3. EFFECTS OF DIAZEPAM

3.1 Introduction

Diazepam belongs to the 'Benzodiazepine' group of the tranquilizers. 'Diazepam' is its non-proprietary name, commercially it is known as valium. Out of the available benzodiazepine tranquilizers diazepam is the most commonly prescribed and used. Its chemical structure is as follows:

\[
\begin{align*}
\text{Cl} & \quad \text{N} & \quad \text{C} & \quad \text{N} \\
\text{CH}_3 & \quad \text{O} & \quad \text{CH}_2 \\
\text{C} & \quad \text{C} & \quad \text{C} \\
\text{Ar} & 
\end{align*}
\]

The molecular weight of diazepam is 285.

Diazepam was first marketed in the year 1961 (Sternbach and Reeder, 1961) and since then it has gained top-most popularity as one of the most important anticonvulsant, hypnotic, sedative, muscle relaxant and anxiolytic drugs (Randall et al., 1961; Cooper, 1963; Hare, 1963; Sellers, 1978; Mandelli et al., 1978). It is approximately five times as potent as chlordiazepoxide as a tranquilizer and skeletal muscle relaxant. In the year 1965 it was introduced in dentistry (Davidau, 1965). More recently diazepam has been used in orthopedic and plastic surgery either in place of anaesthesia or in combination with local analgesia (Adamson et al., 1971; Holst-Nielsen, 1974; Gunther, 1975). It is also useful in treating the acute agitation of alcohol withdrawal in chronic alcoholic patients.

In India total production of diazepam in 1977-78 was 1.00 tonne, which can be taken as an indicative of the extent of its use.
Diazepam has a complex and extensive metabolic fate (Goodman and Gilman, 1975). Absorption after oral administration is quick and complete (Albanus et al., 1977). Similarly quick distribution of diazepam after iv treatment has been shown by Kaplan et al. (1973). Peak plasma level reaches within 30 to 90 minutes. The elimination rate is, however, slow. The elimination half-life ranges from 21 to 37 hours in man after iv treatment (Kaplan et al., 1973). However, age of the individual influences both the absorption and elimination rate after both iv and oral administration. In children an early peak is observed where as in older persons the peak is lower and delayed (Garattini et al., 1973b; Klotz et al., 1975).

Pharmacokinetics in the feto-placental unit has recently been investigated rather extensively. Placental transfer and metabolism of diazepam after maternal administration was demonstrated by a number of workers (Davanagh and Condo, 1964; Erkkola et al., 1974; Kanto and Erkkola, 1974).

Preliminary observation of Verett and Mc Laughlin (1963) suggested also teratogenic effect of the drug in chick embryo.

The pharmacological and therapeutic aspects of diazepam and of benzodiazepines in general have recently been reviewed nicely by a number of workers (Breimer, 1976; Hvidberg and Dam, 1976; Strojny et al., 1977; Mandelli et al., 1978; Sellers, 1978). Thus diazepam is the most widely studied benzodiazepine drug. Most of the works so far done have, however, been limited to the neural
effects of diazepam. Any cellular or sub-cellular studies with diazepam or other benzodiazepines have been confined to brain or nervous tissue (Essman, 1973). Very few studies have been made on the possible cytotoxic and genotoxic effects of diazepam. Ober (1974) described the effects of diazepam on the synthesis of nucleic acids, carbohydrates, chlorophyll and cell nitrogen as well as on the cell multiplication of Scenedesmus obliquus.

Breen and Stenchever (1970) studied the effect of this drug on the ultrastructures of human fibroblasts in culture. Alterations in the organisation of the cytoplasm involving particularly the membraneous elements were noted and these alterations were dose dependent.

During 1969-70 a number of investigations have been done on the clastogenic property of diazepam. Of them the contributions of two laboratories: Stenchever and his co-workers and Staiger and his coworkers, constitute the major bulk. Different results were, however, put forward by these two groups and thus the literature is provided with contradictory reports. Stenchever and his coworkers (Stenchever and Frankel, 1969; Stenchever et al. 1970a) found increased incidence of chromosomal aberrations after in vitro as well as in vivo treatments in man. In contrast, Staiger failed to obtain increase in aberration frequency over the control value when human fibroblasts (Staiger, 1969) and leukocytes (Staiger, 1970) in culture were exposed to various concentrations of the tranquilizer for varied periods (8-96h). Negative results were also reported from in vivo studies. Schmid and Staiger (1969) treated hamsters for 2 weeks with high doses of the drug but
aberration rates of the bone marrow chromosomes were comparable to those of untreated controls. Similarly in rats diazepam failed to produce any increase in the incidence of aberrations of bone marrow chromosomes when compared to controls (Neda et al., 1977). Cohen et al. (1969) and White et al. (1974) could not also detect significant increase of chromosomal aberrations in leukocytes in culture of patients treated with diazepam therapeutically. Plant (Allium fistulosum) chromosomes did not also show any response to this tranquilizer (Zolotareva et al., 1977).

Only one report has so far been made on the effects of this tranquilizer on the meiotic chromosomes and that is a negative report by Stenchever et al. (1974). They could not demonstrate significant difference between the control and treated groups with respect to figures with aneuploids, breaks, rearrangements or shattered chromosomes in CBA mouse.

Thus all the previous studies except the work of Stenchever et al. (1974) on the effects of diazepam on chromosomes are concerned with the metaphase chromosome analysis of somatic cells in vivo and in vitro and they have given conflicting results. In view of the wide spread use of diazepam as medicine and as a drug of habit as well as conflicting results mentioned above we have considered further analysis of its cytotoxic and genotoxic effects with different protocols already indicated. We have also studied its effect on the sperm head morphology and on their production.

3.2 Material and Methods

3.2.1 Cytogenetic assay of bone marrow cells (Metaphase chromosome analysis)

Three sets of experiments as mentioned in the General Material and Methods were conducted. In the 1st set the mice
were treated with a single dose of 0.5 mg (per individual) of the drug and they were killed at 2, 4, 8, 16, 24, 32, 48, 64 and 72 h for chromosome preparation. In the second set the animals were treated with a single dose of 0.25, 0.50, 1.00 or 2.00 mg (per individual) and the chromosome preparation was done at 4 h after the treatment. The mice (males only) of the third set were treated with a single daily dose of 0.5 mg (per individual) for 15 consecutive days and they were killed at the end of 1, 3, 4, 6, 8 and 12 weeks after the last treatment. A portion of the test animals of the third set, however, were constituted by similarly treated males used in the dominant lethal test (vide infra). Details of controls for the three sets and processing were mentioned in General Material and Methods (vide 2.2.1).

3.2.2 Cytogenetic assay of bone marrow cells (Micronucleus test)

Same as mentioned in Material and Methods (General) (Vide 2.2.2).

3.2.3 Cytogenetic assay of male germ cells

Same as mentioned earlier in Material and Methods (General) (vide 2.2.3). In addition, it may be further pointed out here that for this protocol among the test animals a portion (for wks 6, 8 and 12) was constituted by similarly treated males of dominant lethal test and the rests (for wks 1, 3 and 4) were treated separately.

3.2.4 Dominant lethal test

Same as mentioned earlier in Material and Methods (General) (vide 2.2.4). In addition we want to mention here that treatment was started with 20 males. Two of them died during treatment and one each in wks 2 and 3. Four were killed in wk 7 for meiotic study
the result of which is mentioned (vide 3.3.3). Number of females used in different weeks is indicated in Table 9. Data for dominant lethality were collected from 8 post-treatment mating weeks. Pre-implantation loss was not observed.

3.2.5 Sperm count
Same as mentioned earlier in Material and Methods (General) (vide 2.2.5).

3.2.6 Sperm head abnormality
Same as mentioned earlier in Material and Methods (General) (vide 2.2.6).

3.3 Results

3.3.1 Cytogenetic assay of bone marrow cells (Metaphase chromosome analysis)
Qualitative: Since qualitatively the types of aberrations produced in three sets of experiments were almost same no separate description was made for them. Almost all the aberrations encountered were of chromatid type. 'Gap' was the most common type of aberration available. However, among the 'break' type aberrations chromatid break was of common occurrence. The broken fragment of the chromatid of an affected chromosome was found to lie at various places in the metaphase complement (Figs. 1, 2 and 3b, c). Most often it was found to be situated close to its place of origin without having much displacement (Figs. 1a, b, e, g; 2a, b, c, f and 3b). Sometimes, it was situated at various positions in the field (Figs. 1d, 2g, 3c). However, not more than one break in a chromatid of the affected chromosome was observed. On very rare occasion two breaks in two chromatids, one in each, at the non-identical position were found. Iso-chromatid breaks were
Explanation of Fig. 1

Photomicrographs of bone marrow metaphase plates, in part or full, with some structural chromosomal aberrations induced by diazepam.

a, b. Chromatid-type break at the proximal region of chromosome, the broken fragment without displacement.

c. A chromatid break with displaced broken fragment and a fragment of unknown origin.

d. A terminal chromatid deletion, the broken fragment lying apart.

e. Two terminal chromatid breaks involving two different chromosomes, fragment without much displacement.

