations induced by carbaryl:

(a) chromatid gap in q-arm of chromosome 4.
(b) chromatid break in the terminal region of q-arm of chromosome 1.
(c) chromatid breaks in the proximal region of q-arm of chromosome 2.
(d) chromatid break in the q-arm of chromosome 4.
EXPLANATION OF FIGURES

Fig. 74. Histograms showing the frequencies of chromosome aberrations induced by carbaryl in the bone marrow cells of chicks:

(a) after different time intervals.
(b) by acute and chronic exposures.

Fig. 75. Histograms showing the route-response chromosome aberration frequencies in the bone marrow cells of chicks.
Fig 74(a,b) FIXATION TIMES (HOURS)

- (a) % OF CHROMOSOMAL ABERRATION
- (b) % OF CHROMOSOMAL ABBERRATION

Fig 75

ROUTES

Acute
Chronic
Fig. 76. Photomicrographs showing micronuclei and nuclear anomalies in bone marrow and peripheral blood cells of chicks induced by carbaryl:

(a) bone marrow cell showing anaphase division with laggard.
(b) bone marrow cell showing separation of chromosomes connected by chromatin bridge.
(c)-(d) bone marrow erythrocytes with one micronucleus in each.
(e) peripheral blood erythrocyte with unequal separation of chromatin and cytoplasmic material.
(f) peripheral blood erythrocyte showing constriction of the nucleus.
(g)-(h) peripheral blood erythrocytes with one micronucleus in each.
EXPLANATION OF FIGURE

Fig. 77. Histograms showing the frequencies of micronuclei induced by carbaryl in the bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks exposed to different doses through different routes.
EXPLANATION OF FIGURES

Fig. 78. Linear regression analysis on chromosome aberration frequencies in the bone marrow cells of chicks. C in abscissa indicates control.

Fig. 79. Linear regression analysis of micronuclei frequencies in bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks. C in abscissa indicates control.
Fig 78

$r = 0.963 \ (P < 0.01)

b = 0.03$

Fig 79

$r = 0.975 \ (P < 0.01)

b = 0.008$

$r = 0.938 \ (P < 0.05)

b = 0.005$
Table - 26

Frequency of chromosome aberrations induced by carbaryl in the bone marrow cells of chicks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Fixation time (h)</th>
<th>Total no. of cells studied</th>
<th>Chromatid gaps</th>
<th>Chromatid breaks</th>
<th>Chromatid deletions</th>
<th>Total</th>
<th>% of aberrations ± SE⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>1.p.</td>
<td>6</td>
<td>200</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-(1)</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>150</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>6</td>
<td>10</td>
<td>2</td>
<td>12(18)</td>
<td>6.00 ± 0.82**a</td>
</tr>
<tr>
<td>150</td>
<td>1.p.</td>
<td>48</td>
<td>200</td>
<td>3</td>
<td>5</td>
<td>-</td>
<td>5(8)</td>
<td>2.50 ± 1.29</td>
</tr>
<tr>
<td>125</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>2</td>
<td>7</td>
<td>1</td>
<td>8(10)</td>
<td>4.00 ± 0.82*b</td>
</tr>
<tr>
<td>100</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>6(10)</td>
<td>3.00 ± 0.57*a</td>
</tr>
<tr>
<td>30 X 5</td>
<td>1.p.</td>
<td>120</td>
<td>200</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>2(5)</td>
<td>1.00 ± 0.57</td>
</tr>
<tr>
<td>150</td>
<td>p.o.</td>
<td>24</td>
<td>200</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3(5)</td>
<td>1.50 ± 0.50</td>
</tr>
</tbody>
</table>

Results in the parentheses are including of gaps.

⁺ Values are excluding gaps

Result is significantly different from the control at * p < 0.05, ** p < 0.01 (Student's t-test)
Table - 27

Incidence of micronucleated bone marrow cells of chicks induced by carbaryl.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>1.p.</td>
<td>16,000</td>
<td>23</td>
<td>1.44 ± 0.26**</td>
<td>6</td>
<td>0.38 ± 0.16</td>
</tr>
<tr>
<td>125</td>
<td>1.p.</td>
<td>16,000</td>
<td>20</td>
<td>1.25 ± 0.18**</td>
<td>2</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td>100</td>
<td>1.p.</td>
<td>16,000</td>
<td>12</td>
<td>0.75 ± 0.18*</td>
<td>3</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>150</td>
<td>p.o.</td>
<td>16,000</td>
<td>10</td>
<td>0.63 ± 0.31</td>
<td>6</td>
<td>0.38 ± 0.16</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p < 0.05; ** p < 0.01 (Student's t-test)
Results are mean %o ± SE of four chicks/group. Result is significantly different from the control at * p < 0.05; ** p < 0.01 (Student's t-test)

Groups with same superscript differ significantly from each other at p < 0.05 (ANOVA).

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>i.p.</td>
<td>16,000</td>
<td>17</td>
<td>1.06 ± 0.12**a</td>
<td>5</td>
<td>0.31 ± 0.19</td>
</tr>
<tr>
<td>125</td>
<td>i.p.</td>
<td>16,000</td>
<td>12</td>
<td>0.75 ± 0.10*b</td>
<td>4</td>
<td>0.25 ± 0.10</td>
</tr>
<tr>
<td>100</td>
<td>i.p.</td>
<td>16,000</td>
<td>8</td>
<td>0.50 ± 0.10a</td>
<td>3</td>
<td>0.19 ± 0.12</td>
</tr>
<tr>
<td>150</td>
<td>p.o.</td>
<td>16,000</td>
<td>6</td>
<td>0.38 ± 0.16</td>
<td>7</td>
<td>0.44 ± 0.21</td>
</tr>
</tbody>
</table>

Table 28: Incidence of micronucleated peripheral blood cells of chicks induced by carbaryl.
erythrocytes. No significant variations were obtained (ANOVA) among the
doses of bone marrow MN test \( F = 2.94; \, df = 2,9; \, p > 0.05 \), however,
significant variations were observed in peripheral blood MN test \( F = 6.78; \, df = 2,9; \, p < 0.05 \).

**DISCUSSION**

Carbaryl gave positive results for Ames Salmonella test system
and chromosomal aberration test in in vitro mammalian cell culture
(Ishidate et al., 1988). Carbaryl (85 % purity) caused an increased
number of recessive lethals in Drosophila melanogaster but mice fed with
five oral treatments either with 50 or 1000 mg/kg did not increase any
dominant lethals (Epstein et al., 1972). Carbaryl when reacts with
nitrous acid present in the stomach, it leads to the formation of N-
nitrosocarbaryl (Elespuru and Liinsky, 1973). N-nitrosocarbaryl is a
potent bacterial mutagen without metabolic activation and is also
mutagenic in yeast (Elespuru et al., 1974; Siebert and Eisenbrand,
1974). It is also carcinogenic in rats after oral and single
subcutaneous administration (Eisenbrand et al., 1976). N-nitrosocarbaryl
acted as a potent base pair substitution mutagen and showed a relatively
mild frame-shift activity, whereas, the parent compound carbaryl gave
negative results (Regan et al., 1976; FAO/WHO, 1982).

In the present study all the three doses induced significant
aberrations after 24 hours of exposure. The aberration frequency
increased with dose, which indicate that the effects were apparently
dose dependent. It is further envisaged that in all the three doses the
chemical could raise the optimum concentration for mutagenic event.
Generally in genotoxicity studies in order to establish the significance
of positive findings both the availability of the substance in the
target cells and the time of harvest and aberration yield are two
important factors (Galloway et al., 1987; Madle and Lang, 1993).
Probable reasons for such type of concentration and time dependent effects induced by chemicals have been mentioned elsewhere in this thesis (please see monocrotophos).

In a number of animal species, carbaryl is rapidly hydroxylated or hydrolysed and thereafter conjugated and eliminated in the urine as glucuronides or sulphates. Human and rat liver homogenates produced equal amount (3.4 %) of the 4- and 5-hydroxy derivatives of carbaryl. The metabolism and mode of action of carbaryl in chick in vivo test system is not known. However, in in vitro human cell culture N-nitrosocarbaryl formed alkali-sensitive bonds with human DNA whereas in S. typhimurium it acted as a potent base-pair substitution mutagen and relatively mild frame shift activity (WHO, 1986; Regan et al., 1976). So this might be the possible mechanism of action of carbaryl to produce chromosomal aberration in the present test-system.

Micronucleus test is one of the dependable cytogenetic bioassays to screen environmental agents for mutagenicity (Heddle, 1973; Schmid 1976; Savage, 1988). Micronuclei indicate acentric chromosome fragment or whole chromosome laggards during cell division. The significant increase of MN in both the bioassays indicate the clastogenic and/or aneuploidogenic potential of carbaryl in the tested system. In carbamate pesticides, the benzamidazole moiety may act as a base analogue for DNA and act as a spindle poison. They are also considered to be antimutotic agents and cause mitotic arrest, mitotic delay and a low incidence of chromosome delay (WHO, 1986).