f. A chromosome with a chromatid break.

g. Chromatid break in a chromosome with a little displacement of the broken part.
Explanation of Fig. 2

Photomicrographs of some bone marrow metaphase plates with structural chromosomal aberrations induced by diazepam treatment.

a. A chromatid break, the broken piece of the chromatid lying near its place of origin.

b. A portion of a metaphase showing one chromatid break in a chromosome and one gap in a rabbit-ear chromosome.

c, d. Chromosomes with chromatid breaks, the broken fragments not much displaced.

e. A part of a metaphase plate showing a fragment of untraceable origin.

f. Cut-out portion of a metaphase field showing chromatid break, the fragment lying near its place of origin.

g. Part of a metaphase complement with a terminal chromatid deletion in a chromosome, the fragment lying apart.
Explanation of Fig. 3

Photomicrographs of bone marrow metaphases, in part or full showing chromosomal aberrations induced by diazepam.

a. A chromosome with chromosome or iso-chromatid break at distal region.

b. Cut-out portion of a metaphase field showing a chromatid break at the proximal region of a chromosome.

c. Part of a metaphase plate with a terminal chromatid deletion, the fragment having much displaced.

d. An intact metaphase plate with 39 chromosomes.

e. A metaphase plate with 41 chromosomes.
also available (Fig. 3a). Metaphase plate containing two breaks involving two chromosomes (Fig. 1e) or with fragment of untraceable origin was not rare. Usually one chromatid break was found in a cell but the frequency of cells having more than one break or having a mixture of different types of aberrations was not altogether absent (Figs. 1c, 2b).

In experimental set I centromeric separation was not recorded. No aneuploid cell with 42 chromosome was observed. Metaphases with 39 (Fig. 3d) and 41 (Fig. 3e) chromosomes were common.

Quantitative: The quantitative analysis of the data reveals several interesting points. In the 1st set of experiment (Table 1) highest 'breakage' frequency (columns 3-7) was observed at both 4 and 8 h after the treatment. However, the breakage frequencies for 2-16 h did not differ markedly. Similarly, the breakage frequencies for 32-72 h remained almost at the same level. Almost same trend was noticed when total aberration frequencies were considered. However, the breakage as well as total aberration frequencies when considered for 2-72 h varied significantly among themselves (analysis of variance). Another interesting point noted in Table 1 was that for 4-24 h the frequencies of chromatid breaks were more than those of gaps; while the reverse was true for 32-72 h. At 2 h the said frequencies were same. When the data of the treated series were compared with the control values significant increases in both breakage and total aberration frequencies were found at all the sampling hours including 64 and 72 h for which minimum breakage frequencies were recorded due to treatment. With the lapse of time the frequencies for total aberration and breakage were found to decrease and those decreases
were statistically significant at 5% and 1% levels respectively as revealed by correlation coefficient analysis.

Dose-response study (Table 2) exhibited increase of breakage frequencies along with the increase of dose except the case of 1.0 mg dose level. However, the increase was not at all proportional. The highest breakage frequency was noted with 2.0 mg dose level. However, no remarkable variation was noted for 0.5 and 2.0 mg dose levels. If only chromatid breaks were considered the frequency for 0.5 mg dose level was more than double the value for 2.0 mg dose level. In good number of cases small fragments of untraceable origin were encountered with the highest dose level tested (2.0 mg). Those were thought to be resulted from terminal deletion and they influenced the breakage frequencies very much. Compared to the controls the breakage as well as total aberration frequencies increased at all the dose levels and the increases were statistically significant almost in all the cases. With the increase of the dose the effect as calculated from the breakage or total aberration frequencies increased; but that increase was insignificantly correlated with the dose as revealed by correlation coefficient analysis. Analysis of variance showed highly significant differences among the values obtained at different dose levels.

Interestingly no significant increase was noted in the breakage or total aberration or individual aberration frequency over the respective control value at the end of 1, 3, 4, 6 and 8 wks after 15 days repeated treatment (Table 3). The wk 12 data was marginally significant only. Analysis of variance study did not show any significant variation among the values obtained at
Table 2. Frequency distribution of different types of structural aberrations of bone marrow metaphase chromosomes induced by single treatment of diazepam with different doses at 4 h. (Translocation counted as two breaks).

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Cells Scored/Animal</th>
<th>Gaps (1)</th>
<th>Sub-Chromatid br. (2)</th>
<th>Chromatid br. (3)</th>
<th>Iso-Chromatid br. (4)</th>
<th>Fragments (5)</th>
<th>Unequal Chromatid (6)</th>
<th>Translocations (7)</th>
<th>Total of 1-7 Mean % ± S.E.</th>
<th>Total of 3-7 Mean % ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>300/4</td>
<td>9</td>
<td>1</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.66 ± 1.10b</td>
<td>1.33 ± 0.66</td>
</tr>
<tr>
<td>0.50</td>
<td>300/4</td>
<td>4</td>
<td>11</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.66 ± 1.24a</td>
<td>5.66 ± 0.55a</td>
</tr>
<tr>
<td>1.00</td>
<td>300/4</td>
<td>13</td>
<td>7</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9.33 ± 1.15a</td>
<td>2.66 ± 0.47a</td>
</tr>
<tr>
<td>2.00</td>
<td>300/4</td>
<td>12</td>
<td>11</td>
<td>8</td>
<td>-</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>13.99 ± 0.99a</td>
<td>6.33 ± 0.28a</td>
</tr>
<tr>
<td>0.00</td>
<td>3000/30</td>
<td>31</td>
<td>10</td>
<td>16</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>2.03 ± 0.31</td>
<td>0.66 ± 0.15</td>
</tr>
</tbody>
</table>

"t" test: a = p < 0.001, b = p < 0.01

r = 0.84, df=2  
F = 8.783  
df.n₁=3, n₂ = 12  

r = 0.64, df=2  
F = 16.224  
df.n₁=3, n₂ = 12
different weeks after the treatment. Increase of post-treatment period and total aberration frequency or breakage frequency did not exhibit any correlation also.

Numerical changes and centromeric separation of three sets of experiments are presented in Tables 4, 5 and 6. The incidence of polyploidy in all the three sets of experiments remained almost at the control level except two cases: one at wk 1 after 15 days repeated treatment (Table-6) and the other at 2 mg dose level (Table-5). So far the incidence of aneuploidy was concerned, no remarkable variation was noted between the control and treated values in the time-response analysis (Sets I and III, Tables 4 and 6). At 64 h post-treatment a lone case of significant increase in this regard was noticed but the increase was marginally significant only (Table 4). Dose-response analysis (Table 5) exhibited significant increase of aneuploidy at 1.0 and 2.0 mg dose levels.

Marked increase in the frequency of centromeric separation of chromosomes was noted almost in all the cases after the treatment of the drug (Tables 5 and 6). In certain cases the increase was more than 5 times the control value or so. However, no test of significance was done for them.

3.3.2 Cytogenetic assay of bone marrow cells (Micronucleus test)

Qualitative: Most of the affected cells (erythrocytes and nucleated cells) were with one micronucleus (Figs. 4a-j, n & 5a-f). However, cells with more than one micronucleus (MN) were also available (Figs. 4k-m and 5g, h). In certain extreme cases as many as four micronuclei of different sizes were recorded (Fig. 4m) in an erythrocyte and six in a nucleated cell (Fig. 5h). The micronuclei were always round in shape but varied in size (Figs. 4
Explanation of Fig. 4

Cut-out photomicrographs of bone marrow smears showing micronucleated erythrocytes induced by diazepam.

a-c. Polychromatc erythrocytes containing one micronucleus each.

d-j. Normochromatc erythrocytes containing one micronucleus each. Note the size and location of micronuclei.

k. A polychromatc erythrocyte with two micronuclei lying close to each other.

l. Polychromatc erythrocytes with two micronuclei.

m. Polychromatc erythrocyte with four micronuclei of different sizes.

n. A normochromatc erythrocyte with one micronucleus.
Explanation of Fig. 5

Cut-out microphotographs of bone marrow smears showing micronuclei in nucleated cells, multinucleate condition and other divisional abnormalities induced by diazepam.

a-d. Nucleated bone marrow cells containing one micronucleus each.

e. A nucleated cell with one micronucleus at the center of the main nucleus.

f. A nucleated cell with one micronucleus.

g. A nucleated cell with two micronuclei.

h. A cell with six micronuclei of different sizes.

i. A trinucleate cell.

j. A cell at anaphase with some lagging chromosomes.

k. Anaphase bridge and a lagging element.

l. Lagging chromatin element at anaphase in a cell (polar view)

m. Lagging chromatin element at anaphase.
Table 4. Frequency distribution of different types of numerical changes in bone marrow chromosomes induced by single treatment of diazepam (0.5 mg) at different sampling intervals.