More studies in this area should be conducted to know the exact mechanism of action of this chemical, because insecticides are not only used widely in agriculture but also in both domestic and animal farms.
Copper sulphate (CuSO$_4$·5H$_2$O) is a common soluble salt of copper. It is a broad spectrum inorganic pesticide and is variously used as a herbicide and weedicide, particularly against aquatic weeds (Banerjee and Mitra, 1954) as a germicide (Sengupta, 1976) and as a molluscicide (Babu and Rao, 1982).

Copper in trace amounts, is essential for life while excess amount is toxic. Its importance in health and disease is well documented (Linder 1983; Prohaska, 1984; Aaseth and Norseth, 1986). It is well established in the literature that lead and copper reach drinking water through the dissolution of plumbing materials (see Murphy 1993). A number of metals present in the occupational environment have been reported as human carcinogens (Anttila et al., 1993). However, there is no direct positive correlation between Cu exposure and cancer (Howell, 1958).

In spite of the indiscriminate use of synthetic organic pesticides, a number of inorganic pesticides are still widely used in present day agriculture. Copper sulphate has been reported to be mutagenic in E. coli and S. typhimurium (Ishizawa et al., 1978; Hansen and Stern, 1984), cause no DNA damage in prokaryotes (Matsui 1980) and enhance viral transformations (Casto et al. 1979). Its clastogenic effects have been reported in mice in vivo by Bhunya and Pati (1987) and Agarwal et al. (1990); while Tinwell and Ashby (1990) reported its negative effect in mouse bone marrow micronucleus assay. Toxicological data revealed that copper sulphate causes testicular atrophy in Gallus domesticus (Shivanandappa et al., 1983) and has a depressing effect on egg production and ovary weight (Stevenson and Jackson, 1981). There is no record in the literature on the clastogenic effect of copper sulphate in chick in vivo test system. So it was thought imperative to assay the clastogenic potential of copper sulphate (CuSO$_4$) in chick in vivo test system.
MATERIALS AND METHODS

Test chemical: Analytical grade copper sulphate (CuSO₄·5H₂O) (99 % pure) was supplied by Fine-Chem. (India) Ltd. The solvent used was glass double distilled (g.d.d) water.

Dose: Three different doses such as 5, 7.5 and 10 mg/kg b.w. were used for the present experiment. The application volume was 1 ml/100 g b.w.

Experimental protocol: The details of experimental design of BMCA, MNT and statistics have been described in "General materials and methods".

RESULTS

General toxicity: After the administration of the chemical no external toxicity symptoms were marked.

Genotoxicity:

Chromosome aberration: (Figs. 80 a-f, 81, 82 and 85; Table 29)

Qualitative: In the treatment groups, chromosomal aberrations (CA) like chromatid type gaps, breaks and deletions have been scored. Gaps and breaks were more frequent than other types of aberrations. Usually, single aberration occurred per cell.

Quantitative: Quantitatively the two higher doses (10 and 7.5 mg/kg b.w.) induced significant aberrations over the control value in i.p. route. The chemical did not produce significant effect in the p.o. route by the highest dose (10 mg/kg b.w.). In time-response studies the chemical produced significant aberrations after 24 and 48 hours of exposure. Repeated exposures (chronic) of fractionated dose induced less aberration than that of equivalent acute dose and was found to be insignificant. No significant
variations (ANOVA) were obtained among dose-response ($F = 0.702; \text{df} = 2,9; \ p > 0.05$) and time-response ($F = 3.332; \text{df} = 3,12; \ p > 0.05$) analyses.

A tentative assessment revealed that the middle and distal regions of first two chromosomes were more vulnerable to the chemical assault.

**Micronucleus:** (Figs. 83 a-h, 84 and 86; Tables 30-31)

The frequencies of micronuclei induced by copper sulphate with different doses have been presented in tabular form. Compared to controls, all dose levels produced higher incidence of aberrations in the treated series. In bone marrow MN test, the two higher doses (10 and 7.5 mg/kg b.w) in i.p. route and only the highest dose (10 mg/kg b.w) in p.o. route gave significant increase of MN over the controls. In peripheral blood MN test only the two higher doses in i.p. route gave significant results over the control. No significant variations were obtained among the doses of bone marrow MN test ($F = 1.037; \text{df} = 2,9; \ p > 0.05$) and peripheral blood MN test ($F = 1.350; \text{df} = 2,9; \ p > 0.05$). The aberration frequency was more in bone marrow erythrocyte than the peripheral blood erythrocyte micronucleus test. Besides the induction of MN some other nuclear anomalies such as sickle shaped, thinning in the mid region of the nuclei and some cases of vacuolated nuclei were also observed.

**DISCUSSION**

The genotoxicity of Cu compounds has been reported in animal cell culture (Flessel, 1978; Hollstein *et al.*, 1979). It gave negative results in bacterial colorimetric assays- the SOS chromotest (Olivier and Marzin, 1987). Strong clastogenic potential of copper has been reported in plants (Rosen 1964). However, it could not induce SCE frequency over the base line level in *Allium cepa* (Hazen *et al.*, 1988).
EXPLANATION OF FIGURE

Fig. 80. Photomicrographs depicting different types of chromosomal aberrations in the bone marrow cells of chicks induced by copper sulphate:

(a) chromatid gap in the q-arm of chromosome 2.
(b) chromatid break in the q-arm of chromosome 1.
(c) chromatid break and displacement in chromosome 3.
(d) chromatid break in the proximal region of q-arm of chromosome 1.
(e) chromatid break in the q-arm of chromosome 2.
(f) chromatid deletion in Z-chromosome.
EXPLANATION OF FIGURES

Fig. 81. Histograms showing the frequencies of chromosome aberrations induced by copper sulphate in the bone marrow cells of chicks:

(a) after different time intervals.
(b) by acute and chronic exposures.

Fig. 82. Histograms showing the route response chromosome aberration frequencies in the bone marrow cells of chicks.
Fig 81(a,b)  

% of Chromosomal Aberration

- (a) Acute
- (b) Chronic

Fixation Times (Hours)

Fig 82  

% of Chromosomal Aberration

- IP
- PO

Routes
EXPLANATION OF FIGURE

Fig. 83. Photomicrographs of bone marrow and peripheral blood cells showing micronuclei and nuclear anomalies induced by copper sulphate:

(a) bone marrow cell showing extrusion of nucleus.
(b) bone marrow erythrocyte with one large micronucleus.
(c)-(d) bone marrow erythrocytes with one MN in each.
(e)-(h) peripheral blood erythrocytes showing one micronucleus either small or large in each.
Fig. 84. Histograms showing the frequencies of micronuclei induced by carbaryl in the bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks exposed to different doses through different routes.
EXPLANATION OF FIGURES

Fig. 85. Linear regression analysis on chromosome aberration frequencies in the bone marrow cells of chicks. C in abscissa indicates control.

Fig. 86. Linear regression analysis on micronuclei frequencies in bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks. C in abscissa indicates control.
Fig 85

% of Aberration

\[ r = 0.993 \ (P < 0.001) \]
\[ b = 0.36 \]

Fig 86

MN per 1000 Cells

\[ r = 0.989 \ (P < 0.01) \]
\[ b = 0.096 \]

\[ r = 0.994 \ (P < 0.001) \]
\[ b = 0.061 \]

\( \square \) BM
\( \triangle \) PB
## Table 29

Frequency of chromosome aberrations induced by copper sulphate in the bone marrow cells of chicks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Fixation time (h)</th>
<th>No. of cells studied</th>
<th>Chromatid gaps</th>
<th>Chromatid breaks</th>
<th>Chromatid deletions</th>
<th>Total</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.p.</td>
<td>6</td>
<td>200</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>2(3)</td>
<td>1.00 ± 0.58</td>
</tr>
<tr>
<td>10</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>8(11)</td>
<td>4.00 ± 0.82*</td>
</tr>
<tr>
<td>10</td>
<td>1.p.</td>
<td>48</td>
<td>200</td>
<td>7</td>
<td>5</td>
<td>1</td>
<td>6(13)</td>
<td>3.00 ± 0.58*</td>
</tr>
<tr>
<td>7.5</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>7(12)</td>
<td>3.50 ± 0.96*</td>
</tr>
<tr>
<td>5</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>2</td>
<td>5</td>
<td>-</td>
<td>5(7)</td>
<td>2.50 ± 0.96</td>
</tr>
<tr>
<td>2 X 5</td>
<td>1.p.</td>
<td>120</td>
<td>200</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>4(10)</td>
<td>2.00 ± 0.82</td>
</tr>
<tr>
<td>10</td>
<td>p.o.</td>
<td>24</td>
<td>200</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>4(10)</td>
<td>2.00 ± 0.82</td>
</tr>
</tbody>
</table>

Results in the parentheses are including of gaps.