<table>
<thead>
<tr>
<th>Hour</th>
<th>Cells Scored/Cells</th>
<th>Polyploid Mean %±S.E.</th>
<th>Aneuploid cells</th>
<th>Total Aneuploid Mean %±S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animal</td>
<td></td>
<td>38  39  41  42</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>300/5</td>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.99±0.33</td>
<td></td>
<td>0.66±0.33</td>
</tr>
<tr>
<td>4</td>
<td>300/5</td>
<td>9</td>
<td>-  1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.99±0.28</td>
<td></td>
<td>0.33±0.28</td>
</tr>
<tr>
<td>8</td>
<td>300/5</td>
<td>8</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.66±0.47</td>
<td></td>
<td>0.99±0.28</td>
</tr>
<tr>
<td>16</td>
<td>300/5</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.33±0.00</td>
<td></td>
<td>0.33±0.28</td>
</tr>
<tr>
<td>24</td>
<td>300/5</td>
<td>3</td>
<td>-  2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.99±0.28</td>
<td></td>
<td>0.66±0.33</td>
</tr>
<tr>
<td>32</td>
<td>300/5</td>
<td>4</td>
<td>2  1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.33±0.47</td>
<td></td>
<td>0.99±0.55</td>
</tr>
<tr>
<td>48</td>
<td>300/5</td>
<td>6</td>
<td>1  1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.99±0.33</td>
<td></td>
<td>0.66±0.33</td>
</tr>
<tr>
<td>64</td>
<td>300/5</td>
<td>8</td>
<td>2  3  2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.66±0.47</td>
<td></td>
<td>2.33±1.36C</td>
</tr>
<tr>
<td>72</td>
<td>300/5</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.33±0.47</td>
<td></td>
<td>1.00±0.86</td>
</tr>
<tr>
<td>Cont.</td>
<td>3000/30</td>
<td>52</td>
<td>6  14  3</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.73±0.27</td>
<td></td>
<td>0.76±0.18</td>
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</tbody>
</table>

't' test : c = p(0.05)
Table 5. Incidences of centromeric separation and frequency distribution of different types of numerical changes in bone marrow chromosomes induced by single treatment of diazepam with different doses at 4 h.

<table>
<thead>
<tr>
<th>Dose (mg)</th>
<th>Cells scored/Animal</th>
<th>Polyplloid Cells Mean %± S.E.</th>
<th>Aneuploid Cells</th>
<th>Total Aneuploid Mean %± S.E.</th>
<th>Cell with centromeric separation Mean %± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>300/4</td>
<td>7</td>
<td>1.99±0.74</td>
<td>5.99±1.52</td>
<td>4.66±0.74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.33±0.55</td>
<td></td>
<td></td>
<td>4.66±0.57</td>
</tr>
<tr>
<td>0.50</td>
<td>300/4</td>
<td>6</td>
<td>1.99±0.33</td>
<td>9.33±1.49</td>
<td>4.99±0.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.99±0.74</td>
<td></td>
<td></td>
<td>2.33±0.55</td>
</tr>
<tr>
<td>1.00</td>
<td>300/4</td>
<td>7</td>
<td>2.33±0.28</td>
<td>12.99±0.98</td>
<td>6.66±1.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.33±0.98</td>
<td></td>
<td></td>
<td>6.66±0.94</td>
</tr>
<tr>
<td>2.00</td>
<td>300/4</td>
<td>11</td>
<td>2.66±0.94</td>
<td>10.66±1.56</td>
<td>7.66±1.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.66±0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>3000/30</td>
<td>52</td>
<td>0.76±0.18</td>
<td>4.90±0.73</td>
<td>2.43±0.45</td>
</tr>
<tr>
<td>(Cont.)</td>
<td></td>
<td>1.73±0.27</td>
<td></td>
<td></td>
<td>1.23±0.30</td>
</tr>
</tbody>
</table>

't' test : b = p < 0.01, c = p < 0.05
and 5a-h). A considerable proportion of these micronuclei were unusually large indicating that whole chromosome(s) had been lost from the nucleus (Fig. 4h, i, m). The locations of the micronuclei within the cell also varied (Fig. 4). In nucleated cells by nature the nucleus size is always big and cytoplasm is comparatively little and in certain differentiated nucleated cells the nucleus is by nature lobulated. For that reason in certain cases it was difficult for the investigator to differentiate micronuclei from the nuclear lobes. Those cases led to confusion and were not recorded. In addition smear exhibited a number of abnormal mitotic figures like bridge formation (Fig. 5k), lagging chromosome(s)/or fragment(s) (Figs. 5j-m) etc. Binucleate, trinucleate cells (without considering MN) (Fig. 5i) as well as cells with fragmented nuclei were also encountered. Though not quantitatively estimated, compared to the control the incidence of normal and abnormal mitotic figures in the treated series seemed to be higher. Except the non-occurrence of anaphase bridge and lagging elements in the single as well as lowest dose level of double treated series no remarkable difference was noticed between single and double treatment.

Quantitative: Data on the incidence of micronuclei and composition of bone marrow cells in the control and diazepam treated mice are presented in Tables 7 and 29. At intermediate dose level (0.5 mg) the incidence of micronuclei in all the cell types increased significantly (0.1% level). Significant increase in the frequency of micronucleated normocytes was noted at all the dose levels tested. However, in polychromatic erythrocytes the increase was not significant at 0.25 and 1.00 mg dose levels.
Table 7. Results of micronucleus test after diazepam treatment at various dose levels. (Figures in parentheses are MN/Cells scored. For each dose level two treatments were given separated by 24 h).

<table>
<thead>
<tr>
<th>Dose of Animals (mg)</th>
<th>No. of Animals</th>
<th>P.e.c. with MN Mean %±S.E.</th>
<th>N.e.c. with MN Mean %±S.E.</th>
<th>P.+N.e.c. with MN Mean %±S.E.</th>
<th>Nucleated Cells Mean ± S.E.</th>
<th>Erythrocytes/100 nucleated cells Mean ± S.E.</th>
<th>P/N ratio Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>5</td>
<td>0.320±0.022 (32/10000)</td>
<td>0.350±0.031^c (35/10000)</td>
<td>0.335±0.017 (67/20000)</td>
<td>0.360±0.025^c (36/10000)</td>
<td>8.957±0.379^c</td>
<td>0.894±0.017</td>
</tr>
<tr>
<td>0.50</td>
<td>5</td>
<td>0.650±0.096^a (65/10000)</td>
<td>0.600±0.054^a (60/10000)</td>
<td>0.625±0.074^a (125/20000)</td>
<td>0.530±0.090^a (53/10000)</td>
<td>12.418±0.408</td>
<td>0.852±0.018</td>
</tr>
<tr>
<td>1.00</td>
<td>5</td>
<td>0.400±0.024 (40/10000)</td>
<td>0.430±0.041^a (43/10000)</td>
<td>0.415±0.029^a (83/20000)</td>
<td>0.370±0.022 (37/10000)</td>
<td>13.508±0.495^c</td>
<td>0.786±0.027</td>
</tr>
<tr>
<td>0.00 (Cont.)</td>
<td>20</td>
<td>0.297±0.029 (73/23850)</td>
<td>0.220±0.022 (55/23865)</td>
<td>0.262±0.018 (128/47715)</td>
<td>0.249±0.018 (65/25110)</td>
<td>11.383±0.989</td>
<td>0.968±0.099</td>
</tr>
</tbody>
</table>

'r' values 
df = 1

| 'r' test : a = p < 0.001, c = p < 0.05 |
In general the increase was maximum with the intermediate dose (0.50 mg) in poly-, normo- and poly-normochromatic erythrocyte line. Similar well marked increase (at 0.1% level) was also noted with the same dose (0.5 mg) after single treatment (Table-29). Interestingly, when the incidences of micronuclei were compared between single and double treatment series, both erythrocytes and nucleated cells exhibited higher frequency of micronucleated cells after single treatment. Of course in single treatment series the data were scored at 32 h post-treatment, while in the double treatment series at 30 h.

The increase in the frequencies of nucleated bone marrow cells with micronuclei were significant with 0.25 and 0.50 mg but not with 1.00 mg dose. Here also the increase was maximum with the intermediate dose (0.50 mg). Bone marrow depression as revealed by the proportion of total erythrocyte and nucleated cells was found to remain very close to the control value. Differences obtained due to treatment in certain cases were, however, marginally significant only. Attempt was also made to reveal the turnover of poly- and normochromatic erythrocytes by analyzing their ratios. Statistically, however, no significant variation was obtained from the control value at any dose level.

3.3.3 Cytogenetic assay of germ cells

Qualitative - From the qualitative point of view the effects of the drug on the meiotic chromosomes were analysed under three general headings: numerical changes, structural changes and effects on the pairing behaviour. Qualitatively the effects in the control and treated series as well as different test weeks were same except the presence or absence of certain aberration
type(s) in certain test weeks. The numerical changes were divided into two categories, viz. polyploidy and aneuploidy, for convenience. Occurrence of tetraploidy (Fig. 6a) and hexaploidy (Fig. 6b) was very frequent. But odd-polyploidy (3n, 5n or 50) was completely absent. Ploidy at higher level was also available (10n - Fig. 6c) and in one extreme case 18n cell was recorded. Diakinesis and metaphase I plates with 18, 19, 21 or 22 (Fig. 7d, e, f) bivalents instead of usual 20 were also of common occurrence. Those cases were categorised as aneuploidy and peculiarly sex chromosomes were in no way involved in those hypo- and hyperdiploidy. However, no monosomic or trisomic cases were recorded.