+ Values are excluding of gaps.

* Result is significantly different from the control at p < 0.05 (Student's t-test).
Table - 30

Incidence of micronucleated bone marrow cells of chicks induced by copper sulphate.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>i.p.</td>
<td>16,000</td>
<td>17</td>
<td>1.06 ± 0.12**</td>
<td>6</td>
<td>0.38 ± 0.12</td>
</tr>
<tr>
<td>7.5</td>
<td>i.p.</td>
<td>16,000</td>
<td>15</td>
<td>0.94 ± 0.21*</td>
<td>11</td>
<td>0.69 ± 0.26</td>
</tr>
<tr>
<td>5</td>
<td>i.p.</td>
<td>16,000</td>
<td>11</td>
<td>0.69 ± 0.21</td>
<td>2</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td>10</td>
<td>p.o.</td>
<td>16,000</td>
<td>14</td>
<td>0.88 ± 0.07**</td>
<td>8</td>
<td>0.50 ± 0.35</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p<0.05; ** p<0.01 (Student's t-test).
### Table 31

Incidence of micronucleated peripheral blood cells of chicks induced by copper sulphate.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>i.p.</td>
<td>16,000</td>
<td>13</td>
<td>0.81 ± 0.06**</td>
<td>6</td>
<td>0.38 ± 0.07</td>
</tr>
<tr>
<td>7.5</td>
<td>i.p.</td>
<td>16,000</td>
<td>10</td>
<td>0.63 ± 0.07*</td>
<td>9</td>
<td>0.56 ± 0.23</td>
</tr>
<tr>
<td>5</td>
<td>i.p.</td>
<td>16,000</td>
<td>7</td>
<td>0.44 ± 0.21</td>
<td>3</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>10</td>
<td>p.o.</td>
<td>16,000</td>
<td>5</td>
<td>0.31 ± 0.12</td>
<td>4</td>
<td>0.25 ± 0.00</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Results is significantly different from the control at * p<0.05; ** p<0.01 (Student's t-test)
Chromosome aberration is one of the surest parameters for detecting the genotoxic effects induced by environmental chemicals and are considered highly relevant in the human context (ICPEMC 1983; WHO 1985).

From the present results it is evident that CuSO$_4$ is clastogenic in the tested system. Increased frequency of aberrations with the higher dose is due to the better availability of the chemical in the target tissue (Matter, 1976; Behera and Bhunya, 1980). The chemical did not show cumulative effect since repeated fractionated dosing produced less aberration than the equivalent acute dose. It may be envisaged that in fractionated dosing critical concentration of the chemical and/or its metabolites for mutagenic event was not sufficient in the target cells (Matter, 1976; Behera and Bhunya, 1980). Regarding the regional sensitivity to aberrations, it is envisaged that non-random distribution of gaps and breaks in the middle and distal regions of chromosomes one and two might be due to the specific molecular make up of chromosomes. It has been reported that chemicals in general produce localised effects (Kato, 1986b; Alam et al., 1974). Similar type of regional sensitivity has also been observed in mouse chromosomes by Bhunya and Pati (1987).

In the present study lower effect was produced in p.o. route than in i.p. route. It has been reported that copper is absorbed in minute quantities from the mucous surfaces (Sengupta, 1976) which can be attributed to aforesaid result.

The induction of micronuclei in bone marrow cells in response to treatment with test chemicals was recognized as a sensitive indicator of genotoxic potential (Schmid, 1976). The higher frequencies of micronuclei in the treated series clearly indicate the clastogenic effect of CuSO$_4$ in the tested system.

Regarding the mechanism of action of CuSO$_4$ at chromosomal level, it has been reported that copper shows a strong affinity for nucleic acid
components (Eichhorn et al., 1966; Bryan et al., 1981; Agarwal et al., 1989). It has been reported that soluble salts of copper precipitates protein and acts as an astringent (Sengupta, 1976). The mutagenic property of Cu$^{II}$ is due to its property of inducing infidelity in DNA synthesis in vitro and it associates ionically with DNA (Sideris et al., 1981; Patiashvili et al., 1987). Copper does not bind covalently to DNA. Tinwell and Ashby (1990) predicted that the predominance occurrence of gaps and breaks, indicate an indirect (ionic) interaction between Cu$^{II}$ ions and chromatin, rather than its constituent DNA. However, the exact mechanism of action of copper sulphate at molecular level is not yet known.

The result of the present investigation clearly revealed the clastogenic property of copper sulphate in chick in vivo test system. Further work employing different test systems is needed in order to find a definite conclusion for the contradictory results of the chemical in different test systems.
8. TRICHLOROACETIC ACID

Trichloroacetic acid is an organic acid having both medicinal and pesticidal properties. It is widely used as a preemergence herbicide, a peeling agent for wrinkled, sun-damaged skin and tattoos and as common laboratory agent (Ayres, 1964; Piggot and Norris, 1988; Collins, 1989). Its chemical formula is CCl$_3$COOH.

![Structure of trichloroacetic acid](structure.png)

Trichloroacetic acid (TCA), a new contaminant found from the chlorinating water with other organic materials (Correa et al., 1985). Level of TCA and dichloroacetic acid (DCA) in Amherst, Massachusetts drinking water have been found in the range of 100-200 µg/litre (Uden and Miller, 1983). TCA is a major metabolite of trichloroethylene (TCE) and tetrachloroethylene (Coleman et al., 1976; Dekant et al., 1985). TCA is also a metabolic product of CCl$_4$ under reduced oxygen tension (Packer et al., 1978).

The genotoxic property of TCA has not yet been studied widely. However, TCA has been reported to be non-mutagenic (Anderson et al., 1972), but also to be short lived mutagen with DMSO (Nestmann et al., 1980) in the Salmonella spot test. Moriya et al. (1983) reported the cytotoxic effect of TCA in plant system. The genotoxicity of TCA has been reported by Bhunya and Behera (1987) employing different cytogenetic assays like: bone marrow chromosome aberration, micronuclei and sperm head abnormality test. Besides, genotoxicity, other adverse effects of TCA have been reported in the literature. It has been found that, TCA in chlorinated water cause physiological stress in a dragonfly, Somatochlora cingulata (Correa et al., 1985). TCA induces liver peroxisome proliferation (Odum et al., 1988; Parnell et al., 1986) and an increased
incidence of adenomas and hepatocellular carcinomas are observed in mice exposed to TCA or DCA (Herren-Freund et al., 1987). In a ninety day sub-chronic toxicity study TCA induced variable degrees of alternations in the lung and liver of rats (Bhat et al., 1991). There is no report in the literature on the genotoxic effect of TCA in chick in vivo test system. In the present study an endeavour has been made to evaluate the probable genotoxic effect of TCA employing chick in vivo test system.

MATERIALS AND METHODS

Test chemical: The analytical grade of TCA (98% pure) obtained from Glaxo (India) Ltd. was used. Glass double distilled (g.d.d) water was used as the solvent.

Dose: Three different doses 100, 200 and 400 mg/kg b.w. were used for the present experiment. The application volume was 1 ml/100 g b.w.

Experimental Protocol: The details of the experimental design of BMCA, MNT and statistics have been described in "General materials and methods".

RESULTS

General toxicity: The chemical did not produce any clinical signs of external toxicity except some mild shivering just after the administration of the chemical.

Genotoxicity:

Chromosome aberration: (Figs. 87a-f, 88, 89 and 92; Table-32).

Qualitative: Trichloroacetic acid induced aberration including chromatid gaps, breaks, deletions, rings etc. The chemical also induced effects
like stickiness, woolly appearance and corrosiveness on chromosomes. Some cells with chromatid deletions were also observed. Of all types of aberrations chromatid gaps were more frequent.

**Quantitative:** Quantitatively the highest dose (400 mg/kg) induced the highest percentage (5.00 %) of aberration after 24 hours of treatment and repeated exposures of sub-acute dose (chronic) induced a smaller effect (3.00 %) than equivalent acute dose. In time-response study the aberration frequencies were 0.5 %, 5.00 %, 2.00 % after 6, 24 and 48 hours treatment respectively. The highest dose (400 mg/kg) in p.o. route gave significant result over the control value.

The results were generally time and route-responsive, but only the two higher doses in i.p. route gave statistically significant aberration over the control value. No significant variations were observed (ANOVA) among dose-response ($F = 1.141; \text{df} = 2, 9; p > 0.05$) and time-response ($F = 3.35; \text{df} = 3, 12; p > 0.05$) analyses for chromosome aberration assay.

A tentative assessment of the occurrence of gaps and breaks revealed that the middle and terminal regions of larger chromosomes were more susceptible to the chemical.