Among the structural abnormalities were considered only the break type aberrations and translocations. The former comprised chromatid type (Fig. 7a, g) breaks and fragments of untraceable origin (Fig. 7b). No chromosome type break was encountered. A few cases of translocations involving autosomes were also noticed.

In several cases autosomal homologues as well as X and Y were found to separate early in the diakinesis stage (Figs. 6d-f and 7d). In certain instances (Figs. 6d, e) the X and Y were found to lie far away from each other. Similar cases was also noted for autosomes also (Fig. 6f). Among the autosomal bivalents the smaller ones were mostly involved (Fig. 6f) in it.

Quantitative - Data on the meiotic abnormalities induced by diazepam are presented in Table 8. For quantitative analysis the frequencies of numerical and structural abnormalities as well as early dissociation in the treated series were compared with
Explanation of Fig. 6

Photomicrographs of diakinesis and metaphase-I spermatocytes showing various numerical abnormalities and dissociated bivalent induced by diazepam.

a. A tetraploid diakinesis plate.

b. A hexaploid diakinesis plate.

c. A 10n metaphase-I plate with one dissociated X and Y.

d, e. Normal diakinesis plates showing dissociation of sex chromosome bivalents. The dissociated X and Y are variously located.

f. A diakinesis plate with four dissociated autosomal bivalents.
Explanation of Fig. 7

Photomicrographs of diakinesis metaphase-I spermatocytes showing structural and numerical abnormalities and dissociated bivalents induced by diazepam.

a. Part of a diakinesis plate showing one chromatid break in an autosomal bivalent.

b. A diakinesis plate with one extra fragment of unknown origin.

c. Part of a diakinesis plate showing iso-chromatid gap.

d. A metaphase-I plate containing 19 bivalents and dissociated X and Y.

e.f. Diakinesis plates with 19 bivalents.

g. A diakinesis plate with chromatid break in an autosomal bivalent.
Table 8. Frequency distribution of different types of spermatocytic chromosome aberrations at different post-treatment weeks following 15 days repeated treatment of diazepam.

<table>
<thead>
<tr>
<th>Test wks</th>
<th>Set Cells scored/animals</th>
<th>Polyploid cells Mean% ± S.E.</th>
<th>Aneuploid cells Mean% ± S.E.</th>
<th>Breaks Mean% ± S.E.</th>
<th>Translocation Mean% ± S.E.</th>
<th>Dissociation(Mean% ± S.E.) Autosomal X-Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Treat. 150/4</td>
<td>15.82 ± 1.28a</td>
<td>5.92 ± 1.35b</td>
<td>-</td>
<td>-</td>
<td>16.94 ± 7.81 8.92 ± 0.75c</td>
</tr>
<tr>
<td></td>
<td>Cont. 197/4</td>
<td>4.70 ± 0.55</td>
<td>1.19 ± 0.61</td>
<td>0.50 ± 0.43</td>
<td>-</td>
<td>9.27 ± 3.03 4.63 ± 1.34</td>
</tr>
<tr>
<td>3</td>
<td>Treat. 185/4</td>
<td>17.62 ± 4.61c</td>
<td>7.58 ± 1.44</td>
<td>1.00 ± 0.40</td>
<td>-</td>
<td>33.71 ± 10.35 8.34 ± 1.66</td>
</tr>
<tr>
<td></td>
<td>Cont. 209/4</td>
<td>5.96 ± 0.80</td>
<td>2.28 ± 1.45</td>
<td>0.41 ± 0.35</td>
<td>-</td>
<td>15.59 ± 3.97 7.51 ± 3.28</td>
</tr>
<tr>
<td>4</td>
<td>Treat 150/4</td>
<td>32.58 ± 6.14b</td>
<td>1.36 ± 0.57</td>
<td>-</td>
<td>0.79 ± 0.64</td>
<td>27.28 ± 7.15c 5.00 ± 1.52</td>
</tr>
<tr>
<td></td>
<td>Cont. 155/4</td>
<td>5.46 ± 1.36</td>
<td>2.71 ± 1.08</td>
<td>-</td>
<td>-</td>
<td>8.39 ± 1.02 5.67 ± 0.20</td>
</tr>
<tr>
<td>6</td>
<td>Treat. 200/4</td>
<td>16.50 ± 1.47b</td>
<td>4.00 ± 0.70</td>
<td>5.00 ± 1.50c</td>
<td>-</td>
<td>18.00 ± 4.12 16.50 ± 3.11</td>
</tr>
<tr>
<td></td>
<td>Cont. 222/4</td>
<td>6.27 ± 1.35</td>
<td>2.54 ± 0.84</td>
<td>0.50 ± 0.43</td>
<td>-</td>
<td>10.86 ± 2.57 8.62 ± 1.55</td>
</tr>
<tr>
<td>8</td>
<td>Treat. 200/4</td>
<td>15.00 ± 3.35</td>
<td>3.00 ± 1.50</td>
<td>2.00 ± 1.22 1.50 ± 0.43c</td>
<td>-</td>
<td>20.00 ± 4.18c 5.50 ± 1.47</td>
</tr>
<tr>
<td></td>
<td>Cont. 219/4</td>
<td>6.94 ± 0.52</td>
<td>4.00 ± 1.41</td>
<td>0.86 ± 0.44</td>
<td>-</td>
<td>8.08 ± 2.30 5.86 ± 2.50</td>
</tr>
<tr>
<td>12</td>
<td>Treat. 186/4</td>
<td>18.55 ± 1.94a</td>
<td>2.19 ± 0.72</td>
<td>2.00 ± 1.22</td>
<td>-</td>
<td>13.19 ± 3.19 6.38 ± 0.47</td>
</tr>
<tr>
<td></td>
<td>Cont. 200/4</td>
<td>5.74 ± 0.08</td>
<td>1.69 ± 0.87</td>
<td>0.36 ± 0.31</td>
<td>-</td>
<td>6.45 ± 2.82 5.62 ± 2.75</td>
</tr>
</tbody>
</table>

't' test: a = p < 0.001, b = p < 0.01, c = p < 0.05
the respective control values week-wise. The most interesting result was the higher incidence of polyploidy in all the weeks tested and except wk 8 in all the cases the increase was highly significant. In the control series the incidence of polyploidy varied within a narrow range (4.70 - 6.94%). If the control values were pooled the frequency of polyploidy would be 5.88% and if the values of the treated series were compared with that pooled control value data of wk 8 even would have shown significant difference. The highest incidence of polyploidy in the treated series was recorded at wk 4 (32.58%), while in other test weeks it varied between 15.00 - 18.55%.

Though the incidence of aneuploidy was more in certain test weeks of the treated series significant increase was noticed only in wk 1.

Similarly the incidence of dissociation of X and Y in the treated series was significantly high at wk 1 only. In other test weeks the treated values remained almost in the control range. In the treated series the autosomes showed a higher tendency of early separation in almost all the test weeks. However, the data of wk 4 and 8 only exhibited significant increase over their corresponding controls.

The frequency of structural aberrations in general was very low in both control and treated series, even in certain weeks it was completely absent. Only at wk 6 the breakage frequency was significantly higher over the corresponding control value. However, the significance was marginal. Besides, the incidence of breakage in the treated series remained at the control level. In one test week (wk 8), the frequency of translocation became significant
due to the zero value in the corresponding control.

3.3.4 Dominant lethal test

Severe sedative action was marked among the males during the course of treatment, particularly in the early phase. In general the health of the treated individuals got slightly reduced in comparison to the control ones. But this reduction in health was promptly recovered after the discontinuation of the drug.

The number of total implants in the control series ranged from 5 -13 per female and in the treated series the range was 5-12 (excluding the cases of deciduomata). One dead implant per affected female in both control and treated series was of common occurrence. In certain extreme cases in the treated series as many as five dead implants (out of 6 total implants) per female was recorded. While in control the maximum record of dead implants per female was two only. Further, the incidence of intrauterine deaths in the two horns of uterus both in the control and treated series did not differ much. Interestingly, almost all intrauterine deaths took place at early stage of development. In the present investigation for that reason no separation was done for early and late deaths. In total 32 cases of deciduomata were recorded in the treated series and for the want of actual figure of the implants each of them was classed as one dead implant for statistical analysis. If actual figures were known number of dead implants would have been more. In contrast, not a single case of deciduoma was noted in control series.
Table 9 summarizes the data on the dominant lethal test with diazepam. The mating index in the treated series varied from 60.00 - 93.10 while in the control series the range was 70.00 - 93.75. In no mating week the index for treated series differed significantly (χ² test) from that of control. So male fertility remained unaffected after the treatment.

The mean frequencies of living implants per female in the treated series varied between 4.54 - 6.86. The minimum value was exhibited in the 1st week. Then it gradually increased up to wk 4 and after that it fluctuated, though the peak was obtained at wk 7. Statistically, of course this increase showed no correlation with the increase of mating intervals. Contrastly the minimum value recorded in the control series (7.85) was more than the maximum value of the treated series. The differences between the treated and corresponding control values were highly significant ('t' test) in almost all the mating weeks.

So far incidence of dead implants per pregnant female was concerned in the control series, it fluctuated within a narrow range (0.36 - 0.55). On the other hand, in the treated series the highest frequency of dead implants per female (1.29) was recorded in wk 1 and it gradually went on decreasing with lapse of time except the last mating week. Correlation coefficient analysis revealed that this gradual decrease with the lapse of time was statistically significant at 5% level. However, only the 1st week data of the control and treated series differed significantly ('t' test).

In the control series the frequencies of total implantations (dead + living) per female did not vary much with mating intervals. Similarly, the values in the treated series showed variation in
wide range (5.83 - 7.31) this variation did not show any correlation ('r' = 0.09, df = 6) with the mating intervals. When week-wise data were analysed statistically, significant decrease in the treated value from the corresponding control was noted in almost all the test weeks.

In the control series the mutagenic indices of different weeks fluctuated within a limited range. On the other hand, due to the treatment it increased significantly in almost all the mating weeks. The maximum increase was noted in wk 1 where treated value was more than 3 times the corresponding control value. With the lapse of time the mutagenic index in the treated series decreased gradually upto wk 5, then of course it became irregular. However, statistical analysis (correlation coefficient) indicated significant decrease at 5% level in relation to all eight mating weeks.

### 3.3.5 Sperm count

Here data were collected from six different post-treatment weeks (Table 10). Both in the treated and control series right

**Table 10** Effect of diazepam on the epidymal sperm count of mice at different weeks after 15 days repeated treatment. Values are mean number of sperms (heads only) in one WBC chamber of haemocytometer ± S.E. 3-4 animals were utilized in each week.

<table>
<thead>
<tr>
<th>Epididymes</th>
<th>Set</th>
<th>wk 1</th>
<th>wk 3</th>
<th>wk 4</th>
<th>wk 6</th>
<th>wk 8</th>
<th>wk 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
<td>63.18</td>
<td>95.02</td>
<td>58.02</td>
<td>88.80</td>
<td>84.53</td>
<td>104.36</td>
</tr>
<tr>
<td>Right</td>
<td>Control</td>
<td>117.33</td>
<td>106.56</td>
<td>104.98</td>
<td>108.61</td>
<td>111.86</td>
<td>95.03</td>
</tr>
<tr>
<td></td>
<td>Treated</td>
<td>61.54</td>
<td>82.24</td>
<td>74.96</td>
<td>86.74</td>
<td>91.37</td>
<td>85.66</td>
</tr>
<tr>
<td>Left</td>
<td>Control</td>
<td>109.61</td>
<td>109.74</td>
<td>99.85</td>
<td>108.21</td>
<td>103.60</td>
<td>95.67</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>62.36</td>
<td>88.81</td>
<td>66.49</td>
<td>87.77</td>
<td>87.95</td>
<td>95.01</td>
</tr>
<tr>
<td>of Right and Left</td>
<td>Treated</td>
<td>± 4.51</td>
<td>±15.61</td>
<td>±10.53</td>
<td>±7.15</td>
<td>±4.16</td>
<td>±14.71</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>±10.41</td>
<td>±7.77</td>
<td>±9.27</td>
<td>±7.64</td>
<td>±8.22</td>
<td>±4.63</td>
</tr>
</tbody>
</table>

't' test:  c = p < 0.05
and left epididymal sperm counts did not differ markedly at any given post-treatment week. Similarly the values obtained at different weeks did not differ remarkably in both treated and control series. The highest count obtained in the treated series (95.01) was very close to the lowest value recorded in the control series (95.35). Thus in general there was a decrease in the count in the treated series. This decrease was, however, found to be statistically significant only in wk 1 when the data were compared with the corresponding control value. The picture would remain almost unaltered if the control values were pooled.

### 3.3.6 Sperm head abnormality

**Qualitative:** So far qualitative aspect was concerned a variety of abnormal sperm heads involving both size and shape were encountered in the treated series. Arbitrarily they were divided into following types: giant form (Fig. 8a), with flat base (tail end) (Fig. 8b), amorphous (Fig. 8c,d,h,i,k,n-p), triangular (Fig. 8e), without hook (Fig. 8g,l), polyp-type (Fig. 8f,g), semilunar (Fig. 8m), bifurcated acrosomal end (Fig. 8j), and with vacuole (Fig. 8b). Among them giant, amorphous and triangular forms were of frequent occurrence in both treated and control series. Other six types were encountered only in the treated series. However, they were of rare occurrence.

As the grouping of abnormal types were arbitrary it was not possible to have quantitative analysis 'type-wise'. All the abnormal sperm heads were taken together under the heading 'abnormal' for quantitative analysis.

**Quantitative:** In the control series the frequency of abnormal heads ranged from 4.12% to 6.18% (Table 11). The variations among
Explanation of Fig. 8

Cut-out portions photomicrographs showing normal and abnormal sperm heads in diazepam treated mice. Arrows indicate abnormal sperm heads.

a. A giant size sperm head.

b. A sperm head with flat base and another vacuolated sperm head.

c,d. Amorphous type sperm heads.

e. A sperm head with triangular shape.

f. A polyp-type sperm head.

g. A polyp-type and a hook less sperm heads.

h,i. Amorphous types and a number of normal sperm heads.

j. A sperm head with bifurcated acrosomal end.

k. Amorphous type sperm head.

l. A sperm head without acrosomal hook.

m. Semilunar type.

n-p. Amorphous type sperm heads.
Table 11 Effect of diazepam on the incidence of sperm head abnormality in mice at different weeks after 15 days repeated treatment. Values are mean % ± S.E. 4 animals were employed in each week, treated and control. 200 sperm heads were examined from each animal.

<table>
<thead>
<tr>
<th>Set</th>
<th>wk 1</th>
<th>wk 3</th>
<th>wk 4</th>
<th>wk 6</th>
<th>wk 8</th>
<th>wk 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>6.75b</td>
<td>6.50</td>
<td>6.50</td>
<td>14.00b</td>
<td>8.25</td>
<td>6.62</td>
</tr>
<tr>
<td></td>
<td>±0.27</td>
<td>±0.98</td>
<td>±1.06</td>
<td>±2.18</td>
<td>±1.35</td>
<td>±2.71</td>
</tr>
<tr>
<td>Control</td>
<td>4.62</td>
<td>5.18</td>
<td>4.81</td>
<td>4.12</td>
<td>6.18</td>
<td>4.62</td>
</tr>
<tr>
<td></td>
<td>±0.36</td>
<td>±0.35</td>
<td>±0.51</td>
<td>±0.59</td>
<td>±0.32</td>
<td>±0.54</td>
</tr>
</tbody>
</table>

't' test: b = p < 0.01

the different values obtained in different weeks did not differ much. Further, correlation coefficient analysis did not reveal any influence of the weeks on the values obtained in different weeks. So, if all the values of different weeks of control were pooled the mean value would have been 4.92% which is very close to the value obtained by earlier workers (Bruce et al., 1974).

In the treated series the lowest value (6.50%) which was more than the highest value recorded in the control series, was found at wks 3 and 4; while the highest value (14.00) was obtained at wk 6 (Table 11). Thus a general increase in the frequency of abnormal sperm heads was noted in all the test weeks whether compared to the corresponding control value or compared to the pooled mean control value (4.92%). The data of wks 1 and 6 exhibited significant increase over their corresponding control values. However, if pooled control value was considered data of all the weeks except wk 12 would also show significant increase.
3.4 Discussion