**Micronucleus:** (Figs. 90 a-h, 91 and 93; Tables- 33-34)

All the doses produced higher frequencies of MN in the treated series. The two higher doses in bone marrow MN test and the highest dose in peripheral blood MN test gave significant increase of MN over the control in i.p. route. The effect of the chemical in both the micronucleus assays is almost same and the difference of results produced by the different dose regimens is also marginal. No significant variations were obtained (ANOVA) among the doses of bone marrow MN test ($F = 0.44; \text{df} = 2, 9; p > 0.05$) and peripheral blood MN test ($F = 1.463; \text{df} = 2, 9; p > 0.05$). The MN were mostly dot shaped and usually occurred one per cell. Besides the induction of MN, the chemical also induced different types of nuclear anomalies.
EXPLANATION OF FIGURE

Fig. 87. Photomicrographs of bone marrow metaphase spreads showing different types of chromosomal anomalies induced by TCA:

(a) woolly nature of the chromosomes.
(b) chromatid break in the p-arm of chromosome 1 and deletion in Z chromosome.
(c) isochromatid gap in the proximal region of q-arm of chromosome 1.
(d) iso-chromatid gaps in the p-arm and chromatid break in the terminal region of q-arm of chromosome 1.
(e) chromatid break and displacement of the broken portion in chromosome 1.
(f) chromatid gap in the p-arm and chromatid break in the q-arm of chromosome 1.
EXPLANATION OF FIGURES

Fig. 88. Histograms showing the frequencies of chromosome aberrations induced by trichloroacetic acid in the bone marrow cells of chicks:

(a) after different time intervals.
(b) by acute and chronic exposures.

Fig. 89. Histogram showing the route-response chromosome aberration frequencies in the bone marrow cells of chicks.
Fig 88 (a, b)

Fig 89

% of chromosomal aberration vs. fixation times (hours)

(a) % of chromosomal aberration

(b) Acute

Chronic

Fig 89

IP PO Routes
EXPLANATION OF FIGURE

Fig. 90. Photomicrographs of bone marrow and peripheral blood smears depicting micronuclei and nuclear anomaly induced by TCA:

(a) a bone marrow cell showing anaphase separation of chromosomes with chromatin bridges.
(b) bone marrow erythrocyte with one small micronucleus.
(c) bone marrow erythrocytes with one large micronucleus.
(d) bone marrow erythrocyte with one small micronucleus.
(e) peripheral blood erythrocyte with one small micronucleus.
(f) peripheral blood erythrocyte with one large micronucleus.
(g)-(h) peripheral blood erythrocytes with a small micronucleus in each.
EXPLANATION OF FIGURE

Fig. 91. Histograms showing the frequencies of micronuclei induced by TCA in the bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks exposed to different doses through different routes.
Fig 91

DOSE (mg/kg)

MN PER 1000 CELLS

BM
PB

100
200
400
400

Fig 91
EXPLANATION OF FIGURES

Fig. 92. Linear regression analysis on chromosome aberration frequencies in the bone marrow cells of chicks. C in abscissa indicates control.

Fig. 93. Linear regression analysis on micronuclei frequencies in bone marrow (BM) and peripheral blood (PB) erythrocytes of chicks. C in abscissa indicates control.
Fig. 92

Fig. 93
Table - 32

Frequency of chromosome aberrations induced by trichloroacetic acid in the bone marrow cells of chicks.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Fixation time (h)</th>
<th>Total no. of cells studied</th>
<th>Chromatid gaps</th>
<th>Isochromatid gaps</th>
<th>Chromatid breaks</th>
<th>Isochromatid breaks</th>
<th>Deletions</th>
<th>Exchange</th>
<th>Total</th>
<th>% of aberrations ± SE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>1.p.</td>
<td>6</td>
<td>200</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1(4)</td>
<td>0.50 ± 0.50</td>
</tr>
<tr>
<td>400</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>10(15)</td>
<td>5.00 ± 1.29*</td>
</tr>
<tr>
<td>400</td>
<td>1.p.</td>
<td>48</td>
<td>200</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>4(10)</td>
<td>2.00 ± 1.41</td>
</tr>
<tr>
<td>200</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7(13)</td>
<td>3.50 ± 0.50**</td>
</tr>
<tr>
<td>100</td>
<td>1.p.</td>
<td>24</td>
<td>200</td>
<td>6</td>
<td>1</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5(11)</td>
<td>2.50 ± 1.50</td>
</tr>
<tr>
<td>80X5</td>
<td>1.p.</td>
<td>120</td>
<td>200</td>
<td>5</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6(11)</td>
<td>3.00 ± 0.58</td>
</tr>
<tr>
<td>400</td>
<td>p.o.</td>
<td>24</td>
<td>200</td>
<td>3</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>4(7)</td>
<td>2.00 ± 0.82*</td>
</tr>
</tbody>
</table>

Results in the parentheses are including of gaps
+ Values are excluding of gaps.