3.4.1 Cytogenetic assay of bone marrow cells (Metaphase chromosome analysis)

Both time-response and dose-response analyses of the bone marrow metaphase chromosomes after single treatment exhibited significantly higher breakage frequencies in the treated series. The period of maximum response in diazepam (0.5 mg, single dose) treated mice was 4-8 h post treatment and the value was about 9 times the control one. Similarly a 10 fold (approx.) increase in the breakage frequency was noted with the highest dose (2.0 mg) in the dose-response study. It thus clearly reveals the clastogenic capacity of diazepam. Almost all the breaks observed during the period of 4-8 h post-treatment after single dose were of chromatid type. The fact that diazepam caused the most pronounced chromosome damage within 8 h of treatment suggests that the later part of the cell cycle, i.e. late S or G2, is the most sensitive stage to the mutagenic activity of diazepam or its active agent. In the present study diazepam at doses of 0.5 mg and above caused significant increase in chromosome damage to bone marrow cells. So, it seems the lowest single dose to increase significantly the breakage frequency of bone marrow chromosomes in mouse system is about 0.50 mg. It is pointed out that single dose of 0.5 mg/individual used here exceeds the human therapeutic dose by a factor of about 20 only. So our results of time-response and dose-response studies after single treatment on bone marrow chromosomes are in agreement with those of Stenchever and Rankel (1969) and Stenchever et al. (1970a) but contradict other earlier works (Staiger, 1969, 1970; Schmid and Staiger, 1969; Cohen et al., 1969; White et al., 1974; Neda et al., 1977).
Stenchever and Frankel (1969) using 72 h human lymphocyte cultures found that the cells exposed to 10 \( \mu g/ml \) or 20 \( \mu g/ml \) of the drug for the entire 72 h were associated with increased aberrations. Similarly, a significant increase in aberration frequency was observed (Stenchever et al., 1970) in leukocytes of one out of 23 patients receiving repeated doses of diazepam. The above patient had received 30 mg/day for 18 months and when restudied 6 months after stopping the drug use the aberration rate came to the control range. While, Staiger tested its effects on chromosomes of human fibroblasts (Staiger, 1969) and leukocytes (Staiger, 1970) in culture but could not find increase of aberration rates with various concentrations (12.5 \( \mu g/ml \) - 50 \( \mu g/ml \)) and with exposure times ranging from 8 h - 4 days. Four in vivo experiments also gave negative results. Schmid and Staiger (1969) treated Chinese hamsters for two weeks with high doses of diazepam (300 and 900 mg/kg) and chromosome preparations of bone marrow cells were made 5-8 h after the last dose. The incidence of chromosome aberrations in those animals did not differ appreciably from that of the untreated control. Similar negative reports were also made by Neda et al. (1977) who treated rats with daily doses of 200 and 500 mg/kg for 1, 5 and 10 days and showed no increase in the incidence of chromosome aberrations when compared with the values obtained from untreated control rates. In vivo tests were also conducted on human subjects. Cohen et al. (1969), White et al. (1974) failed to obtain chromosomal aberration in leukocytes of patients who received diazepam therapeutically. In the former case the patients were treated with therapeutic doses for 36-72 months, while in the latter observation was done after single therapeutic dose. However,
the experimental designs of both the studies with human subjects mentioned above were complicated for simultaneous use of other drugs. Thus all the earlier works were concerned with the metaphase chromosome analysis of somatic cells in vitro and in vivo and most of them showed negative clastogenic effect.

Absence of chromosome damaging effect was also noted in the present study from metaphase chromosome analysis after repeated treatment of the same dose (0.50 mg) for 15 days (Table 3). In that part of the study chromosome preparation was done at the end of wks 1, 3, 4, 6, 8 and 12 after the last treatment. Thus mouse system also provided both positive and negative results and time following treatment seems to be a key factor for the differential results obtained in the present study. This is clearly evident in single dose series where the highest breakage frequency in the bone marrow cells was obtained at 4 and 8 h post-treatment and it went on decreasing with lapse of time. At 72 h the value was only 3 times the control one. In the repeated dose regimen the minimum gap between treatment and processing was one week. Schmid and Staiger (1969) also noted importance of the time period between treatment and processing for its clastogenic efficiency.

Its effect on the incidence of polyploidy and aneuploidy was found to be negative in single treatment (time-response and dose-response) as well as repeated treatment series in the bone marrow cells except certain stray cases.

It will be of considerable interest to discuss the probable reasons for such contradictions. We will take up this point later.
3.4.2 Cytogenetic assay of bone marrow cells (Micronucleus test)

In several studies it has been shown that micronucleus test (MNT) is comparable to, or even more sensitive and reliable than, metaphase scoring in the screening of chemical agents for chromosomal aberrations (Boller and Schmid, 1970; Matter and Schmid, 1971; Heddle, 1973; Ledebur and Schmid, 1973; Matter and Grauwiler, 1974; Maier and Schmid, 1976; Schmid, 1976; Seiler, 1976). The principle of MNT has been mentioned earlier. In the present investigation poly- and normochromatic erythrocytes as well as nucleated bone marrow cells were considered. However, during scoring of the latter, as mentioned earlier, due to the presence of very big nucleus and comparatively little cytoplasm in certain cells (myeloblast, myelocytes, erythroblasts) separate existence of MN became doubtful. Similarly confusion arose regarding the reality of MN in certain cells with lobulated nucleus (neutrophils). Though those doubtful cases were not recorded the data on nucleated cells were surely influenced by it. For that we do not want to put much importance on the incidence of MN in the nucleated cells.

The higher incidence of MN in erythrocytes, in which incidence is more relevant (Ledebur and Schmid, 1973; Schmid, 1976) indicates positive effects of the drug. So the results of MNT agree well with those of cytogenetic assay based on metaphase chromosome analysis after single treatment. Dose-response analysis in two types of erythrocytes exhibited parallelism. The highest response was obtained with 0.5 mg dose for both the types of erythrocytes and the values were more than 2 times the corresponding
control values. Normocytes are older than polychromatic erythrocytes by about 24 hours, and only such agents capable of interfering with the mitotic process are known to enhance the frequency of micronucleated normocytes (Matter and Grauwiler, 1974; Sailer, 1976; Schmid, 1976). So higher incidence of micronucleated normocytes at all the dose levels in the present study indicate such type of action of diazepam. As positive evidences for such kind of mutagenic action peculiar features in the bone marrow preparation like rather large MN, large size of polychromatic erythrocytes, and large number of normal and abnormal mitotic figures in the nucleated cells can be cited. Abnormal mitotic figures included bridge formation and lagging pieces of chromatin materials in anaphases. Though these two anaphasic abnormalities are not quite convincing evidences of spindle inhibition, their appearance in the preparation strengthens the belief that the drug acts as spindle poison. Further, mitotic poisons are capable of producing large MN, because only through such agents whole chromosome(s) will be lost during mitosis in appreciable number and subsequently be manifested as large MN. Large size polychromatic erythrocytes are resulted at high doses due to polyploidy and subsequent elimination of the polyploid nucleus. Of the 3 dose levels tested, 2 higher doses yielded significant increase in the incidence of MN. Interestingly highest frequency of micronucleated erythrocytes was obtained with the intermediate dose (0.5 mg). In this regard data of the MNT are comparable with the data of corresponding doses of metaphase chromosome analysis. Thus doseresponse analysis here reveals a curve characteristic of spindle poisons (Maier & Schmid, 1976; Sailer, 1976). Although the
present experiment suffers from the limitation for having data for 3 dose levels only, the highest incidence of MN with the intermediate dose strengthens further the explanation that diazepam acts as a spindle poison.

As it is manifested by the ratio of erythrocytes and nucleated cells the drug exerts slight bone marrow depressive effect with the highest dose. Such type of action with highest dose is also evidenced from lowering of P/N ratio (which is insignificant marginally only). These data on bone marrow composition and P/N ratio also support our dose response data on the incidence of MN. These facts demonstrate induction of mitotic inhibition by diazepam with the highest dose. In this regard our results are in agreement with those of Stenchever and Frankel (1969), Stenchever et al. (1970) and Ober (1974).

Though its action on the mitotic apparatus is well documented, its clastogenic activity, however, cannot be completely ruled out. Instances of such chemicals acting on mitotic spindle as well as on chromosomes are not rare (Manna and Parida, 1965). Moreover, it is not known if these two activities are caused by the same agent or by different agents (Parent drug and its metabolites). Diazepam is converted into two major metabolites (N-desmethyldiazepam and Oxazepam); conversion of diazepam into desmethyldiazepam is very quick but the next step is very slow. Naturally demonstration of diazepam induced MN within 30 h rules out possible potentials of the second metabolite. Since late S or G2 chromosomes are susceptible to diazepam or its active agent (N-desmethyldiazepam) for clastogenicity (as revealed from bone marrow metaphase chromosome analysis) much difference in the incidence of MN in two types of erythrocytes is not expected also.
3.4.3 Cytogenetic assay of male germ cells

The incidence of breakage as well as translocation in the spermatocyte chromosomes after the treatment of diazepam was not very much striking, though in certain cases the increases in the frequency in the treated series were significantly higher over their corresponding control values. As the spermatocytes in mice remain for three weeks (Oakberg, 1956, 1960; Cattanach, 1966) aberrations induced in the spermatocytic chromosomes at least towards the end of treatment would have been expressed in the first week. Lack of effect in the first week clearly indicates non-susceptibility of spermatocytic chromosomes of mice to diazepam. Similarly lack of chromosomal aberrations in wks 3 and 4 also indicates resistance of the spermatogonial chromosomes. The incidence of breakage is slightly higher in last three test weeks in comparison to the first three weeks, but a definite pattern is lacking. Marginally significant increase of frequencies in certain weeks may be supposed to be accidental with little or no biological significance. It is clearly evidenced in case of translocation at wk 8 where the treated value was marginally significant over the 'nil' value obtained in the corresponding control. Our supposition of the non-susceptibility of spermatocytic and spermatogonial chromosomes was also supported by the dominant lethal test discussed later. Our result also supports the findings of Stenchever et al. (1974b) who could not detect increase of aberration in meiotic chromosomes of mice after 21 days consecutive treatment.
The higher incidence of polyploidy in almost all the test weeks is noteworthy. So far the author is aware, occurrence of polyploidy due to diazepam treatment was noted in no earlier report. It was not also demonstrated in the bone marrow cells by the present workers (Das and Kar, 1977; see also section 3.3.1).