Result is significantly different from the control at * p<0.05; ** p<0.01 (Student's t-test)
Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p < 0.05; ** p < 0.01 (Student's t-test)
Table - 34

Incidence of micronucleated peripheral blood cells of chicks induced by TCA

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Total no. of cells studied</th>
<th>Cells with MN</th>
<th>% of aberrations ± SE</th>
<th>Cells with NA</th>
<th>% of aberrations ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>i.p.</td>
<td>16,000</td>
<td>11</td>
<td>0.69 ± 0.12*</td>
<td>5</td>
<td>0.31 ± 0.16</td>
</tr>
<tr>
<td>200</td>
<td>i.p.</td>
<td>16,000</td>
<td>9</td>
<td>0.56 ± 0.21</td>
<td>1</td>
<td>0.06 ± 0.06</td>
</tr>
<tr>
<td>100</td>
<td>i.p.</td>
<td>16,000</td>
<td>7</td>
<td>0.44 ± 0.21</td>
<td>9</td>
<td>0.56 ± 0.19</td>
</tr>
<tr>
<td>400</td>
<td>p.o.</td>
<td>16,000</td>
<td>8</td>
<td>0.50 ± 0.20</td>
<td>3</td>
<td>0.19 ± 0.12</td>
</tr>
</tbody>
</table>

Results are mean % ± SE of four chicks/group.

Result is significantly different from the control at * p < 0.05 (Student's t-test)
DISCUSSION

Trichloroacetic acid was reported to be non-mutagenic in Salmonella spot test at low doses but lethal at higher concentrations (Andersen et al., 1972). It has been reported to be a short lived mutagen with DMSO in Salmonella by Nestmann et al. (1980). In all the assay the highest dose produced the highest frequencies of aberrations. In chromosome aberration assay highest effect was produced after 24 hours with the highest dose and relatively lower effects were observed 6 and 48 hours after treatment and with lower doses. The decrease in aberration frequency with lower doses and at latter stages is probably due to non-availability of the critical concentration of genetically reactive metabolites at the target molecules (DNA and protein) and due to elimination of metabolites from the body with time (Tates and Natarajan, 1976). A critical concentration of the reactive metabolites of chemical compounds in the target tissue/cell is extremely important for the production of any mutagenic event (Matter, 1976). Again decrease in number of aberrant cells at later hours might also be due to the cell death and the mechanics of mitotic transmission. In the present investigation the highest dose (400 mg/kg b.w.) gave significant yield of aberration in both the routes. However, relatively higher frequency of aberration was produced by i.p. treatment with TCA than oral. This apparent difference in the production of aberration frequency may roughly be correlated with the higher elimination due to poor absorption of the chemical administered orally, as a result of which relatively higher frequency of aberration was produced by i.p. administration than oral. The cytogenetic effects induced by repeated treatment with fractionated doses was less than the effect induced by the equivalent dose administered once. This may be due to constant elimination of the chemical from the body by ongoing complex metabolic processes. Similar types of results have been reported earlier in mice (Schmid et al., 1971; Behera and Bhunya, 1989; Bhunya and Pati, 1990) and in chicks (Bhunya and Jena, 1992).
The micronucleus test is comparable to or even more sensitive and reliable than metaphase scoring in the screening of chemical agents for mutagenicity in vivo (Matter and Schmid, 1971; Schmid, 1976; Maier and Schmid, 1976). The higher frequency of MN in the erythrocytes of bone marrow and peripheral blood clearly indicated the clastogenic effect of TCA in the tested system.

The mechanism of action of TCA with chromosomes at molecular level is not clearly known. It has been reported earlier that acidic pH can induce chromosomal aberrations in cultured mammalian cells (Brusick, 1986; Cifone et al., 1987) or root meristem tissues of Vicia faba (Zura and Grant, 1981). In the process of metabolism, trichloroacetic acid can be hydrolysed to yield more highly oxygenated acids. It has been suggested further that TCA could react with sulfhydryl groups of proteins and amino acids to form an alkylated product (Freed and Montgomery, 1969).

In the present study TCA was found to be mutagenic in chick in vivo test system. Further studies in other test systems including human in vitro systems are needed for better evaluation of its mutagenic potential. Since it has been found to be mutagenic in the tested system, its cautious handling and restricted use are suggested.