The factors presumably responsible for polyploidy in male germ cells have been reviewed in detail by Hulten et al. (1970). It has been known since long that cells of the male germ line placed in saline or any hypotonic solution show increased incidence of 'polyploidy' by fusion of contiguous cells (Fawcett et al., 1959; Dym and Fawcett, 1971). Such type of artifactual production of 'polyploid' spreads has been reported in 14 strains of mice by Beatty et al. (1975). In fact the germ cells in the present study yielded a large number of 'polyploid' spreads even in the control series, the incidence is much more than the value obtained from the bone marrow. The phenomenon of fusion due to hypotonic pre-treatment may thus have some role for the higher incidence of 'polyploidy' in control mice here. However, tremendous increase of its frequency in the treated series cannot be explained with this artifactual production only. Ford and Evans (1971) have considered artifactual origin, however, to be unlikely. Again in the present study tetraploidy was of frequent occurrence, odd polyploidy was completely absent which may be considered as an indicative of occurrence of true polyploidy in spermatogonial stages. The lone case of 18 n spermatocyte-I stage mentioned above might have arisen due to fusion of both ploidy and fusion. "Superimposition" of the cells as sometimes thought to be a possible explanation (Beatty et al., 1975) cannot account for characteristically identical stages of the chromosomes of a polyploid stage.
Effects of phenothiazine tranquilizers (Adams, 1975; Polson and Adams, 1973) as well as diazepam (Breen and Stenchever, 1970) on the membrane systems of cell, viz: nuclear and cell membrane, endoplasmic reticulum, golgi, etc. are well known. Recently chlorpromazine, a phenothiazine tranquilizer, has been reported to cause microtubular disruption just like colchicine or vinblastine (Thyberg et al., 1977). Colchicine like effect of diazepam as characterized by the occurrence of polyploidy in meiotic cells and centromeric separation of bone marrow chromosomes is supposed to be due to its possible effects on the microtubules. Our micronucleus test mentioned earlier (vide supra) also suggested its effect on the spindle structure. However, in the bone-marrow cells after repeated treatment significant increase in polyploidy was noticed at wk 1 only. Its absence in subsequent weeks may be presumed to be due to the fact that the affected cells were killed or simply eliminated in the circulation by the time of fixation. Dose-response analysis in the bone marrow cells did not also reveal remarkably higher incidence of polyploidy, probably because of the short period (4 hr) of exposure of the cells to the drug. Absence of polyploidy in the time-response analysis of the bone marrow cells after single treatment may be attributed to the low dose (0.50 mg) which is incapable of producing spindle disruption as revealed from micronucleus test. Polyploidy revealed in the spermatocytes after repeated treatment was certainly resulted from the effect of the drug on the spindle structure of spermatogonial cells.

Regarding aneuploidy, incidence was not striking except the first two test weeks, in other weeks it was in the control range. Though the 'lower tail' comprising less than 20 bivalents is assumed
to contain some artifactual chromosome loss during preparation
it is expected to contain some true aneuploids also as it is
evident from the 'upper tail' comprising 21 and 22 bivalents.
The incidence as such is more in the germ cells in comparison to
the bone marrow cells. The reason may be same as assumed in the
case of polyploidy. This aneuploidy may be attributed to the
spermatogonial aneuploidy, though it is unable to explain all the
data.

Miklos (1974) stressed the importance of proper association
between sex chromosomes in order to ensure a normal course of
spermatogenesis. Similarly Beechey (1973), Forejt and Gregorova
(1977) reported sterility in mouse being associated with
dissociation of X and Y. Similar phenomenon may be assumed to hold
good in case of autosomes also. At the starting of scoring a good
number of dissociation cases were noticed and obviously they were
scored. However, quantitative analysis could not reveal very
remarkable elevation in the incidence in the treated series. In
three cases only the treated values were significantly higher, that
too marginally. For that reason undue importance is not given to
the dissociation cases.

3.4.4 Dominant lethal test

Though sensitivity of dominant lethal test (DLT) has
recently been questioned (Green and Springer, 1973) it has been
used extensively particularly in mouse during the last 10 years
to assess the mutagenicity of a wide variety of substances. Rather,
owing to its involvement with the germ cells it has become one of
the important screening systems currently used in mutagenicity
testing. So far the author is aware diazepam has not been tested
with DLT. However, this test in the present investigation had some limitation. Only one dose level (0.5 mg) — minimum but highly effective dose as revealed from the bone marrow cytogenetic study was employed.

The frequency of total implants per female in the control series ranged from $8.26 \pm 0.32$ to $8.70 \pm 0.29$, which is very close to the values obtained in mice by other workers (Ehling et al., 1968, 1972; Generoso et al., 1978; Favor and Crenshaw, 1978; and others). Further, our data on the incidence of dead implants per female in the control which varied between $0.36 \pm 0.16$ and $0.55 \pm 0.09$ was also in agreement with those of others (Lorke and Machemer, 1974; Petersen and Legator, 1973 and others). Significant increase in the mutagenic indices and dead implants per dam in certain weeks and significant decrease in the incidence of total as well as live implants per dam clearly demonstrate dominant lethality due to the drug treatment. The frequency of dead implants per dam would have been more, though mutagenic index would remain unaltered, if implantation data for resorption (decidualmata) cases could have been known. Absence of resorption case in the entire control series is also an indication of the effectiveness of the drug in the treated series and it is particularly effective in the first week. This is also evidenced by the higher incidence of resorption in early weeks. Oakberg (1960), Cattanach (1966) and Ghosal and Mukherjee (1971) determined the times required for the gametogenic stages to develop into mature sperms and to reach the ejaculate in mice. It helps us to know the sensitivity of any particular stage to certain chemical. First week post-injection sperms are treated as spermatozoa of vas and epididymes, second
post-injection sperms as testicular sperms and late spermatids, and third week post-injection sperms as early spermatids. Induction of structural and numerical changes of chromosomes in the parental germ cells is believed to be the genetic basis for dominant lethality (Bateman and Epstein, 1971). The increase of dominant lethality in early weeks in this study does indicate that diazepam or its active agent (s), like EMS, TEM and several other chemical mutagens (see Ehling et al., 1968, 1972; Cattanach et al., 1968; Leonard et al., 1971; Partington and Bateman, 1964; Rohrborn 1970; Cox and Lyon, 1975; Ray et al., 1974; Arnold et al., 1976; Hastings et al., 1976), affects the post-meiotic sperm cells and the effect is reduced with subsequent matings. However, it is not possible at present to pin-point the exact stage susceptible to the drug due to 15 days treatment. Differential foetal lethality observed in different post-treatment weeks has led to the suggestion that different stages in spermatogenesis have varied sensitivities to the drug perhaps because of dissimilar metabolic states. This is in contrast with the unspecific type of effects of ionizing radiations (Ehling, 1971). Since intrauterine death involved mostly the early foetus the effect appears to be specific for the early post-implantation stages of embryonic development.

Dominant lethal test in the present investigation dealt with the post-implantation loss only. But the significant decrease in the total implantation per dam is judged to reflect certain pre-implantation loss also. Certain degree of pre-implantation loss is also evidenced from simultaneous decrease of live implants and total implants per dam in almost all the weeks without being accompanied with significant increase of dead implants (except wk 1)
Mating indices in different weeks among controls fluctuated between the two extreme values (70.00 and 95.00), while treated mice showed a trend of gradual increase of mating index in relation to the increase of mating intervals. In general, however, we find a tendency of lower mating index in the treated mice, though the differences were not striking. From these observations it seems that the drug has very little effect on the mating behaviour or fertility of males. Slight decrease in mating index that we find may be attributed to the sedative action of the drug.

The results of the present study are in good agreement with those of our cytogenetic findings on bone marrow cells after single treatment. Legator and Malling (1969) also suggested that the usefulness of DLT can be enhanced by carrying out side by side cytogenetic studies. The increased intrauterine death noted here have been assumed to be caused by chromosomal abnormalities in the male germ cells. The lack of effect in the bone marrow chromosomes after repeated treatment may be, as mentioned earlier, due to long gap between treatment and processing of the material, by that time the affected cells are assumed to be killed or to have come in the circulation, or due to some phenomenon related to the development of tolerance. Though repeated treatment regimen was also used here the effect accounted may be attributed to the initial treatment which affected early spermatids or so. This assumption may be substantiated if we look to our data on meiotic chromosome analysis where the spermatocytic and spermatogonial cells exhibited considerable amount of resistance to the drug. Anyway, repeated treatment has made the subject more complicated as it was assumed by others also (Brewen and Preston, 1978), and further studies are
needed with single treatment.

3.4.5 Sperm count

Counting of epididymal sperm was undertaken as an ancillary experiment to dominant lethal test. Walton (1927) reported in rabbit that the litter size depended on the density of the sperm suspension. It has been reported by Searle and Beechey (1974) that if the sperm count in mice comes below 10% level of the normal count complete sterility is resulted, which may be evidenced by sterile mating. Our dominant lethal test did not account for complete sterility at any test week. However, in the present investigation the count was significantly reduced after diazepam treatment at wk 1 only when it came to almost 50% level of the corresponding control value. With the lapse of time the count increased almost gradually and at wk 12 it came to the control range. The depletion was not so severe as recorded with acute X-irradiation (Bateman, 1958; Searle and Beechey, 1974). But the remarkable depletion in sperm count at wk 1 may have some bearing with the lower mating index recorded in the dominant lethal test. The reason for this depletion is not, however, clear. It may be resulted from lower testicular sperm production or resorption of spermatozoa in the epididymis. Anyway, the most adverse effect of the drug was thus manifested at wk 1, which indicates susceptibility of post-meiotic germ cells. These data are, therefore, in good agreement with those of dominant lethal test.

3.4.6 Sperm head abnormality

X-ray as well as different chemical agents well known for their clastogenic and mutagenic activity have been shown to produce marked increase in sperm abnormalities (Oakberg and DiMinno, 1960;
MacLeod et al., 1964; Bruce et al., 1974; Wyrobek and Bruce, 1978). Wyrobek and Bruce (1975) compared the sperm abnormality inducing action of 25 chemicals with their mutagenic, teratogenic and carcinogenic activities. Recently Wyrobek and Bruce (1978) have provided a list of 60 chemicals tested for their sperm abnormality inducing action in mice. So far our knowledge goes effects of benzodiazepine tranquilizers on the sperm head morphology have not been reported in any organism as yet. Although the criteria of sperm morphology includes different parts of a sperm, analysis becomes easier if limited to head abnormalities only, since head shape is most insensitive to preparational damage.

So far qualitative aspect of abnormal sperm head morphology is concerned in control and diazepam treated mice some common types of abnormalities were recorded. However, a few new types were encountered only in the treated animals. Their frequencies were also very low. Naturally it will not be unreasonable to assume that they were not typical for the treatment of the drug. In the control animals the frequencies of sperm head abnormalities in different weeks fluctuated within a very narrow range and their mean was very close to the value typical for the strain (Bruce et al., 1974). The frequency of sperm head abnormality increased in all the test weeks after the treatment, but the increase was remarkably significant at wk 6 only. If the treated values would have been compared with the pooled mean control value (4.92) significant differences would have been found in some other weeks also. Anyway, the effect persists upto wk 12 even, though the level remains less pronounced. So, we can rank diazepam with antimetabolites like hydroxyurea, imuran, IUdR, aminopterine, etc. which are known to
produce also less marked effect on sperm morphology (Wyrobek and Bruce, 1975). However, in all those cases maximum effect was noticed at wk 4. But with regard to sperm head abnormality producing capacity diazepam differs from two other drugs of abuse viz. aspirin and phenobarbital which failed to produce any adverse effect on sperm morphology (Wyrobek and Bruce, 1978). From the remarkably high incidence of testicular sperm head abnormality obtained at wk 6 it would be reasonable to assume that the drug exerted its effect for the induction of abnormality on sperm head when the cells were in spermatogonial stage. However, dose-response analysis and single treatment may give a clear cut picture on the effectiveness of the drug and stage sensitivity.

3.4.7 General

Thus in contrast to several negative reports mentioned earlier our data on metaphase chromosome analysis of bone marrow cells after single treatment, MNT, DLT reveal beyond doubt that diazepam has little, though not severe, clastogenic effect. Its action on the spindle structure has also been revealed from meiotic chromosome analysis and MNT.

A decade has already elapsed since the initial report of a possible 'genetic' effect of diazepam was described. But no definite answer has yet been emerged; the reports so far made are contradictory. With the chemical mutagenesis this type of contradiction is not at all new. In this regard as an example the case of LSD can be cited, which perhaps poses the highest degree of contradictions (Cohen and Shiloh, 1978). It will be of considerable interest if we now discuss the probable reasons of contradiction, which may be of different types as summerised by Cohen and Shiloh (1978) in
connection with LSD. However, the reasons for differential results cannot be explained satisfactorily until we know the pharmacokinetics of diazepam as well as its mechanism of action at the molecular level. Though by now we have sufficient knowledge on the former, the latter is still obscure. So far the author is aware literature provides only one report (Ober, 1974) on the mode of action of diazepam at the molecular level. Ober (1974) studied the effect of the drug on the rate of cell division and on the synthesis of DNA, RNA, carbohydrate, etc. in Scenedesmus obliquus in vitro. The cell division was completely stopped with $10^{-4}$ M diazepam. In the presence of the tranquilizer at the concentration of 80 $\mu$M the synthesis of both RNA and DNA was shown to be greatly reduced. This decrease in RNA and DNA synthesis was suggested as one of the possible reasons for the inhibition of cell division. He also showed that the metabolic inhibition was dose dependent and complete at 80 $\mu$M concentration. Again cells after complete metabolic inhibition induced by 80 $\mu$M diazepam returned to the normal level 24 h after the withdrawal of the drug. We do not know as yet which step is exactly affected by diazepam to cause inhibition. In the present study we are not much concerned with the inhibitory action of diazepam because we have not studied this aspect very specifically. But we cannot go away without it. Inhibition of DNA synthesis and ultimately metabolic inhibition induced by the drug may be supposed to be a reason for failure to obtain positive effect in certain earlier experiment. In both the two earlier works conducted on laboratory mammals in vivo (Schmid and Staiger, 1969) Neda et al., 1977) very high doses were used. The doses used by Schmid and Staiger (1969) were 300-1000 times the human therapeutic
dose and they observed the effect at 5-8 h after the treatment. Similarly Neda et al. (1977) treated rats with doses which were 200-500 times the human therapeutic dose and observed the effect on 1.5 and 10 days after the treatment. So it is not quite improbable that after the use of high doses the affected cells did not reach to the metaphase stage and exhibit effect. In contrast in our case 0.5 mg, per individual dose was almost equivalent to 20 times the human therapeutic dose. This type of phenomenon is not new in chemical mutagenesis. Chemicals like deoxyadenosine, 5-Flurodeoxyuridine, etc. are well known to produce such type of effect (Kihlman, 1966; Manna and Das, 1975). Again it is highly probable that inhibition of DNA synthesis is the cause for its clastogenic effect as is found with several other inhibitors of DNA synthesis (Kihlman, 1966).

Recent knowledge on pharmacokinetics of diazepam may be of some help in understanding the problem. Pharmacologically diazepam is a widely studied benzodiazepine. Pharmacological action of diazepam is found to vary according to the route of administration and oral treatment has been proved to be most effective (Mandelli et al., 1978). The mean plasma half-life of diazepam is about 31 h (Sellers, 1978). Its absorption is very rapid and complete, particularly after oral administration, the plasma concentration peaking about 1 h after ingestion. Diazepam undergoes bio-transformation as mentioned below and desmethyldiazepam is its main biologically active metabolite. Desmethyldiazepam has longer half-life than the parent drug (50.9 h) (Sellers, 1978). The first step of the biotransformation is very rapid and almost complete (98%) and its excretion, biliary and/or urinary, is negligible (2%)
(Hvidberg and Dam 1976). So the clastogenic effect of diazepam after single treatment at early hour may be due to the parent drug or its active metabolite. Gradual decrease of the clastogenic effect with time as revealed from metaphase chromosome analysis of bone marrow cells after single treatment also supports the assumption.

Naturally the time period between treatment and processing seems to be an important factor for the expression of clastogenic effect. Lack of effect on the bone marrow chromosomes here after repeated treatment may be supposed to be due to long gap between treatment and processing. The affected bone marrow cells might have come out in the circulation by the time of analysis. Failure to obtain any effect on human chromosomes by Cohen et al., (1969) and White et al. (1974) as well as on rat chromosomes by Neda et al. (1977) may be explained in the same line. Further, pharmacologically single and repeated treatment give differential results. A diminuation of both diazepam and desmethyldiazepam steady state plasma levels has been shown during continuous treatment, suggesting an autoinduction phenomenon (Kanto et al., 1974). Another possible explanation for lower plasma concentration is the accumulation of
those two compounds in erythrocytes after prolonged treatment (Zingales, 1973). The most striking feature is the report that the values measured over a period of 2-5 years were 1/5 - 1/10th those found after 15 days treatment (Kanto et al., 1974). Role of metabolic autoinduction discussed above seems to be a more likely explanation. Observation of Klotz et al. (1975), however, seems to suggest some degree of saturation for diazepam after multiple dosing. Anyway, absence of effect after repeated treatment in the present as well as earlier investigation (Schmid and Staiger, 1969) may also be explained from the lowering of the plasma level of diazepam or its active metabolite. In this connection we may refer to the work of Swain et al. (1980) who studied the effect of morphine sulphate on the bone marrow chromosomes of mice after single treatment and seven days repeated treatment of the same dose and found that the breakage frequency got markedly reduced after repeated treatment. They could not give exact explanation for it but repeated treatment and thereby development of tolerance had been suggested to be responsible for the decrease. However, it is difficult to interpret the results of in vitro experiments. Staiger (1969, 1970) and Stenchever and Frankel (1969) followed almost identical experimental design but obtained opposite results. Thus with these limited data we are not in a position to put forward any explanation for the inconsistencies on the clastogenic effects of diazepam. Over and above, differences in animals (species), dosages used, exposure period, etc. are too great for comparison. Many more researches are needed to solve the problem.